IN-BIOS[9,5]000 2022

Experimental Design

- from a HTS perspective

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Oct 18, 2022

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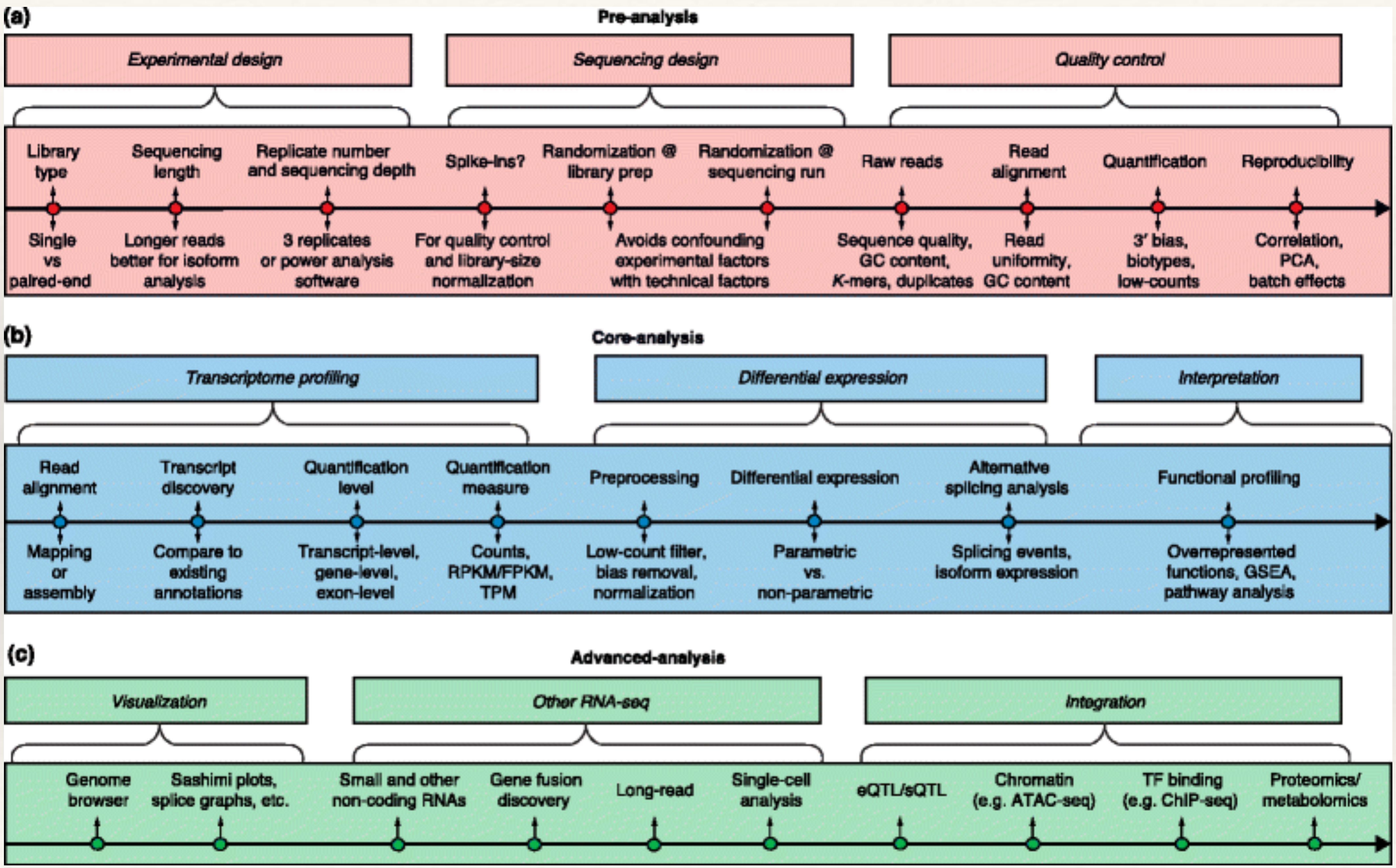
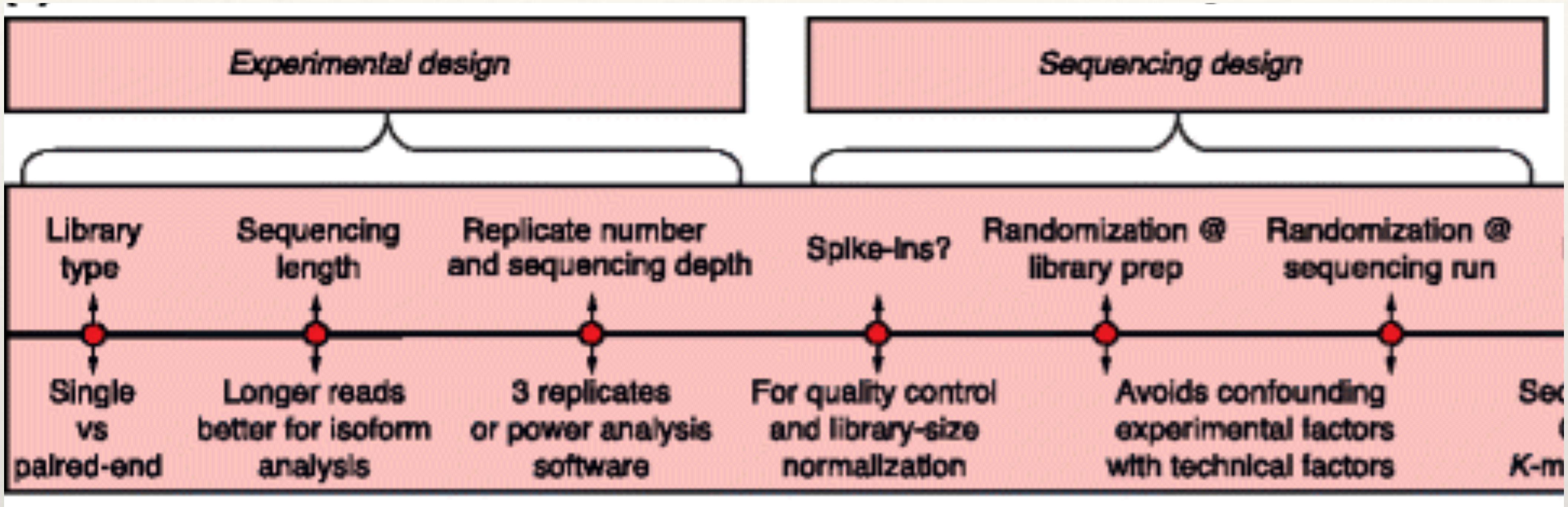
RNA seq analysis pipeline



A survey of best practices for RNA-seq data analysis
https://doi.org/10.1186/s13059-016-0881-8

Experimental design



Design of the experiment and **sequencing plan** have a direct effect on downstream analyses and interpretation of data

A survey of best practices for RNA-seq data analysis
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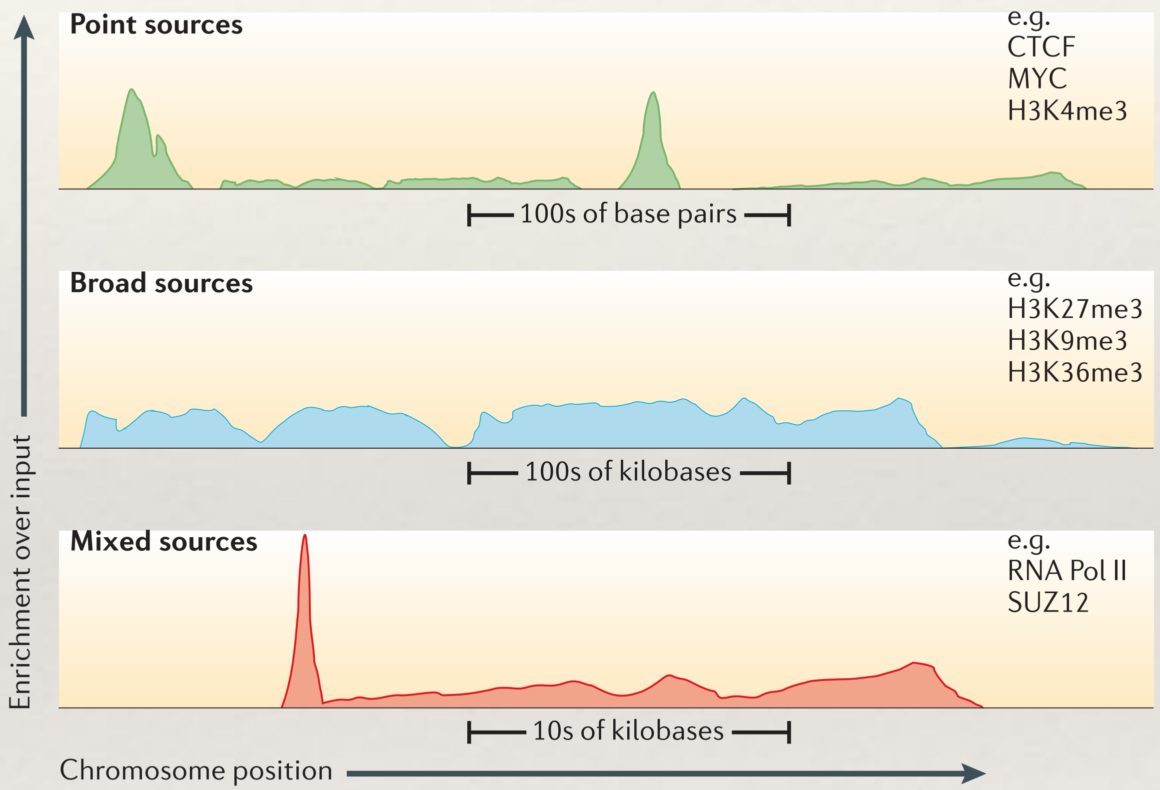
Experimental design

- Biological question
- Platform choice
- Technology variation
    - Technical bias
        - Run/Lane bias
        - Index/barcode bias
        - Duplicates
        - Error rates
- Sample variation
    - PCR amplification?
- Sequencing depth
- Data analysis
- Species-specific information
    - Is there a genome sequence available??
    - Genome size (c-value)
    - genomesize.com

Biological question

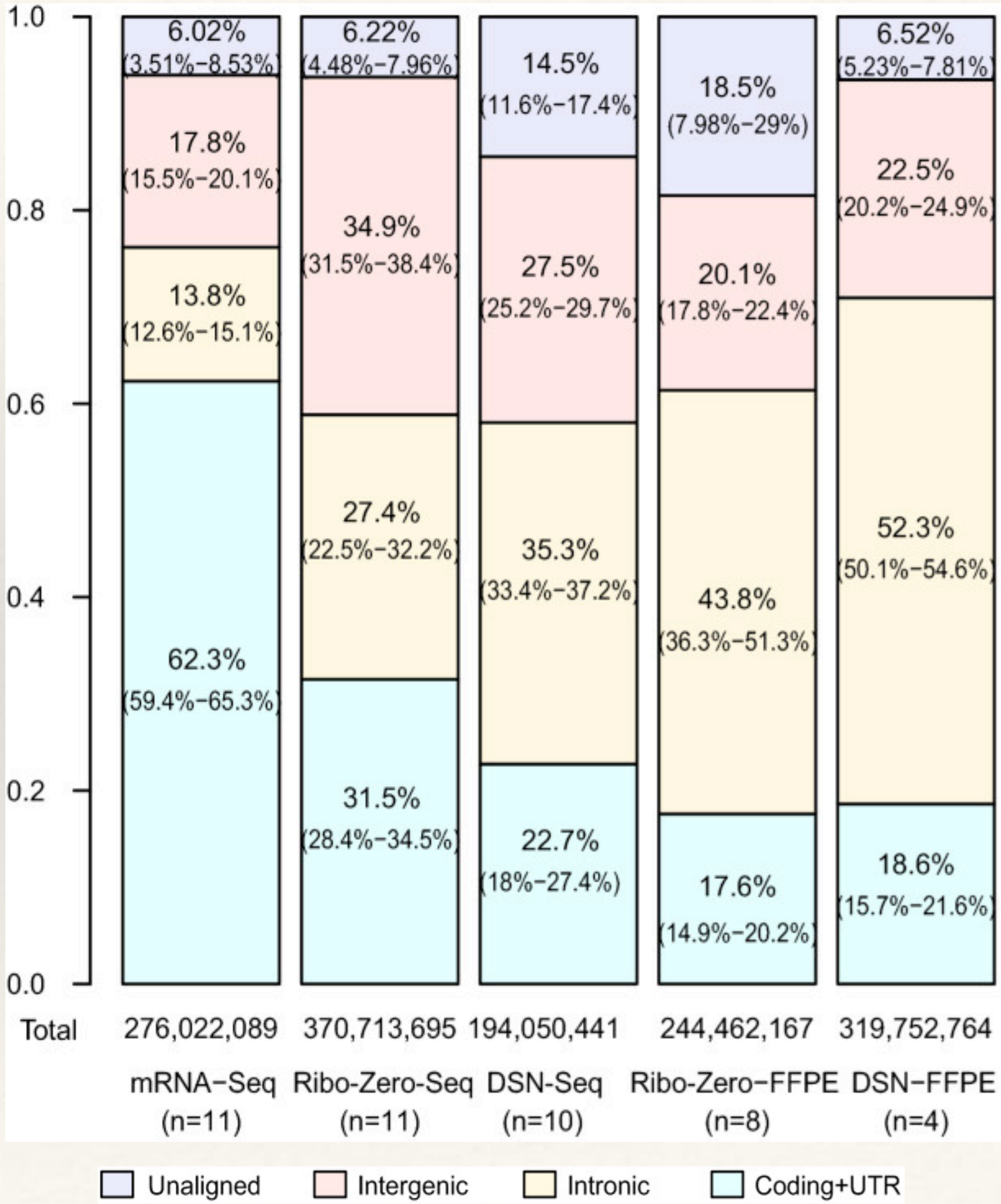
- **Know your targets**
  - Whole genome
  - Targeted (re)seq
    - Exome
    - ChIP-seq



Nature Reviews Genetics 15, 121–132 (2014) doi:10.1038/nrg3642

Biological question

- **Know your targets**
  - RNA-seq
    - rRNA depleted?
    - polyA enriched?
    - microRNA



BMC Genomics 2014, **15**:419 doi:10.1186/1471-2164-15-419

Experimental design

- Short or long fragments
- Short or long reads
- Single or paired end
- Multiplexing
  - single or dual index
  - more barcodes?
- Library prep method
- Depth required
- Coverage required
- Replicates
  - biological
  - technical

Platform choice: Read length

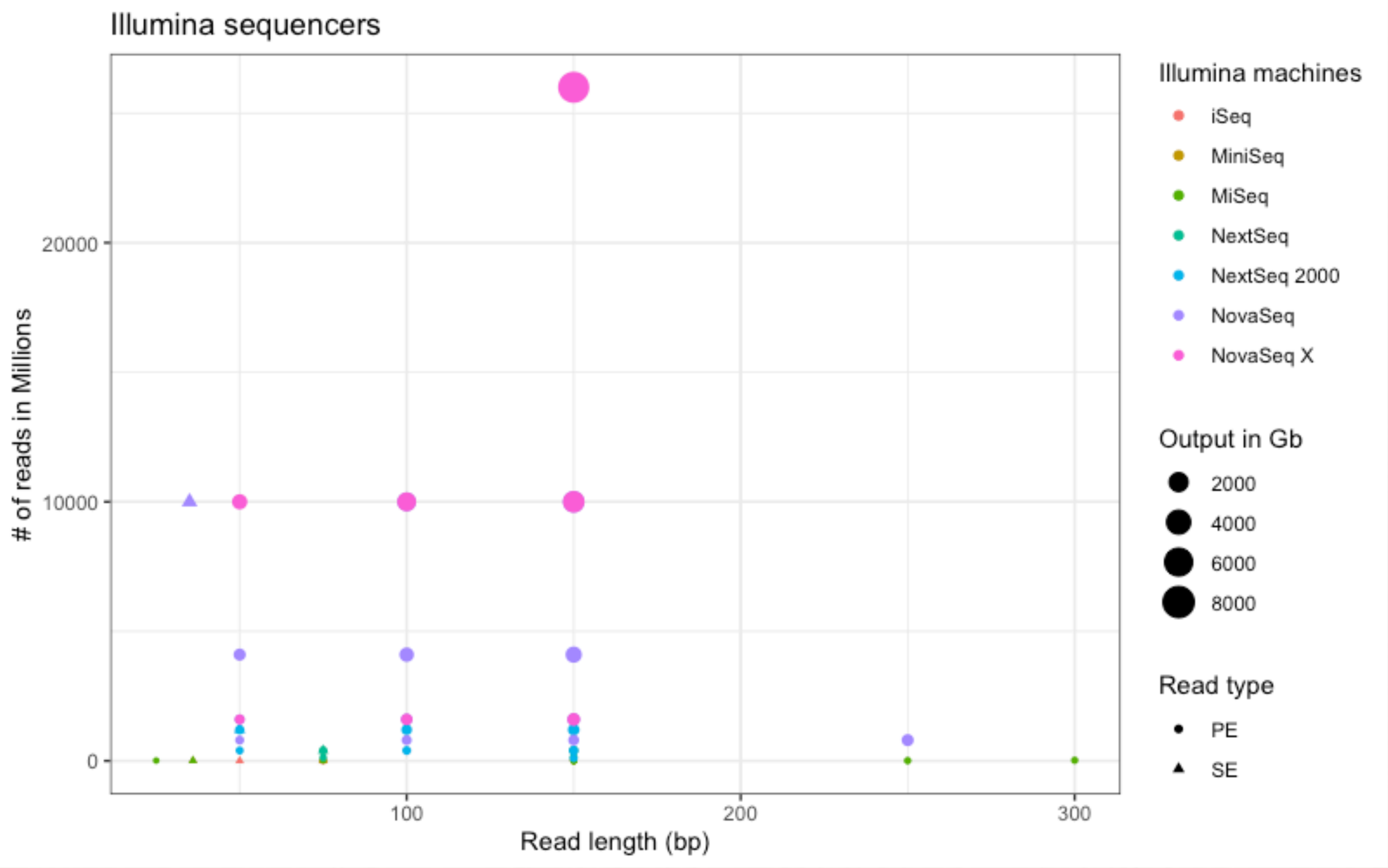
MiniSeq
MiSeq
NextSeq
HiSeq series
NovaSeq
NovaSeq X

Roche 454
SOLiD
Ion Torrent

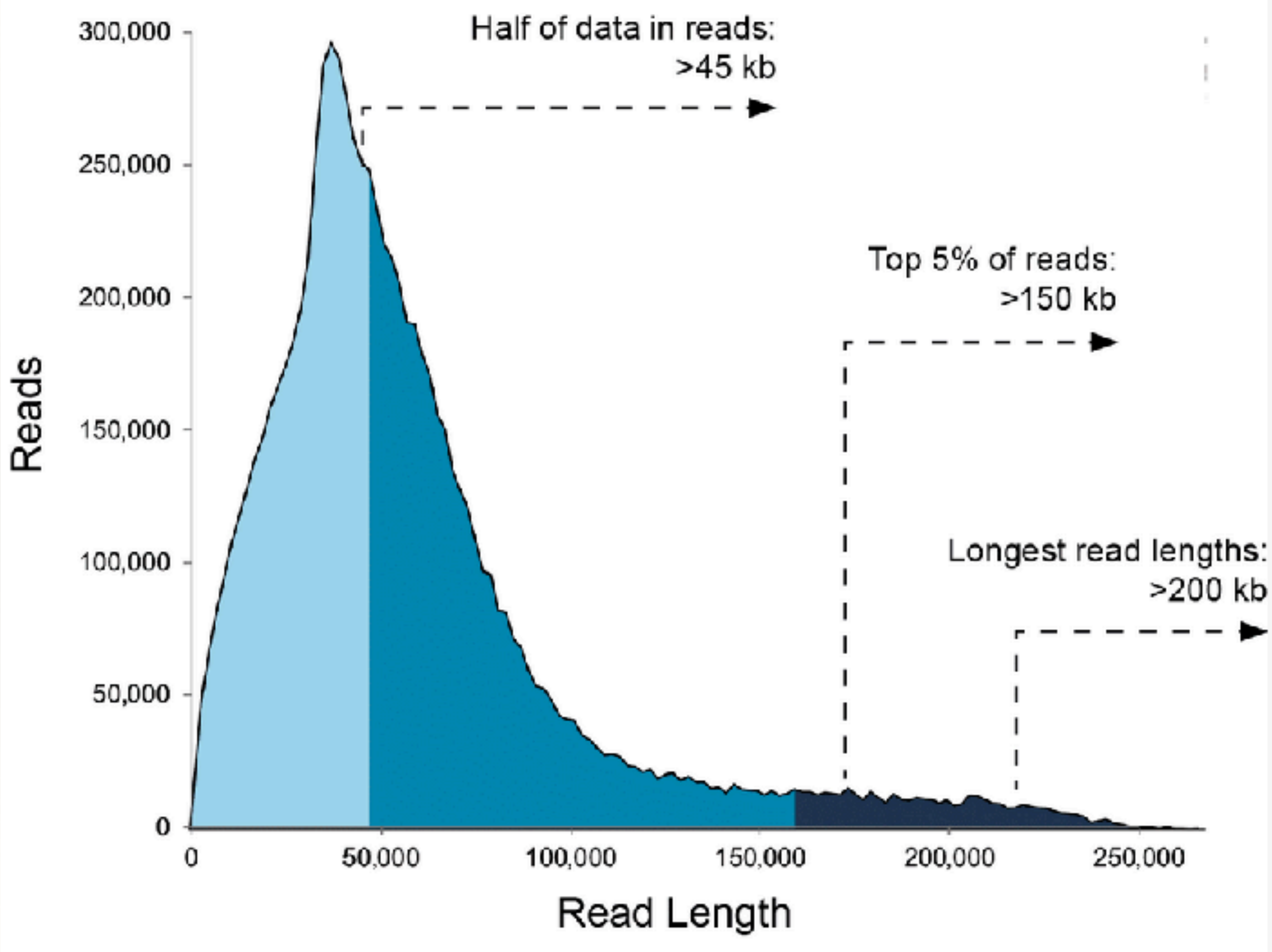
RS II
Sequel

MinION
Flongle
GridION
PromethION P2/solo
PromethION 24/48

Illumina data output



Pacbio/Nanopore data output



Indexing

- Dual index possible
- Dual internal barcodes possible
  - multiplex up to 4000 samples.

Fadrosh *et al. Microbiome* 2014, **2**:6
http://www.microbiomejournal.com/content/2/1/6

Microbiome

METHODOLOGY **Open Access**

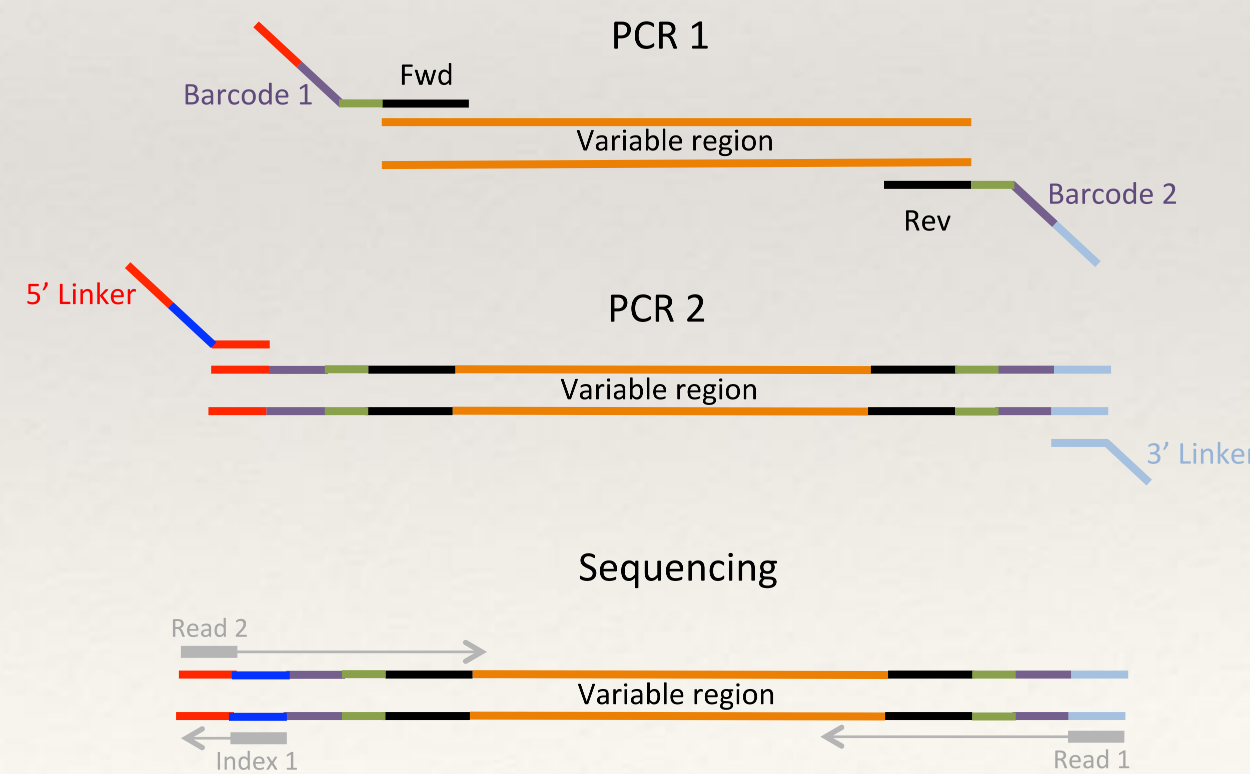
An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform

Douglas W Fadrosh[1†], Bing Ma[1†], Pawel Gajer[1], Naomi Sengamalay[1], Sandra Ott[1], Rebecca M Brotman[2] and Jacques Ravel[1*]

Sequencing Cycle

	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
Sample 1	C	C	T	A	A	A	C	T	A	C	G	G	A	C	T	C	C	T	A	C	G	G	G	A	G	G	C	A	G	C
Sample 2	G	T	G	G	T	A	T	G	G	G	A	G	T	A	C	T	C	C	T	A	C	G	G	G	A	G	G	C	A	G
Sample 3	T	G	T	T	G	C	G	T	T	T	C	T	G	T	A	C	T	C	C	T	A	C	G	G	G	A	G	G	C	A
Sample 4	A	C	A	G	C	C	A	C	C	C	A	T	C	G	A	A	C	T	C	C	T	A	C	G	G	G	A	G	G	C
Sample 5	G	T	T	A	C	G	T	G	G	T	T	G	A	T	G	A	A	C	T	C	C	T	A	C	G	G	G	A	G	G
Sample 6	T	A	C	C	G	G	C	T	T	G	C	A	T	G	C	G	A	A	C	T	C	C	T	A	C	G	G	G	A	G
Sample 7	C	A	C	C	T	T	A	C	C	T	T	A	G	A	G	T	G	G	A	C	T	C	C	T	A	C	G	G	G	A
Sample 8	T	T	A	A	C	T	G	G	A	A	G	C	C	C	T	G	T	G	G	A	C	T	C	C	T	A	C	G	G	G

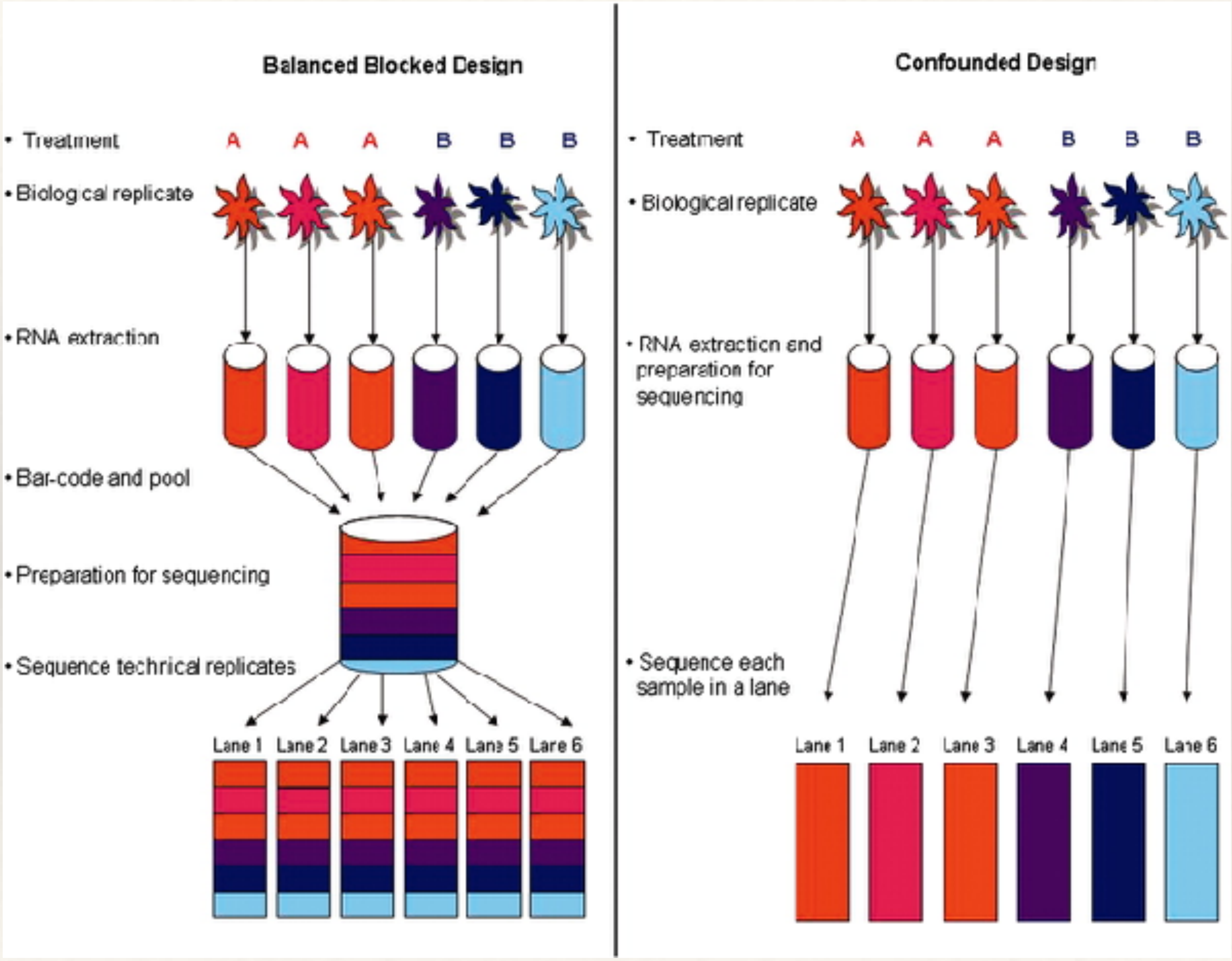
Index 1
Heterogeneity Spacer
16S rRNA gene sequence



Technical bias

- Lane/flowcell bias
- Index/barcode bias
- Batch effect

- Randomisation is key

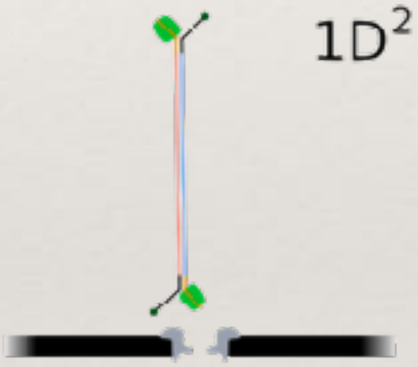


http://www.genetics.org/content/185/2/405.long

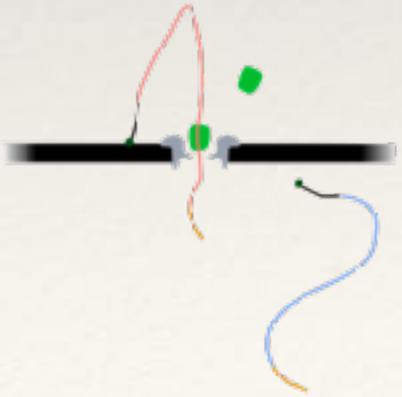
Error rates

- Illumina has low error rates
- Pacbio and Oxford Nanopore have relatively high error rates

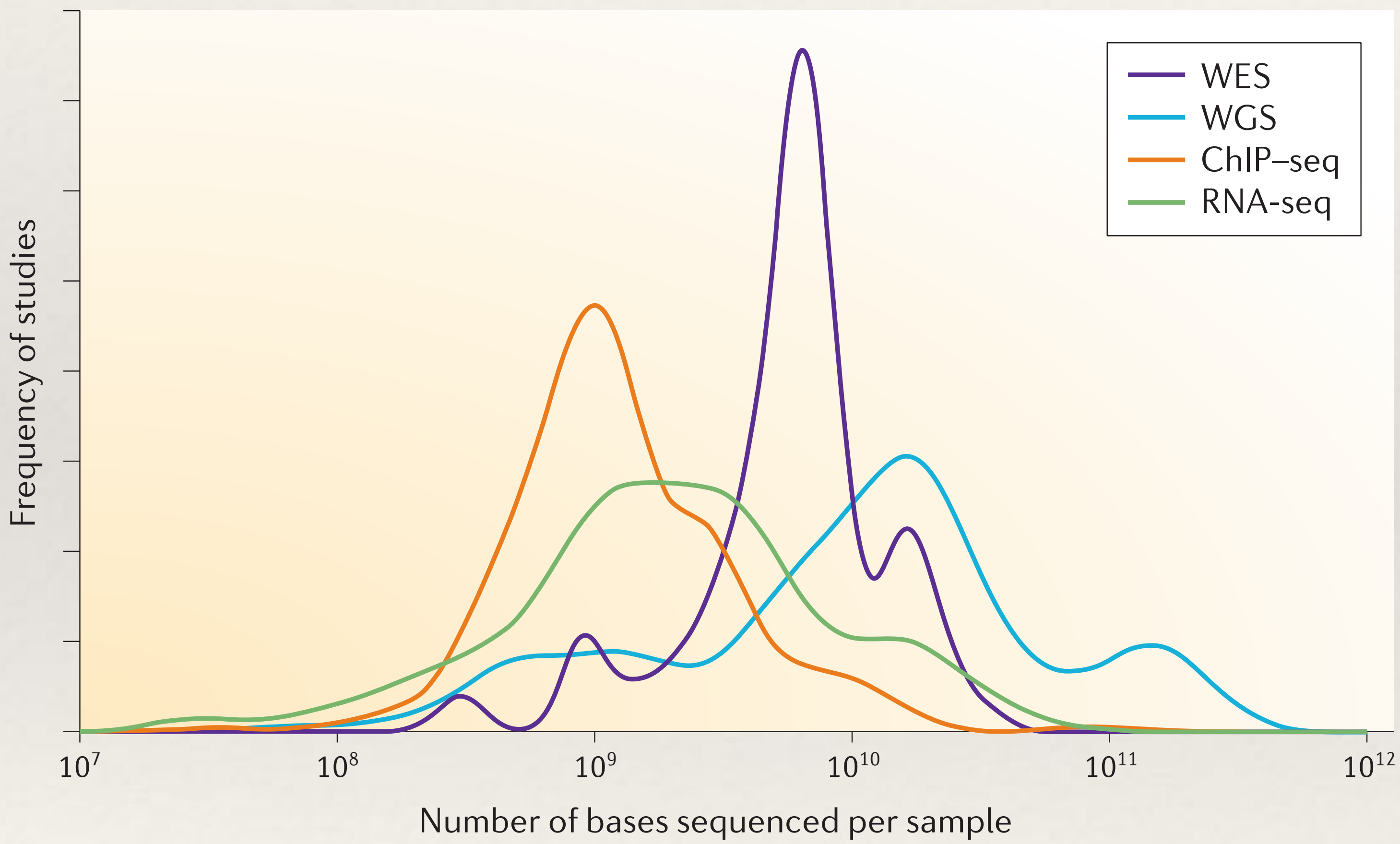
  - Cyclic sequencing can reduce the error rate in Pacbio
  - 1D^2 sequencing can reduce the error rate in Oxford Nanopore

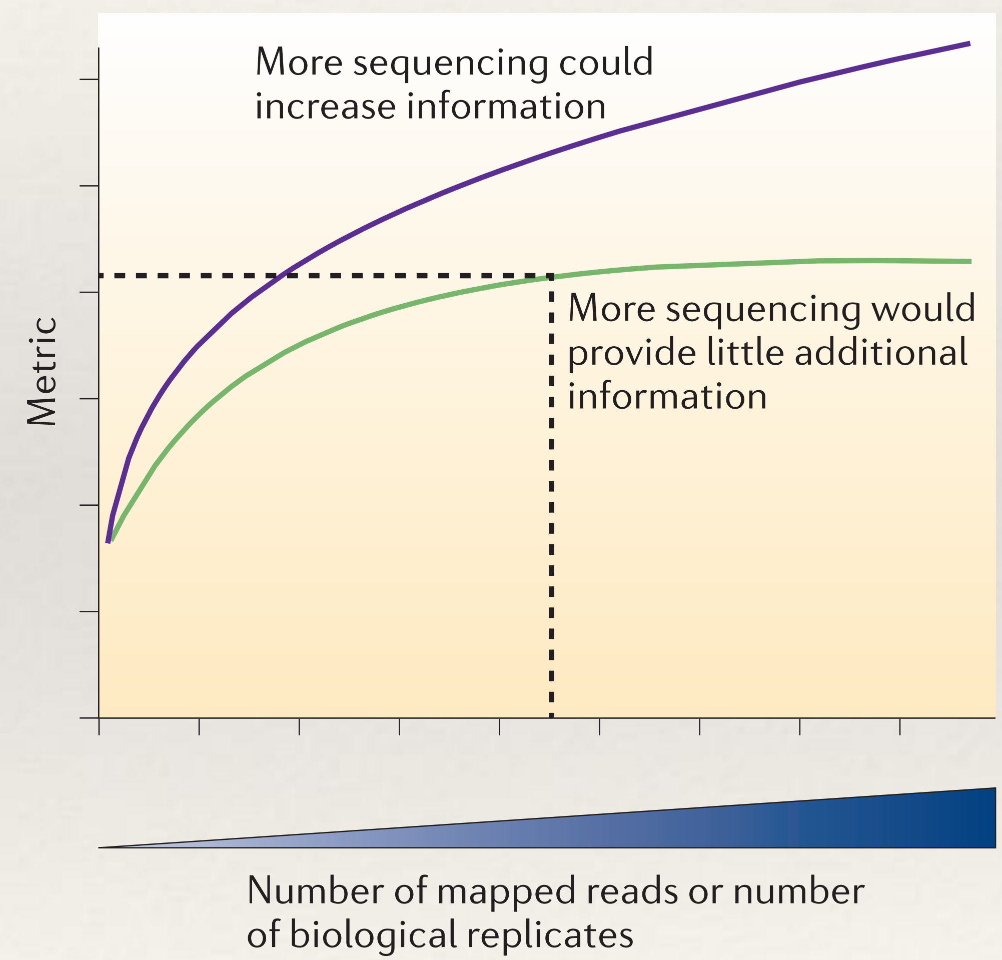


- Deep sequencing is used to correct for errors



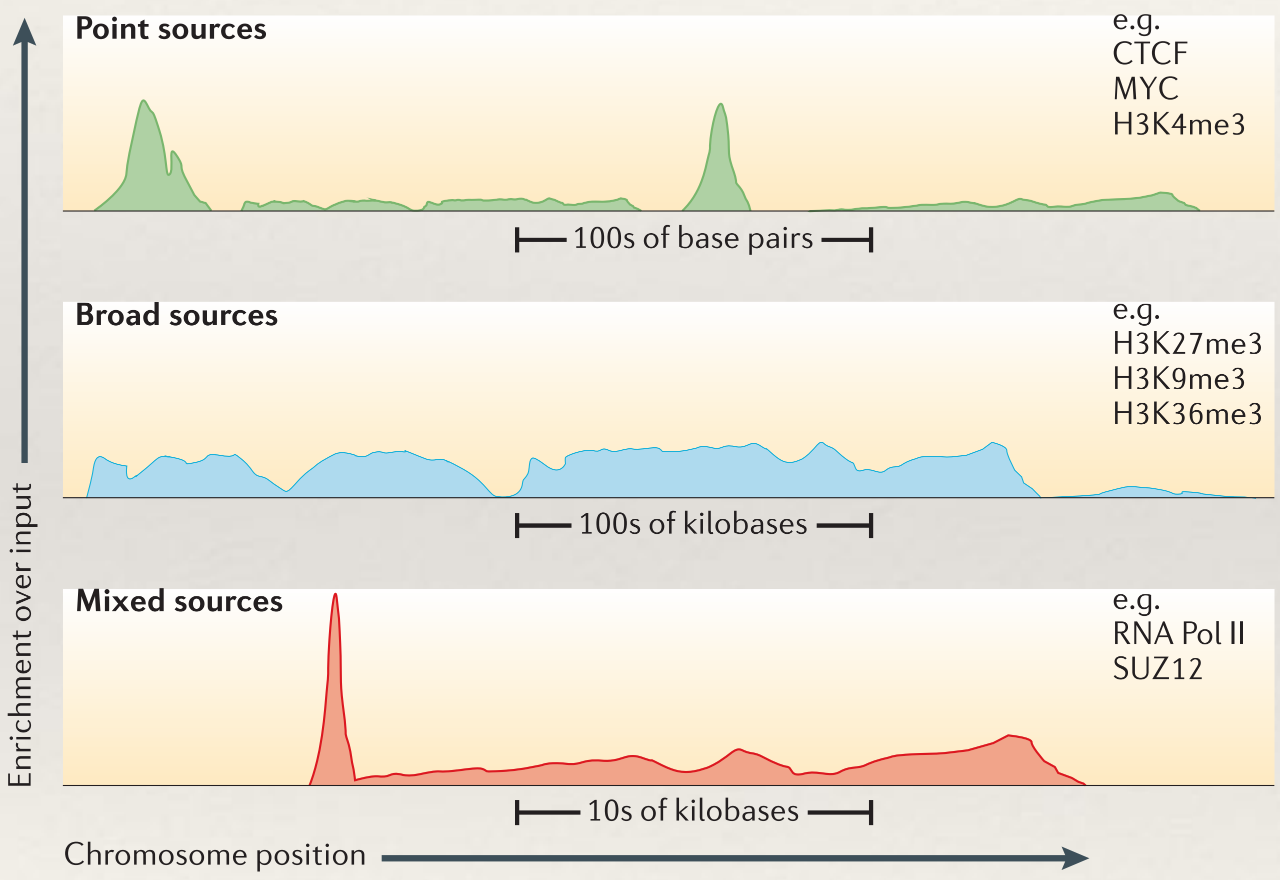
Sequencing depth and coverage





Sequencing depth and coverage
https://doi.org/10.1038/nrg3642

Sequencing depth and coverage



Techniques	Read counts in representative studies
DNaseI-seq and FAIRE–seq	20–50 million
CLIP–seq	7.5 million; 36 million
iCLIP and PAR–CLIP	8 million; 14 million
CHiRP and CHART	26 million
4C	1–2 million
ChIA–PET	20 million
5C	25 million
Hi-C	>100 million
MeDIP–seq	60 million
CAP–seq	>20 million
ChIP–seq	>10 million per sample (point source); >20 million per sample (broad source)

Sequencing depth and coverage
https://doi.org/10.1038/nrg3642

Replicates and Depth

- Sound experimental design
- Number of replicates
  - Biological variation
  - Technical replicates - not so important
- Sequencing depth

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 %

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Replicates vs Depth

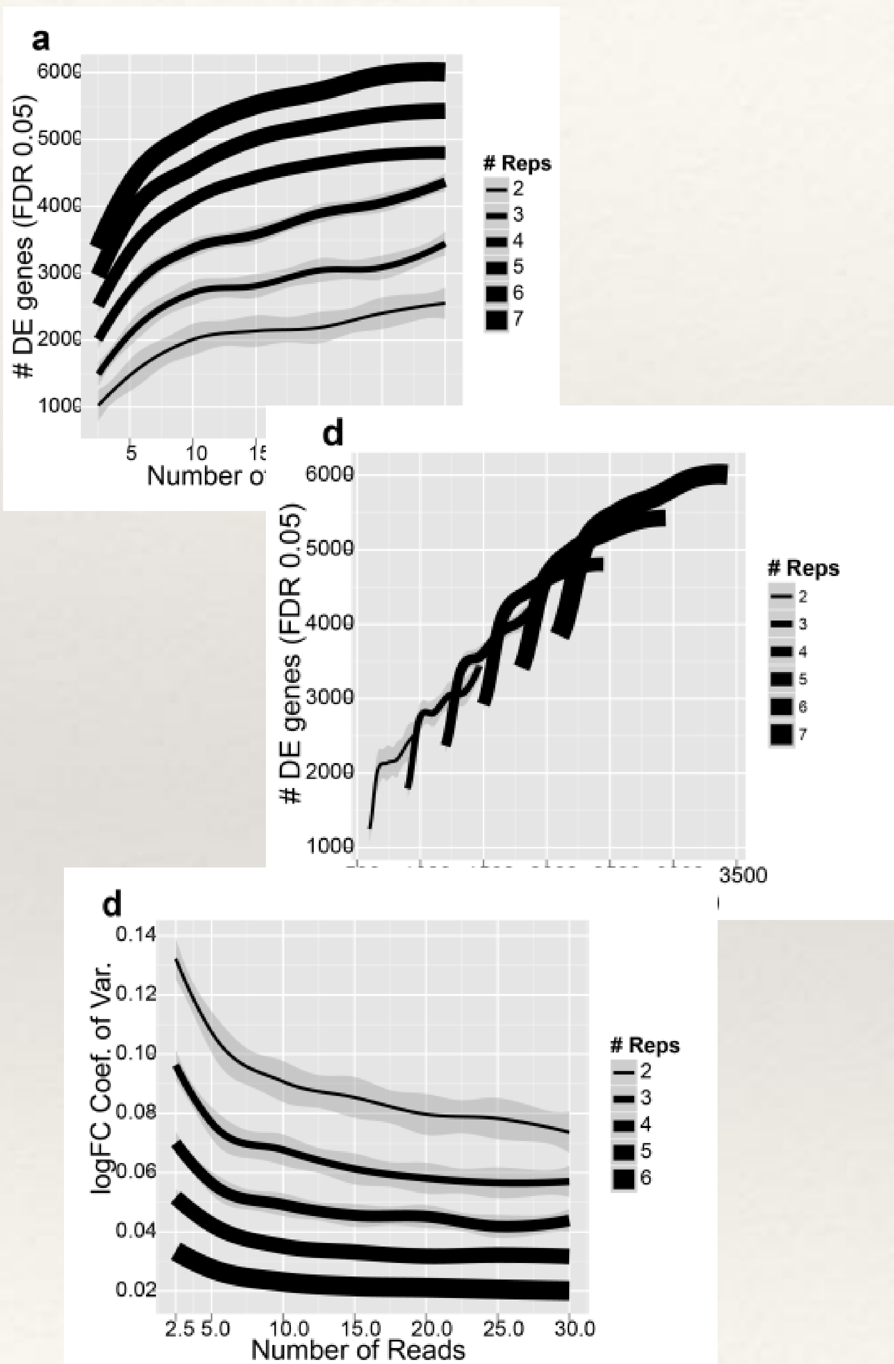


Table 1. Cost efficiency for power to detect DE genes (cost per 1% power given each experimental design where the variables are). Assumptions made during calculations are described in Methods. * indicates lowest cost per 1% power in each replication level. Units are in dollars.

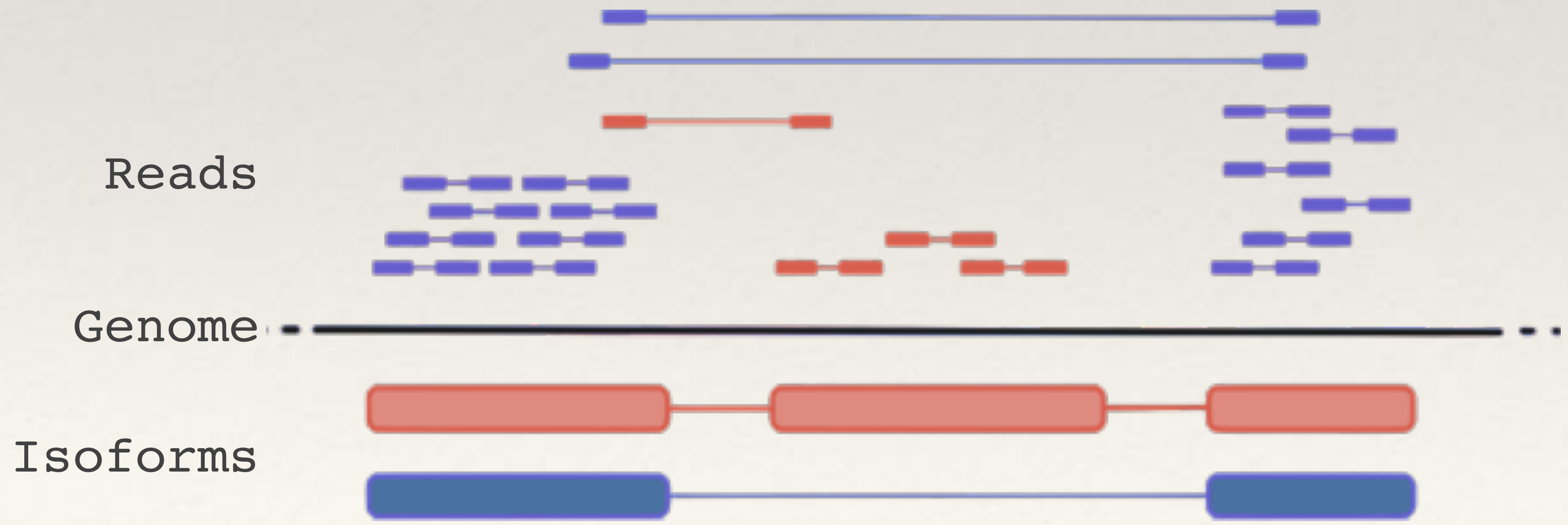
Relative Cost	2.5M	5M	10M	15M	20M	25M	30M
2 replicates	24.2	17.2	14.4*	15.8	16.7	17.0	17.8
3 replicates	23.4	17.2	15.3*	16.3	17.1	18.5	19.4
4 replicates	23.1	17.7	16.5*	17.5	18.6	19.8	21.2
5 replicates	23.8	19.0	18.1*	19.4	21.0	22.8	24.9
6 replicates	25.0	20.7	20.6*	22.4	24.6	27.0	29.4
7 replicates	26.8	23.0*	23.5	26.0	28.7	31.5	34.3

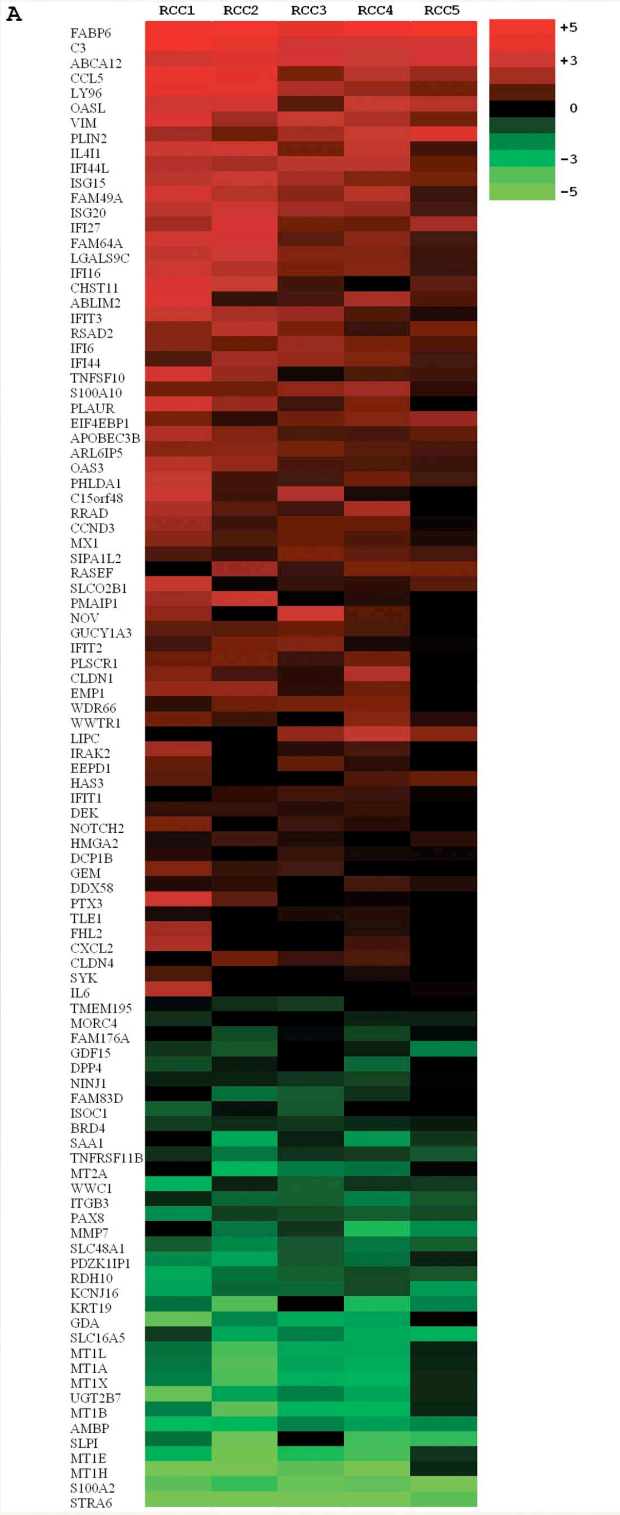
http://www.doi.org/10.1093/bioinformatics/btt688

Depth: example

- RNA sequencing
  - Highly expressed known transcripts
  - Novel isoforms
  - Low expressed / rare transcripts

More depth





Design prior to sequencing

- Sources of variation
  - Dynamic range - Not all samples get sequenced the same way
  - Technical variation - biases inherent to the technology
  - Biological variation
- Controlling for variation
  - Randomisation
  - Blocking: Pool and sequence across several lanes
  - Replication

Pre-processing

- Remove sequencing adapters
- Trim / remove low quality reads
- Remove sequencing spike-ins (PhiX for Illumina), if any

➡ Make sure paired end data is always paired and in correct order!

Simple truth

To consult the statistician after an experiment is finished is often merely to ask him (her) to conduct a post mortem examination. He (she) can perhaps say what the experiment died of.

- Ronald Fischer