NextGen Sequencing: Experimental Planning and Data Analysis

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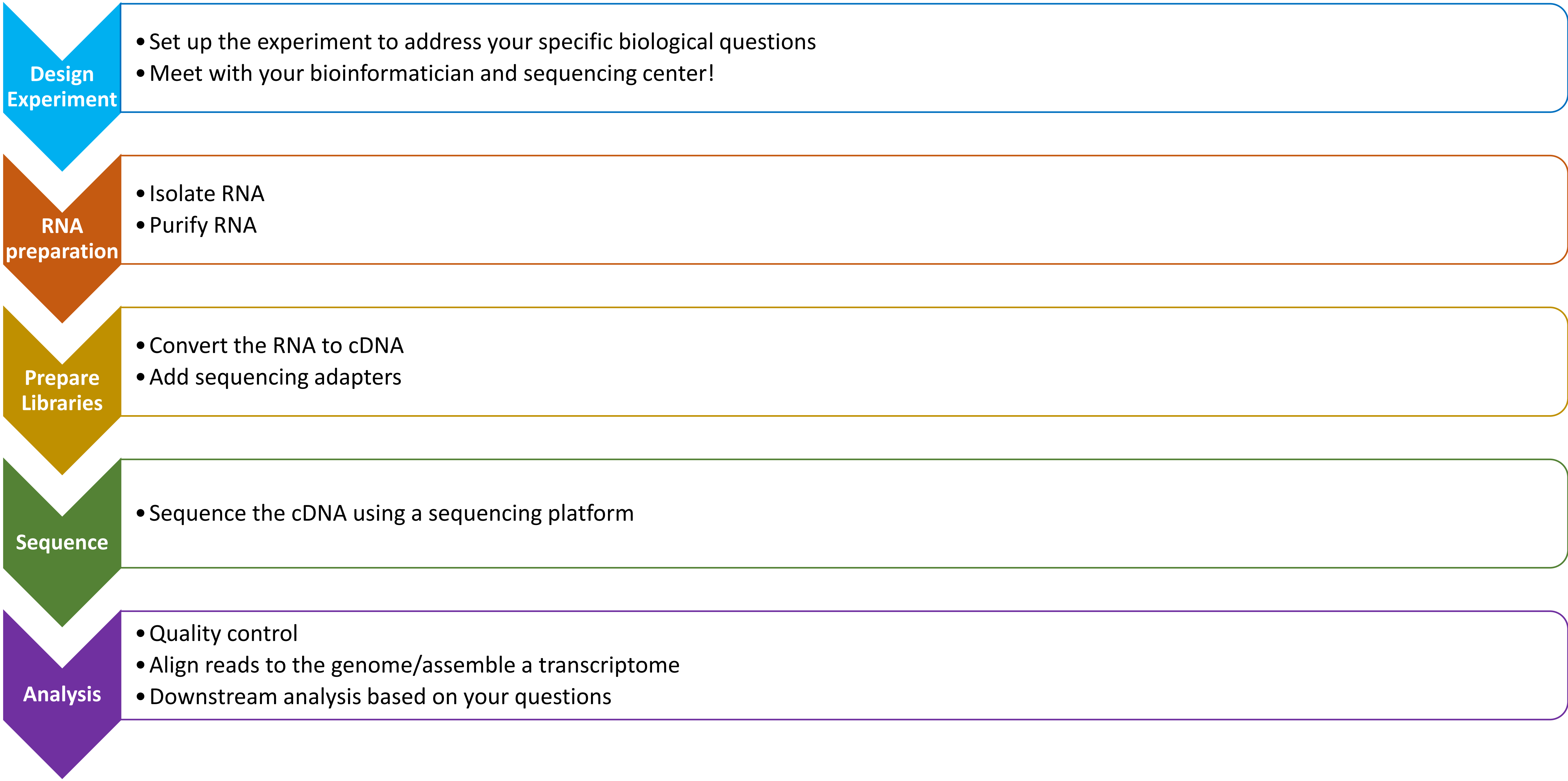
A Next Generation Sequencing (NGS) Refresher

- Became commercially available in 2005
- Construction of a sequencing library → clonal amplification to generate sequencing features
- High degree of parallelism
- Uses micro and nanotechnologies to reduce size of sample components
  - Reduces reagent costs
  - Enables massively parallel sequencing reactions
- Revolutionary: has brought high speed to genome sequencing
  - Changed the way we do research, medicine

RNA Sequencing

- High-throughput sequencing of RNA
- Allows for quantification of gene expression and differential expression analyses
- Characterization of alternative splicing
- Annotation
  - Goal is to identify genes and gene architecture
- *de novo* transcriptome assembly
  - no genome sequence necessary!

RNA-seq workflow



Design Experiment

- Set up the experiment to address your specific biological questions
- Meet with your bioinformatician and sequencing center!

RNA preparation

- Isolate RNA
- Purify RNA

Prepare Libraries

- Convert the RNA to cDNA
- Add sequencing adapters

Sequence

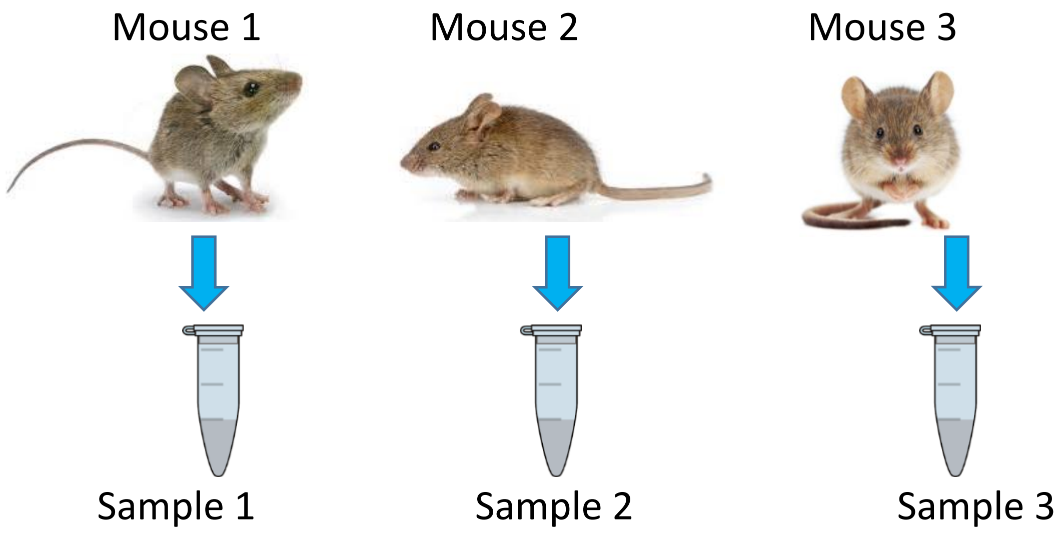
- Sequence the cDNA using a sequencing platform

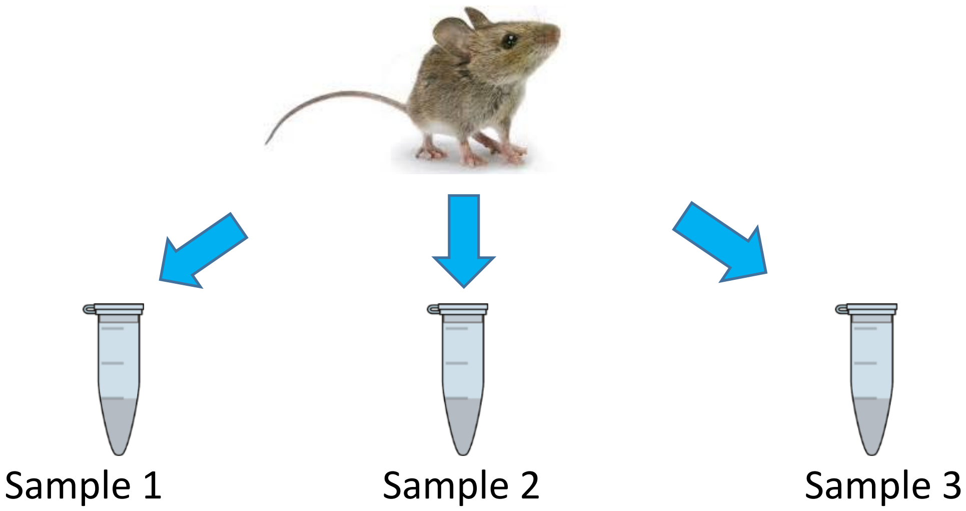
Analysis

- Quality control
- Align reads to the genome/assemble a transcriptome
- Downstream analysis based on your questions

Replication

- Number of replicates depends on various factors:
  - Cost, complexity of experimental design (how many factors are of interest), availability of samples
- Biological Replicates
  - Sequencing libraries from multiple independent biological samples
  - Very important in RNA-seq differential expression analysis studies
  - At least 3 biological replicates needed to more accurately calculate statistics such as p-values
- Technical Replication
  - Sequencing multiple libraries from the same biological sample
  - Allows estimation of non-biological variation
  - Not generally necessary in RNA-seq experiments
  - Technical variation is more of an issue only for lowly expressed transcripts

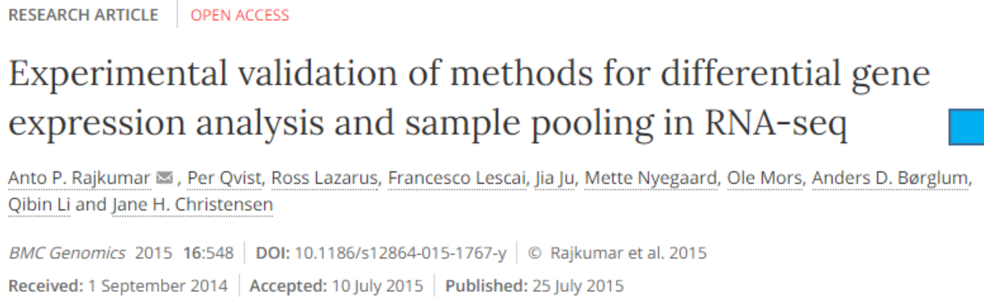




Design Experiment

Pooling Samples in RNA-seq

- Can be beneficial if tissue is scare/enough RNA is tough to obtain
- Utilizes more samples, could increase power due to reduced biological variability
- Danger is of a pooling bias (a difference between the value measured in the pool and the mean of the values measured in the corresponding individual replicates)
- Can get a positive result due to only one sample in the pool
- Might miss small alterations that might disappear when only 1 sample has a different transcriptome profile than others in the pool
- Generally it is better to use one biological replicate per sample
- If you must pool, try to use the same amount of material per sample in the pool, use stringent FDR cutoffs, and many biological reps per pool



RESEARCH ARTICLE | OPEN ACCESS

Experimental validation of methods for differential gene expression analysis and sample pooling in RNA-seq

Anto P. Rajkumar, Per Qvist, Ross Lazarus, Francesco Lescai, Jia Ju, Mette Nyegaard, Ole Mors, Anders D. Børglum, Qibin Li and Jane H. Christensen

BMC Genomics 2015 16:548 | DOI: 10.1186/s12864-015-1767-y | © Rajkumar et al. 2015

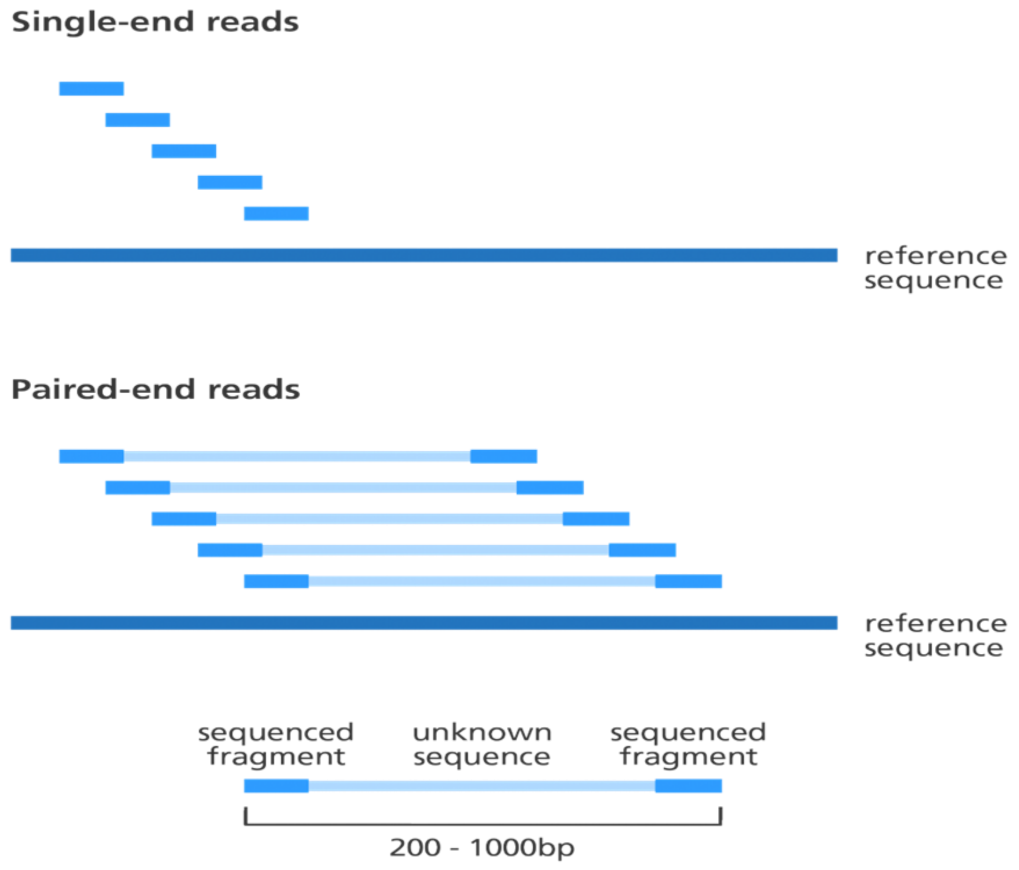
Received: 1 September 2014 | Accepted: 10 July 2015 | Published: 25 July 2015

Evaluated validity of two pooling strategies (3 or 8 biological replicates per pool; two pools per group). Found pooling bias and low positive predictive value of DE analysis in pooled samples.

Design Experiment

Single-end versus paired-end

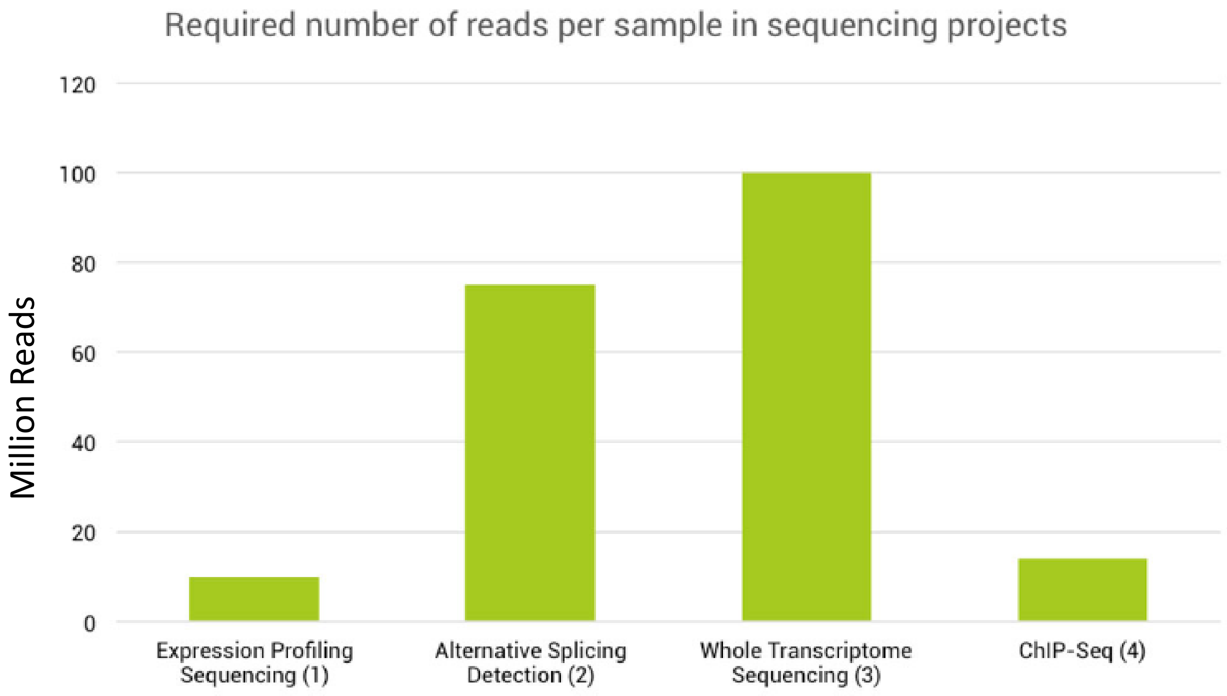
- Reads = the sequenced portion of cDNA fragments
- Single-end= cDNA fragments are sequenced from only one end (1x100)
- Paired-end= cDNA fragments are sequenced from both ends (2x100)
- Paired-end is important for de novo transcriptome assembly and for identifying transcriptional isoforms
- Less important for differential gene expression if there is a good reference genome
- Don’t use paired-end reads for sequencing small RNAs…
- Note on read-length: long reads are important for de novo transcript assembly and for identifying transcriptional isoforms, not required for differential gene expression if there is a good reference genome



Design Experiment

Sequencing Depth – How deep should I sequence?

- Depth= (read length)(number of reads) / (haploid genome length)
- Each library prep method suffers from specific biases and results in uneven coverage of individual transcripts → in order to get reads spanning the entire transcript more reads (deeper sequencing) is required
- Depends on experimental objectives
    - Differential gene expression? Get enough counts of each transcript such that accurate statistical inferences can be made
    - De novo transcriptome assembly? Maximize coverage of rare transcripts and transcriptional isoforms
    - Annotation?
    - Alternative splicing analysis?



1) Liu Y., et al., RNA-seq differential expression studies: more sequence or more replication? Bioinformatics 30(3):301-304 (2014) 2) Liu Y., et al., Evaluating the impact of sequencing depth on transcriptome profiling in human adipose. Plos One 8(6):e66883 (2013) 3) Bentley, D. R. et al. Accurate whole human genome sequencing using reversible terminator chemistry. Nature 456, 53–59 (2008) 4) Rozowsky, J.et al., PeakSeq enables systematic scoring of ChIP-seq experiments relative to controls. Nature Biotech. 27, 65-75 (2009).

Strand Specificity

- Strand-specific= you know whether the read originated from the + or – strand
- Important for *de novo* transcript assembly
- Important for identifying true anti-sense transcripts
- Less important for differential gene expression if there is a reference genome
- Knowledge of strandedness may help assign reads to genes adjacent to one another but on opposite strands

RNA-seq experimental design summary

- Very important step - if done incorrectly no amount of statistical expertise can glean information out of your data!!!
- Biological replicates
  - For differential expression I generally recommend at least 3 – allows you to estimate variance and p-values
- Technical replicates
  - Generally not necessary in RNA-seq experiments
- Depth of sequencing
  - Depends on your experimental goals and organism!
- Length of reads
  - Longer reads = better alignments
  - Longer reads = more expensive
- Paired-end or single-end?
  - Paired-end = better alignment
  - Paired-end = more expensive
- Pooling – Not ideal but sometimes necessary
- Strand-specific?
  - Definitely for antisense transcript identification and *de novo* transcriptome assembly
  - Not necessary for differential gene expression on an organism with a well-characterized reference genome

Experimental Design

Perfect World

- Reads as long a possible
- Paired-end
- Sequence as deeply as possible to detect novel transcripts (100-200M)
- As many replicates as possible
- Preferably run a small pilot experiment first to see how many replicates are needed given the effect size

http://scotty.genetics.utah.edu/scotty.php

Real World

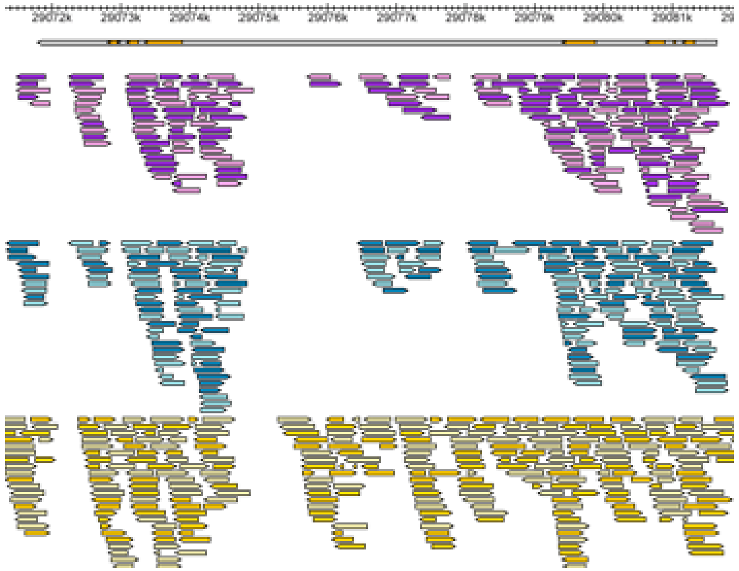
- Determine what your goals are and what treatments you are interested in; plan accordingly
- For a simple differential gene expression experiment on a human you could get away with single-end, 75-100bp reads, with n=3 biological replicates, sequenced to ~30 million reads/sample (1 lane of sequencing for a simple control vs treatment 6 sample design)

Design Experiment

Microarray versus RNA-Seq

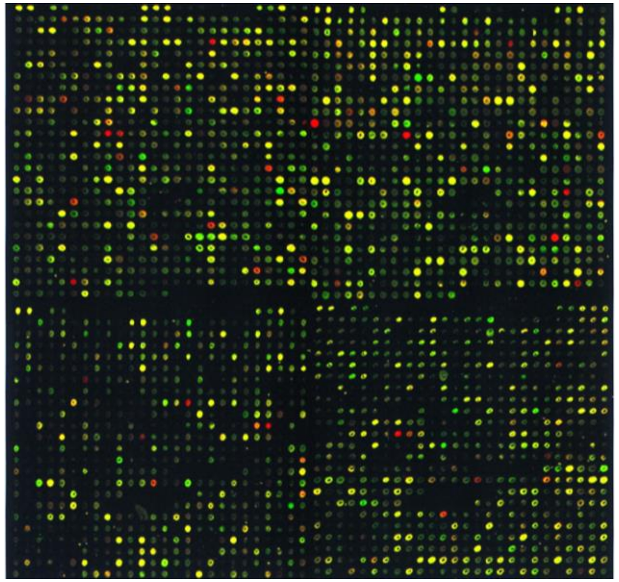
RNA-seq

- Counts (discrete data)
- Negative binomial distribution used in statistical analysis
- No genome sequence needed
- Can be used to characterize novel transcripts/splice forms
- Metric: Counts (quantitative)



Microarray

- Continuous data
- Normal distribution used in statistical analysis
- Genome must be sequenced
- Uses DNA hybridizations – sequence info needed
- Metric: Relative intensities

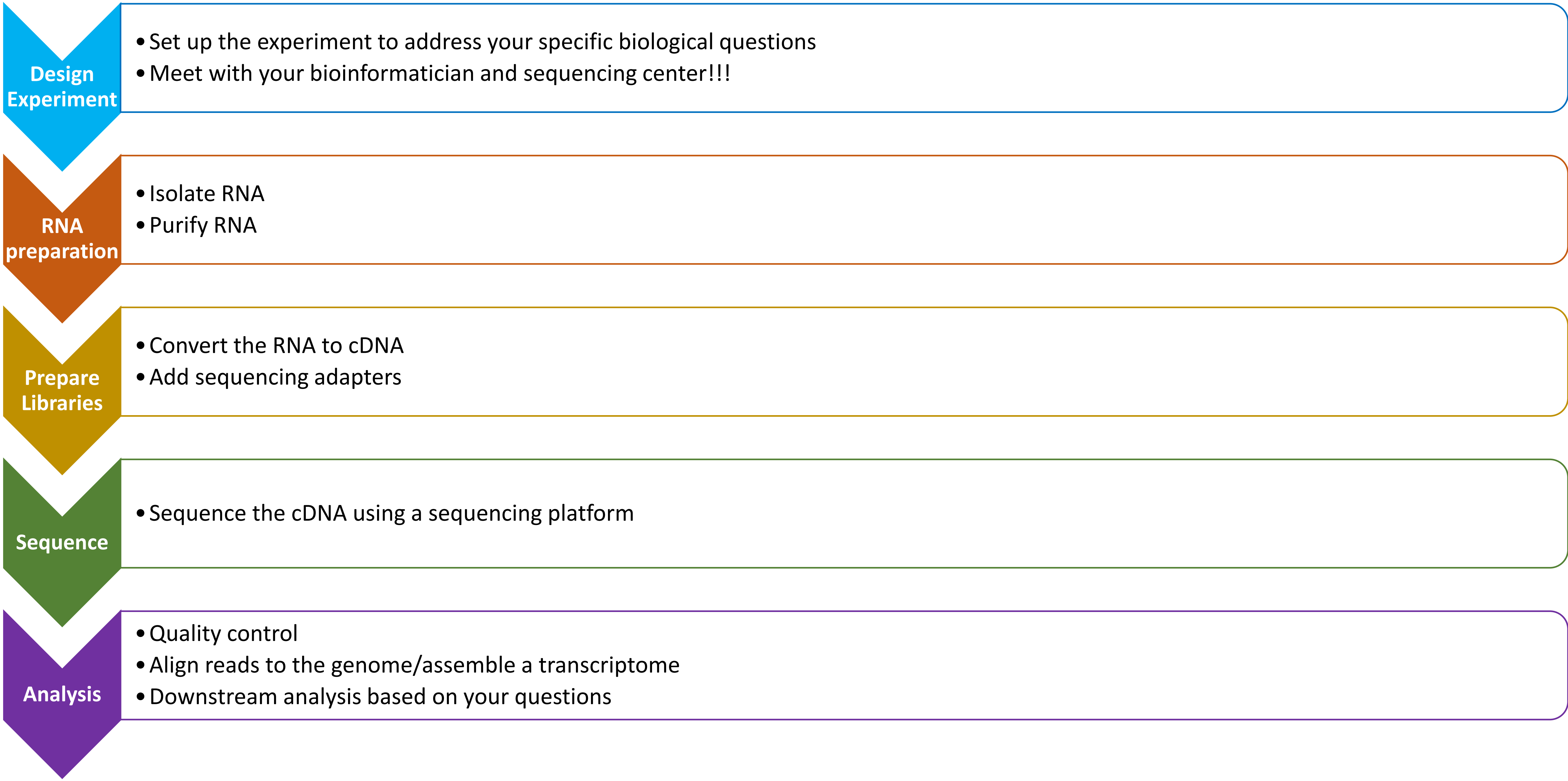


Design Experiment

Do I use Microarray or Sequencing?

- What expertise is available?
  - Is your lab already set up for microarrays? Does your bioinformatician prefer to analyze next gen data? What are people in your department familiar with? Is there someone who can help you troubleshoot problems?
- Cost → microarrays are cheaper
- At what levels are the transcripts of interest likely to be expressed at?
  - Microarrays indicate relative rather than absolute expression
    - This can be problematic for accurate estimation of expression levels of very highly or lowly expressed transcripts
- Does your organism of interest have a well characterized genome?
- Data analysis: how confident are you in your ability to analyze the data?
  - Microarrays have been around for a lot longer and so microarray analysis has more user-friendly tools

RNA-seq workflow



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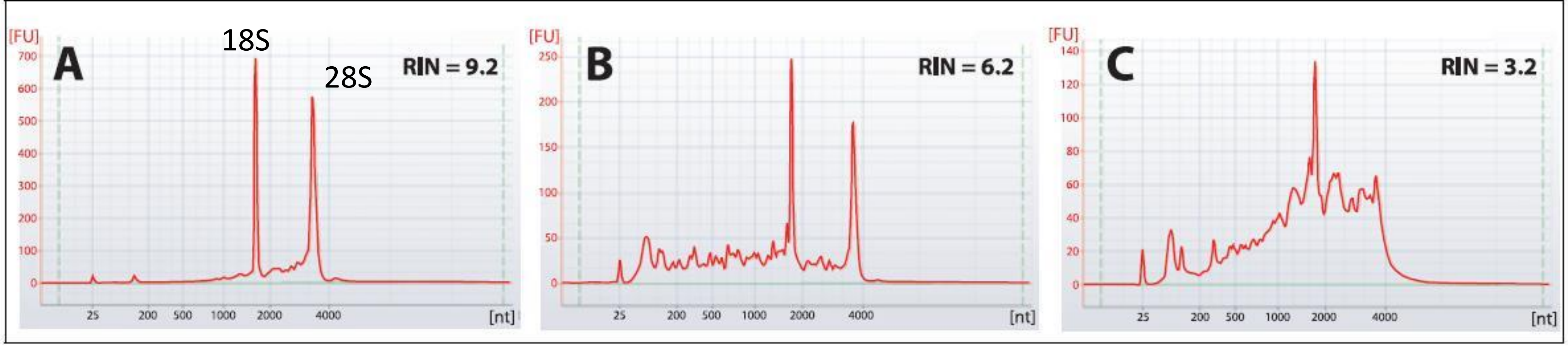
RNA preparation

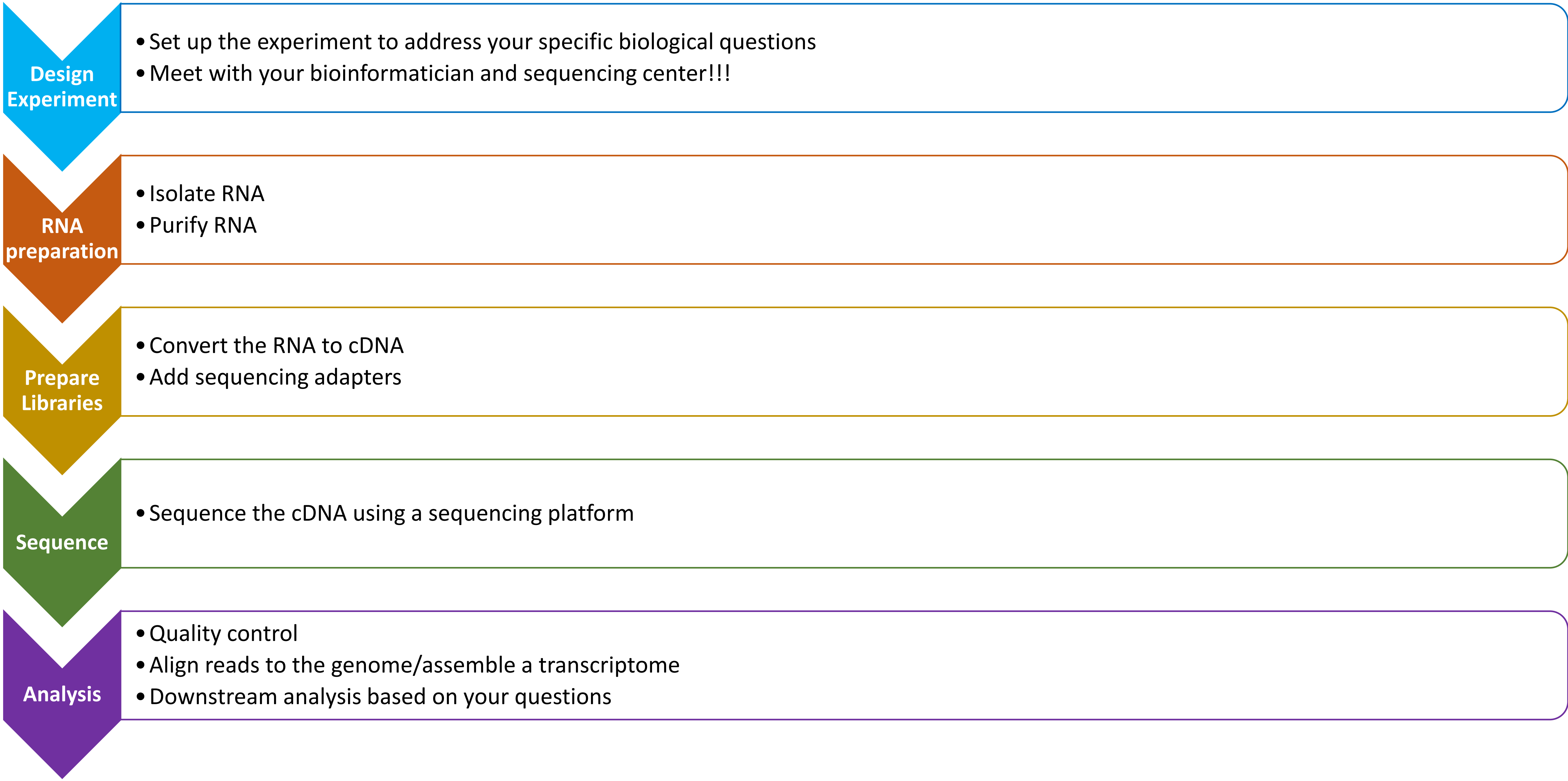
RNA extraction, purification, and quality assessment

Figure 2.1 Example Agilent Bioanalyzer Electropherograms from three different total RNAs of varying integrity. Panel [A] represents a highly intact total RNA (RIN = 9.2), panel [B] represents a moderately intact total RNA (RIN = 6.2), and panel [C] represents a degraded total RNA sample (RIN = 3.2).



- RIN= RNA integrity number
- Generally, RIN scores >8 are good, depending on the organism
- Important to use high RIN score samples, particularly when sequencing small RNAs to be sure you aren't simply selecting degraded RNAs

RNA-seq workflow



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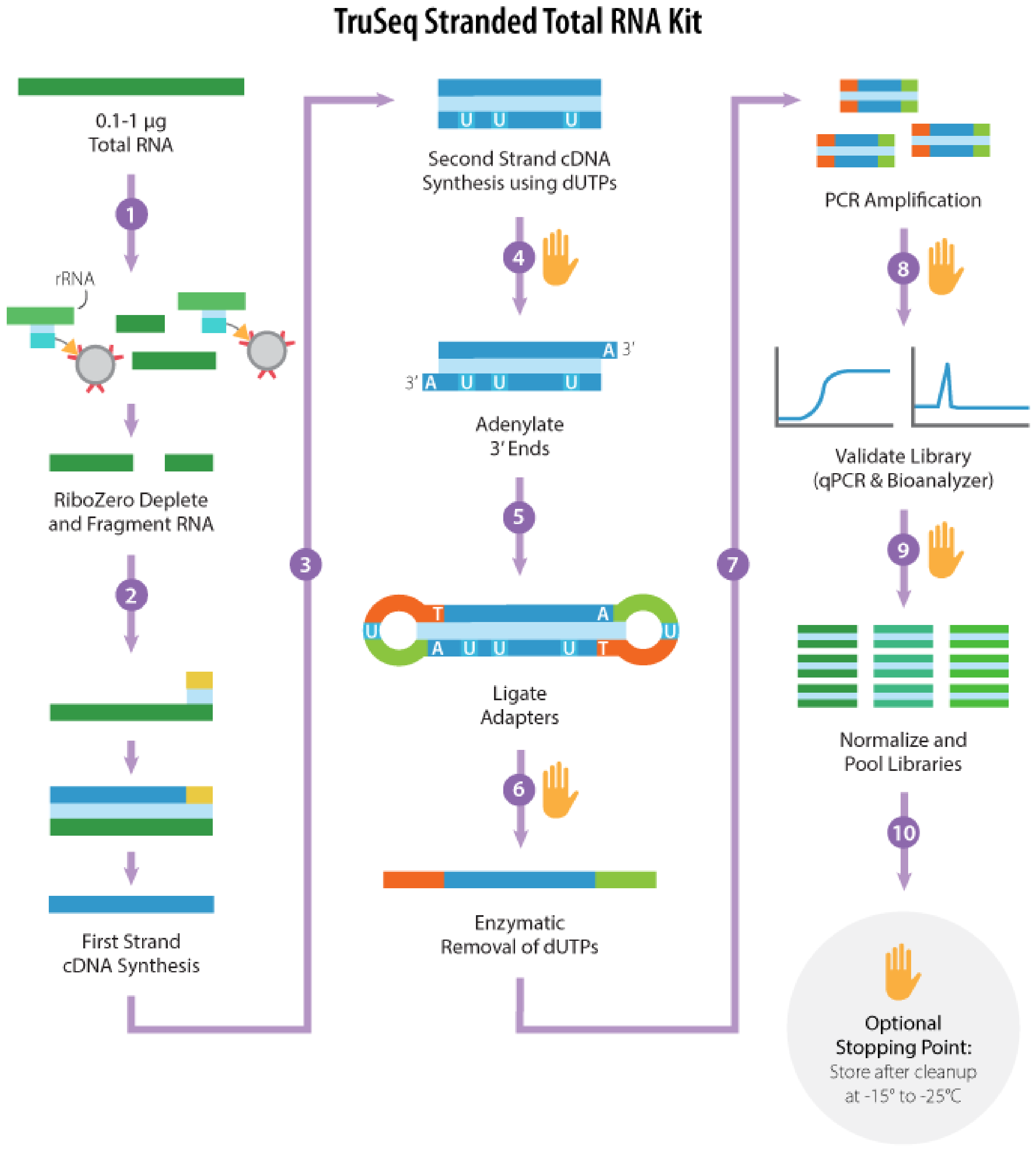
Target Enrichment

- It is necessary to select which RNAs you sequence
- Total RNA generally consists of >80% rRNA (Raz et al. ,2011)
  - If rRNA not removed, most reads would be from rRNA
- Size selection – what size RNAs do you want to select? Small RNAs? mRNAs?
- Poly A selection= method of isolating Poly(A+) transcripts, usually using oligo-dT affinity
- Ribodepletion = depletes Ribosomoal RNAs using sequence-specific biotin-labeled probes

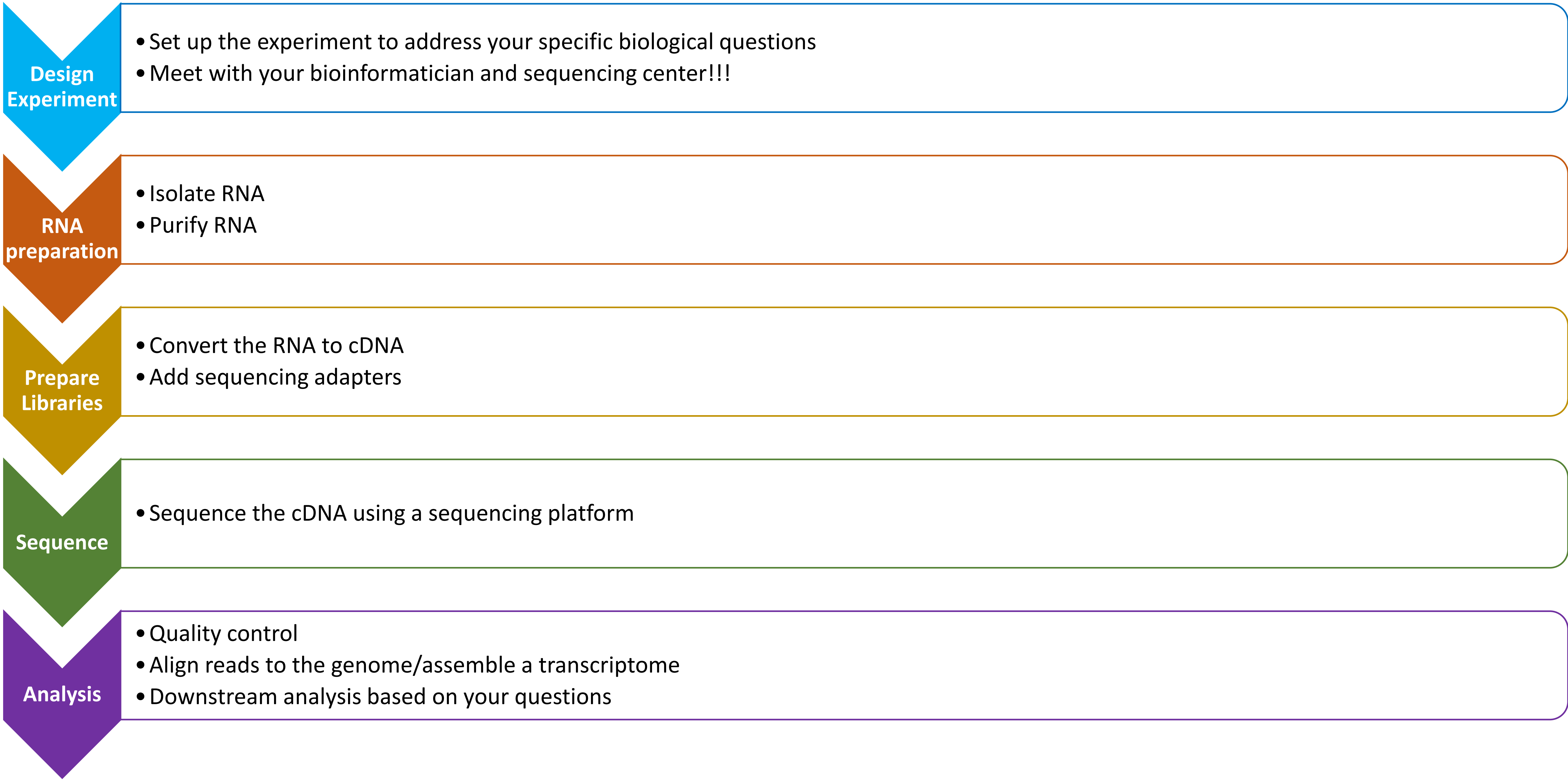
Prepare Libraries

Library Prep

- Before a sample can be sequenced, it must be prepared into a sample library from total RNA.
- A library is a collection of fragments that represent sample input
- Different methods exist, each with different biases



RNA-seq workflow



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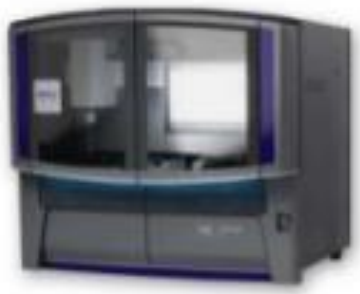
Sequence

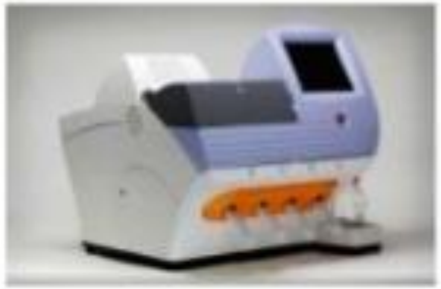
Next Generation Sequencing Platforms

- 454 Sequencing / Roche
  - GS Junior System
  - GS FLX+ System
- Illumina (Solexa)
  - HiSeq System
  - Genome analyzer IIx
  - MySeq
- Applied Biosystems – Life Technologies
  - SOLiD 5500 System
  - SOLiD 5500xl System
- Ion Torrent
  - Personal Genome Machine (PGM)
  - Proton

GS FLX 454
(ROCHE)

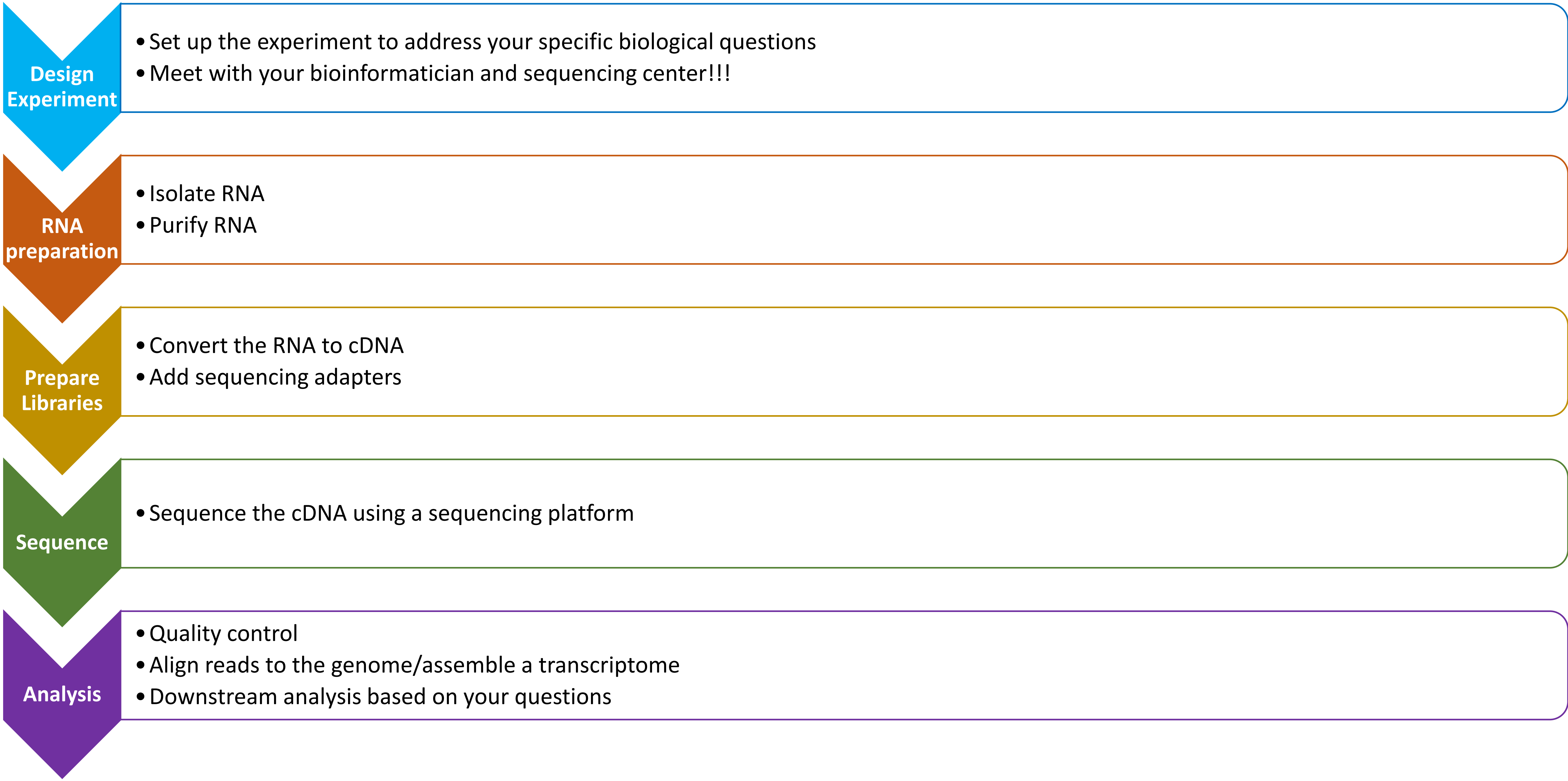
HiSeq 2000
(ILLUMINA)


5500xl SOLiD
(ABI)


Ion TORRENT

https://youtu.be/HMyCqWhwB8E

RNA-seq workflow



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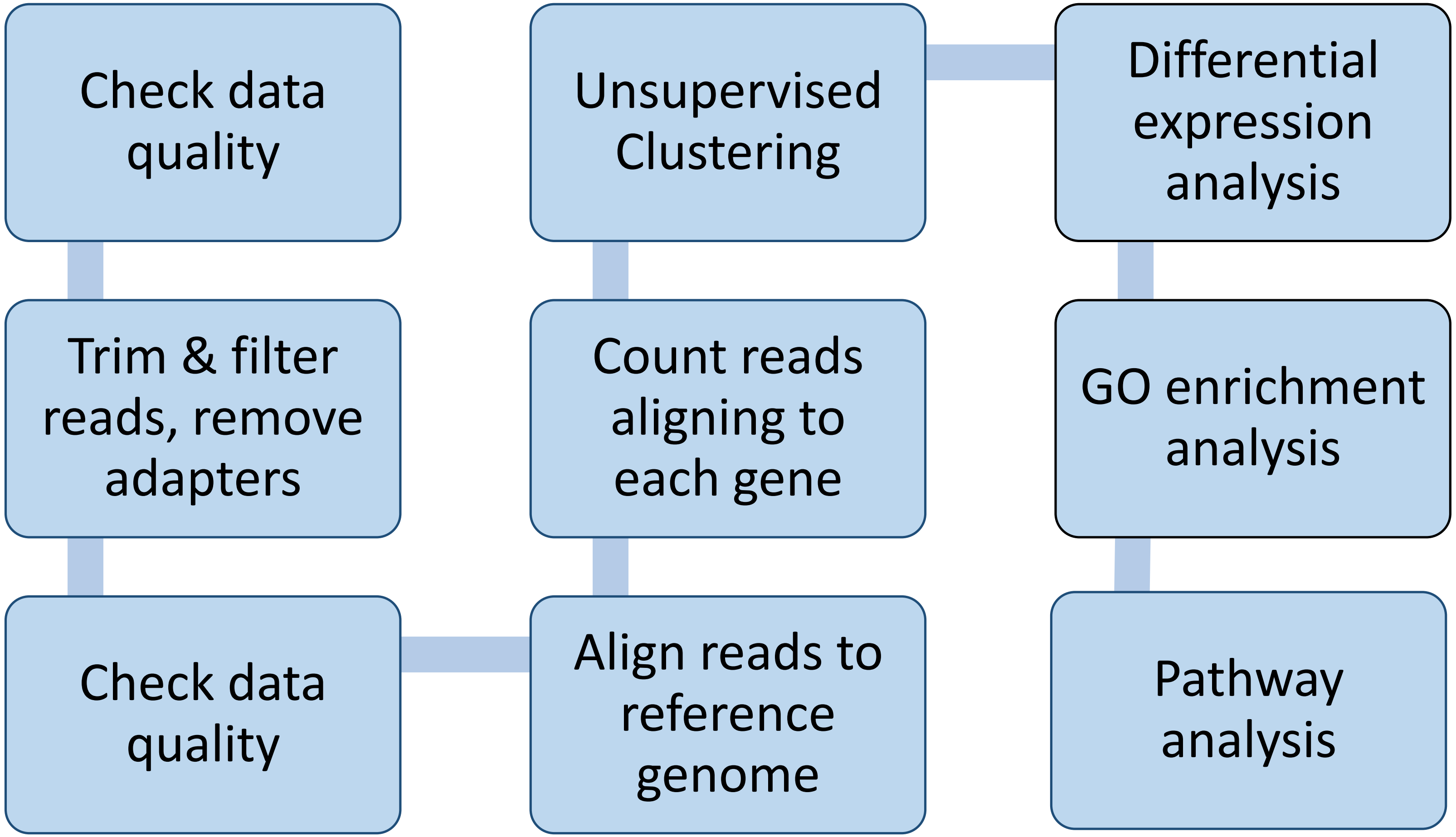
Analysis

Standard Differential Expression Analysis



Check data quality

Trim & filter reads, remove adapters

Check data quality

Align reads to reference genome

Count reads aligning to each gene

Unsupervised Clustering

Differential expression analysis

GO enrichment analysis

Pathway analysis

Analysis

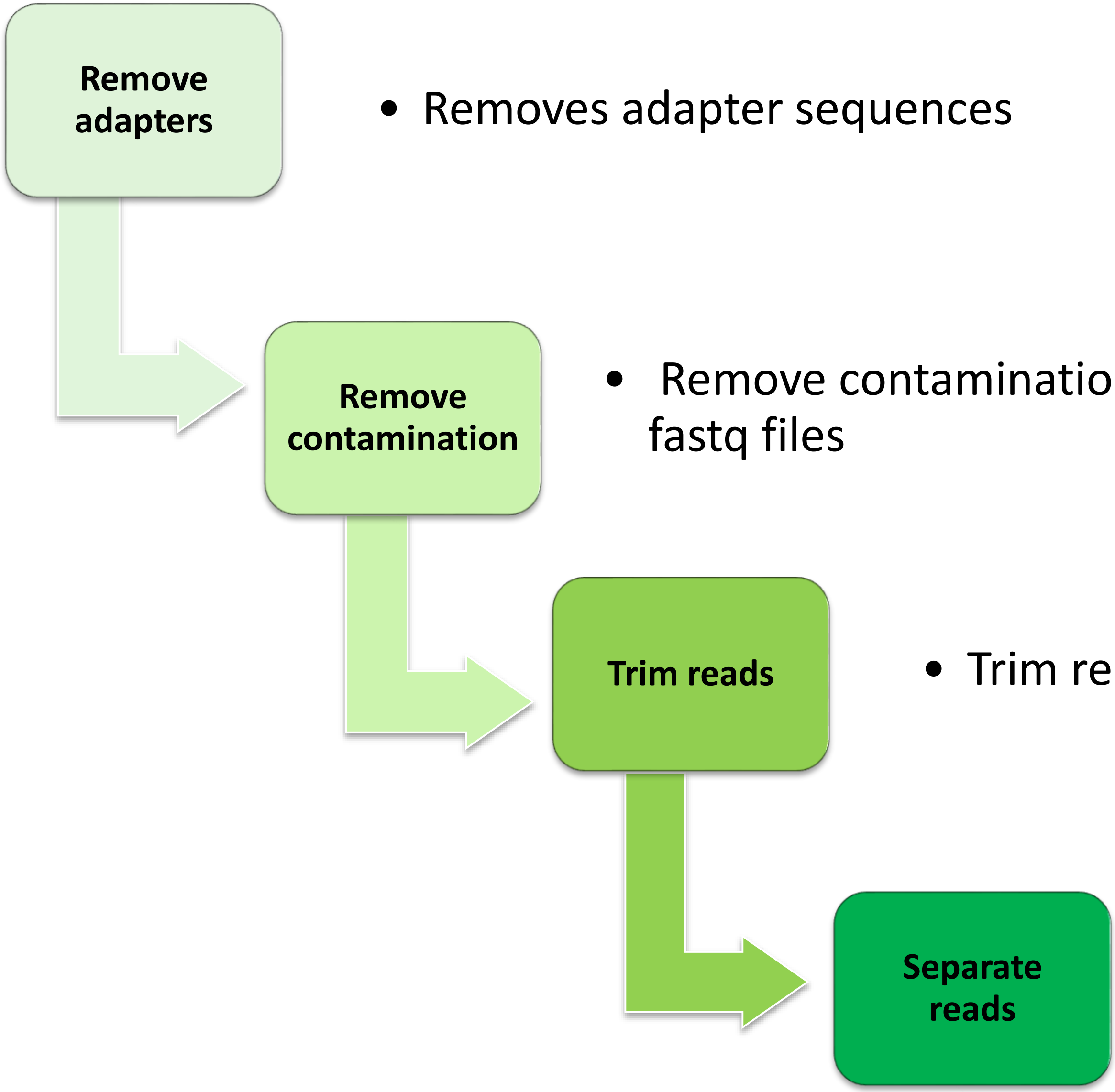
File formats - FASTQ files – what we get back from the sequencing center

- <u>This is usually the format your data is in when sequencing is complete</u>
- Text files
  - Contains both sequence and base quality information
- Phred score = Q = $-10\log_{10}P$
  - P is base-calling error probability
- Integer scores converted to ASCII characters
- Example:

```
@ILLUMINA:188:C03MYACXX:4:1101:3001:1999 1:N:0:CGATGT
TACTTGTTACAGGCAATACGAGCAGCTTCCAAAGCTTCACTAGAGACATTTTCTTTCTCCCAACTCACAAGATGAACACAAAATGGAAACT
+
1=DDFFFHHHHHJJDGHHHIJIJIIJJIJIIIGIIGJIIIJCHEIIJGIJJIJIIJIJIFGGGGGIJIFFBEFDC>@@BB?A9@3;@(553>@>C(59:?
```

Analysis

Data Cleaning: a Multistep Process



Remove adapters

Remove contamination

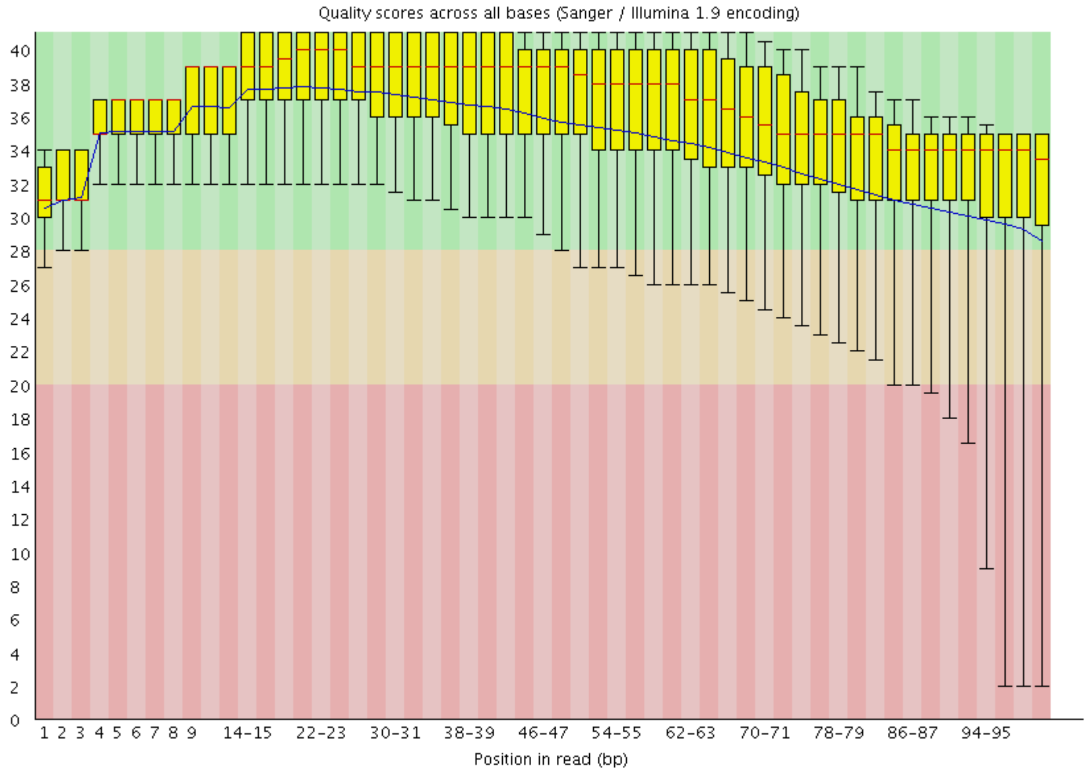
Trim reads

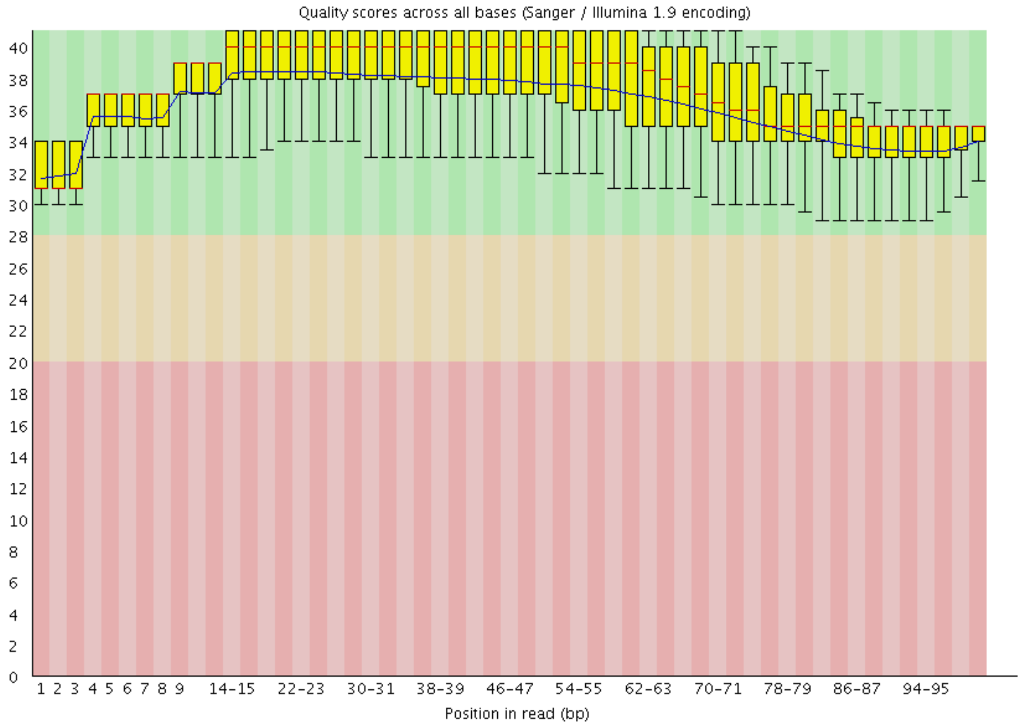
Separate reads

- Removes adapter sequences
- Remove contamination from fastq files
- Trim reads based on quality
- Separate reads into paired and unpaired

Analysis

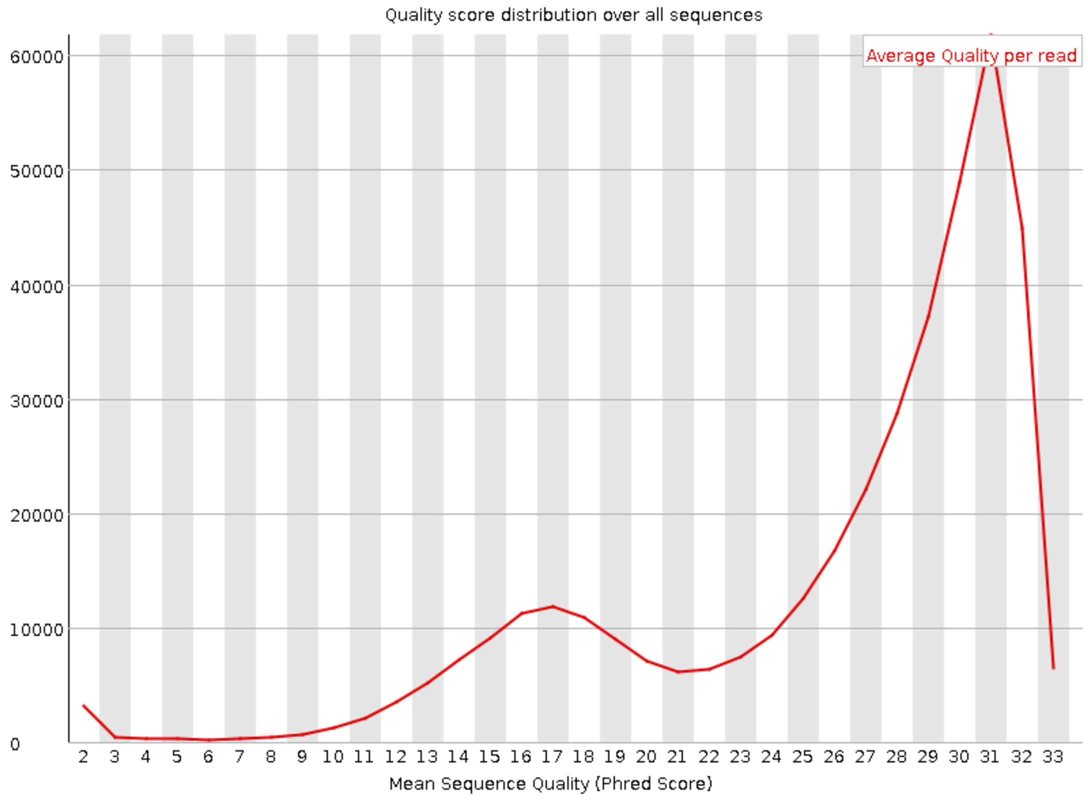
Quality Control – Per Base Sequence Quality

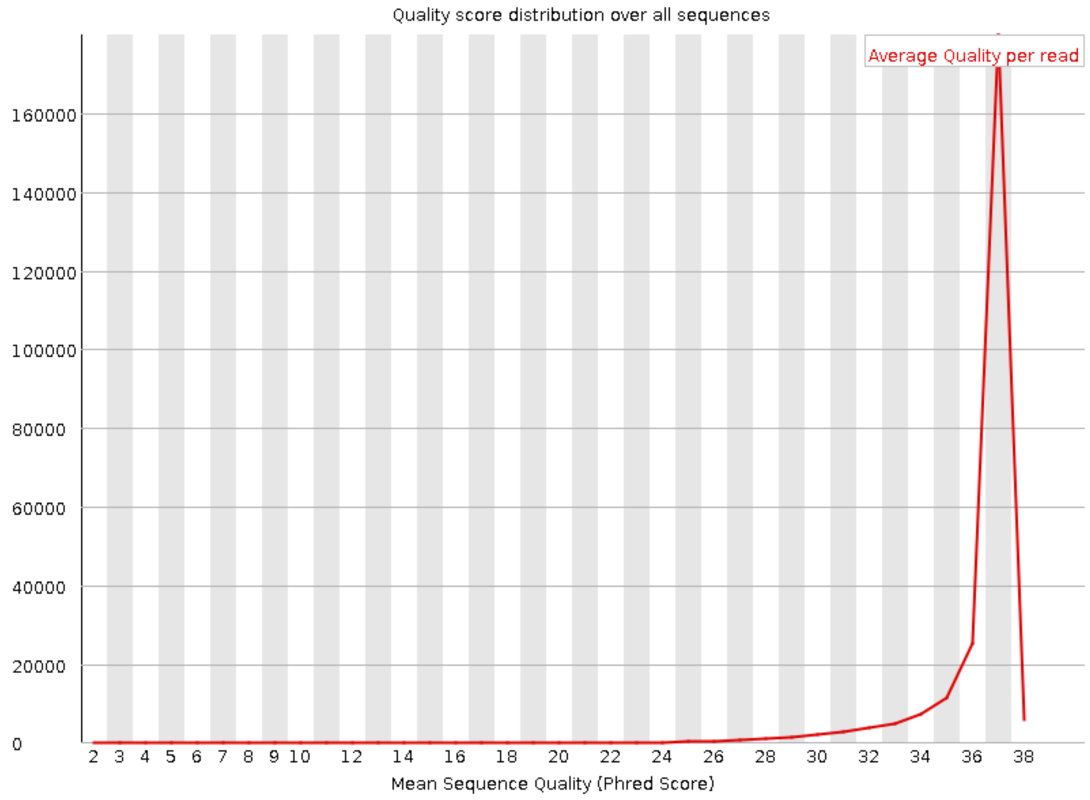




Analysis

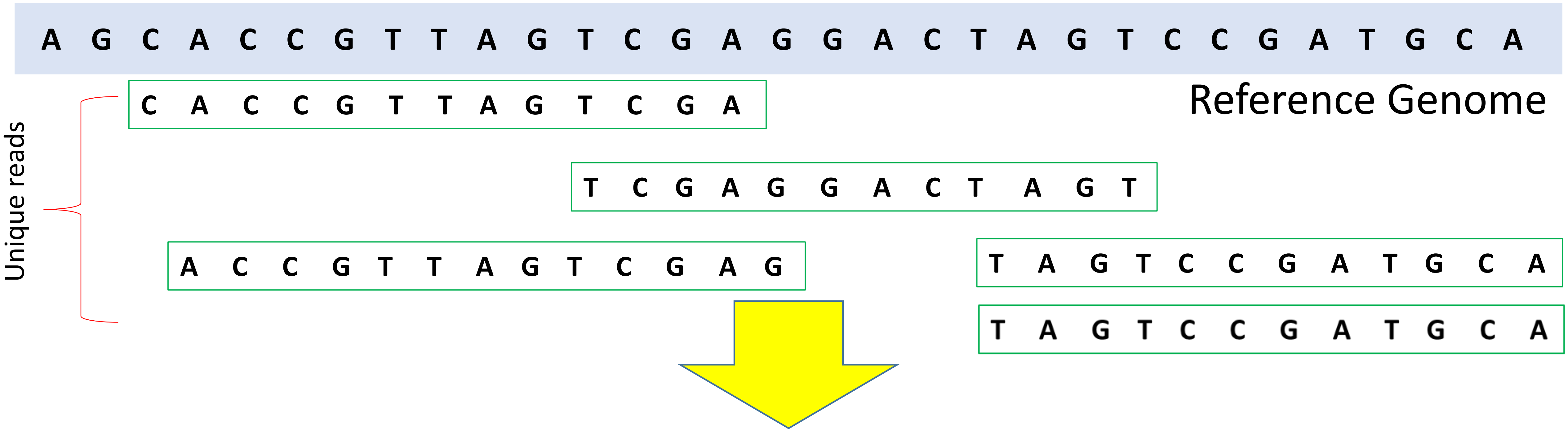
Quality Control – Per Sequence Quality Scores





Analysis

Aligning Reads to a Reference



AGCACCGTTAGTCGAGGACTAGTCCGATGCA

Reference Genome

Unique reads

CACCGTTAGTCGA

TCGAGGACTAGT

ACCGTTAGTCGAG

TAGTCCGATGCA

TAGTCCGATGCA

	Sample 1	Sample 2	…….	Sample N
Gene 1	145	176	…….	189
Gene 2	13	27	…….	19
…….	…….	…….	…….	…….
Gene G	28	30	…….	20

File formats: FASTA files

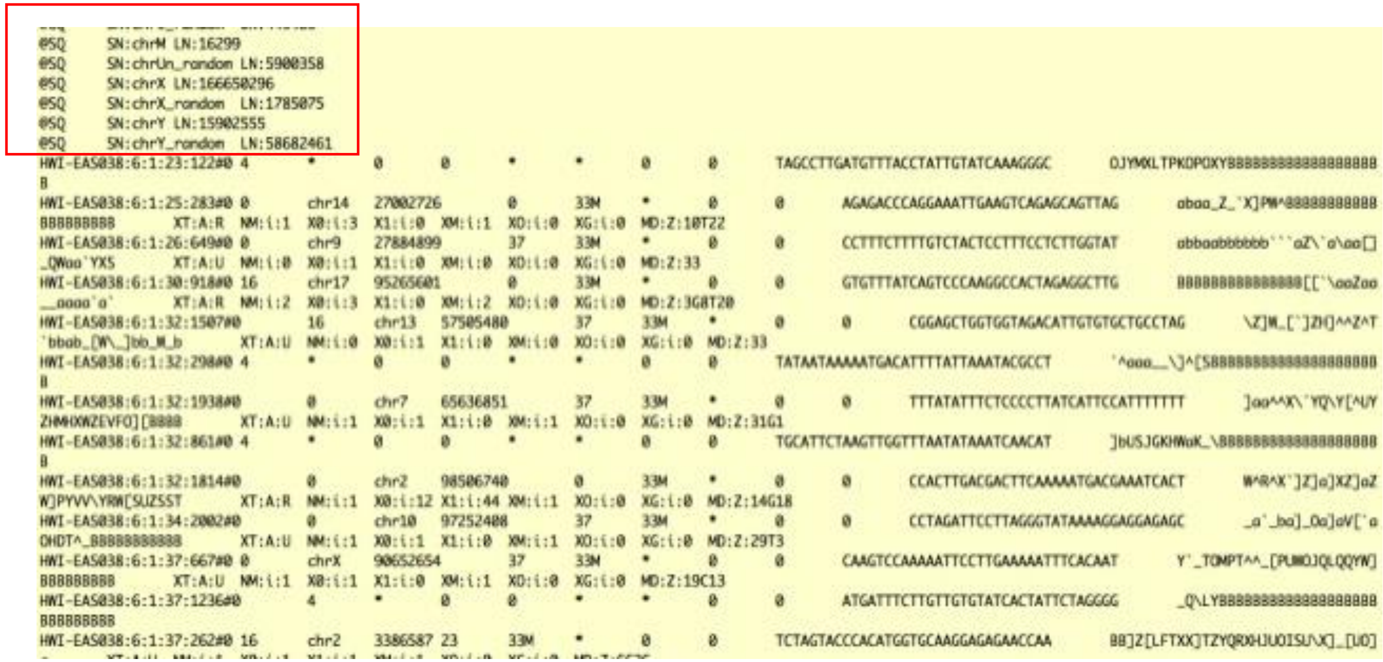
- Text file with sequences (amino acid or nucleotides)
- First line per sequence begins with > and information about sequence
- Example:

```
>comp2_c0_seq1
GCGAGATGATTCTCCGGTTGAATCAGATCCAGAGGCATGTATATATCGTCTGCAAAATGCTAGAAA
CCCTCATGTGTGTAATGCAGTGCATTCATGAAAACCTTGTAAGCTCACGTGTCGCTGACTGTCTGA
GAACCGACTCGCTAATGTTCCATGGAGTGGCTGCATACATCACAGATTGTGATTCCAG
GTTGCGAGACTATTTGCAGGATGCATGCGAGCTGATTGCCTATTCCTTCTACTTCTTAAATAAAGTA
AGAGC
```

Analysis

File formats: BAM and SAM files

- SAM file is a tab-delimited text file that contains sequence alignment information
- This is what you get after aligning reads to the genome
- BAM files are simply the binary version (compressed and indexed version) of SAM files → they are smaller
- Example:



```
@SQ     SN:chrM LN:16299
@SQ     SN:chrUn_random LN:5900358
@SQ     SN:chrX LN:166650296
@SQ     SN:chrX_random  LN:1785075
@SQ     SN:chrY LN:15902555
@SQ     SN:chrY_random  LN:58682461
HWI-EAS038:6:1:23:122#0 4       *       0       0       *       *       0       0       TAGCCTTGATGTTTACCTATTGTATCAAAGGGC       OJYMXLTPKOPOXYBBBBBBBBBBBBBBBBBBB
B
HWI-EAS038:6:1:25:283#0 0       chr14   27002726        0       33M     *       0       0       AGAGACCCAGGAAATTGAAGTCAGAGCAGTTAG       abaa_Z_`X]PW^BBBBBBBBBBB
BBBBBBBBB       XT:A:R  NM:i:1  X0:i:3  X1:i:0  XM:i:1  XO:i:0  XG:i:0  MD:Z:10T22
HWI-EAS038:6:1:26:649#0 0       chr9    27884899        37      33M     *       0       0       CCTTTCTTTTGTCTACTCCTTTCCTCTTGGTAT       abbaabbbbbb```aZ\`a\aa[]
_QWaa`YXS       XT:A:U  NM:i:0  X0:i:1  X1:i:0  XM:i:0  XO:i:0  XG:i:0  MD:Z:33
HWI-EAS038:6:1:30:918#0 16      chr17   95265601        0       33M     *       0       0       GTGTTTATCAGTCCCAAGGCCACTAGAGGCTTG       BBBBBBBBBBBBBBBB[[`\aaZaa
__aaaa`a`       XT:A:R  NM:i:2  X0:i:3  X1:i:0  XM:i:2  XO:i:0  XG:i:0  MD:Z:3G8T20
HWI-EAS038:6:1:32:1507#0        16      chr13   57505480        37      33M     *       0       0       CGGAGCTGGTGGTAGACATTGTGTGCTGCCTAG       \Z]W_[`]ZH]^^Z^T
`bbab_[W\_]bb_W_b       XT:A:U  NM:i:0  X0:i:1  X1:i:0  XM:i:0  XO:i:0  XG:i:0  MD:Z:33
HWI-EAS038:6:1:32:298#0 4       *       0       0       *       *       0       0       TATAATAAAAATGACATTTTATTAAATACGCCT       `^aaa__\]^[SBBBBBBBBBBBBBBBBBBB
B
HWI-EAS038:6:1:32:1938#0        0       chr7    65636851        37      33M     *       0       0       TTTATATTTCTCCCCTTATCATTCCATTTTTTT       ]aa^^X\`YQ\Y[^UY
ZHMHXWZEVFO][BBBB       XT:A:U  NM:i:1  X0:i:1  X1:i:0  XM:i:1  XO:i:0  XG:i:0  MD:Z:31G1
HWI-EAS038:6:1:32:861#0 4       *       0       0       *       *       0       0       TGCATTCTAAGTTGGTTTAATATAAATCAACAT       ]bUSJGKHWaK_\BBBBBBBBBBBBBBBBBBB
B
HWI-EAS038:6:1:32:1814#0        0       chr2    98506740        0       33M     *       0       0       CCACTTGACGACTTCAAAAATGACGAAATCACT       W^R^X`]Z]a]XZ]aZ
W]PYVV\YRW[SUZSST       XT:A:R  NM:i:1  X0:i:12 X1:i:44 XM:i:1  XO:i:0  XG:i:0  MD:Z:14G18
HWI-EAS038:6:1:34:2002#0        0       chr10   97252408        37      33M     *       0       0       CCTAGATTCCTTAGGGTATAAAAGGAGGAGAGC       _a`_ba]_Oa]aV[`a
OHDT^_BBBBBBBBBBB       XT:A:U  NM:i:1  X0:i:1  X1:i:0  XM:i:1  XO:i:0  XG:i:0  MD:Z:29T3
HWI-EAS038:6:1:37:667#0 0       chrX    90652654        37      33M     *       0       0       CAAGTCCAAAAATTCCTTGAAAAATTTCACAAT       Y`_TOMPT^^_[PUMOJQLQQYW]
BBBBBBBBB       XT:A:U  NM:i:1  X0:i:1  X1:i:0  XM:i:1  XO:i:0  XG:i:0  MD:Z:19C13
HWI-EAS038:6:1:37:1236#0        4       *       0       0       *       *       0       0       ATGATTTCTTGTTGTGTATCACTATTCTAGGGG       _Q\LYBBBBBBBBBBBBBBBBBBB
BBBBBBBBB
HWI-EAS038:6:1:37:262#0 16      chr2    3386587 23      33M     *       0       0       TCTAGTACCCACATGGTGCAAGGAGAGAACCAA       BB]Z[LFTXX]TZYQRXHJUOISU\X]_[UO]
```

Header lines (begin with "@")

Alignment section

Analysis

Terminology

- Counts = (X_i) the number of reads that align to a particular feature *i* (gene, isoform, miRNA…)
- Library size= (N) number of reads sequenced

Units

- FPKM = Fragments per kilobase of exon per million mapped reads
  - Takes length of gene (l_i) into account
  - $\mathrm{FPKM}_{\mathrm{i}}=(X_i\,/l_i{*}N){*}10^9$
- CPM = Counts Per Million mapped reads
  - $\mathrm{CPM}_{\mathrm{i}}= X_i\,/N{*}10^6$
- FDR = False Discovery Rate (the rate of Type I errors – false positives); a 10% FDR means that 10% of your differentially expressed genes are likely to be false positives
  - we must adjust for multiple testing in RNA-seq statistical analyses to control the FDR

Caveats

- If you have zero counts it does not necessarily mean that a gene is not expressed at all
  - Especially in single-cell RNA-seq
- RNA and protein expression profiles do not always correlate well
  - Correlations vary wildly between RNA and protein expression
  - Depends on category of gene
  - Correlation coefficient distributions were found to be bimodal between gene expression and protein data (one group of gene products had a mean correlation of 0.71; the another had a mean correlation of 0.28)
    - Shankavaram et. al, 2007

Many tools exist for differential expression

- Cufflinks
  - Tests both isoform and gene differential expression
  - Corrects for differences in sequencing depth and transcript length
  - Allows comparison of genes across samples and between different genes within the same sample
- DESeq2
  - Tests gene differential expression
  - Corrects for differences in transcript pool and extreme outliers to allow better across-sample comparability
  - Allows comparison of genes across samples
  - More conservative
- edgeR
  - Tests gene differential expression
  - Corrects for differences in transcript pool and extreme outliers to allow better across-sample comparability
  - Allows comparison of genes across samples
  - Less conservative

Downstream analysis: What to do with your gene list

- Annotate DEG
- Find biological processes that are enriched amongst the DEGs
- Pathway analysis
- Clustering analyses
- Biological Validation

GO terms

- GO Consortium (Gene Ontology Consortium) seeks to provide consistent descriptions of gene products across databases
  - Started as a collaboration between FlyBase (Drosophila), Saccharomyces Genome Database (SGD), and the Mouse Genome Database (MGD)
  - Now incorporates many more databases
- Comprised of 3 structured ontologies that describe gene products in terms of associated:
  - Biological processes (operations or sets of molecular events with a defined beginning and end)
  - Cellular components (the parts of a cell or its extracellular environment)
  - Molecular functions (activities of a gene product at a molecular/biochemical level – such as “catalysis” or “binding”)

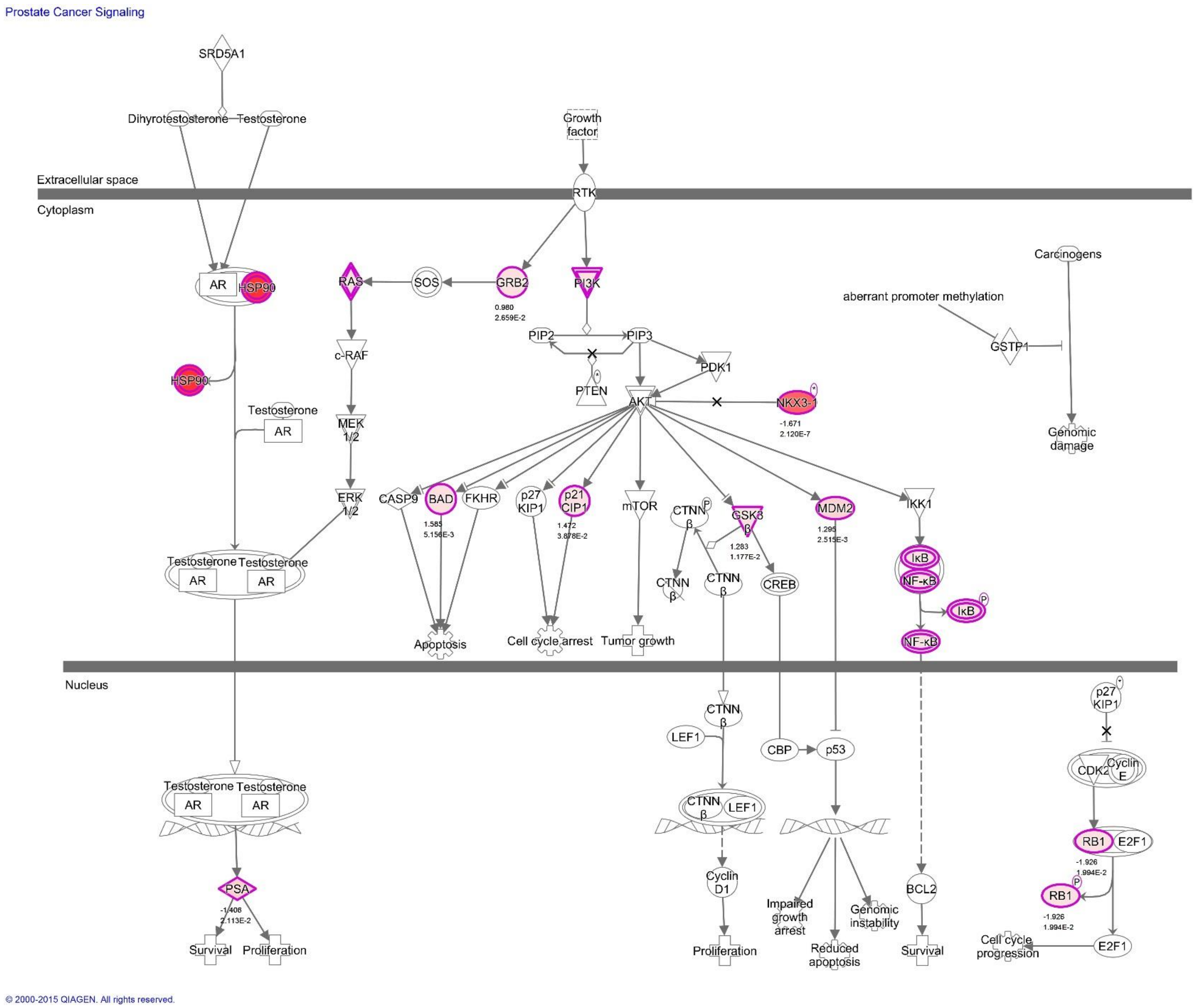
GO term enrichment analysis

- Which GO terms are enriched in my list of interesting genes?
  - Find GO terms that are over-represented or under-represented using annotations for your gene set
- Fisher’s Exact Test/Hypergeometric test often used
  - Compare GO terms mapping to gene list with GO terms mapping to a background/reference gene list (such as all the genes in the yeast genome)
- Numerous tools exist for GO term enrichment analysis (the following require no computational background and have GUIs):
  - AmiGO
  - OBO-edit
  - AgriGO
  - BLAST2GO
  - DAVID
  - BiNGO

Analysis

Pathway Analysis

Identify pathways with a significant number of differentially expressed genes – this gives information on potential pathways that are affected by the treatment


Prostate Cancer Signaling

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Thank you!

Any questions?