Introduction to NGS Analysis

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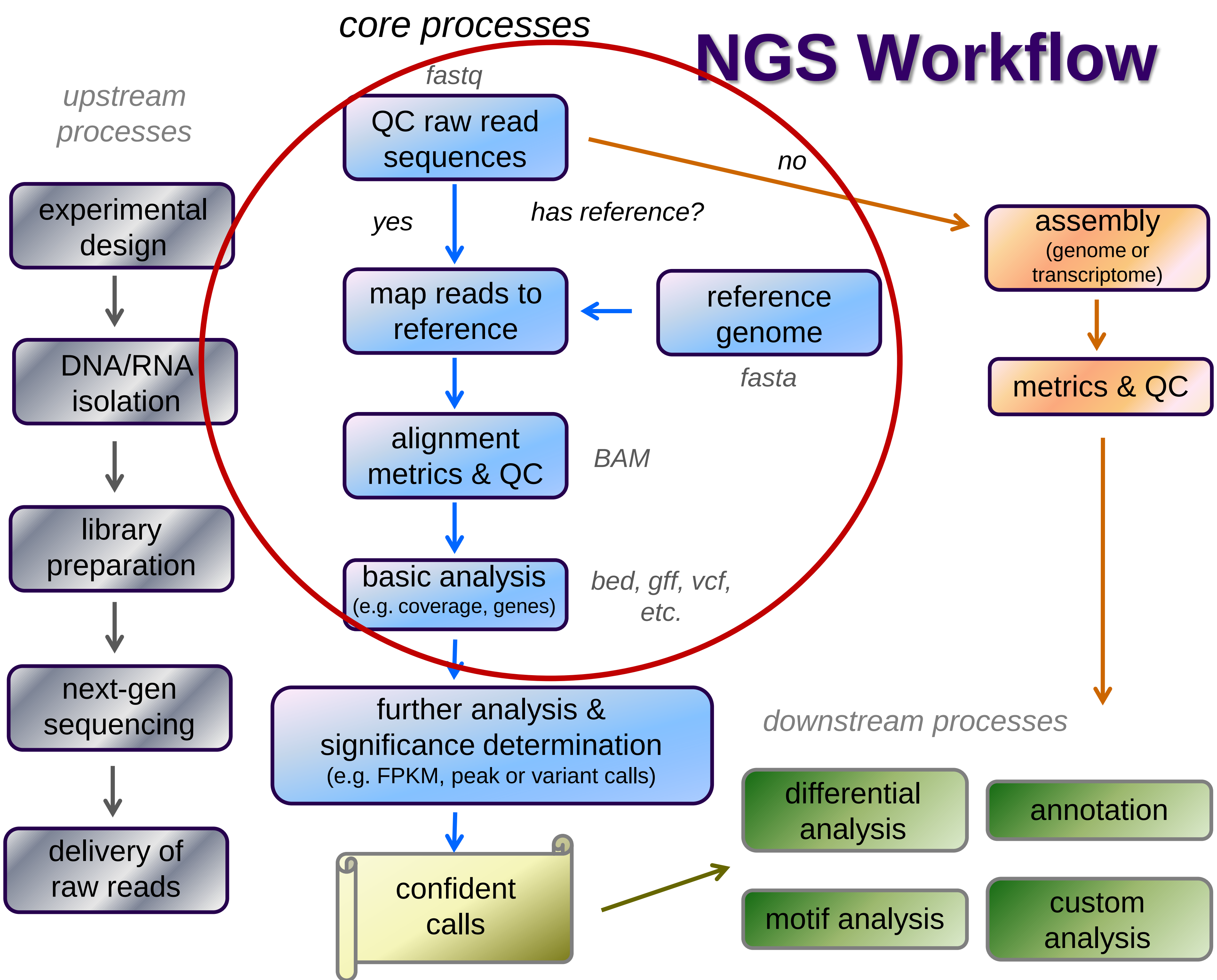
Outline

- NGS overview & terminology
- The FASTQ format
- Raw data QC and preparation
- Alignment to a reference

NGS Overview and Terminology

- NGS workflow overview
- Sequencing terminology & considerations

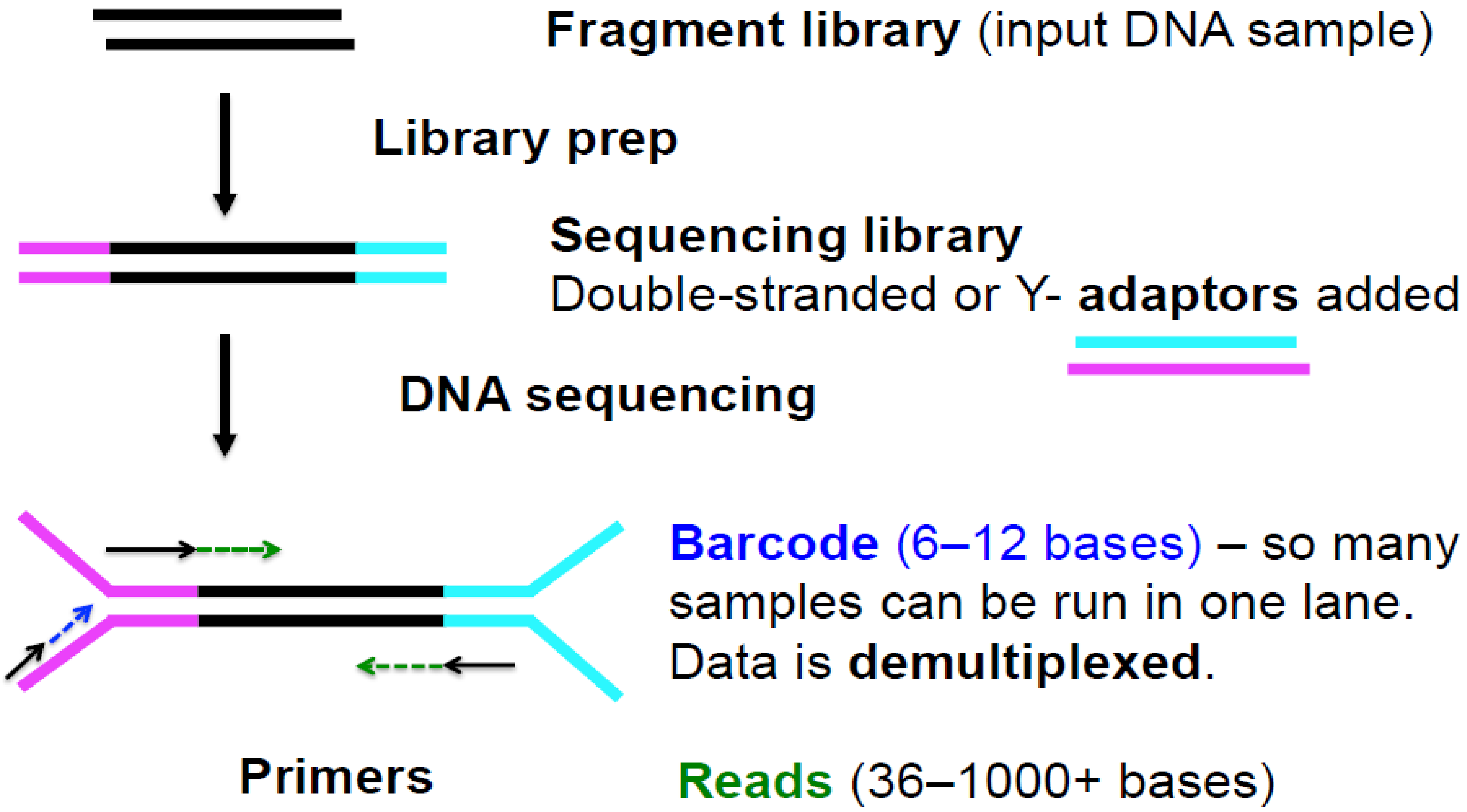
NGS Workflow



Sequencing technologies

- Illumina (Solexa) now dominent
  - Official Illumina video
  - Another Illumina video
  - Broad Center GA Boot Camp
- Many others
  - Comparison of NGS technologies (Liu et al., 2012) http://www.hindawi.com/journals/bmri/2012/251364/

Read sequence terminology



Fragment library (input DNA sample)

Library prep

Sequencing library
Double-stranded or Y- **adaptors** added

DNA sequencing

Barcode (6–12 bases) – so many samples can be run in one lane. Data is **demultiplexed**.

Primers

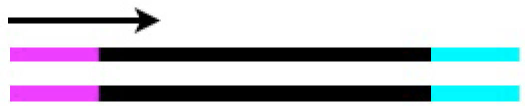
Reads (36–1000+ bases)

- Adapter areas include primers, barcode
  - sequencing facility will have more information

https://wikis.utexas.edu/display/GSAF/Illumina+-+all+flavors

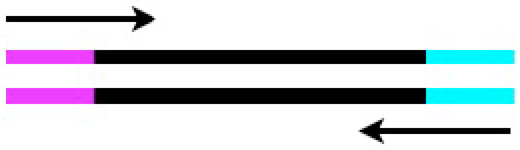
Types of Illumina sequencing

single-end



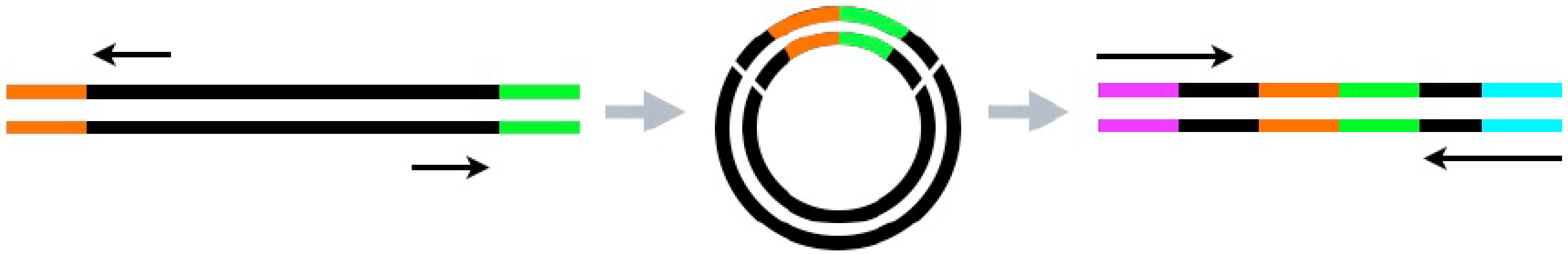
independent reads

paired-end



two inwardly oriented reads separated by ~200 nt

mate-paired



two outwardly oriented reads separated by ~3000 nt

Sequencing depth

- No single answer to how much depth is adequate
- Depends on:
  - genome size
    - prokaryotes – a few Kilobases
    - lower eukaryotes – some number of Megabases
    - higher eukaryotes – Gigabases
  - library fragment enrichment
    - e.g. ChIP-seq or RIP-seq
  - theoretical library complexity
    - genomic resquencing ***vs*** 4c
  - desired sensitivity
    - e.g. looking for rare mutations

Library complexity is primarily a function of experiment type

higher complexity

less enrichment for specific sequences

lower sequence duplication expected
more sequencing depth required

genomic

MNase-seq

exon capture

RNA-seq

ChIP-seq

miRNA-seq

… and
- *sequencing depth*
- *genome size*

more enrichment for specific sequences

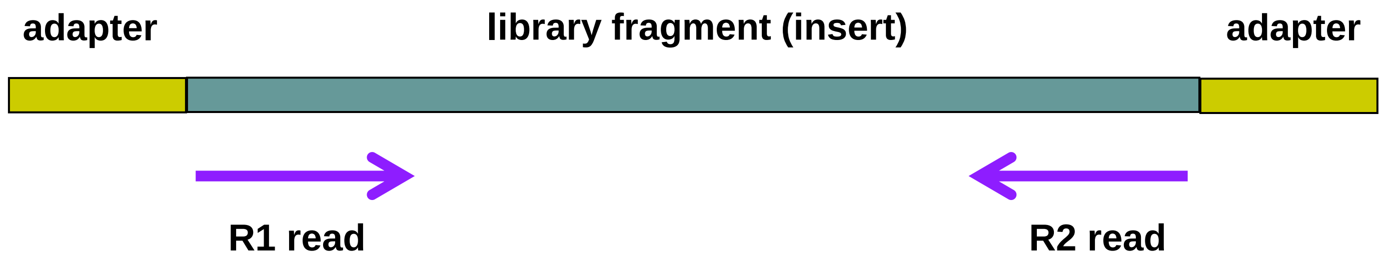
higher sequence duplication expected
less sequencing depth required

lower complexity

Reads and Fragments

- With paired-end sequencing, keep in mind the distinction between
  - the library ***fragment*** that was sequenced
    - also called ***inserts***
  - the ***sequence reads*** (R1 & R2) you receive
    - also called ***tags***

- There is considerable confusion of terminology in this area!
  - Be sure you request depth in ***read pairs*** for paired-end sequencing



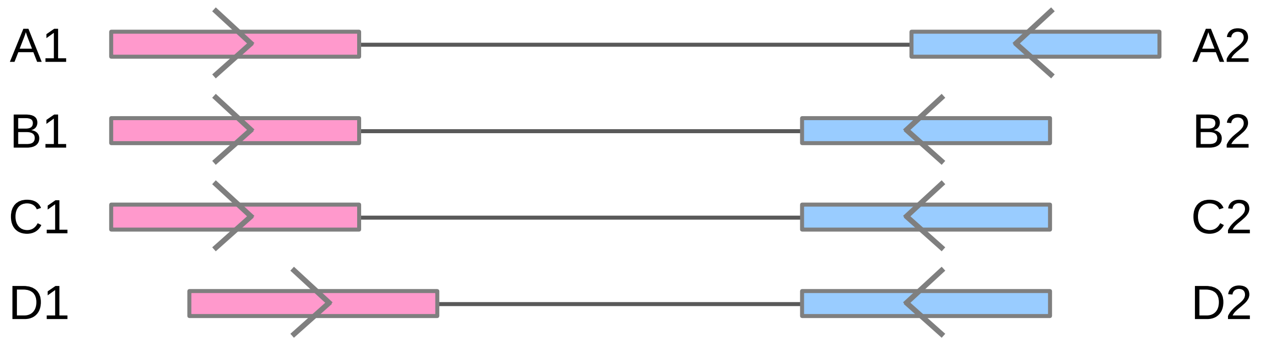
Single end vs Paired end

- ***paired end*** (PE) reads can be mapped more reliably
  - especially against lower complexity genomic regions
    - when one member of a read pair does not align well, it can still be "rescued" if its mate maps well
  - they also provide more bases around a locus
    - e.g. for analysis of polymorphisms
  - actual fragment sizes can be determined
    - from the alignment records for each dual-mapping "proper pair"
  - they also help distinguish the true complexity of a library
    - by clarifying which *fragments* are duplicates

- ***but*** PE reads are more expensive – and larger
  - more storage space and processing time required

Read vs fragment duplication

- Consider the 4 fragments below
  - 4 R1 reads (pink), 4 R2 reads (blue)
- Duplication when only 1 end considered
  - A1, B1, C1 have identical sequences, D1 different
    - 2 unique + 2 duplicates = 50% duplication rate
  - B2, C2, D2 have identical sequences, A2 different
    - 2 unique + 2 duplicates = 50% duplication rate
- Duplication when both ends considered
  - fragments B and C are duplicates (same external sequences)
    - 3 unique + 1 duplicate = 25% duplication rate



The FASTQ format

FASTQ files

- Nearly all sequencing data now delivered as FASTQ files
  - usually compressed to save space
    - (**gzip**'d, with **.gz** file extension)
  - best practice: leave them that way!
    - 3x to 6x space saving
    - most tools handle **gzip**'d FASTQ
- Paired-end sequencing data comes in 2 FASTQs
  - one each for R1 and R2 reads

    Sample_MyTubeID_L008_R1.fastq.gz

    Sample_MyTubeID_L008_R2.fastq.gz
  - ***order of reads is identical***
    - aligners rely on this "name ordering" for PE

FASTQ format

- Text format for storing sequence and quality data
  - http://en.wikipedia.org/wiki/FASTQ_format
- 4 lines per sequence:
  1. ***@read name***
  2. ***called base sequence* (ACGTN)**

     always 5’ to 3’; usually excludes 5’ adapter/barcode
  3. ***+optional read name***
  4. ***base quality scores encoded as text characters***
- FASTQ representation of a single, 50 base R1 sequence

```
@HWI-ST1097:97:D0WW0ACXX:8:1101:2007:2085 1:N:0:ACTTGA
ATTCTCCAAGATTTGGCAAATGATGAGTACAATTATATGCCCCAATTTACA
+
?@@?DD;?;FF?HHBB+:ABECGHDHDCF4?FGIGACFDFH;FHEIIIB9?
```

FASTQ read names

- Illumina read names encode information about the source cluster
  - unique identifier (“fragment name”) begins with @, then:
    - sequencing machine name
    - lane number
    - flowcell grid coordinates
    - R1, R2 reads will have the same name
  - a space separates the name from extra read information:
    - end number (1 for R1, 2 for R2)
    - two qualtiy fields (N = *not* QC failed)
    - barcode sequence

```
@HWI-ST1097:97:D0WW0ACXX:8:1101:2007:2085 1:N:0:ACTTGA
@HWI-ST1097:97:D0WW0ACXX:8:1101:2007:2085 2:N:0:ACTTGA
```

FASTQ quality scores

http://www.asciitable.com/

```
Quality character   !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJ
                    |         |         |         |         |
ASCII Value         33        43        53        63        73
Base Quality (Q)    0         10        20        30        40
```

Probability of Error = $10^{-Q/10}$

- Base qualties expressed as ***Phred*** scores
  - log scaled, higher = better
  - 20 = $1/10^2$ = 1/100 errors, 30 = $1/10^3$ = 1/1000 errors
- In older FASTQ files, ASCII offsets may differ
  - modern Sanger format shown above
  - see http://en.wikipedia.org/wiki/FASTQ_format for others

Multiple lanes

- Sometimes the sequencing facility splits your sample across lanes
  - one submitted sample may be delivered as multiple FASTQ files

    Lane1: Sample_MyTubeID_L001_R1.fastq.gz, Sample_MyTubeID_L001_R2.fastq.gz

    Lane8: Sample_MyTubeID_L008_R1.fastq.gz, Sample_MyTubeID_L008_R2.fastq.gz

- Your sample may be re-run to "top off" requested read depth
  - be careful with the file names!
    - if run in the same lane, the FASTQ file names will be the same

    1st run: Sample_MyTubeID_L003_R1.fastq.gz

    2nd run : Sample_MyTubeID_L003_R1.fastq.gz

- Best practice
  - keep original data in separate directories by date & project
  - process data from multiple lanes separately for as long as possible
    - e.g., through alignment, then merge the sorted BAMs
    - identical sequences from different lanes can be considered unique (non-duplicates)

Data QC & preparation

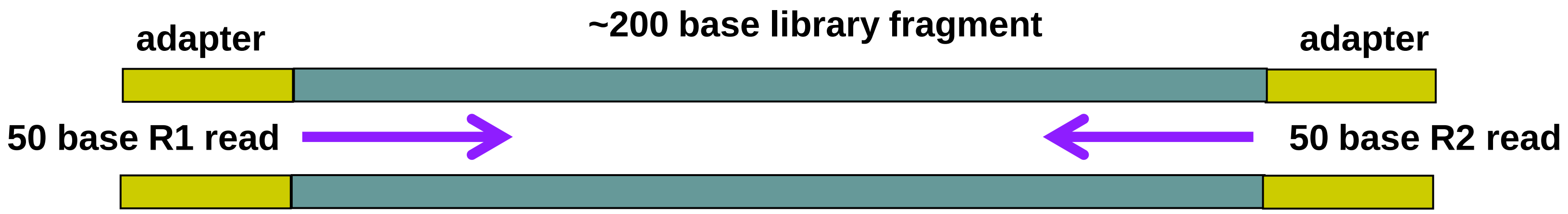
- QC of raw sequences with **FastQC** tool
- Dealing with adapters

Raw sequence quality control

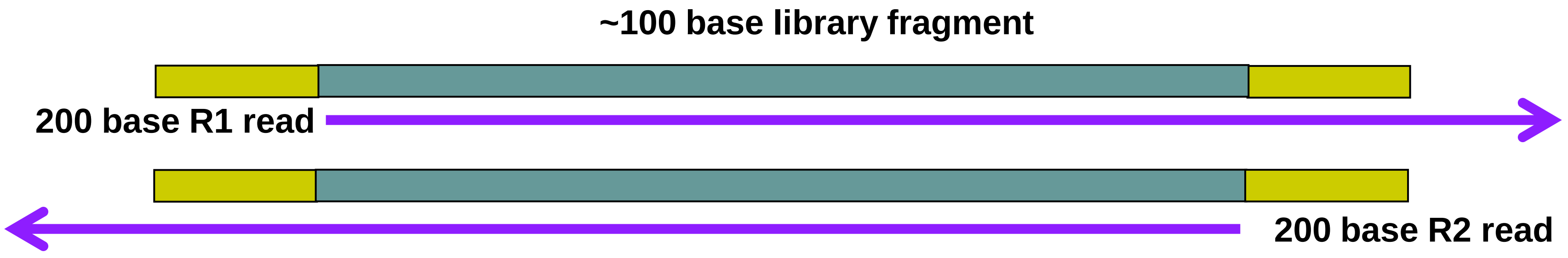
- Critical step! Garbage in = Garbage out
  - general sequence quality
    - base quality distributions
    - sequence duplication rate
  - trim 3’ adapter sequences?
    - important for RNAseq
  - trim 3’ bases with poor quality?
    - important for *de novo* assembly
  - other contaminents?
    - technical – samples sequenced on other lanes
    - biological – rRNA in RNAseq
- Know your data
  - sequencing center pre-processing
    - 5’ barcode removal; QC-failed reads filtered
  - PE reads? relative orientations?
  - technology specfic issues?
    - e.g. PAR clip should produce C→T transitions

3’ Adapter contamination

A. *reads short compared to fragment size (no contamination)*



B. *Reads long compared to library fragment (3’ adapter contamination)*



FastQC

- Quality Assurance tool for FASTQ sequences
- Can run as interactive tool or command line
- Input:
  - FASTQ file(s)
  - run on both R1, R2 files
- Output:
  - directory with html & text reports
    - **fastqc_report.html**
    - **fastqc_data.txt**

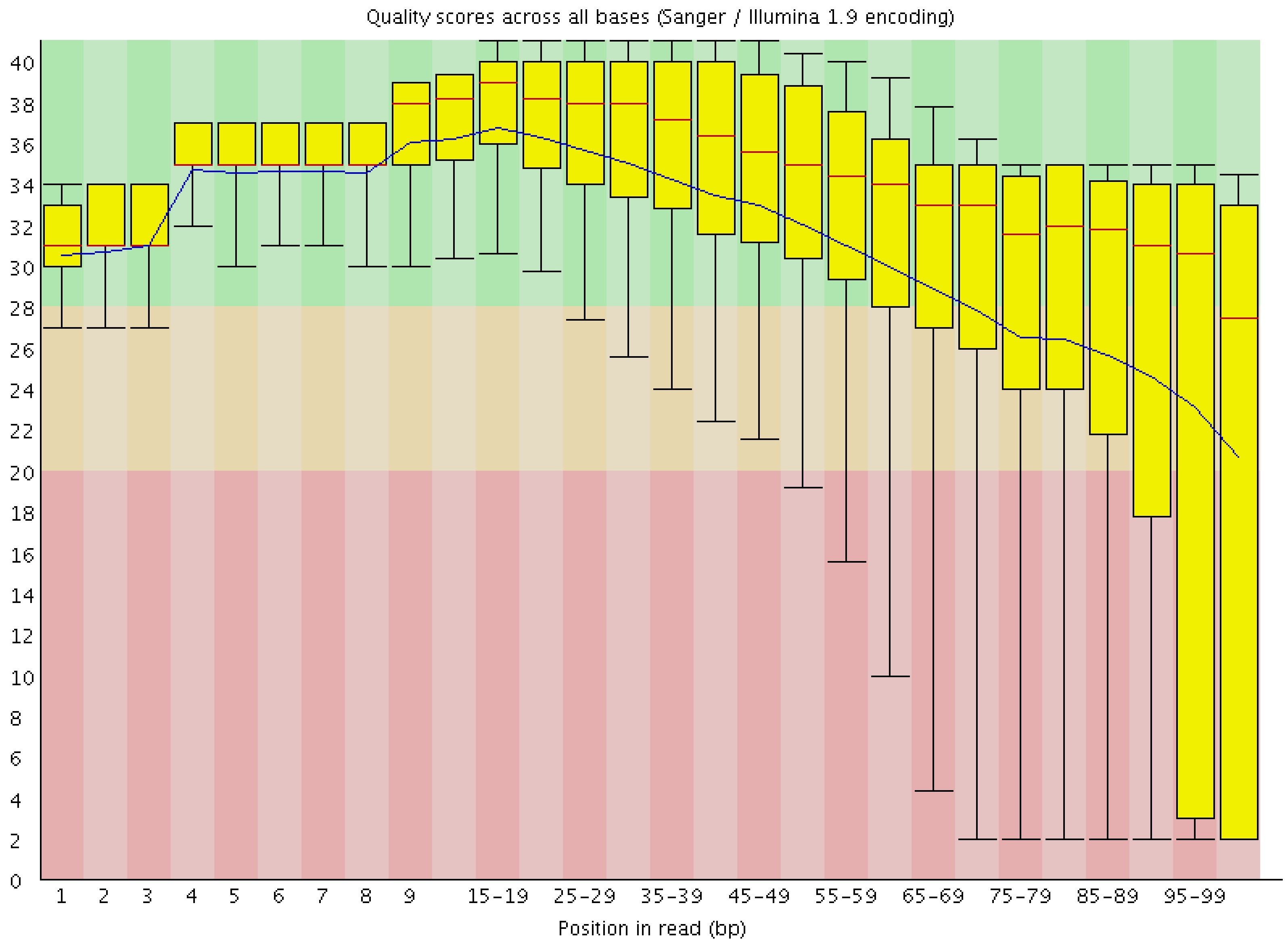
FastQC resources

- FastQC website:
  http://www.bioinformatics.babraham.ac.uk
- FastQC report documentation:
  http://www.bioinformatics.babraham.ac.uk/projects/fastqc/Help/3%20Analysis%20Modules/
- Good Illumina dataset:
  http://www.bioinformatics.babraham.ac.uk/projects/fastqc/good_sequence_short_fastqc/fastqc_report.html
- Bad Illumina dataset:
  http://www.bioinformatics.babraham.ac.uk/projects/fastqc/bad_sequence_fastqc/fastqc_report.html
- Real Yeast ChIP-seq dataset:
  http://web.corral.tacc.utexas.edu/BioITeam/yeast_stuff/Sample_Yeast_L005_R1.cat_fastqc/fastqc_report.html

Most useful FastQC reports

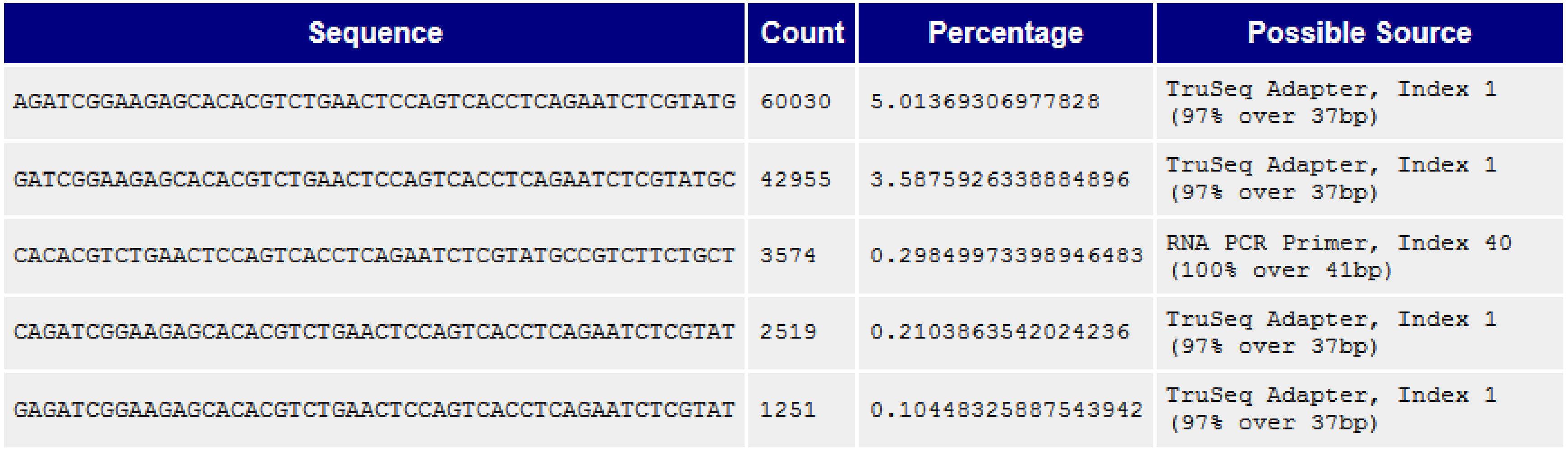
- Should I trim low quality bases?
  - *Per-base sequence quality Report*
    - based on *all* sequences
- Do I need to remove adapter sequences?
  - *Overrepresented sequences Report*
    - based on *1st 200,000* sequences
- How complex is my library?
  - *Sequence duplication levels Report*
    - estimate based on *1st 200,000* sequences

FastQC Per-base sequence quality report



FastQC Overrepresented sequences report

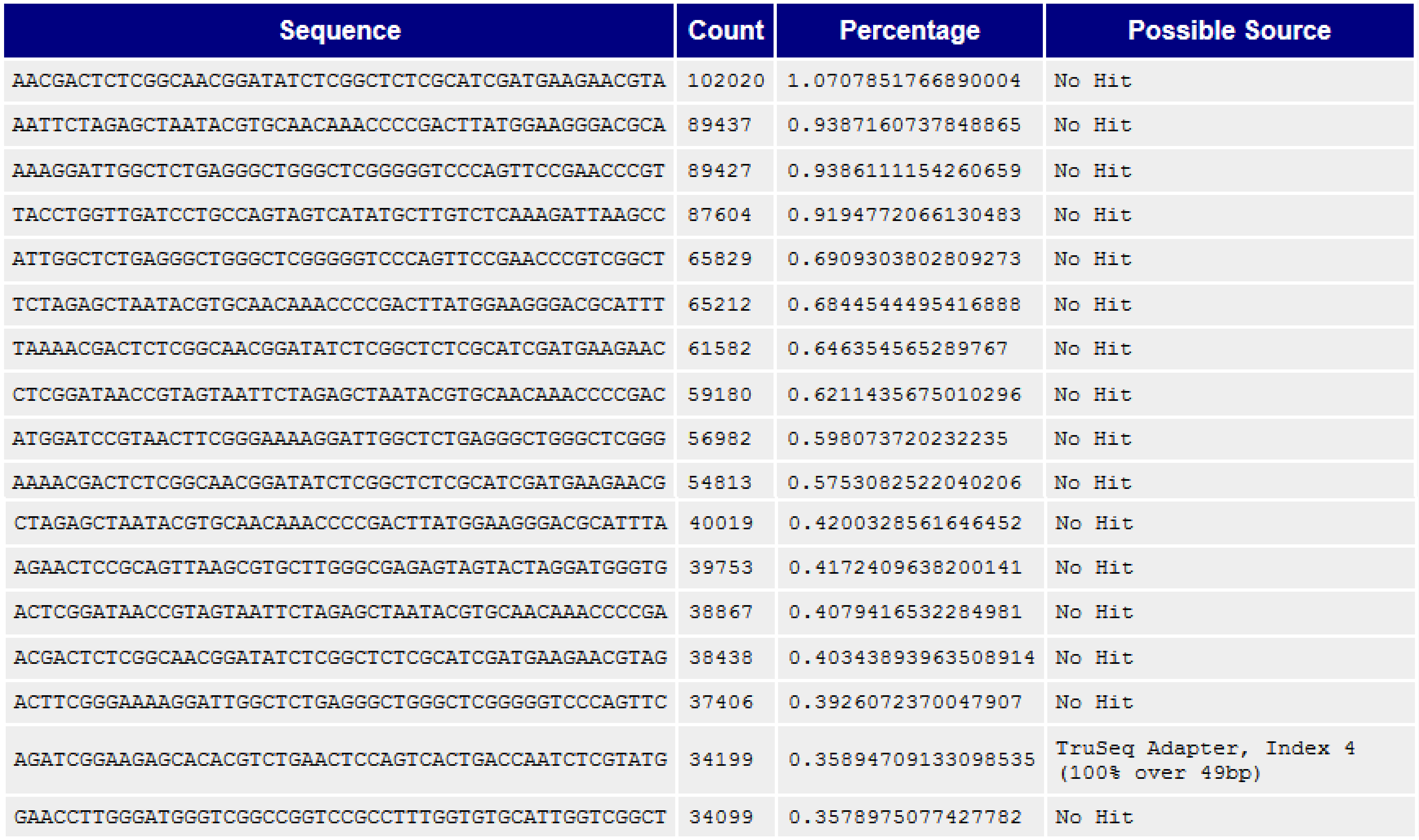
- **FastQC** knows Illumina adapter sequences
- Here ~9-10% of sequences contain adapters
    - calls for trimming



Sequence	Count	Percentage	Possible Source
AGATCGGAAGAGCACACGTCTGAACTCCAGTCACCTCAGAATCTCGTATG	60030	5.01369306977828	TruSeq Adapter, Index 1 (97% over 37bp)
GATCGGAAGAGCACACGTCTGAACTCCAGTCACCTCAGAATCTCGTATGC	42955	3.587592633884896	TruSeq Adapter, Index 1 (97% over 37bp)
CACACGTCTGAACTCCAGTCACCTCAGAATCTCGTATGCCGTCTTCTGCT	3574	0.29849973398946483	RNA PCR Primer, Index 40 (100% over 41bp)
CAGATCGGAAGAGCACACGTCTGAACTCCAGTCACCTCAGAATCTCGTAT	2519	0.210386354202436	TruSeq Adapter, Index 1 (97% over 37bp)
GAGATCGGAAGAGCACACGTCTGAACTCCAGTCACCTCAGAATCTCGTAT	1251	0.10448325887543942	TruSeq Adapter, Index 1 (97% over 37bp)

Overrepresented sequences

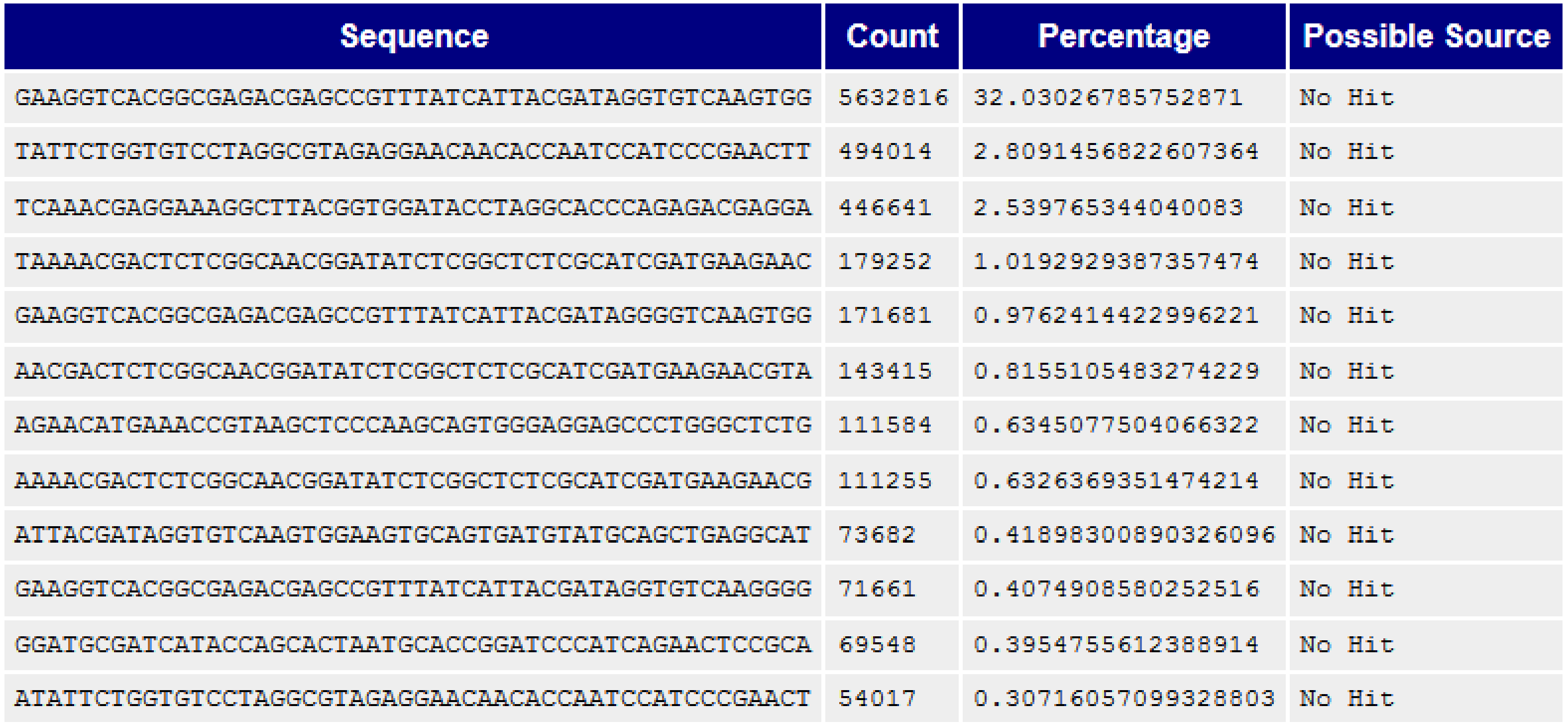
- Here < 1% of sequences contain adapters
  - trimming optional



Sequence	Count	Percentage	Possible Source
AACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTA	102020	1.0707851766890004	No Hit
AATTCTAGAGCTAATACGTGCAACAAACCCCGACTTATGGAAGGGACGCA	89437	0.9387160737848865	No Hit
AAAGGATTGGCTCTGAGGGCTGGGCTCGGGGGTCCCAGTTCCGAACCCGT	89427	0.938611154260659	No Hit
TACCTGGTTGATCCTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCC	87604	0.9194772066130483	No Hit
ATTGGCTCTGAGGGCTGGGCTCGGGGGTCCCAGTTCCGAACCCGTCGGCT	65829	0.6909303802809273	No Hit
TCTAGAGCTAATACGTGCAACAAACCCCGACTTATGGAAGGGACGCATTT	65212	0.6844544495416888	No Hit
TAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAAC	61582	0.646354565289767	No Hit
CTCGGATAACCGTAGTAATTCTAGAGCTAATACGTGCAACAAACCCCGAC	59180	0.6211435675010296	No Hit
ATGGATCCGTAACTTCGGGAAAAGGATTGGCTCTGAGGGCTGGGCTCGGG	56982	0.598073720232235	No Hit
AAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACG	54813	0.5753082522040206	No Hit
CTAGAGCTAATACGTGCAACAAACCCCGACTTATGGAAGGGACGCATTTA	40019	0.4200328561646452	No Hit
AGAACTCCGCAGTTAAGCGTGCTTGGGCGAGAGTAGTACTAGGATGGGTG	39753	0.4172409638200141	No Hit
ACTCGGATAACCGTAGTAATTCTAGAGCTAATACGTGCAACAAACCCCGA	38867	0.4079416532284981	No Hit
ACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAG	38438	0.40343893963508914	No Hit
ACTTCGGGAAAAGGATTGGCTCTGAGGGCTGGGCTCGGGGGTCCCAGTTC	37406	0.3926072370047907	No Hit
AGATCGGAAGAGCACACGTCTGAACTCCAGTCACTGACCAATCTCGTATG	34199	0.35894709133098535	TruSeq Adapter, Index 4 (100% over 49bp)
GAACCTTGGGATGGGTCGGCCGGTCCGCCTTTGGTGTGCATTGGTCGGCT	34099	0.3578975077427782	No Hit

Overrepresented sequences

- Here nearly 1/3 of sequences some type of non-adapter contamination
  - **BLAST** the sequence to identify it



Sequence	Count	Percentage	Possible Source
GAAGGTCACGGCGAGACGAGCCGTTTATCATTACGATAGGTGTCAAGTGG	5632816	32.03026785752871	No Hit
TATTCTGGTGTCCTAGGCGTAGAGGAACAACACCAATCCATCCCGAACTT	494014	2.8091456822607364	No Hit
TCAAACGAGGAAAGGCTTACGGTGGATACCTAGGCACCCAGAGACGAGGA	446641	2.539765344040083	No Hit
TAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAAC	179252	1.0192929387357474	No Hit
GAAGGTCACGGCGAGACGAGCCGTTTATCATTACGATAGGGGTCAAGTGG	171681	0.9762414422996221	No Hit
AACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTA	143415	0.8155105483274229	No Hit
AGAACATGAAACCGTAAGCTCCCAAGCAGTGGGAGGAGCCCTGGGCTCTG	111584	0.6345077504066322	No Hit
AAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACG	111255	0.6326369351474214	No Hit
ATTACGATAGGTGTCAAGTGGAAGTGCAGTGATGTATGCAGCTGAGGCAT	73682	0.41898300890326096	No Hit
GAAGGTCACGGCGAGACGAGCCGTTTATCATTACGATAGGTGTCAAGGGG	71661	0.4074908580252516	No Hit
GGATGCGATCATACCAGCACTAATGCACCGGATCCCATCAGAACTCCGCA	69548	0.3954755612388914	No Hit
ATATTCTGGTGTCCTAGGCGTAGAGGAACAACACCAATCCATCCCGAACT	54017	0.30716057099328803	No Hit

Dealing with adapters

- Three main options:
  1. Hard trim all sequences by specific amount
  2. Remove adapters specifically
  3. Peform a local (vs global) alignment

Hard trim by specific length

- E.g. trim 100 base reads to 50 bases
- ***Pro:***
  - Can eliminate vast majority of adapter contamination
  - Fast, easy to perform
  - Low quality 3’ bases also removed
- ***Con:***
  - Removes information you may want
    - e.g. splice junctions for RNAseq, coverage for mutation analysis
  - Not suitable for very short library fragments
    - e.g. miRNA libraries

Trim adapters specifically

- ***Pro:***
  - Can eliminate vast majority of adapter contamination
  - Minimal loss of sequence information
    - still ambiguous: are 3’-most bases part of sequence or adapter?
- ***Con:***
  - Requires knowledge of insert fragment structure and adapters
  - Slower process; more complex to perform
  - Results in heterogenous pool of sequence lengths
    - can confuse some tools (rare)

FASTQ trimming

- Tools:
  - **cutadapt** – https://code.google.com/p/cutadapt/
  - **trimmomatic** – http://www.usadellab.org/cms/?page=trimmomatic

- Features:
  - hard-trim specific number of bases
  - trimming of low quality bases
  - specific trimming of adapters
  - support for trimming paired end read sets
    - typically reads less than a specified length *after trimming* are discarded
    - leads to different sets of R1 and R2 reads unless care taken
      - aligners do not like this!

Local vs global alignment

- ***Global***
  - requires query sequence to map ***fully*** (end-to-end) to reference
- ***Local***
  - allows a ***subset*** of the query sequence to map to reference

global *(end-to-end) alignment of query*

local *(subsequence) alignment of query*

```
     CACAAGTACAATTATACAC          CTAGCTTATCGCCCTGAAGGACT
TACATACACAAGTACAATTATACACAGACATTAGTTCTTATCGCCCTGAAAATTCTCC
```

reference sequence

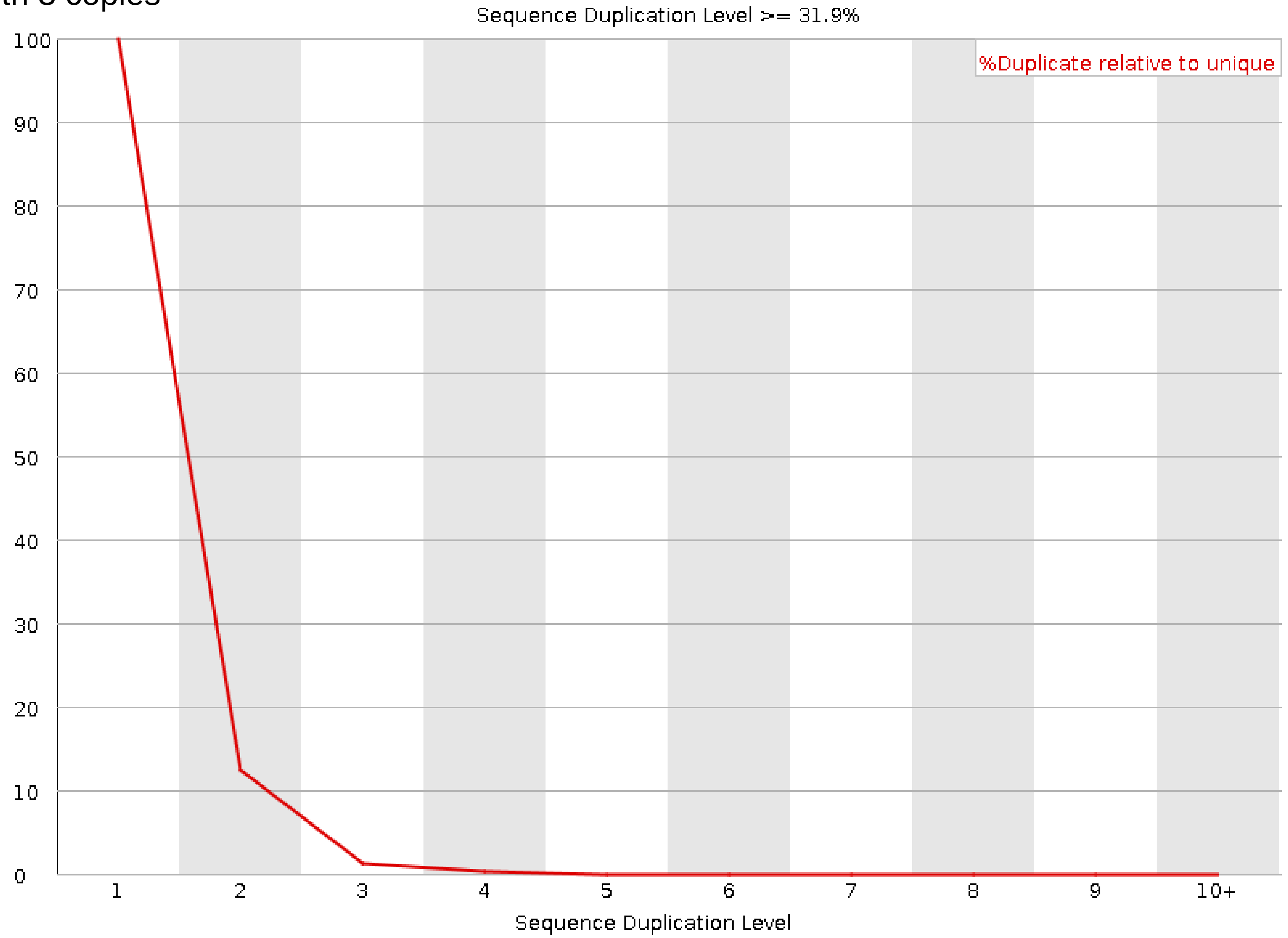
Peform local alignment

- ***Pro:***
  - mitigates adapter contamination while retaining full query sequence
  - minimal ambiguity
    - still ambiguous: are 5'/3'-most bases part of sequence or adapter?
- ***Con:***
  - not supported by many aligners
    - e.g. not by the **tophat** splice-aware aligner for RNAseq
  - slower alignment process
  - more complex post-alignment processing may be required
- Aligners with local alignment support:
  - **bwa mem**
  - **bowtie2 --local**

FastQC Sequence duplication report
Yeast ChIP-seq

for every 100 unique sequences
there are:
- ~12 sequences w/2 copies
- ~1-2 with 3 copies

some duplication expected due to IP enrichment

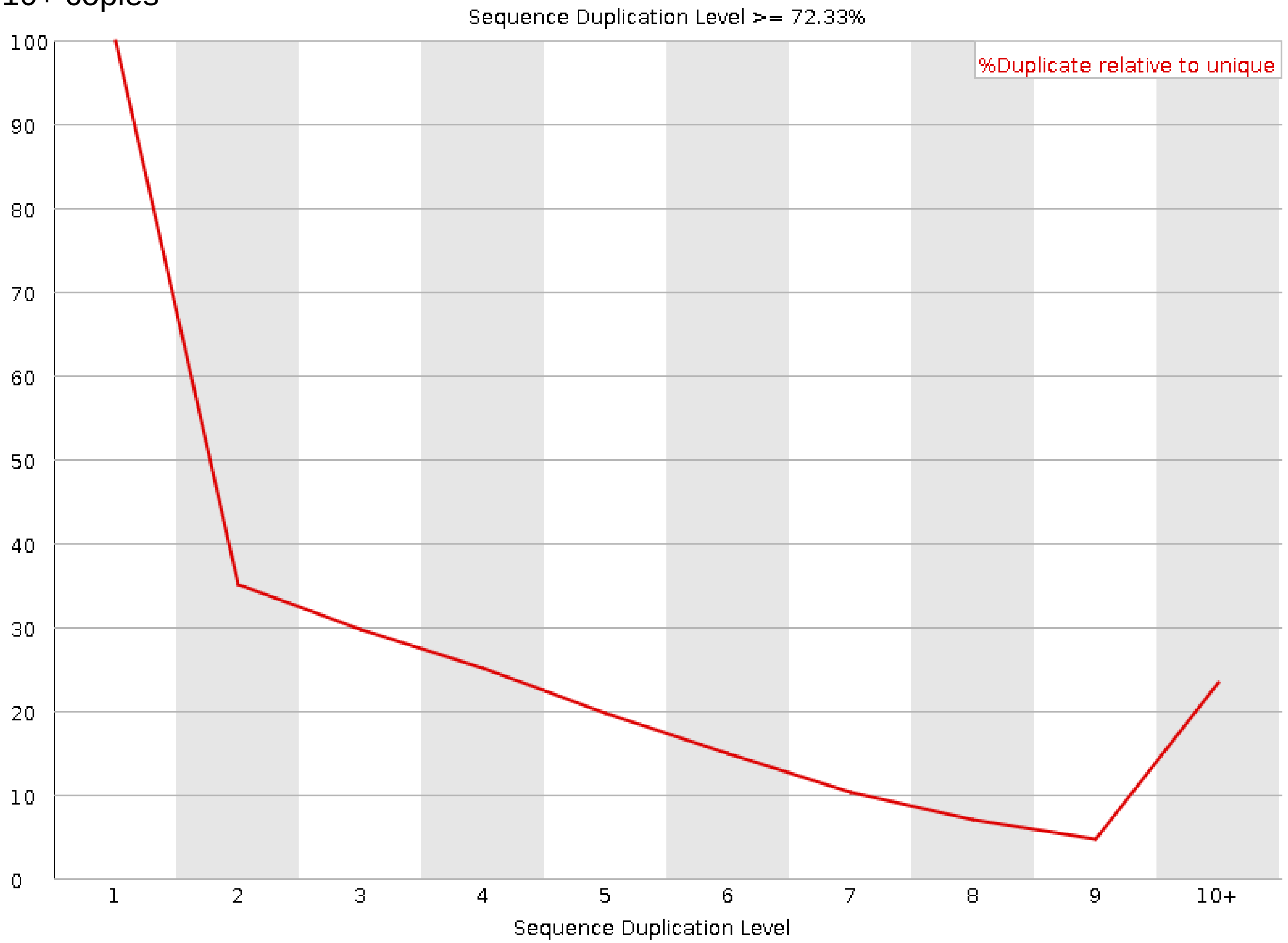


Sequence duplication report

Yeast ChIP-exo

for every 100 unique sequences
there are:
- ~35 sequences w/2 copies
- ~22 with 10+ copies

success! protocol expected to have high duplication



Library complexity is primarily a function of experiment type

higher complexity

less enrichment for specific sequences

genomic

MNase-seq

exon capture

RNA-seq

ChIP-seq

miRNA-seq

more enrichment for specific sequences

lower complexity

lower sequence duplication expected
more sequencing depth required

… and
- *sequencing depth*
- *genome size*

higher sequence duplication expected
less sequencing depth required

Alignment to a reference genome

- Alignment overview & concepts
- Preparing a reference genome
- Alignment steps

Short Read Aligners

- Short read mappers determine the placement of query sequences against a known reference
  - **BLAST**:
    - one query sequence (or a few)
    - many matches for each
  - short read aligners
    - many millions of query sequences
    - want only one "best" mapping (or a few)
  - many such aligners available
    http://en.wikipedia.org/wiki/List_of_sequence_alignment_software
- We use 2 of the most popular
  - **bwa** (Burrows Wheeler Aligner) by Heng Li
    http://bio-bwa.sourceforge.net/
  - **bowtie2** – part of the Johns Hopkins Tuxedo suite of tools
    http://bowtie-bio.sourceforge.net/bowtie2/manual.shtml

Aligner criteria

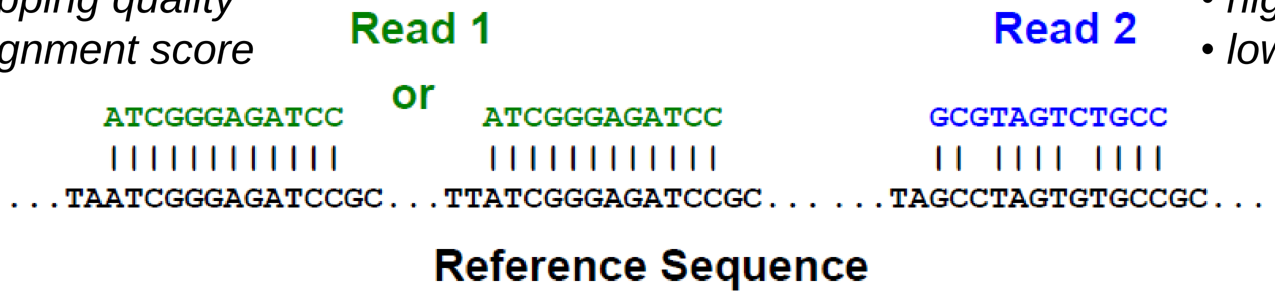
- Adoption and currency
  - widspread use by bioinformatics community
  - still being actively developed
- Features
  - well understood algorithm(s)
  - support for a variety of input formats and read lengths
  - detection of indels and gaps
  - makes use of base qualities
  - handling of multiple matches?
- Usability
  - configurability and transparency of options
  - ease of installation and use
- Resource requirements
  - speed (“fast enough”)
  - scalability (takes advantage of multiple processors)
  - reasonable memory footprint

Mapping vs Alignment

- ***Mapping*** determines one or more "seed" positions (a.k.a "hits") where a read shares a subsequence with the reference
- ***Alignment*** starts with the seed and determines how read bases are best matched, base-by-base, around the seed
- Mapping quality and alignment scores are both reported
- High ***mapping quality*** ≠ High ***alignment score***
  - ***mapping quality*** describes ***positioning***
    - reflects the probability that the read is *incorrectly* mapped to the reported location
    - is a Phred score: $\mathtt{P(mis\text{-}mapped)} = 10^{\mathtt{-mappingQuality/10}}$
    - reflects the complexity/information content of the sequence ("mappability")
  - ***alignment score*** describes ***fit***
    - reflects the correspondence between the read and the reference sequences

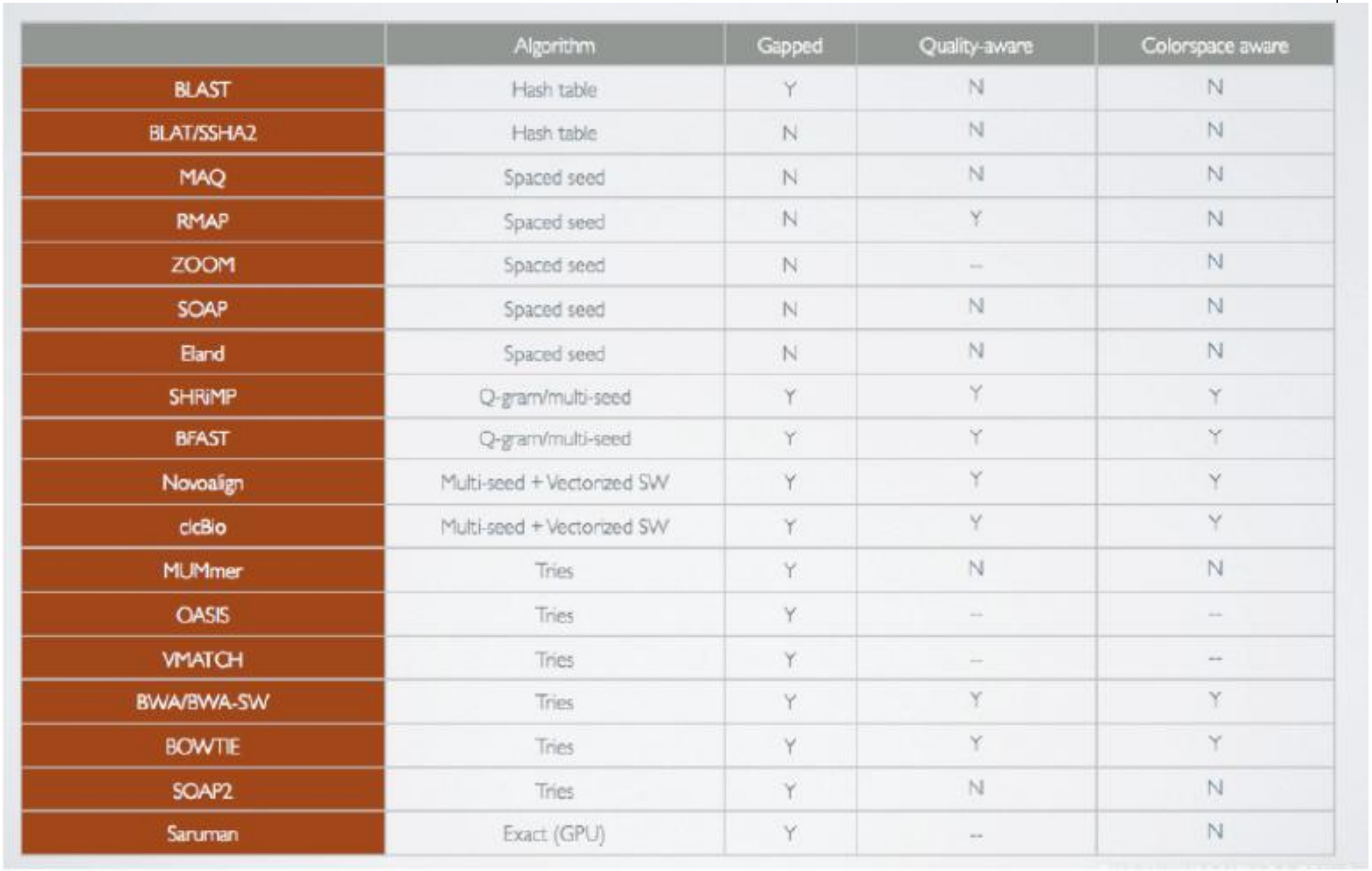
- *low mapping quality*
- *high alignment score*

- *high mapping quality*
- *low alignment score*



Some Aligners

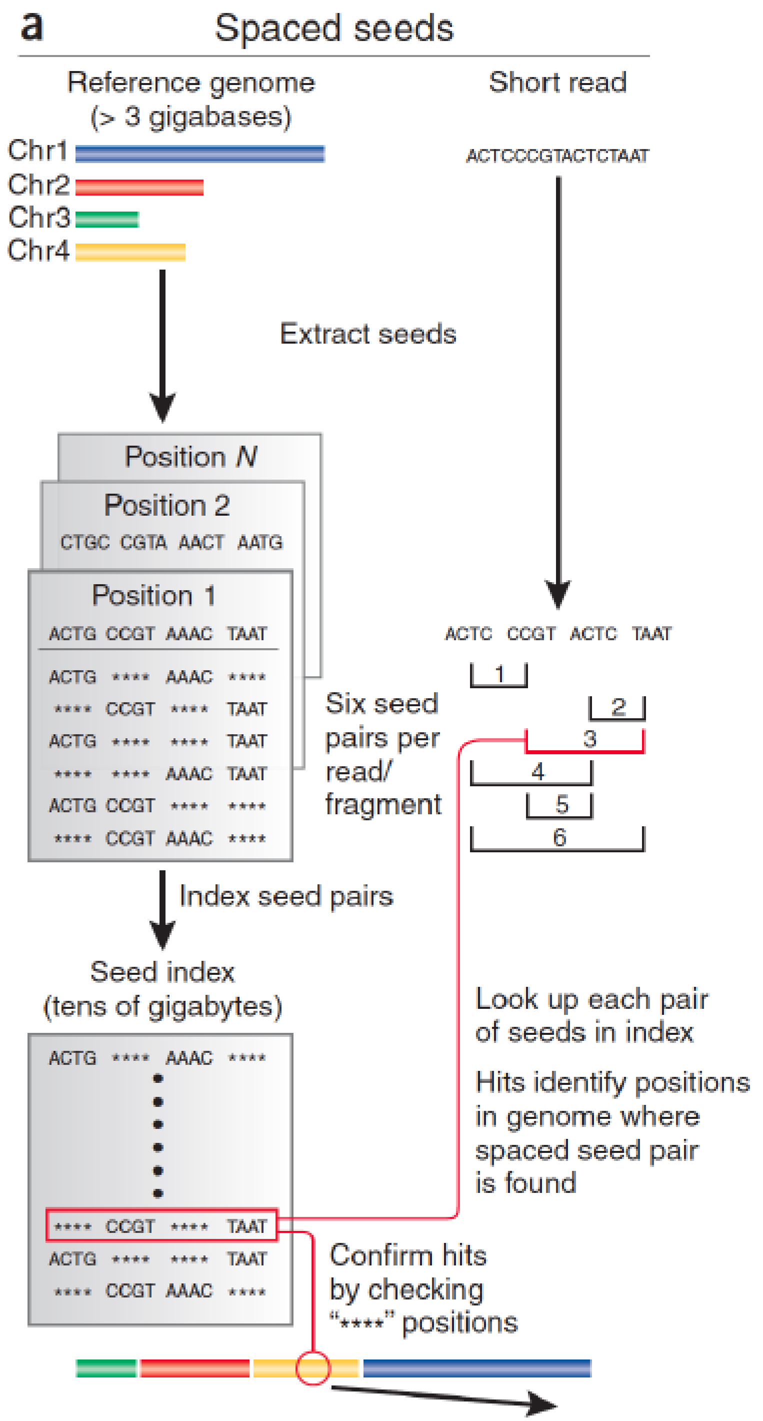
Two main mapping algorithms: *spaced seeds*, *suffix-array tries*



	Algorithm	Gapped	Quality-aware	Colorspace aware
BLAST	Hash table	Y	N	N
BLAT/SSHA2	Hash table	N	N	N
MAQ	Spaced seed	N	N	N
RMAP	Spaced seed	N	Y	N
ZOOM	Spaced seed	N	--	N
SOAP	Spaced seed	N	N	N
Eland	Spaced seed	N	N	N
SHRiMP	Q-gram/multi-seed	Y	Y	Y
BFAST	Q-gram/multi-seed	Y	Y	Y
Novoalign	Multi-seed + Vectorized SW	Y	Y	Y
clcBio	Multi-seed + Vectorized SW	Y	Y	Y
MUMmer	Tries	Y	N	N
OASIS	Tries	Y	--	--
VMATCH	Tries	Y	--	--
BWA/BWA-SW	Tries	Y	Y	Y
BOWTIE	Tries	Y	Y	Y
SOAP2	Tries	Y	N	N
Saruman	Exact (GPU)	Y	--	N

courtesy of Matt Vaughn, TACC

<u>trie</u> = tree structure for fast text re<u>trie</u>val.



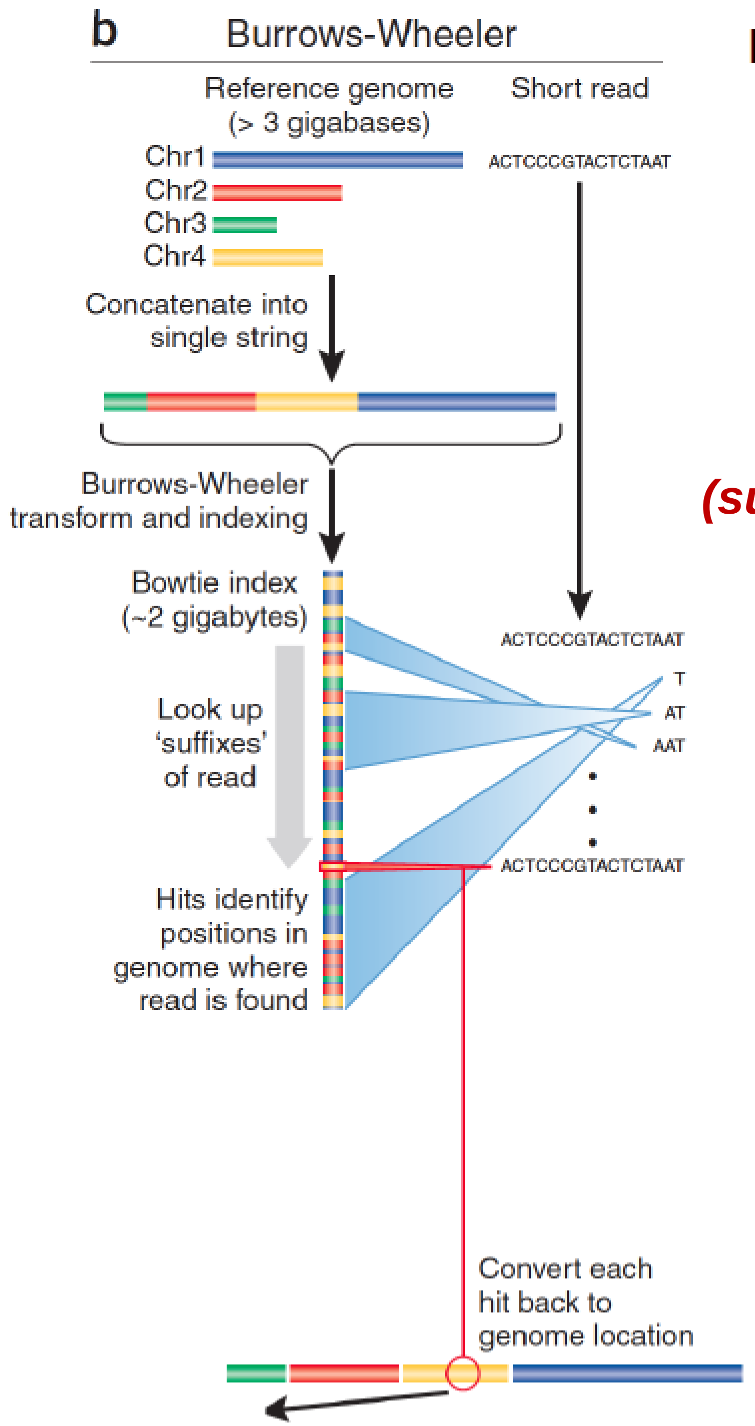
Hash table enables lookup of exact matches.

Subsequence	Reference Positions
ATAGCTAATCCAAA	2341, 2617264
ATAGCTAATCCAAT	
ATAGCTAATCCAAC	134, 13311, 732661,
ATAGCTATCCAAAG	
ATAGCTAATCCATA	
ATAGCTAATCCATT	3452
ATAGCTAATCCATC	
ATAGCTATCCAATG	234456673

Table is sorted and complete so you can jump immediately to matches. (But this can take a lot of memory.)

May include N bases, skip positions, etc.

Trapnell, C. & Salzberg, S. L. How to map billions of short reads onto genomes. *Nature Biotech.* **27**, 455–457 (2009).

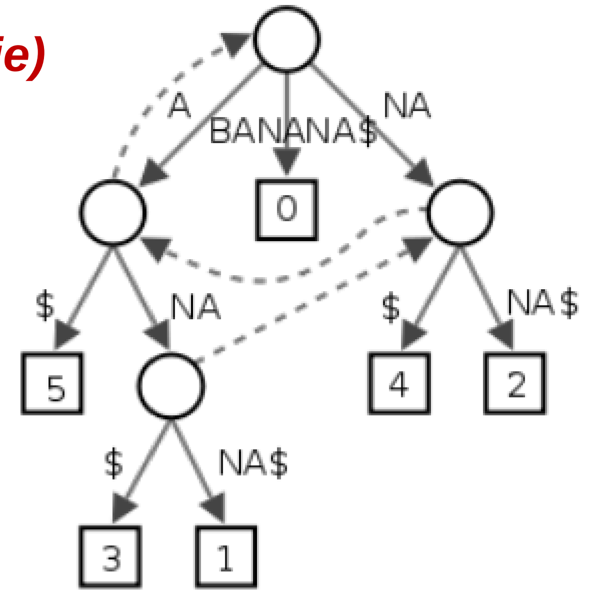

b
Burrows-Wheeler
Reference genome (> 3 gigabases)
Short read
Chr1
Chr2
Chr3
Chr4
ACTCCCGTACTCTAAT
Concatenate into single string
Burrows-Wheeler transform and indexing
Bowtie index (~2 gigabytes)
ACTCCCGTACTCTAAT
Look up 'suffixes' of read
T
AT
AAT
ACTCCCGTACTCTAAT
Hits identify positions in genome where read is found
Convert each hit back to genome location

Burrows-Wheeler transform compresses sequence.

Input	SIX.MIXED.PIXIES.SIFT.SIXTY.PIXIE.DUST.BOXES
Output	TEXYDST.E.IXIXIXXSSMPPS.B..E.S.EUSFXDIIOIIIT

Suffix tree enables fast lookup of subsequences.

(suffix array trie)



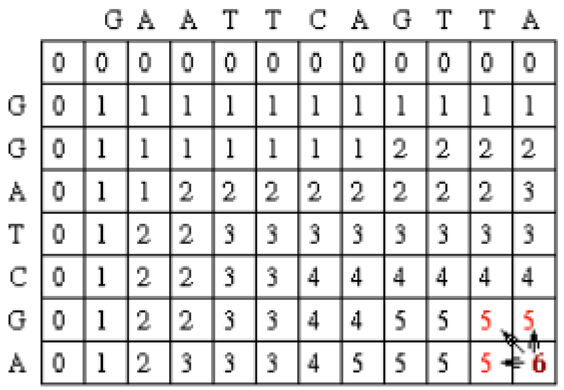
http://en.wikipedia.org/wiki/Suffix_tree

Exact matches at all positions below a node.

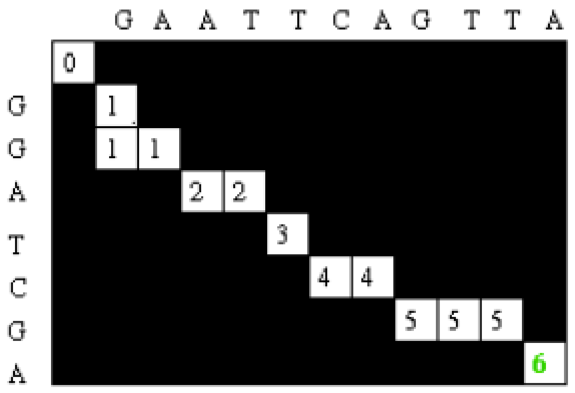
Trapnell, C. & Salzberg, S. L. How to map billions of short reads onto genomes. *Nature Biotech.* **27**, 455–457 (2009).

Alignment via dynamic programming

- Dynamic programming algorithm (Smith-Waterman | Needleman-Wunsch)



		G	A	A	T	T	C	A	G	T	T	A
	0	0	0	0	0	0	0	0	0	0	0	0
G	0	1	1	1	1	1	1	1	1	1	1	1
G	0	1	1	1	1	1	1	1	2	2	2	2
A	0	1	1	2	2	2	2	2	2	2	2	3
T	0	1	2	2	3	3	3	3	3	3	3	3
C	0	1	2	2	3	3	4	4	4	4	4	4
G	0	1	2	2	3	3	4	4	5	5	5	5
A	0	1	2	3	3	3	4	5	5	5	5	6



```
G _ A A T T C A G T T A
|     |   | |   |     |
G G _ A _ T C _ G _ _ A
```

- **Alignment score = Σ**
  - match reward
  - base mismatch penalty
  - gap open penalty
  - gap extension penalty
  - rewards and penalties may be adjusted for quality scores of bases involved

Reference sequence

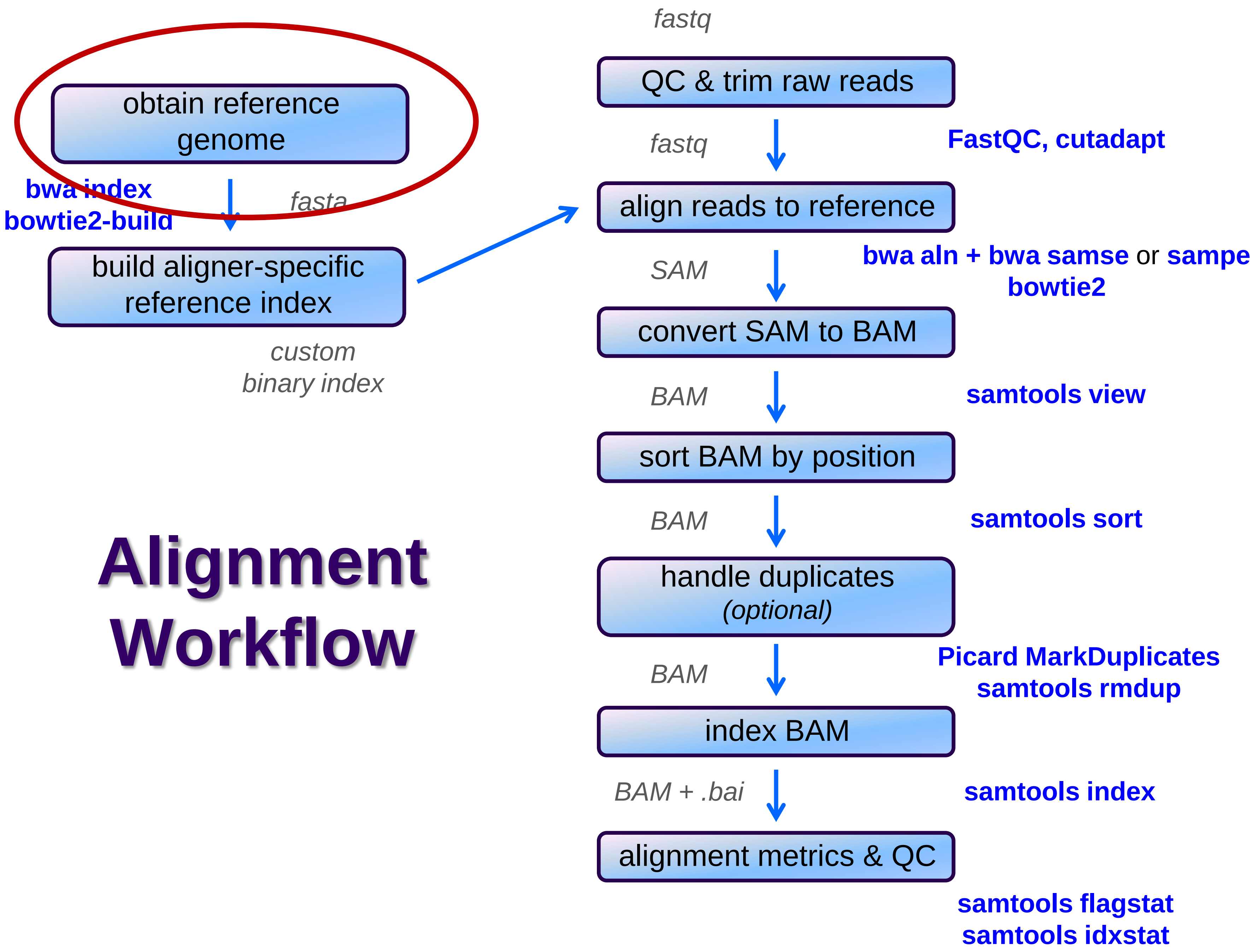
```
ATTTGCGATCGGATGAAGACGAA
|||||||||||||||
ATTTGCGATCGGATGTTGACTTT

ATTTGCGATCGGATGAAGACG..AA
|||||||||||||||XX|||Xii||
ATTTGCGATCGGATGTTGACTTTAA
```

Paired End mapping

- Having paired-end reads improves mapping
  - mapping one read with high confidence anchors the pair
    - even when its mate read alone maps several places equally
- There is an expected insert size distribution based on the DNA fragment library
  - only one of a pair might map ***(singleton/orphan)***
  - both reads can map within the most likely distance range ***(proper pair)***
  - both reads can map but with an unexpected insert size or orientation ***(discordant pair)***
- The insert size is reported in the alignment record for both proper and discordant pairs

Alignment Workflow



Obtaining a reference

- What is a reference?
  - any set of named sequences
    - e.g. names are chromosome names
    - technically refered to as “contigs”
- Assembled genomes
  - Ensembl, UCSC for eukaryotes
    - FASTA files (**.fa, .fasta**)
  - GenBank, NCBI for prokaryotes/microbes
    - Records contain both fasta sequences and annotations
- Any set of sequences of interest, e.g:
  - transcriptome (set of gene sequences)
  - rRNA/tRNA genes (for filtering)
  - miRNA hairpin sequences from miRBase

FASTA format

- FASTA files contain a set of sequence records
  - sequence name line
    - always starts with **>**
      - followed by name and other (optional) descriptive information
  - one or more sequence line(s)
    - never starts with **>**
- Mitochondrial chromosome sequence, human hg19

```
>chrM
GATCACAGGTCTATCACCCTATTAACCACTCACGGGAGCTCTCCATGCAT
TTGGTATTTTCGTCTGGGGGGTGTGCACGCGATAGCATTGCGAGACGCTG
GAGCCGGAGCACCCTATGTCGCAGTATCTGTCTTTGATTCCTGCCTCATT ...
```

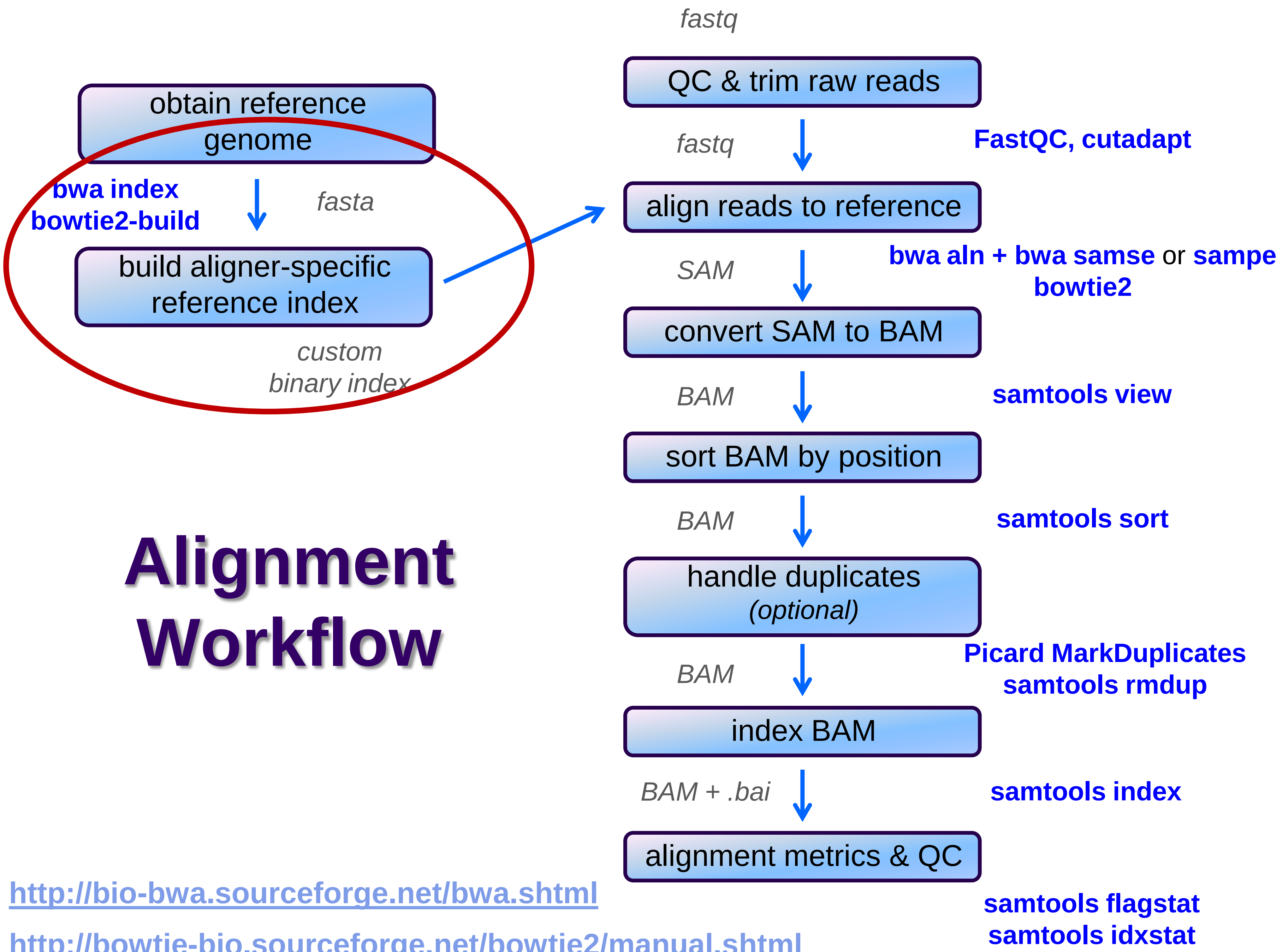
- Let-7e miRNA, human miRBase v21

```
>hsa-let-7e MI0000066 Homo sapiens let-7e stem-loop
CCCGGGCTGAGGTAGGAGGTTGTATAGTTGAGGAGGACACCCAAGGAGATCACTATACGG
CCTCCTAGCTTTCCCCAGG
```

Reference considerations

- Is it appropriate to your study?
  - close enough to your species? complete?
- Does it contain repeats? What kinds?
  - know this up front or you will be confused
- From which source? And which version?
  - UCSC hg19 vs Ensembl GRCh37
- What annotations exist?
  - references lacking feature annotations are much more challenging
- Watch out for sequence name issues!
  - sequence names are different between UCSC/Ensembl
    - e.g. “chr12” vs “12”
  - annotation sequence names must match names in your reference!
  - long sequence names can cause problems
    - rename: `>hsa-let-7e_MI0000066_Homo_sapiens_let-7e stem-loop`
    - to: `>hsa-let-7e`

Alignment Workflow



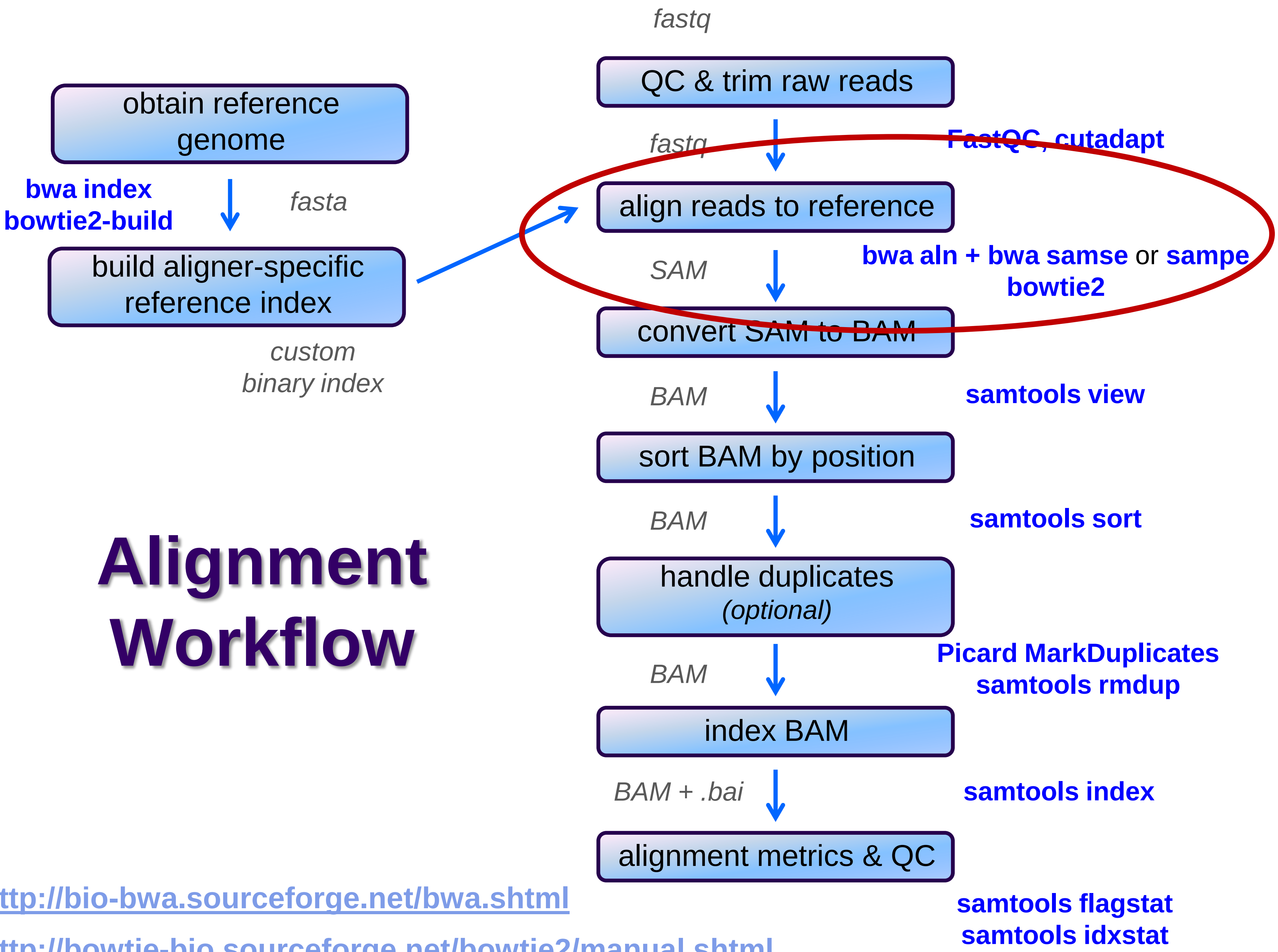
http://bio-bwa.sourceforge.net/bwa.shtml

http://bowtie-bio.sourceforge.net/bowtie2/manual.shtml

Building a reference index

- Index format is specific to each aligner
  - may take several hours to build
    - but you build each index once, use for multiple alignments
- Input:
  - a FASTA file
- Output:
  - a number of binary files the aligner will use
- Best practice:
  - build each index in its own appropriately named directory, e.g.
    - **refs/bowtie2/UCSC/hg19**
    - **refs/bwa/Ensembl/GRCh37**

Alignment Workflow



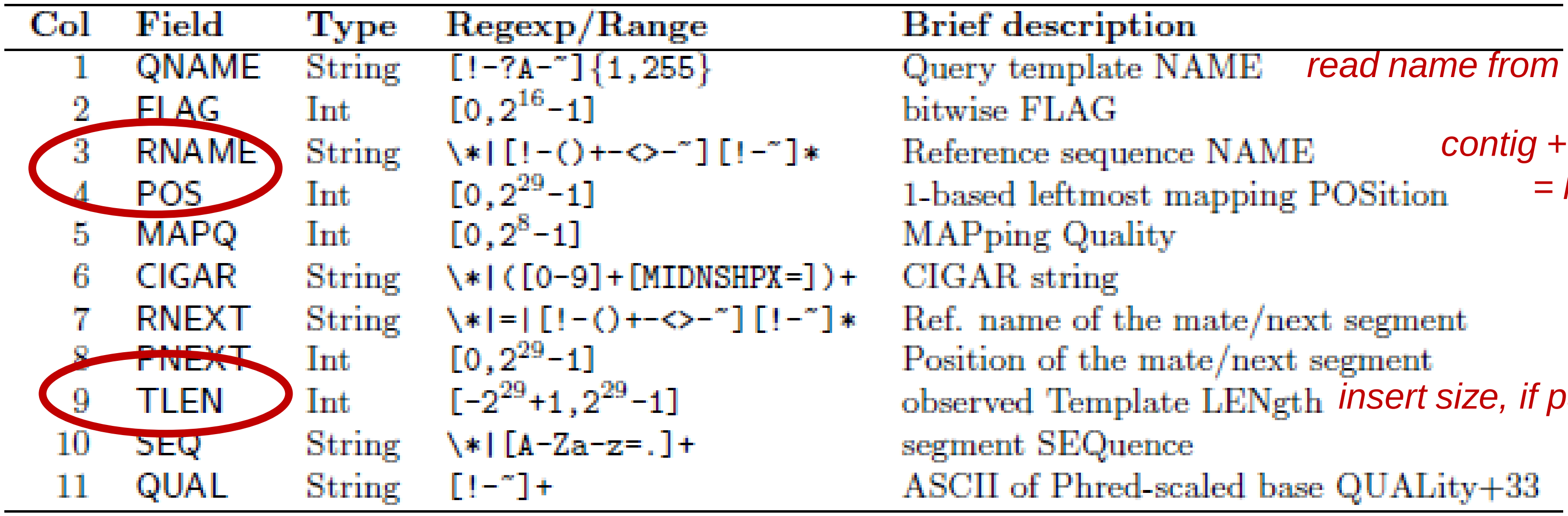
http://bio-bwa.sourceforge.net/bwa.shtml

http://bowtie-bio.sourceforge.net/bowtie2/manual.shtml

SAM file format

- Aligners take FASTQ as input, output alignments in SAM format
  - community file format that describes how reads align to a reference
    - can also include unmapped reads
  - the Bible: http://samtools.github.io/hts-specs/SAMv1.pdf
- SAM file consists of:
  - a header
    - includes reference sequence names and lengths
  - alignment records, one for each sequence read
    - alignments for R1 and R2 reads have separate records, with fields that refer to the mate
    - 11 fixed fields + extensible-format **key:type:value** tuples

SAM file format
Fixed fields



Col	Field	Type	Regexp/Range	Brief description
1	QNAME	String	`[!-?A-~]{1,255}`	Query template NAME
2	FLAG	Int	$[0,2^{16}-1]$	bitwise FLAG
3	RNAME	String	`\*|[!-()+-<>-~][!-~]*`	Reference sequence NAME
4	POS	Int	$[0,2^{29}-1]$	1-based leftmost mapping POSition
5	MAPQ	Int	$[0,2^{8}-1]$	MAPping Quality
6	CIGAR	String	`\*|([0-9]+[MIDNSHPX=])+`	CIGAR string
7	RNEXT	String	`\*|=|[!-()+-<>-~][!-~]*`	Ref. name of the mate/next segment
8	PNEXT	Int	$[0,2^{29}-1]$	Position of the mate/next segment
9	TLEN	Int	$[-2^{29}+1,2^{29}-1]$	observed Template LENgth
10	SEQ	String	`\*|[A-Za-z=.]+`	segment SEQuence
11	QUAL	String	`[!-~]+`	ASCII of Phred-scaled base QUALity+33

read name from fastq

contig + start
= locus

insert size, if paired

```
SRR030257.264529     99  NC_012967    1521    29   34M2S    =    1564    79
    CTGGCCATTATCTCGGTGGTAGGACATGGCATGCCC
    AAAAAA;AA;AAAAAA??A%.;?&'3735',()0*,
    XT:A:M  NM:i:3  SM:i:29  AM:i:29  XM:i:3  XO:i:0  XG:i:0  MD:Z:23T0G4T4

SRR030257.2669090    147 NC_012967    1521    60   36M      =    1458    -99
    CTGGCCATTATCTCGGTGGTAGGTGATGGTATGCGC
    <<9:<<AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
    XT:A:U  NM:i:0  SM:i:37  AM:i:37  X0:i:1  X1:i:0  XM:i:0  XO:i:0  XG:i:0  MD:Z:36
```

SAM format – Bitwise flags

Bit	Description	
0x1	template having multiple segments in sequencing	*1 = part of a read pair*
0x2	each segment properly aligned according to the aligner	*1 = "properly" paired*
0x4	segment unmapped	*1 = read did **not** map*
0x8	next segment in the template unmapped	*1 = mate did **not** map*
0x10	SEQ being reverse complemented	*1 = minus strand read*
0x20	SEQ of the next segment in the template being reversed	*1 = mate on minus strand*
0x40	the first segment in the template	*1 = R1 read*
0x80	the last segment in the template	*1 = R2 read*
0x100	secondary alignment	*1 = secondary possible hit*
0x200	not passing quality controls	
0x400	PCR or optical duplicate	*1 = marked as duplicate*

```
SRR030257.264529    99   NC_012967   1521   29   34M2S   =   1564   79
    CTGGCCATTATCTCGGTGGTAGGACATGGCATGCCC
    AAAAAA;AA;AAAAAA??A%.;?&'3735',()0*,
    XT:A:M  NM:i:3  SM:i:29  AM:i:29  XM:i:3  XO:i:0  XG:i:0  MD:Z:23T0G4T4

SRR030257.2669090   147  NC_012967   1521   60   36M     =   1458   -99
    CTGGCCATTATCTCGGTGGTAGGTGATGGTATGCGC
    <<9:<<AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
    XT:A:U  NM:i:0  SM:i:37  AM:i:37  X0:i:1  X1:i:0  XM:i:0  XO:i:0  XG:i:0  MD:Z:36
```

```
Decimal     Hex
     99   = 0x63
   = 64   = 0x40
   + 32   + 0x20
   +  2   + 0x02
   +  1   + 0x01

    147   = 0x93
  = 128   = 0x80
  +  16   + 0x10
  +   2   + 0x02
  +   1   + 0x01
```

http://broadinstitute.github.io/picard/explain-flags.html

Sometimes a CIGAR is just a way of describing how a read is aligned…

```
Ref   CTGGCCATTATCTC--GGTGGTAGGACATGGCATGCCC
Read         aaATGTCGCGGTG.TAGGAggatcc
```

2S5M2I4M1D5M6S

	Op	BAM	Description
	M	0	alignment match (can be a sequence match or mismatch)
	I	1	insertion to the reference
	D	2	deletion from the reference
*	N	3	skipped region from the reference
	S	4	soft clipping (clipped sequences present in SEQ)
*	H	5	hard clipping (clipped sequences NOT present in SEQ)
*	P	6	padding (silent deletion from padded reference)
*	=	7	sequence match
*	X	8	sequence mismatch

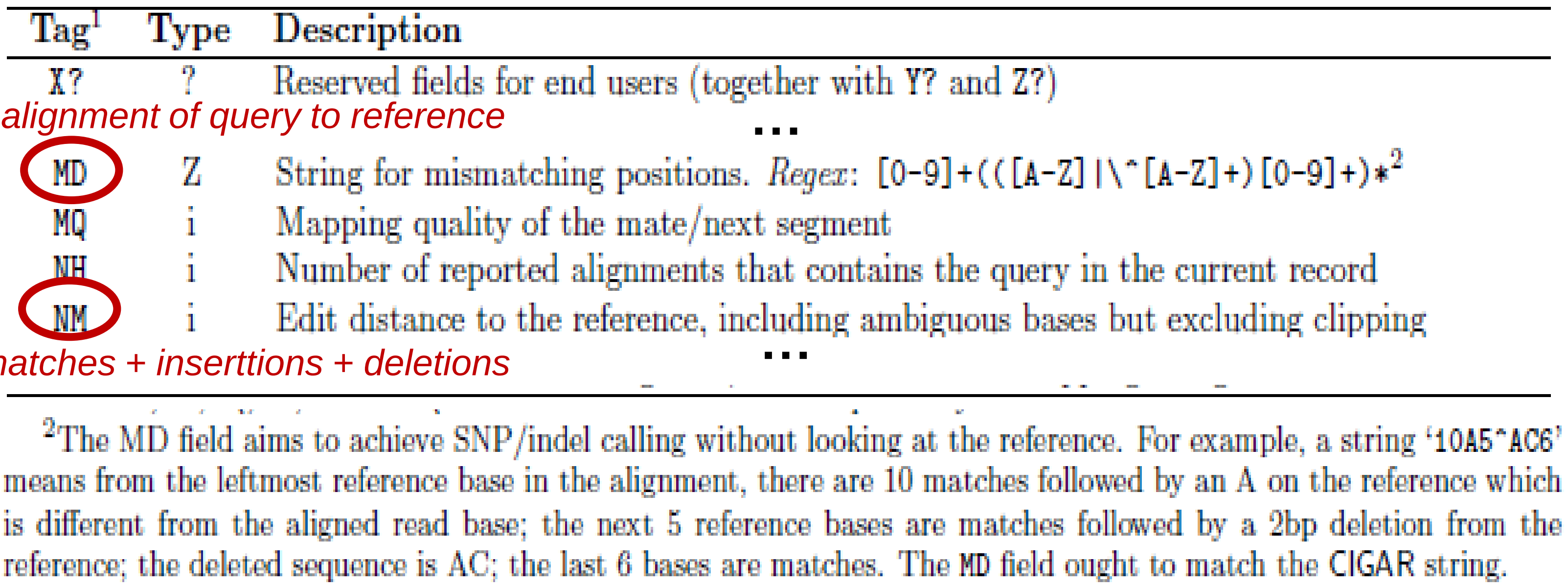
“N” indicates splicing event in tophat RNAseq BAMs

*Rarer / newer

CIGAR = "Concise Idiosyncratic Gapped Alignment Report"

SAM file format

key:type:value tuples



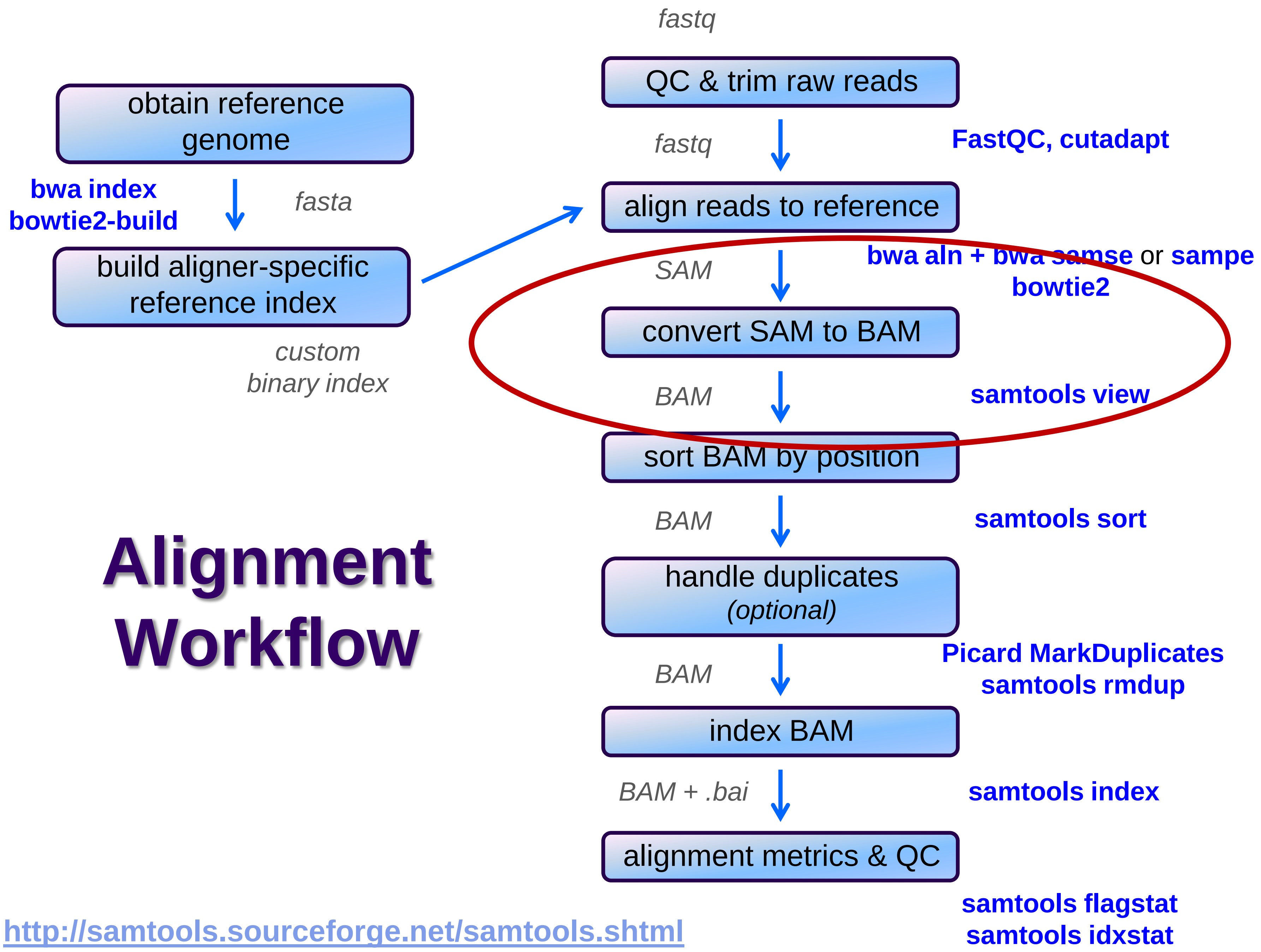
Tag[1]	Type	Description
X?	?	Reserved fields for end users (together with Y? and Z?)
		…
MD	Z	String for mismatching positions. *Regex*: `[0-9]+(([A-Z]|\^[A-Z]+)[0-9]+)*`[2]
MQ	i	Mapping quality of the mate/next segment
NH	i	Number of reported alignments that contains the query in the current record
NM	i	Edit distance to the reference, including ambiguous bases but excluding clipping
		…

details alignment of query to reference

# mismatches + inserttions + deletions

[2] The MD field aims to achieve SNP/indel calling without looking at the reference. For example, a string '`10A5^AC6`' means from the leftmost reference base in the alignment, there are 10 matches followed by an A on the reference which is different from the aligned read base; the next 5 reference bases are matches followed by a 2bp deletion from the reference; the deleted sequence is AC; the last 6 bases are matches. The `MD` field ought to match the CIGAR string.

```
SRR030257.264529     99   NC_012967    1521     29   34M2S    =     1564     79
     CTGGCCATTATCTCGGTGGTAGGACATGGCATGCCC
     AAAAAA;AA;AAAAAA??A%.;?&'3735',()0*,
     XT:A:M  NM:i:3  SM:i:29  AM:i:29  XM:i:3  XO:i:0  XG:i:0  MD:Z:23T0G4T4
```



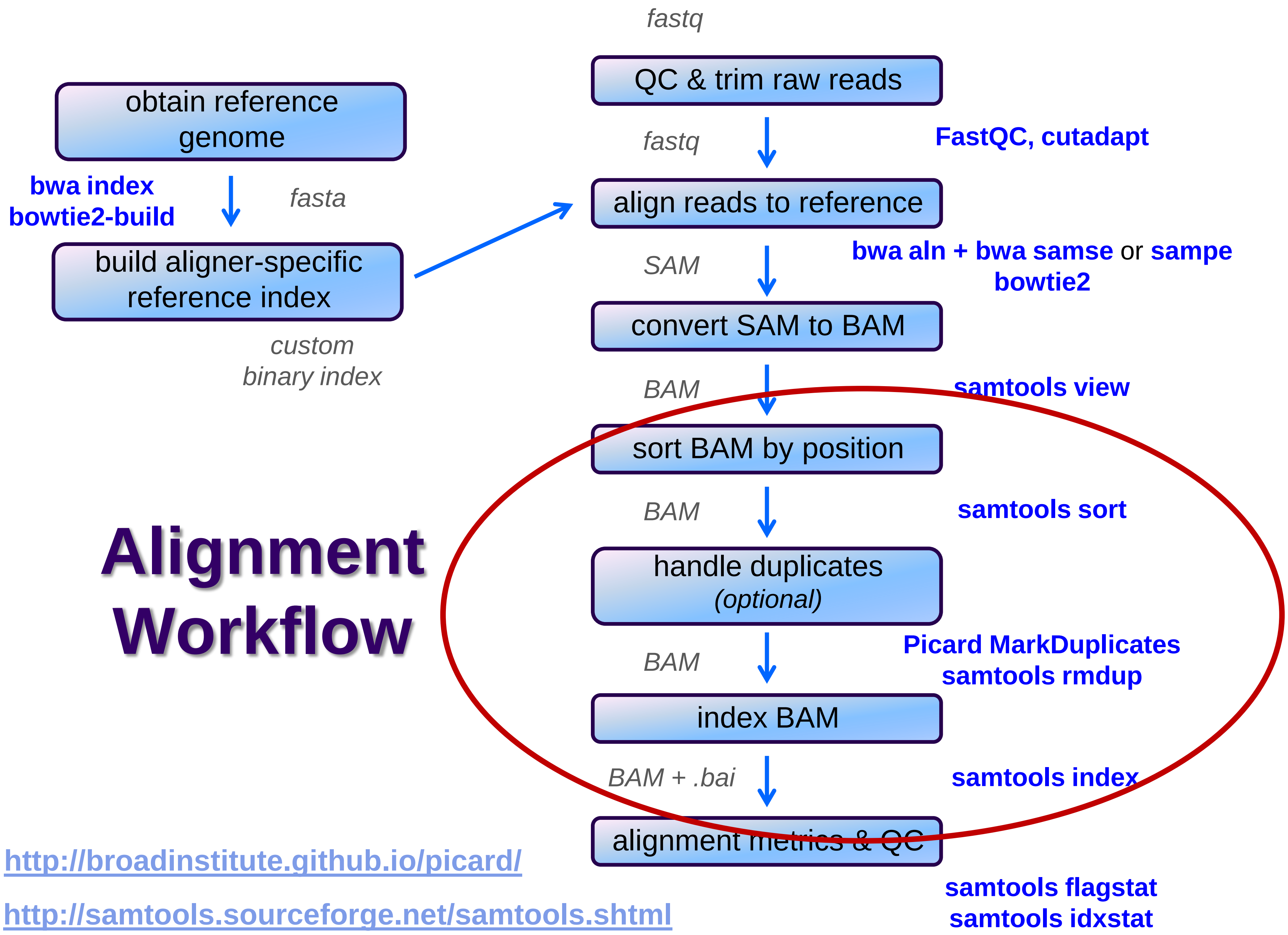
Alignment Workflow

http://samtools.sourceforge.net/samtools.shtml

SAM / BAM files

- SAM and BAM are two forms of the same data
  - SAM – Sequence Alignment Map
    - plain text format
  - BAM – Binary Alignment Map
    - ***same data*** in a custom compressed (**gzip**'d) format
- Differences
  - BAMs are ***much*** smaller than SAM files due to compression
  - BAM files support fast random access; SAM files do not
    - requires the BAM file to be indexed
  - most tools support BAM format and may require indexing
- Best practices
  - remove intermediate SAM and BAM files created during alignment and only save the final sorted, indexed BAM
  - keep your alignment artifacts (BAM, statistics files, log files) separate from the original FASTQ files
    - alignments can be easily re-generated; raw sequences cannot

Alignment Workflow



http://broadinstitute.github.io/picard/

http://samtools.sourceforge.net/samtools.shtml

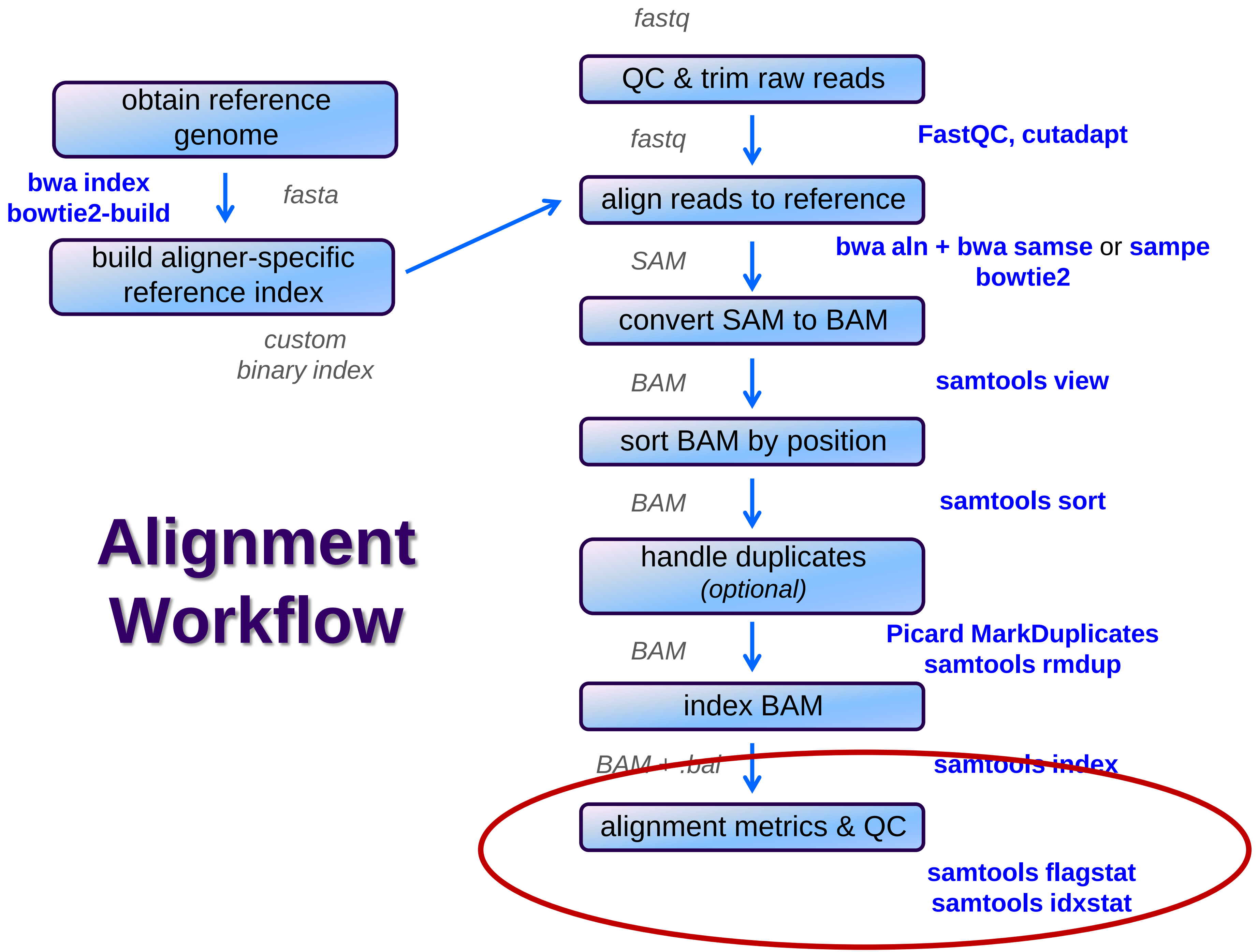
Sorting / indexing BAM files

- SAM created by aligner contains read records in ***name order***
  - same order as read names in the input FASTQ file
    R1, R2 have adjacent SAM records
  - SAM → BAM conversion does not change the name-sorted order
- Sorting BAM puts records in ***locus order***
  - by contig name then start position
    - contig name order given in SAM/BAM header
    - based on order of sequences in FASTA used to build reference
  - sorting is *very* compute and I/O intensive
    - can take several hours for large BAM
- Indexing a locus-sorted BAM allows fast random access
  - creates a binary alignment index file (**.bai**)
  - quite fast

Handling Duplicates

- Optional step, but very important for many protocols
- Definition of duplicates:
  - single end reads or singleton/discordant alignment
    - alignments start at the same location and have the same length
  - properly paired reads
    - pairs have same external coordinates
- Two choices for handling:
  - **samtools rmdup** – ***removes*** duplicates entirely
    - faster, but data is lost
    - does not properly handle data from multiple lanes
  - **Picard MarkDuplicates** – ***flags*** duplicates only
    - slower, but all alignments are retained
    - alignments from different lanes/replicates are handled properly
  - both tools are quirky in their own ways

Alignment Workflow



Alignment metrics

- **samtools flagstat**
  - simple statistics based on alignment record flag values
    - total sequences (R1+R2), total mapped
    - number properly paired
    - number of duplicates (0 if duplicates were not marked)

```
30146426 + 0 in total (QC-passed reads + QC-failed reads)
13532165 + 0 duplicates
28804693 + 0 mapped (95.55%:-nan%)
30146426 + 0 paired in sequencing
15073213 + 0 read1
15073213 + 0 read2
28546786 + 0 properly paired (94.69%:-nan%)
28712992 + 0 with itself and mate mapped
91701 + 0 singletons (0.30%:-nan%)
64973 + 0 with mate mapped to a different chr
50382 + 0 with mate mapped to a different chr (mapQ>=5)
```

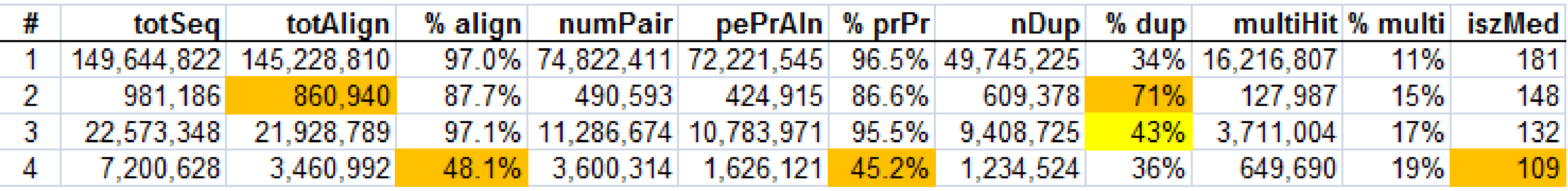
Computing average insert size

- Needed for RNAseq alignment using **tophat**
- Simple **awk** script that computes average insert size for a BAM
  - **-F 0x4** filter to **samtools view** says only consider mapped reads
    - technically “not unmapped”
  - the **-f 0x2** filter says consider only properly paired reads
    - they have reliable “insert size" values in column 9
  - insert size values are negative for minus strand reads
    - can ignore because each proper pair should have one plus and one minus strand alignment

```
samtools view -F 0x4 -f 0x2 my_pe_data.bam | awk \
  'BEGIN{ FS="\t"; sum=0; nrec=0; }
   { if ($9 > 0) {sum += $9; nrec++;} }
   END{ print sum/nrec; }'
```

Interpreting alignment metrics

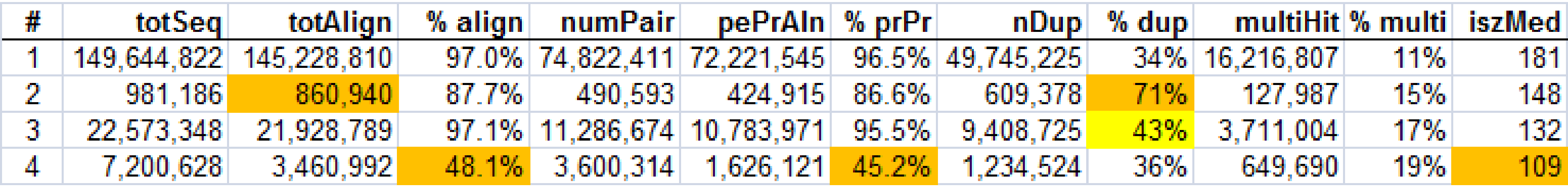
- Table below is taken from a spreadsheet I keep on all our alignments
  - all are yeast paired-end read datasets from ChIP-seq experiments
- Alignment rates
  - samples 1-3 have excellent alignment rates & good rates of proper pairing
  - sample 4
    - has an unusually low alignment rate for a ChIP-seq dataset
    - has a median insert size of only 109, and these were un-trimmed 50 bp reads
    - could 3' adapter contamination be affecting the alignment rate?
      - try re-aligning the sequences after trimming, say to 30 bases
      - see if the alignment rate improves



#	totSeq	totAlign	% align	numPair	pePrAln	% prPr	nDup	% dup	multiHit	% multi	iszMed
1	149,644,822	145,228,810	97.0%	74,822,411	72,221,545	96.5%	49,745,225	34%	16,216,807	11%	181
2	981,186	860,940	87.7%	490,593	424,915	86.6%	609,378	71%	127,987	15%	148
3	22,573,348	21,928,789	97.1%	11,286,674	10,783,971	95.5%	9,408,725	43%	3,711,004	17%	132
4	7,200,628	3,460,992	48.1%	3,600,314	1,626,121	45.2%	1,234,524	36%	649,690	19%	109

Interpreting alignment metrics

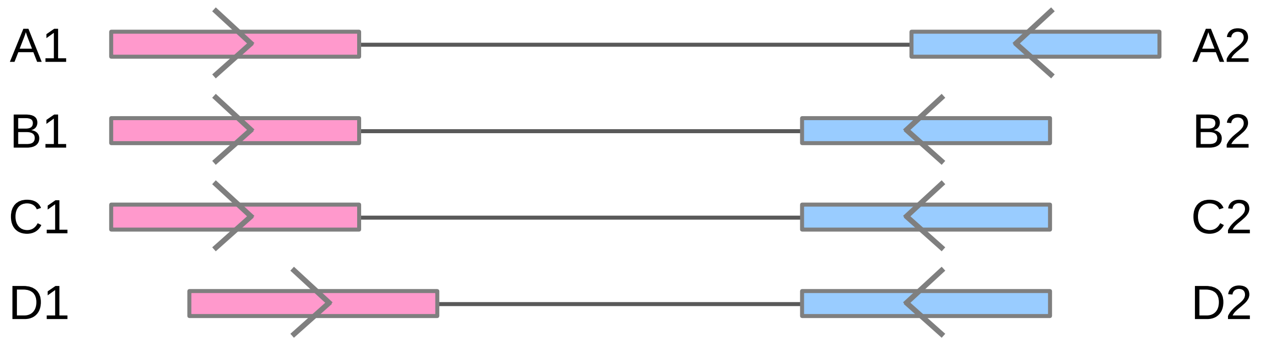
- Duplication rates
  - sample 1 is incredibly deeply sequenced (yeast genome only ~12 Mbase)
    - has a *very* low duplication rate considering
    - turns out this is a control dataset (Mock ChIP), so is a great control to use (wonderfully complex!)
  - sample 2 is not very deeply sequenced but has a high duplication rate (71%)
    - subtracting duplicates from total aligned leaves only ~250,000 non-dup reads
      - not enough for further analysis (prefer 500,000+)
  - sample 3 has reasonable sequencing depth with substantial duplication (43%)
    - still leaves plenty of non-duplicate reads (> 12 million)



#	totSeq	totAlign	% align	numPair	pePrAln	% prPr	nDup	% dup	multiHit	% multi	iszMed
1	149,644,822	145,228,810	97.0%	74,822,411	72,221,545	96.5%	49,745,225	34%	16,216,807	11%	181
2	981,186	860,940	87.7%	490,593	424,915	86.6%	609,378	71%	127,987	15%	148
3	22,573,348	21,928,789	97.1%	11,286,674	10,783,971	95.5%	9,408,725	43%	3,711,004	17%	132
4	7,200,628	3,460,992	48.1%	3,600,314	1,626,121	45.2%	1,234,524	36%	649,690	19%	109

Read vs fragment duplication

- Consider the 4 fragments below
  - 4 R1 reads (pink), 4 R2 reads (blue)
- Duplication when only 1 end considered
  - A1, B1, C1 have identical sequences, D1 different
    - 2 unique + 2 duplicates = 50% duplication rate
  - B2, C2, D2 have identical sequences, A2 different
    - 2 unique + 2 duplicates = 50% duplication rate
- Duplication when both ends considered
  - fragments B and C are duplicates (same external sequences)
    - 3 unique + 1 duplicate = 25% duplication rate



Alignment wrap up

- Many tools involved
  - choose one (or two) and learn their options well
- Many steps are involved in the full alignment workflow
  - important to go through manually a few times for learning
    - but gets tedious quickly!
  - best practice
    - automate series of complex steps by wrapping into a ***pipeline script***
    - e.g. **bash**, **perl** or **python** script
- For UT folks with TACC accounts
  - I have a set of TACC-aware alignment pipeline scripts
    - plus a set of pre-build reference indexes

Final thoughts

- Good judgement comes from experience

  unfortunately…

- Experience comes from bad judgement!

- So go get started making your 1st 1,000 mistakes….