High-throughput sequencing: Alignment and related topic

Simon Anders
EMBL Heidelberg

HTS Platforms

- Established platforms
  - Illumina HiSeq, ABI SOLiD, Roche 454
- Newcomers: Benchtop machines
  - 454 GS Junior, Illumina MiSeq, IonTorrent PGM

Applications of HTS

- Sequencing of (genomic) DNA
  - de-novo sequencing
  - resequencing (variant finding)
  - enrichment sequencing (ChIP-Seq, MeDIP-Seq, ...)
  - targeted sequencing (exome sequencing, ...)
  - CCC-like (4C, HiC)
  - metagenomics
- Sequencing of RNA (actually: cDNA)

Applications of HTS

- Sequencing of (genomic) DNA
- Sequencing of RNA (actually: cDNA)
  - whole transcriptome*: RNA-Seq, Tag-Seq, ...
  - enriched fraction: HITS-CLIP, ...
  - labeled material: DTA, ...

* or: polyadenylated fraction

HTS: Bioinformatics challenges

Solutions specific to HTS are required for

- assembly
- alignment
- statistical tests (counting statistics)
- visualization
- segmentation
- ...

Two types of experiments

- Discovery experiments
  - finding all possible variant
  - getting an inventory of all transcripts
  - finding all binding sites of a transcription factor
- Comparative experiments
  - comparing tumour and normal samples
  - finding expression changes due to a treatment
  - finding changes in binding affinity

Assembly and Alignment

- First step in most analyses is the *alignment* of reads to a genome
- Except the point is to get the genome: *de-novo assembly*
- Special cases: Transcriptome assembly, metagenomics

The data funnel: ChIP-Seq, non-comparative

- Images
- Base calls
- Alignments
- Enrichment scores
- Location and scores of peaks (or of enriched regions)
- Summary statistics
- Biological conclusions

The data funnel: Comparative RNA-Seq

- Images
- Base calls
- Alignments
- Expression strengths of genes
- Differences between these
- Gene-set enrichment analyses
-

Where does Bioconductor come in?

- Processing of the images and determining of the read sequencest
  - typically done by core facility with software from the manufacturer of the sequencing machine
- Aligning the reads to a reference genome (or assembling the reads into a new genome)
  - Done with community-developed stand-alone tools.
- Downstream statistical analysis.
  - Write your own scripts with the help of Bioconductor infrastructure.

Alignment

Alignment

- Many different aligners:

  Eland, Maq, Bowtie, BWA, SOAP, SSAHA, TopHat, SpliceMap, GSNAP, Novoalign, ...

- Main differences:
  - Publication year, maturity, development after publication, popularity
  - usage of base-call qualities, calculation of mapping qualities
  - Burrows-Wheeler index or not
  - speed-vs-sensitivity trade-off
  - suitability for RNA-Seq (“spliced alignment”)
  - suitability for special tasks (e.g., color-space reads, bisulfite reads, variant injection, local re-alignment, ...)

Alignment: Workflow

- Preparation: Generate an *index* from FASTA file with the genome.

- Input data: FASTQ files with raw reads (demultiplexed)
- Alignment
- Output file: SAM file with alignments

Raw reads: FASTQ format

"FASTA with Qualities"

Example:

```
@HWI-EAS225:3:1:2:854#0/1
GGGGGGAAGTCGGCAAAATAGATCCGTAACTTCGGG
+HWI-EAS225:3:1:2:854#0/1
a`abbbbabaabbbababb^``[aaa`_N]b^ab^```a
@HWI-EAS225:3:1:2:1595#0/1
GGGAAGATCTCAAAAACAGAAGTAAAACATCGAACG
+HWI-EAS225:3:1:2:1595#0/1
a`abbbababbbabbbbbbabb`aaababab\aa_`
```

FASTQ format

Each read is represented by four lines:

- '@', followed by read ID
- sequence
- '+', optionally followed by repeated read ID
- quality string:
  - same length as sequence
  - each character encodes the base-call quality of one base

FASTQ format: quality string

- If p is the probability that the base call is wrong,

  the Phred score is:

$$Q = -10 \log_{10} p$$

- The score is written with the character whose ASCII code is $Q+33$ (Sanger Institute standard).

- Before SolexaPipeline version 1.8, Solexa used instead the character with ASCII code $Q+64$.
- Before SolexaPipeline version 1.3, Solexa also used a different formula, namely $Q = -10 \log_{10} (p/(1-p))$

FASTQ: Phred base-call qualities

quality score Q_{phred}	error prob. p	characters
0 .. 9	1 .. 0.13	!"#$%&'()*
10 .. 19	0.1 .. 0.013	+,-./01234
20 .. 29	0.01 .. 0.0013	56789:;<=>
30 .. 39	0.001 .. 0.00013	?@ABCDEFGH
40	0.0001	I

Quality scales

```
 SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS.....................................................
 .........................XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX.....................
 ............................IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII.....................
 ..............................JJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJ.....................
 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)
L - Illumina 1.8+ Phred+33,  raw reads typically (0, 41)
```

[Wikipedia article “FASTQ format”]

FASTQ and paired-end reads

Convention for paired-end runs:

The reads are reported two FASTQ files, such that the n^{th} read in the first file is mate-paired to the n^{th} read in the second file. The read IDs must match.

Alignment output: SAM files

A SAM file consists of two parts:

- Header
  - contains meta data (source of the reads, reference genome, aligner, etc.)
  - Most current tools omit and/or ignore the header.
  - All header lines start with “@”.
  - Header fields have standardized two-letter codes for easy parsing of the information
- Alignment section
  - A tab-separated table with at least 11 columns
  - Each line describes one alignment

A SAM file

[...]

HWI-EAS225_309MTAAXX:5:1:689:1485 0 XIII 863564 25 36M * 0 0
GAAATATATACGTTTTTATCTATGTTACGTTATATA CCCCCCCCCCCCCCCCCCCCCCCC4CCCB4CA?AAA< NM:i:0
X0:i:1 MD:Z:36

HWI-EAS225_309MTAAXX:5:1:689:1485 16 XIII 863766 25 36M * 0 0
CTACAATTTTGCACATCAAAAAAGACCTCCAACTAC =8A=AA784A9AA5AAAAAAAAAAA=AAAAAAAAAA NM:i:0
X0:i:1 MD:Z:36

HWI-EAS225_309MTAAXX:5:1:394:1171 0 XII 525532 25 36M * 0 0
GTTTACGGCGTTGCAAGAGGCCTACACGGGCTCATT CCCCCCCCCCCCCCCCCCCCCC?CCACCACA7?<??? NM:i:0
X0:i:1 MD:Z:36

HWI-EAS225_309MTAAXX:5:1:394:1171 16 XII 525689 25 36M * 0 0
GCTGTTATTTCTCCACAGTCTGGCAAAAAAAAGAAA 7AAAAAA?AA<AA?AAAAA5AAA<AAAAAAAAAAAA NM:i:0
X0:i:1 MD:Z:36

HWI-EAS225_309MTAAXX:5:1:393:671 0 XV 440012 25 36M * 0 0
TTTGGTGATTTTCCCGTCTTTATAATCTCGGATAAA AAAAAAAAAAAAAAA<AAAAAAAA<AAAA5<AAAA3 NM:i:0
X0:i:1 MD:Z:36

HWI-EAS225_309MTAAXX:5:1:393:671 16 XV 440188 25 36M * 0 0
TCATAGATTCCATATGAGTATAGTTACCCCATAGCC ?9A?A?CC?<ACCCCCCCCCCCCCCCCCCACCCCCCC NM:i:0
X0:i:1 MD:Z:36

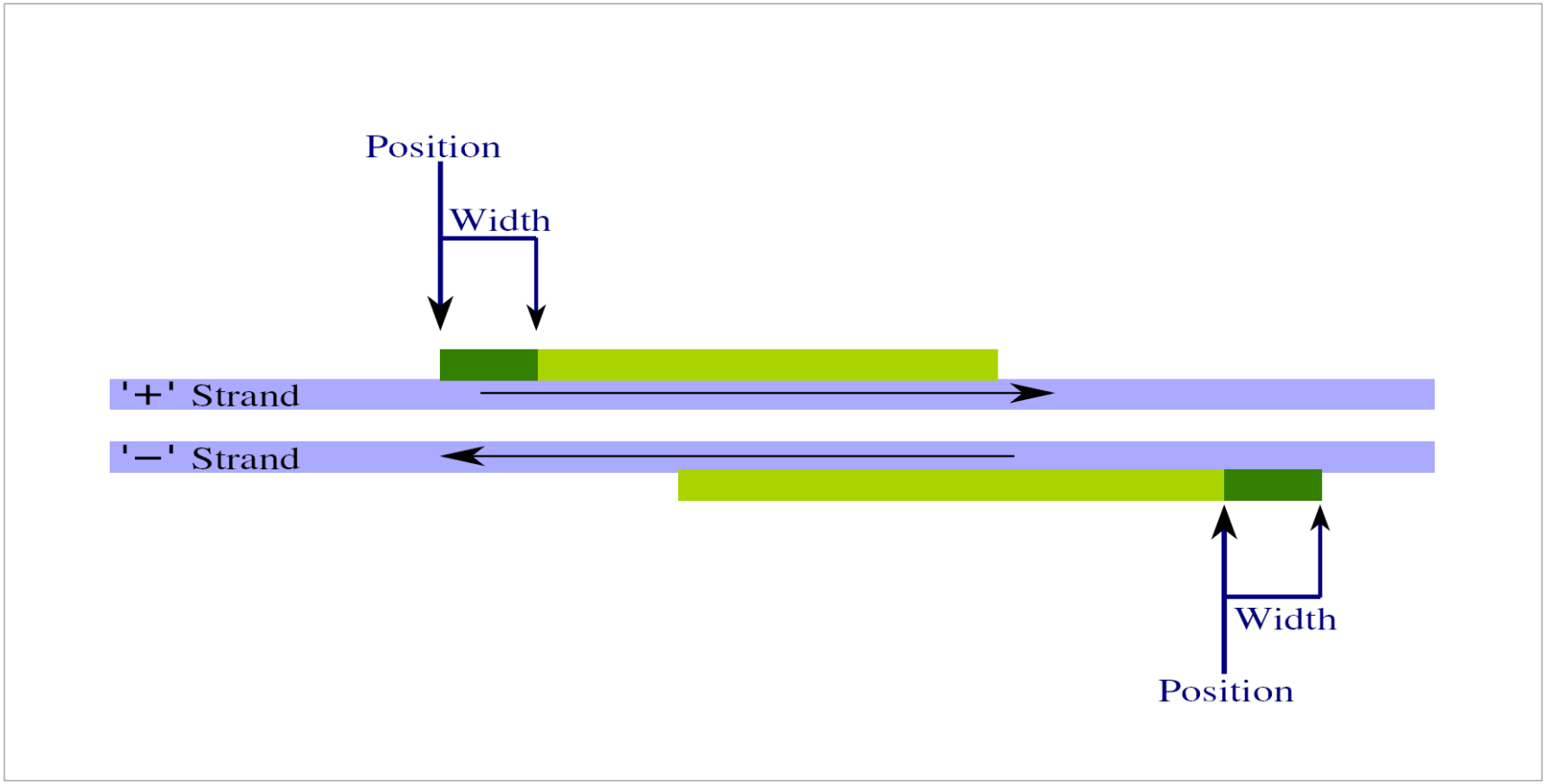
[...]

SAM format: Alignment section

The columns are:

- QNAME: ID of the read (“query”)
- FLAG: alignment flags
- RNAME: ID of the reference (typically: chromosome name)
- POS: Position in reference (1-based, left side)
- MAPQ: Mapping quality (as Phred score)
- CIGAR: Alignment description (gaps etc.) in CIGAR format
- MRNM: Mate reference sequence name [for paired end data]
- MPOS: Mate position [for paired end data]
- ISIZE: inferred insert size [for paired end data]
- SEQ: sequence of the read
- QUAL: quality string of the read
- extra fields

Reads and fragments



SAM format: Flag field

FLAG field: A number, to be read in binary

bit	hex	decimal	
0	00 01	1	read is a paired-end read
1	00 02	2	read pair is properly matched
2	00 04	4	read has not been mapped
3	00 08	8	mate has not been mapped
4	00 10	16	read has been mapped to “-” strand
5	00 20	32	mate has been mapped to “-” strand
6	00 40	64	read is the first read in a pair
7	00 80	128	read is the second read in a pair
8	01 00	256	alignment is secondary
9	02 00	512	read did had not passed quality check
10	04 00	1024	read is a PCR or optical duplicate

SAM format: Optional fields

last column

- Always triples of the format TAG : VTYPE : VALUE
- some important tag types:
  - NH: number of reported aligments
  - NM: number of mismatches
  - MD: positions of mismatches

SAM format: CIGAR strings

Alignments contain gaps (e.g., in case of an indel, or, in RNA-Seq, when a read straddles an intron).

Then, the CIGAR string gives details.

Example: “M10 I4 M4 D3 M12” means

- the first 10 bases of the read map (“M10”) normally (not necessarily perfectly)
- then, 4 bases are inserted (“I4”), i.e., missing in the reference
- then, after another 4 mapped bases (“M4”), 3 bases are deleted (“D4”), i.e., skipped in the query.
- Finally, the last 12 bases match normally.

There are further codes (N, S, H, P), which are rarely used.

SAM format:
paired-end and multiple alignments

- Each line represents one *alignments*.
- Multiple alternative alignments for the same read take multiple lines. Only the read ID allows to group them.
- Paired-end alignments take two lines.

- All these reads are not necessarily in adjacent lines.

sorted SAM/BAM files

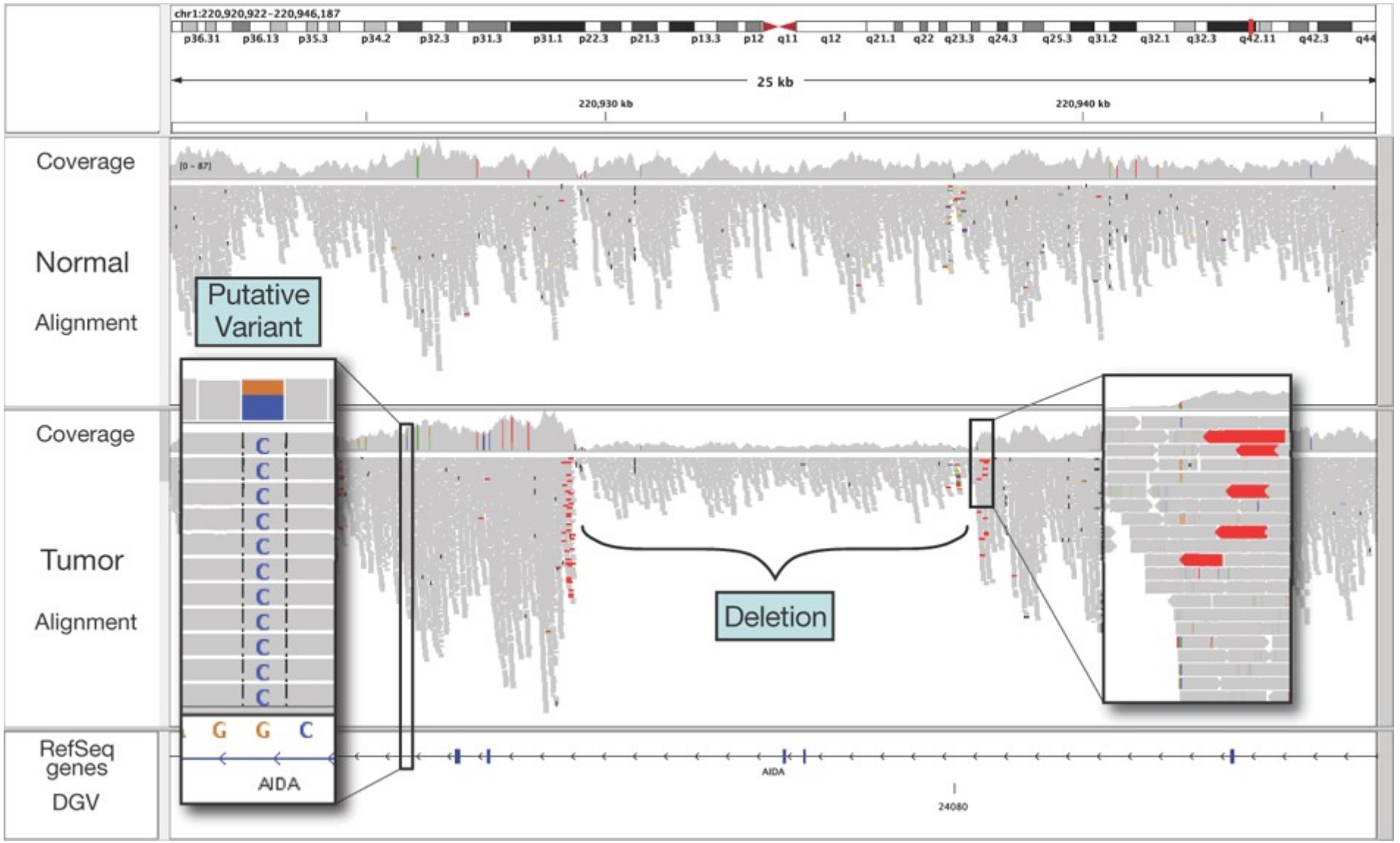
- Text SAM files (.sam): standard form
- BAM files (.bam): binary representation of SAM
  - more compact, faster to process, random access and indexing possible
- BAM index files (.bai) allow random access in a BAM file that is sorted by position.

SAMtools

- The SAMtools are a set of simple tools to
  - convert between SAM and BAM
  - sort and merge SAM files
  - index SAM and FASTA files for fast access
  - calculate tallies (“flagstat”)
  - view alignments (“tview”)
  - produce a “pile-up”, i.e., a file showing
    - local coverage
    - mismatches and consensus calls
    - indels

- The SAMtools C API facilitates the development of new tools for processing SAM files.

Visualization of SAM files

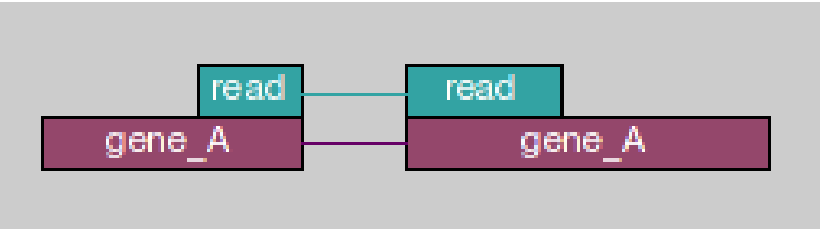


Integrative Genomics Viewer (IGV): Robinson et al., Broad Institute

Special considerations for RNA-Seq

RNA alignment

- Only few aligners (e.g., TopHat, GSNAP, SpliceMap) deal with spliced read.
- Use these for RNA-Seq data.

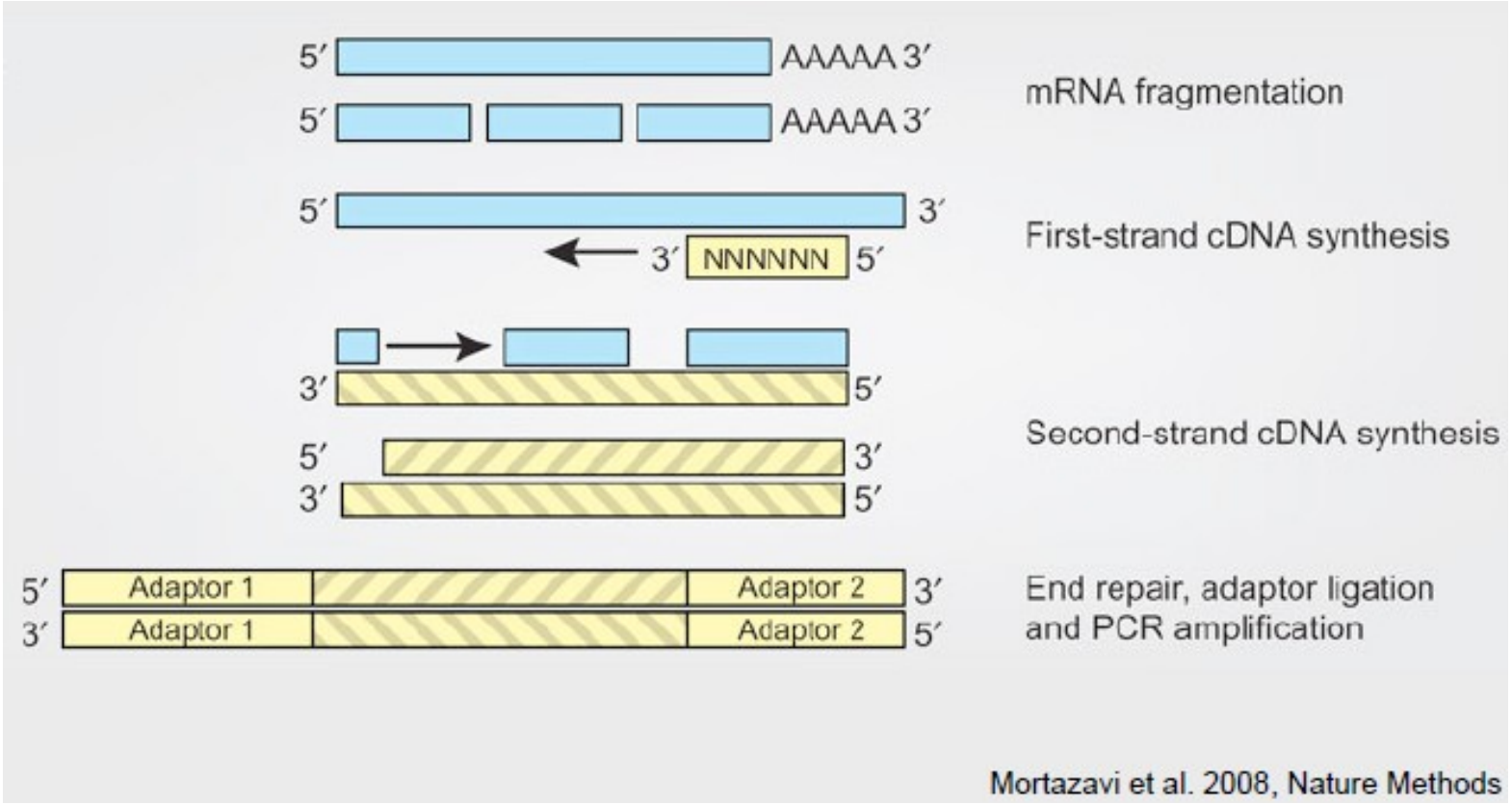


Strand-specific protocols

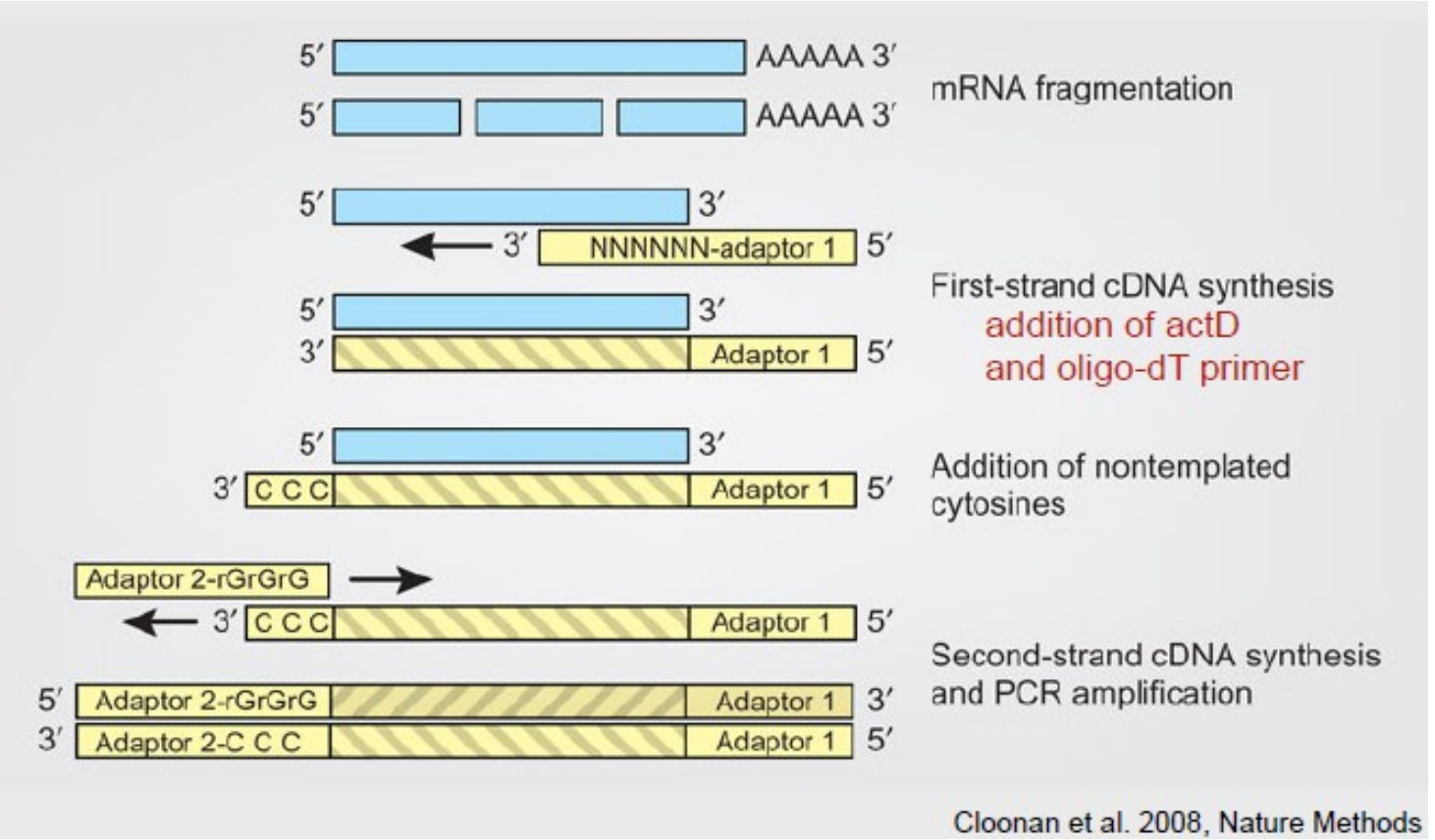
- Standard RNA-Seq loses strand information.
- If you want to distinguish sense from anti-sense transcripts, you need a strand-specific one.

- Make sure you know whether the library you analyse is strand-specific.

Solexa standard protocol for RNA-Seq



Strand-specific RNA-Seq with random hexamer priming

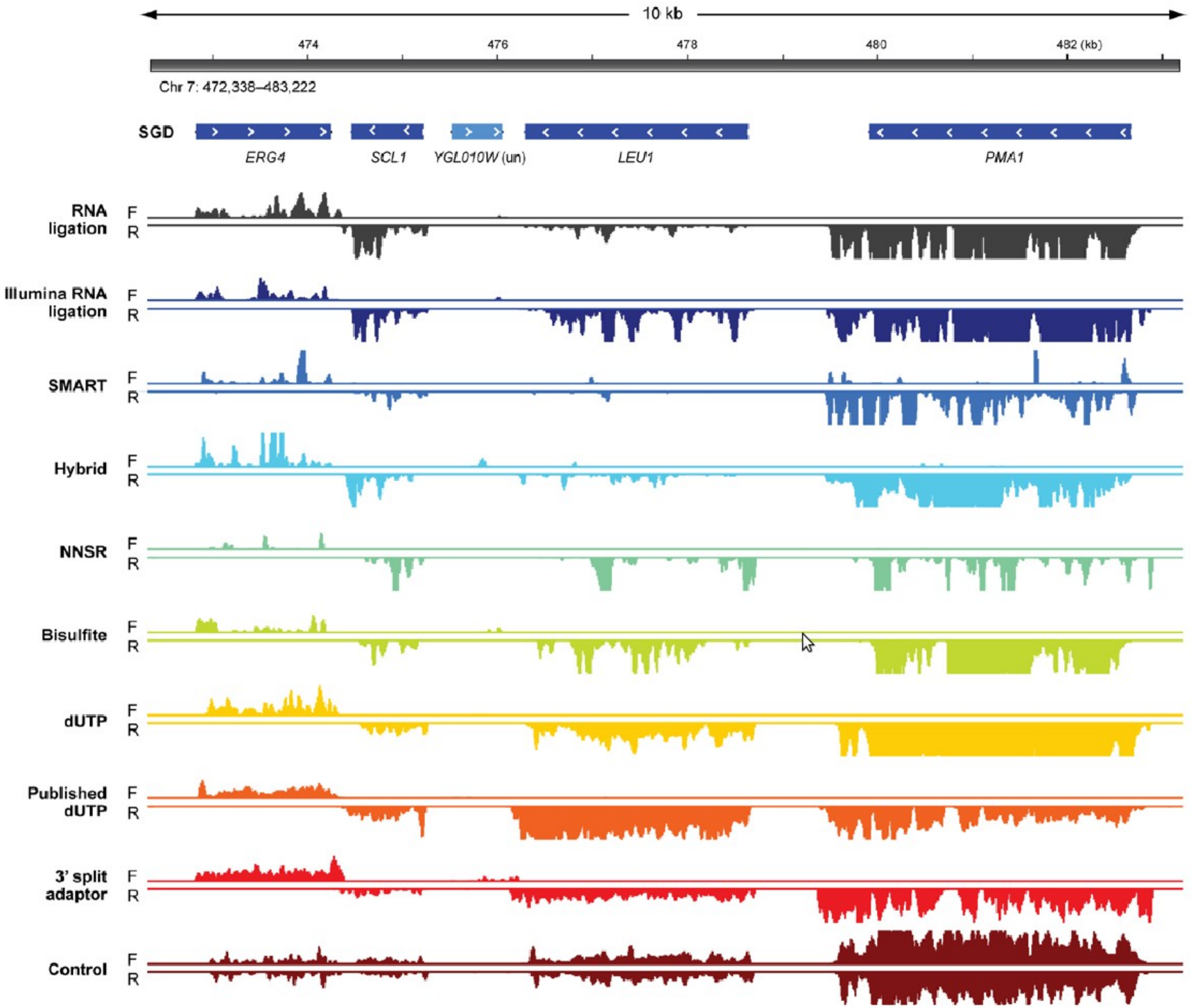


Cloonan et al. 2008, Nature Methods

Coverage in RNA-Seq

- When sequencing genomic DNA, the coverage seems reasonably even.

- In RNA-Seq, this quite different



Levin et al., Nature Methods, 2010

*