5 - Data QC and Preparation

Wednesday afternoon

Bernardo J. Clavijo
Richard Smith-Unna
Gonzalo Garcia

TGAC

Living on a biased environment

Review Article

Library preparation methods for next-generation sequencing: Tone down the bias

Erwin L. van Dijk[a,*], *Yan Jaszczyszyn*[b], *Claude Thermes*[a]

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Table 1 – Sources of bias in DNA-seq library preparation and suggestions for improvement.

Description	Suggestions for improvement	Reference
Fragmentation of chromatin Bias due to more efficient sonication of euchromatin than heterochromatin	Second fragmentation after IP to concentrate fragments in the optimal size range	Mokry et al 2010 [5]
Size selection Bias due to heating agarose gel slices in chaotropic salt buffer	Melt agarose gel slices at room temperature	Quail et al 2008 [6]
PCR Bias due to preferential amplification of templates with neutral GC%	1. Use Kapa HiFi rather than Phusion polymerase. 2. For AT-rich genomes, use lower extension temperatures and/or use the PCR additive TMAC 3. For GC-rich genomes, use extended denaturation times and/or use the additive betaine 4. For the amplification of minute quantities of genomic DNA (single cell), use MDA rather than PCR	Quail et al 2012 [12] Oyola et al 2012 [13] Aird et al 2011[3] Dabney et al 2012 [14]

Table 2 – Sources of bias in RNA-seq library prep and suggestions for improvement.

Description	Suggestion for improvement	Reference
RNA extraction using Trizol: selective loss of GC poor or highly structured small RNAs at low RNA concentrations	Use similar RNA concentrations for samples that are to be compared or avoid Trizol extraction altogether. Use alternative protocols such as the MirVana miRNA isolation kit.	Kim et al., 2012 [43]
Ribosomal RNA (rRNA) depletion/ mRNA enrichment: bias due to exonuclease targeting partially degraded mRNAs	Use subtractive hybridization rather than exonuclease treatment to deplete rRNAs	He et al., 2010 [26]
RNA fragmentation by RNase III: not completely random, leading to reduced complexity	Use chemical treatment (e.g. zinc) rather than RNAse III for RNA fragmentation	Wery et al., 2013 [28]
Random hexamer priming bias: not completely random	A read count reweighing scheme was proposed that adjusts for the bias and makes the distribution of reads more uniform	Hansen et al., 2010 [29]
Reverse transcription: antisense artefacts (especially in the SMART and the NSR methods)	Add actinomycin D to the reaction (not possible for the SMART method)	Perocchi et al., 2007 [38] Levin et al., 2010 [25]
Adapter ligation bias due to substrate preferences of T4 RNA ligases	Use adapters with random nucleotides at the extremities to be ligated.	Jayaprakash et al., 2011 [34] Sorefan et al., 2013 [31] Sun et al., 2011 [35] Zhuang et al., 2012 [33]
Reduced ligation efficiency due to RNA modifications	If necessary, perform the appropriate enzymatic treatments to generate a 5' monophosphate and a 3' OH. For RNAs with a 2'O-methyl modification use adjusted ligation reaction conditions	Munafo and Robb, 2010 [46]

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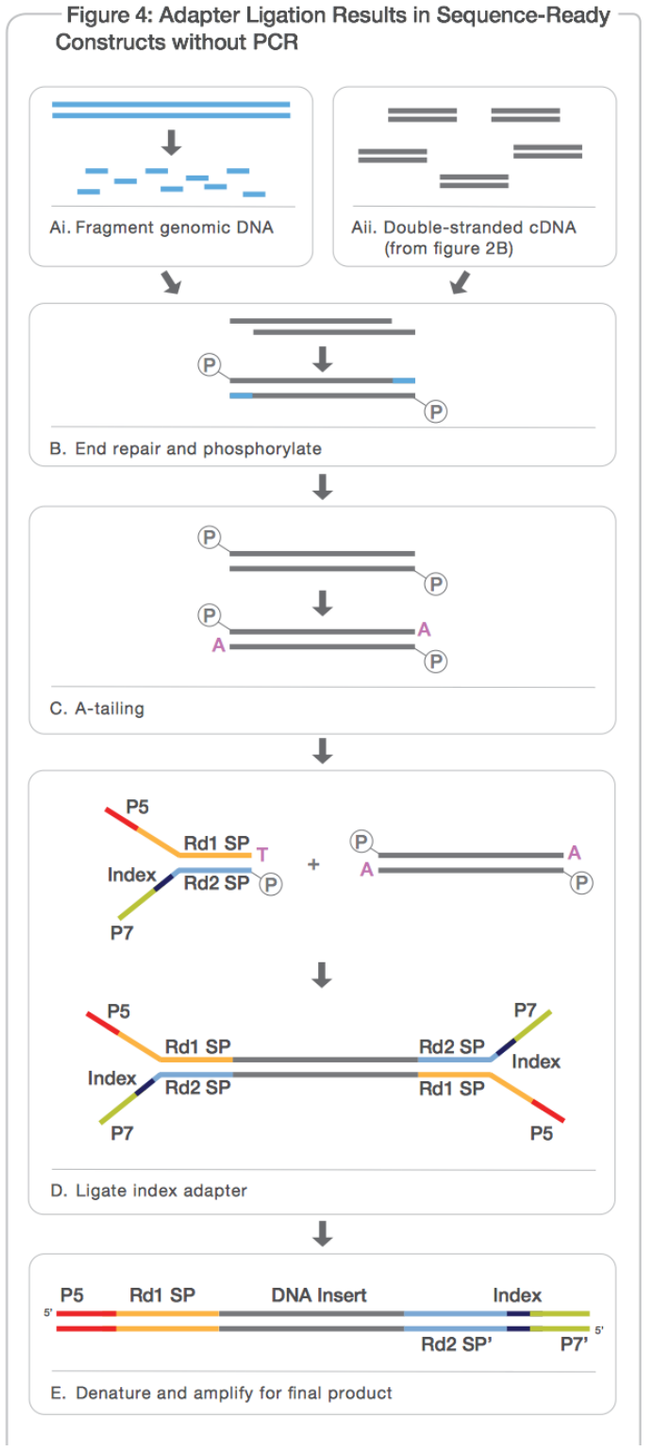
Sample and library preparation: a source of bias



- DNA/RNA extraction techniques have bias:
  - And sample quality limit sequencing!
- Samples are never pure.
- PCR generates further bias.
- No chemical reaction is perfect, nor complete.
- You can learn what your typical biases are:
  - Assess them.
  - Take their impact into account.
  - Try to get better data produced.

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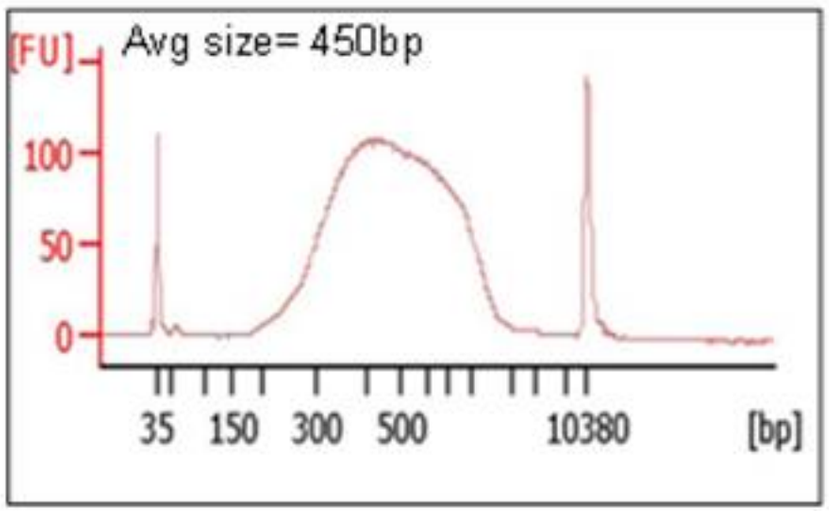
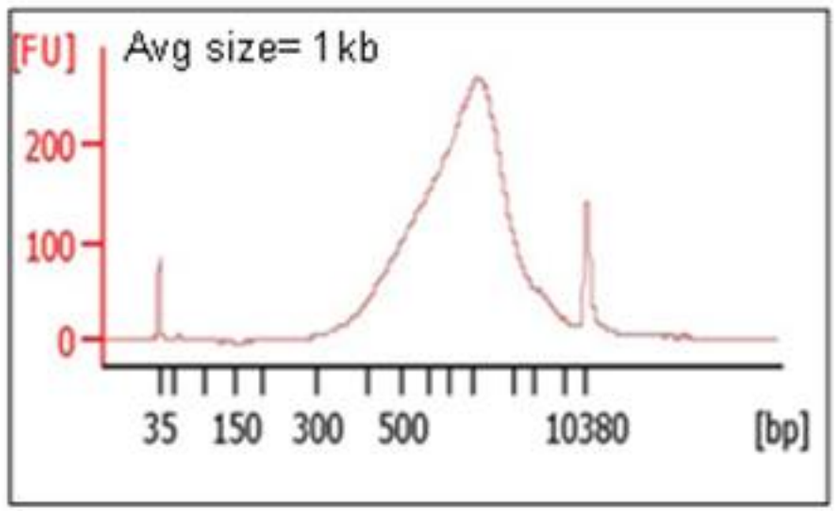
Do not neglect the QC data from the lab





- Concentrations.
- Sample contamination.
- Fragment sizes!

Re: ENQ-[redacted]
Bernardo Clavijo (TGAC)
Sent: Monday, 11 May 2015 14:21
To: [redacted] (JIC); [redacted] (TGAC); [redacted] (TGAC)

Great guys! As you know I love to offload my work to the lab, so basically I make you generate fantastic data so I get to just press a button and deliver a nice result.

Much appreciated,

bj

From: "[redacted] (JIC)" <[redacted]@jic.ac.uk>
Date: Monday, 11 May 2015 14:19
To: "[redacted] (TGAC)" <[redacted]@tgac.ac.uk>, Bernardo Clavijo <bernardo.clavijo@tgac.ac.uk>, "[redacted] (TGAC)" <[redacted]@tgac.ac.uk>
Subject: RE: ENQ-1004

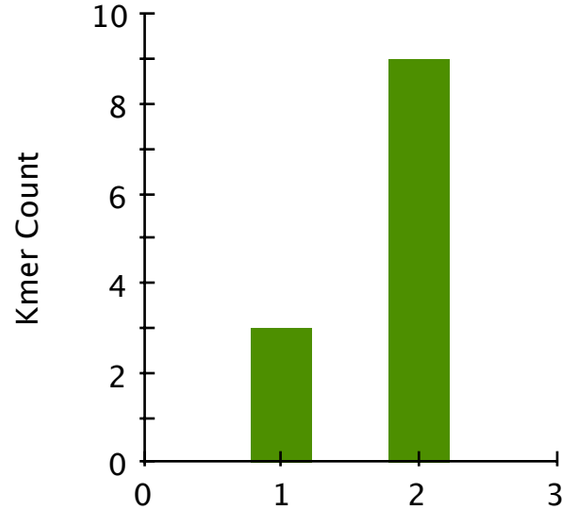
Mine too! Will make more DNA this week, we still have [redacted]

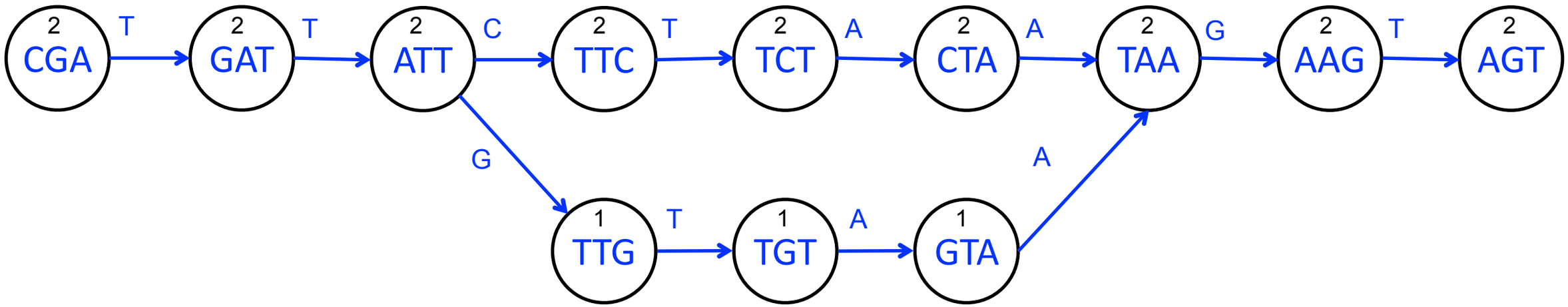
Cheers
[redacted]

Counting kmers

```
>seq1
TTCTAAGT
>seq2
CGATTCTA
```

```
>seq3
CGATTGTAAGT
```





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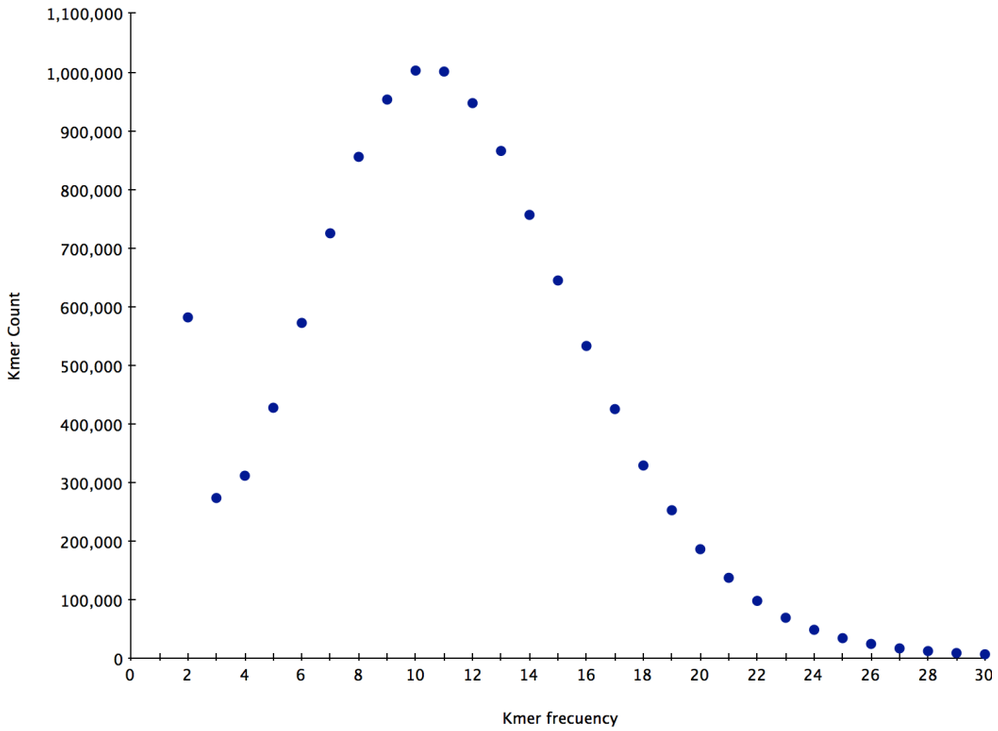
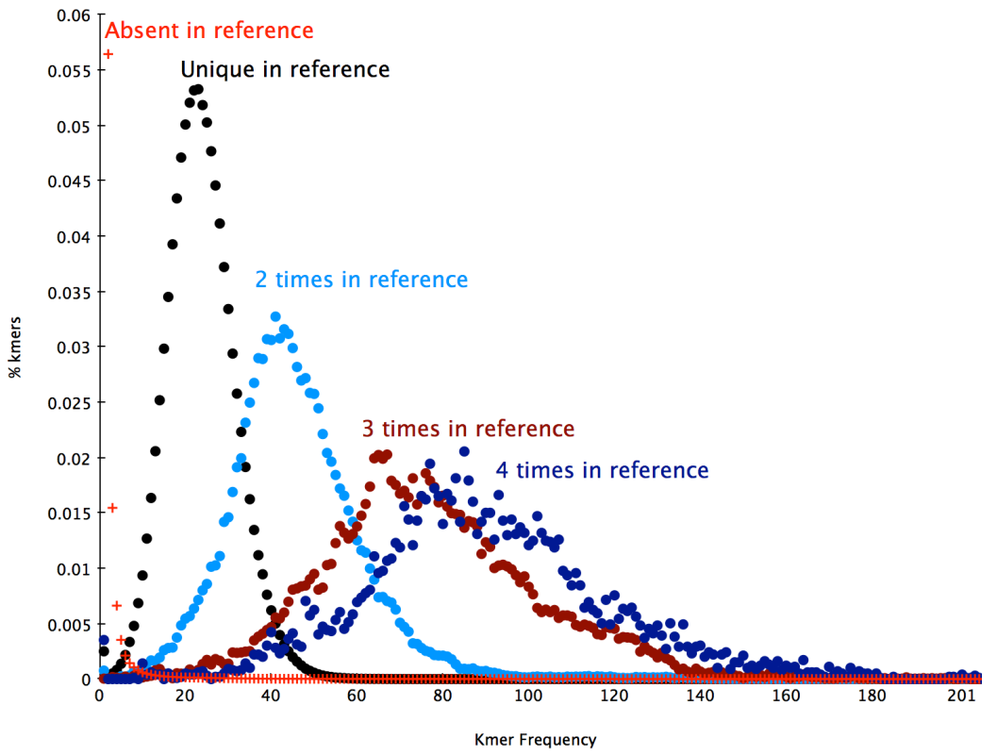
The kmer spectrum… and its dissection.





- We typically use jellyfish to kmer-count.
- You can “read”:
  - Kmer coverage.
  - Genome size.
  - Errors vs. Good kmers.
- Comparing different spectrum (KAT):
  - Is a reference free library assessment.
  - Runs fast.
  - Gives at least a better vs. worse result.

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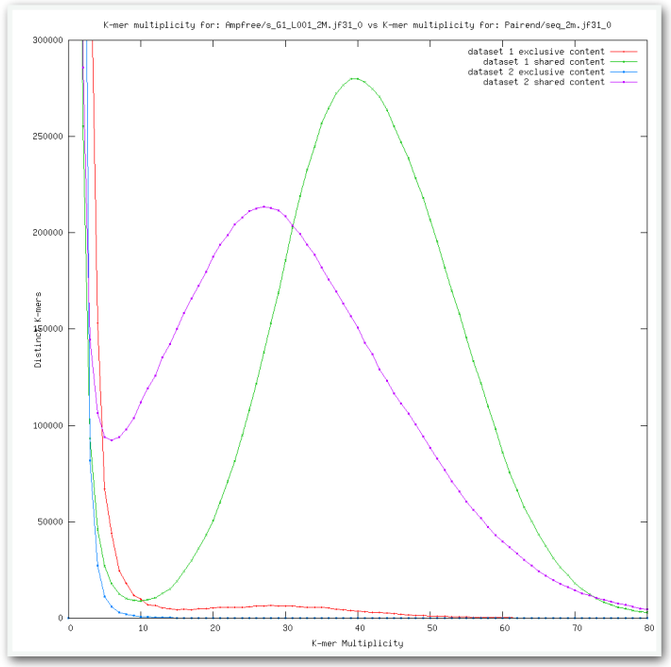
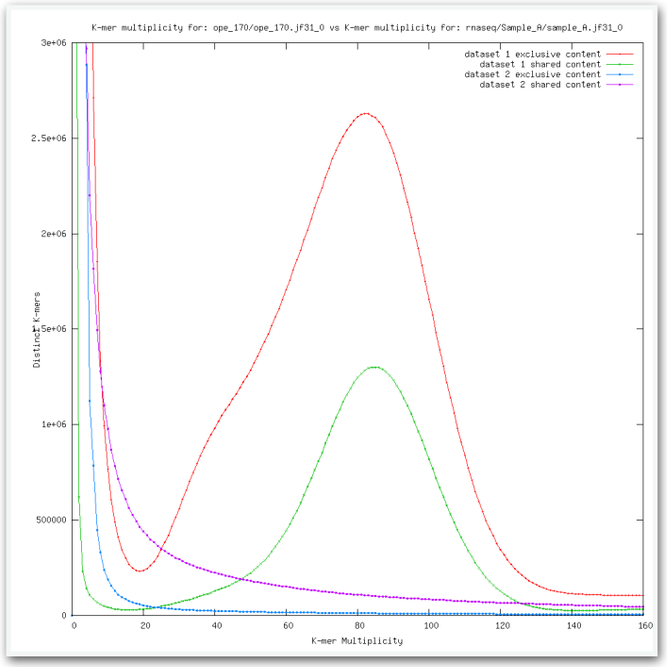
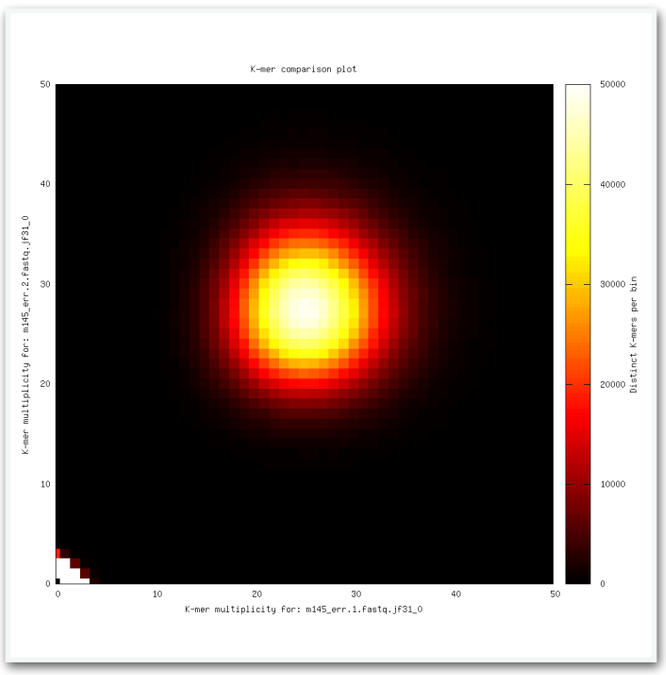
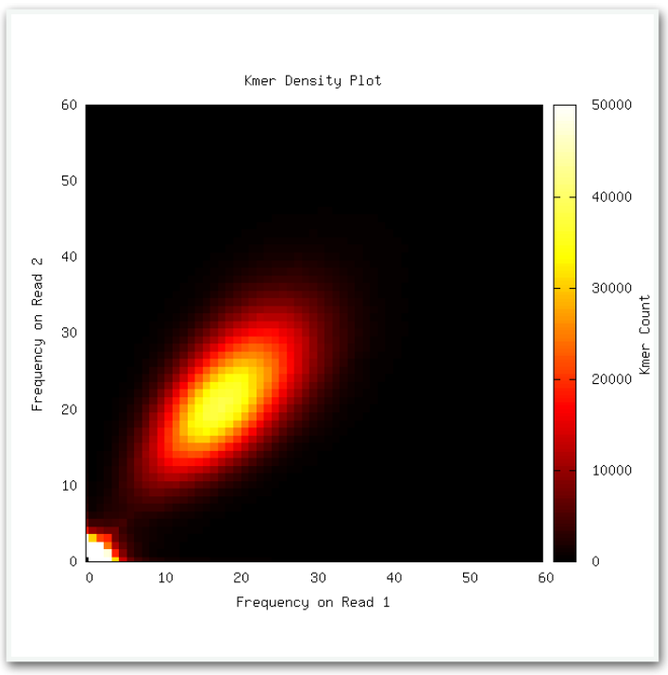
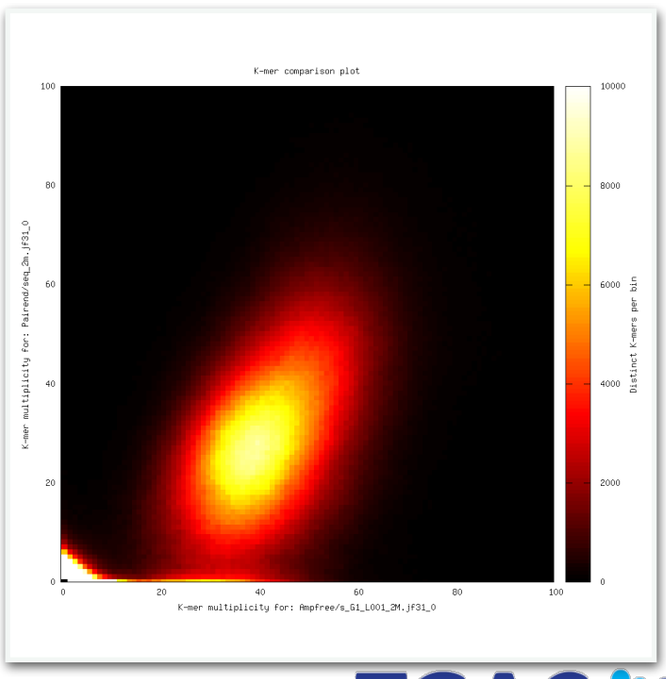
Internal and external library coherence (KAT)



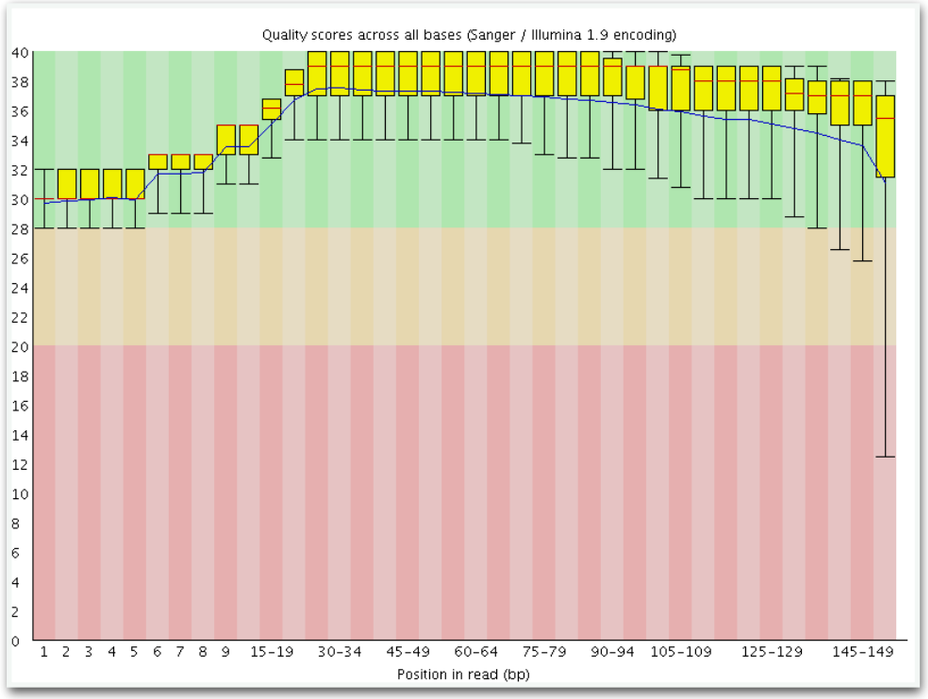


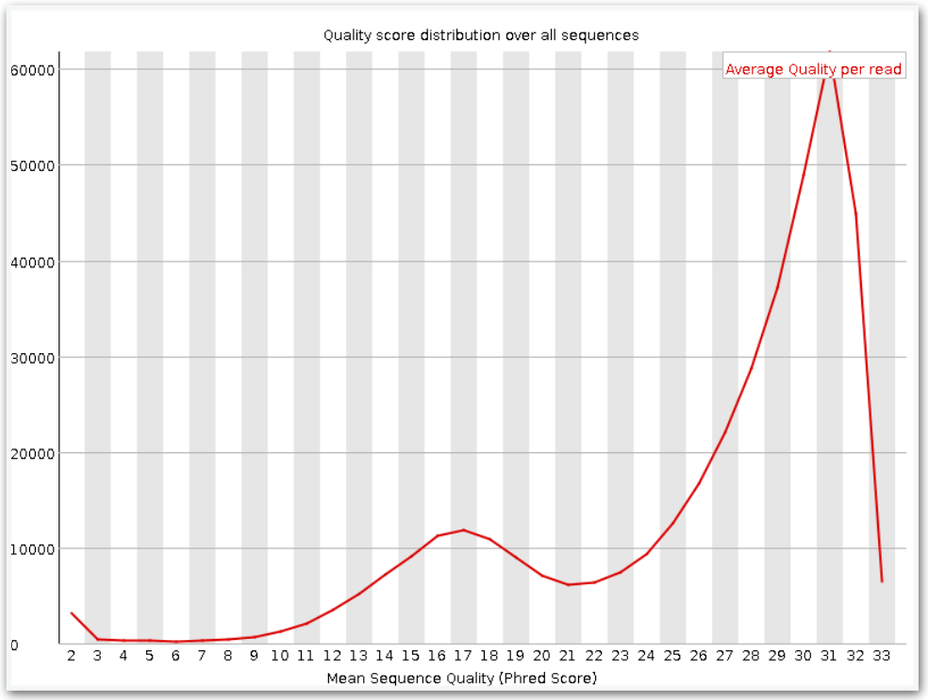


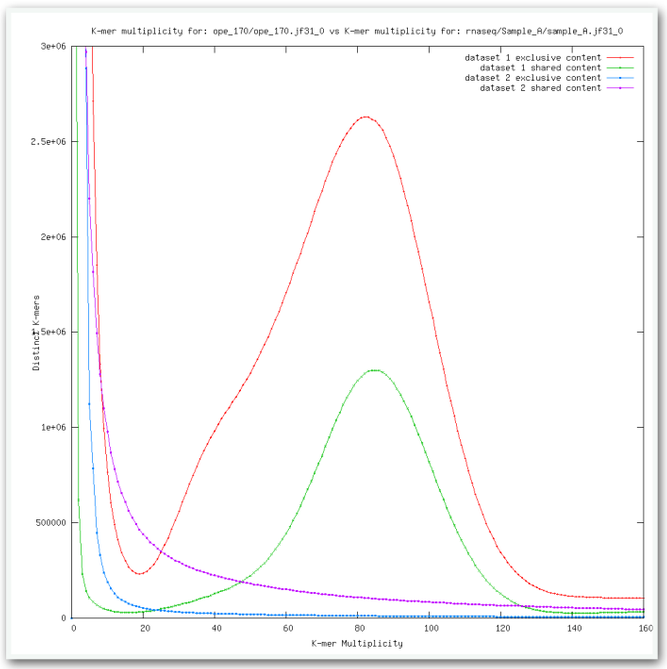


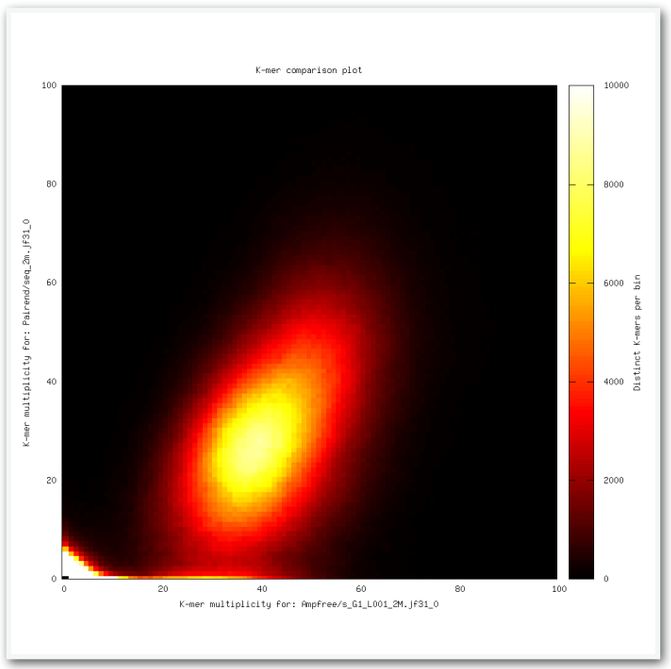


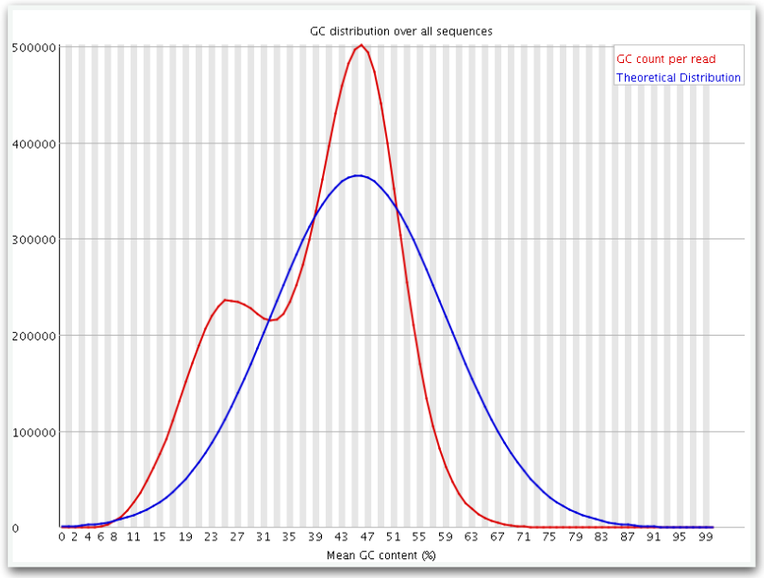
Do **QC before** performing the **analysis**











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PreQC

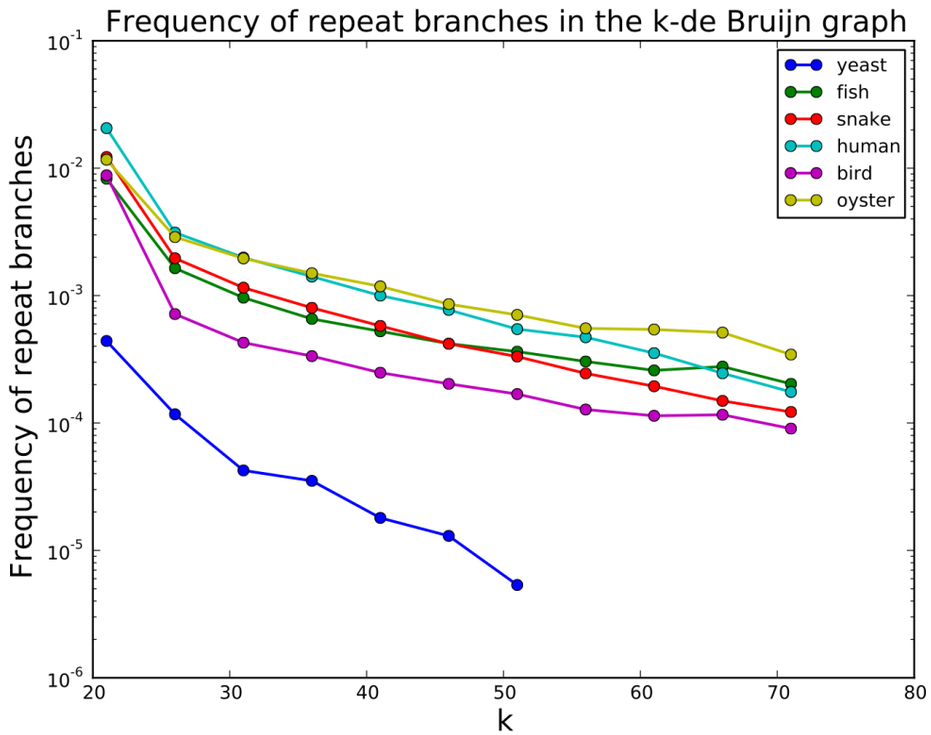


Figure 2: The estimated repeat branch rate for each genome as a function of k. The yeast data stops at k=51 as the number of repeat branches found falls below the minimum threshold for emitting an estimate.

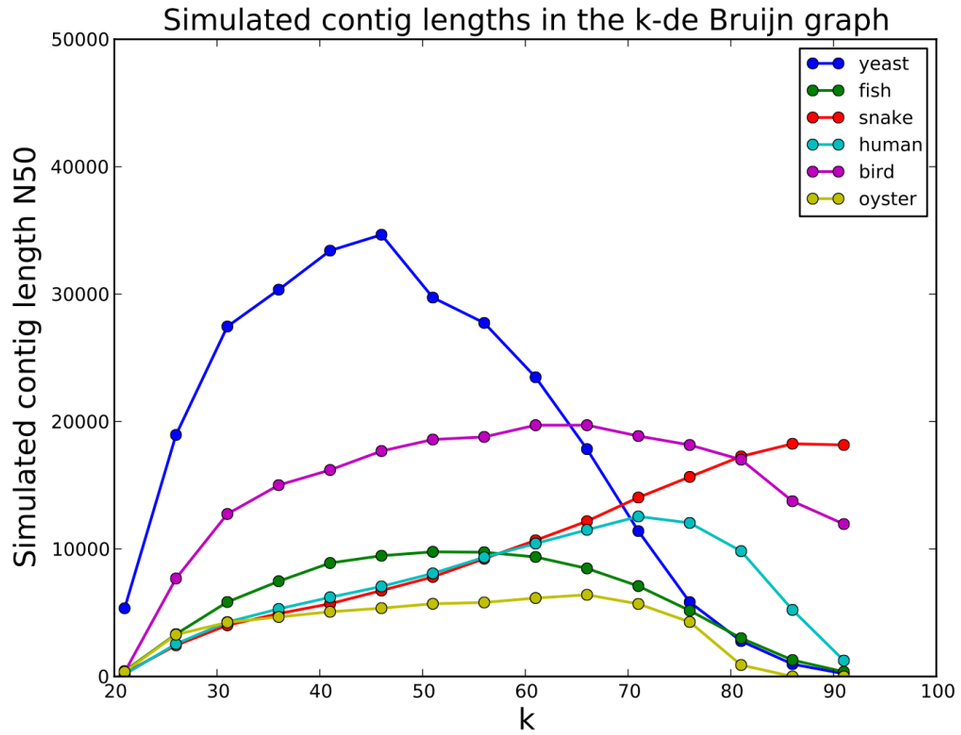
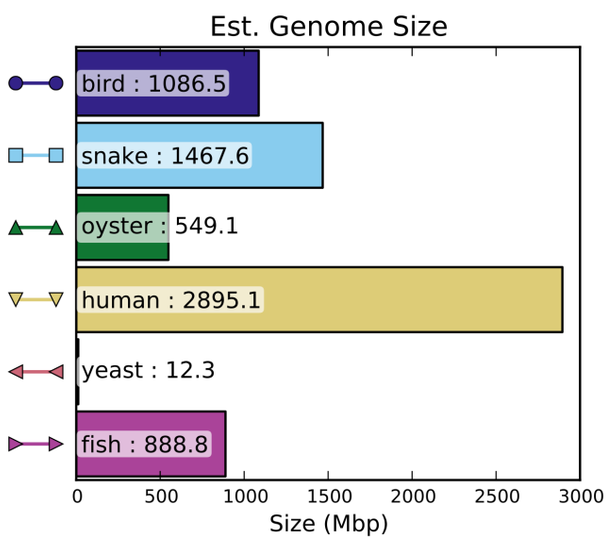


Figure 5: The N50 length of simulated contigs for k from 21 to 91, in increments of 5



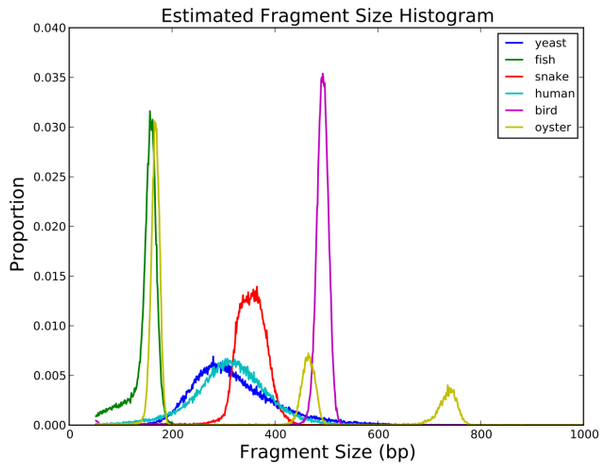


Figure 8: The estimated paired-end fragment size for each data set. The oyster data set is a mixture of three libraries.

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Read preparation:

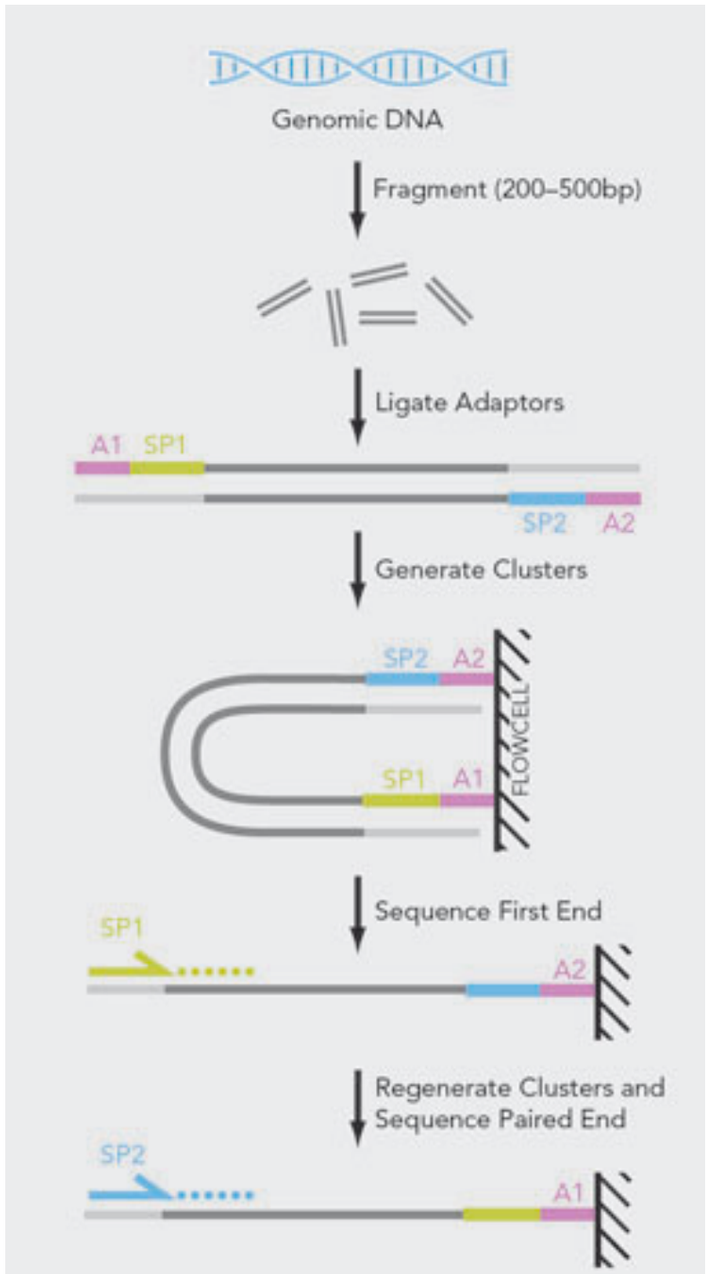
- **Adaptor trimming:** if you have lots of adaptor sequence.
  - But SPECIALLY if you have linkers from LMP (check Nextclip).
- **Pair joining:** allows higher k on overlapping reads. Might loose longer frags.
- **Quality trimming:** only if your data is terrible and you are short of memory.
- **Error correction:** once it miscorrects, all subsequent processing is tainted.
  - Your approach should be able to cope with errors, EC is just one option.
  - Pacbio reads are a special case, more about that later.
- **Deduplication:** hard to do right, sometimes needed, scaffolders handle it.
- **Digital normalisation:** rna-* / meta-*, and if you understand what it does.
- **IN GENERAL:** illumina is better than it used to be. Keep it in mind.

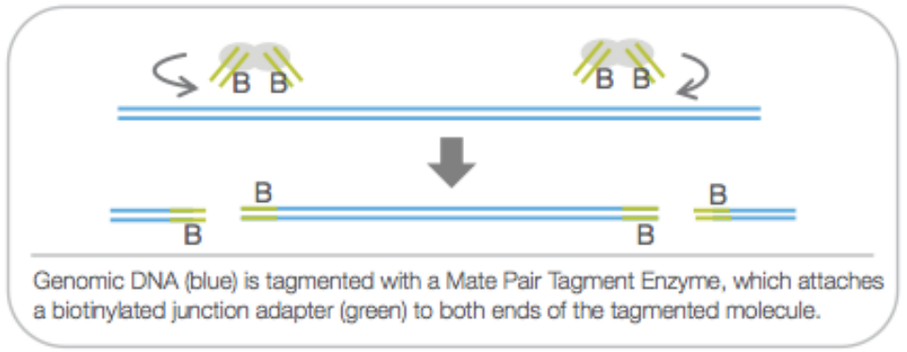
Questions?

Preparing LMP data…

… an exceptionally successful example.

Paired End & Long Mate Paired Libraries





BIOINFORMATICS **APPLICATIONS NOTE** *Vol. 30 no. 4 2014, pages 566–568*
doi:10.1093/bioinformatics/btt702

Sequence analysis Advance Access publication December 2, 2013

NextClip: an analysis and read preparation tool for Nextera Long Mate Pair libraries

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The Genome Analysis Centre (TGAC), Norwich Research Park, Norwich NR4 7UH, UK
Associate Editor: Michael Brudno

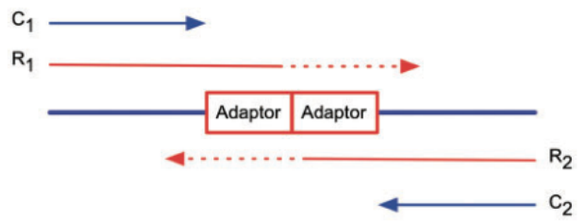

Fig. 1. Nextera mate pair fragments are formed by the joining of two junction adaptors. Reads R_1 and R_2 are produced from both ends and are clipped at the adaptor to produce C_1 and C_2

Table 1. ABySS *A. thaliana* assembly with and without NextClip clipping.

Reads used for assembly	Contig N50	Scaffold N50
Paired End only	15,627	21,939
PE and all raw LMP	15,627	15,628
PE and NextClip processed A,B,C cat.	15,627	245,226

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Yeast Assemblies

```
n       n:500   n:N50   min     N80     N50     N20     max     sum
17783   7370    2171    500     744     1205    2024    6372    8031482 yeast_pe_k91-unitigs.fa
9030    4941    1079    500     1306    2708    5235    17385   9789489 yeast_pe_k91-contigs.fa
8810    4842    1037    500     1336    2753    5478    82405   9811356 yeast_pe_k91-scaffolds.fa
n       n:500   n:N50   min     N80     N50     N20     max     sum
17783   7370    2171    500     744     1205    2024    6372    8031482 yeast_pe_raw_lmp1_k91-unitigs.fa
9030    4941    1079    500     1306    2708    5235    17385   9789489 yeast_pe_raw_lmp1_k91-contigs.fa
8571    4662    1024    500     1434    2898    5409    17385   9850977 yeast_pe_raw_lmp1_k91-scaffolds.fa
n       n:500   n:N50   min     N80     N50     N20     max     sum
17783   7370    2171    500     744     1205    2024    6372    8031482 yeast_pe_clipped_lmp1_abc_k91-unitigs.fa
9030    4942    1079    500     1306    2708    5235    17385   9789862 yeast_pe_clipped_lmp1_abc_k91-contigs.fa
6907    2988    232     500     1967    10653   25702   74395   9843053 yeast_pe_clipped_lmp1_abc_k91-scaffolds.fa
```

What if… I assembly the LMP library **alone**?

```
n       n:500   n:N50   min     N80     N50     N20     max     sum
17783   7370    2171    500     744     1205    2024    6372    8031482 yeast_pe_k91-unitigs.fa
9030    4941    1079    500     1306    2708    5235    17385   9789489 yeast_pe_k91-contigs.fa
8810    4842    1037    500     1336    2753    5478    82405   9811356 yeast_pe_k91-scaffolds.fa
n       n:500   n:N50   min     N80     N50     N20     max     sum
17783   7370    2171    500     744     1205    2024    6372    8031482 yeast_pe_raw_lmp1_k91-unitigs.fa
9030    4941    1079    500     1306    2708    5235    17385   9789489 yeast_pe_raw_lmp1_k91-contigs.fa
8571    4662    1024    500     1434    2898    5409    17385   9850977 yeast_pe_raw_lmp1_k91-scaffolds.fa
n       n:500   n:N50   min     N80     N50     N20     max     sum
17783   7370    2171    500     744     1205    2024    6372    8031482 yeast_pe_clipped_lmp1_abc_k91-unitigs.fa
9030    4942    1079    500     1306    2708    5235    17385   9789862 yeast_pe_clipped_lmp1_abc_k91-contigs.fa
6907    2988    232     500     1967    10653   25702   74395   9843053 yeast_pe_clipped_lmp1_abc_k91-scaffolds.fa
n       n:500   n:N50   min     N80     N50     N20     max     sum
2740    644     92      502     17019   36185   75693   144003  11.5e6  yeast_clipped_lmp1_abc_k91-unitigs.fa
1831    158     20      502     78404   188426  377063  431579  11.91e6 yeast_clipped_lmp1_abc_k91-contigs.fa
1759    104     12      502     135419  328003  648583  1026799 11.91e6 yeast_clipped_lmp1_abc_k91-scaffolds.fa
```


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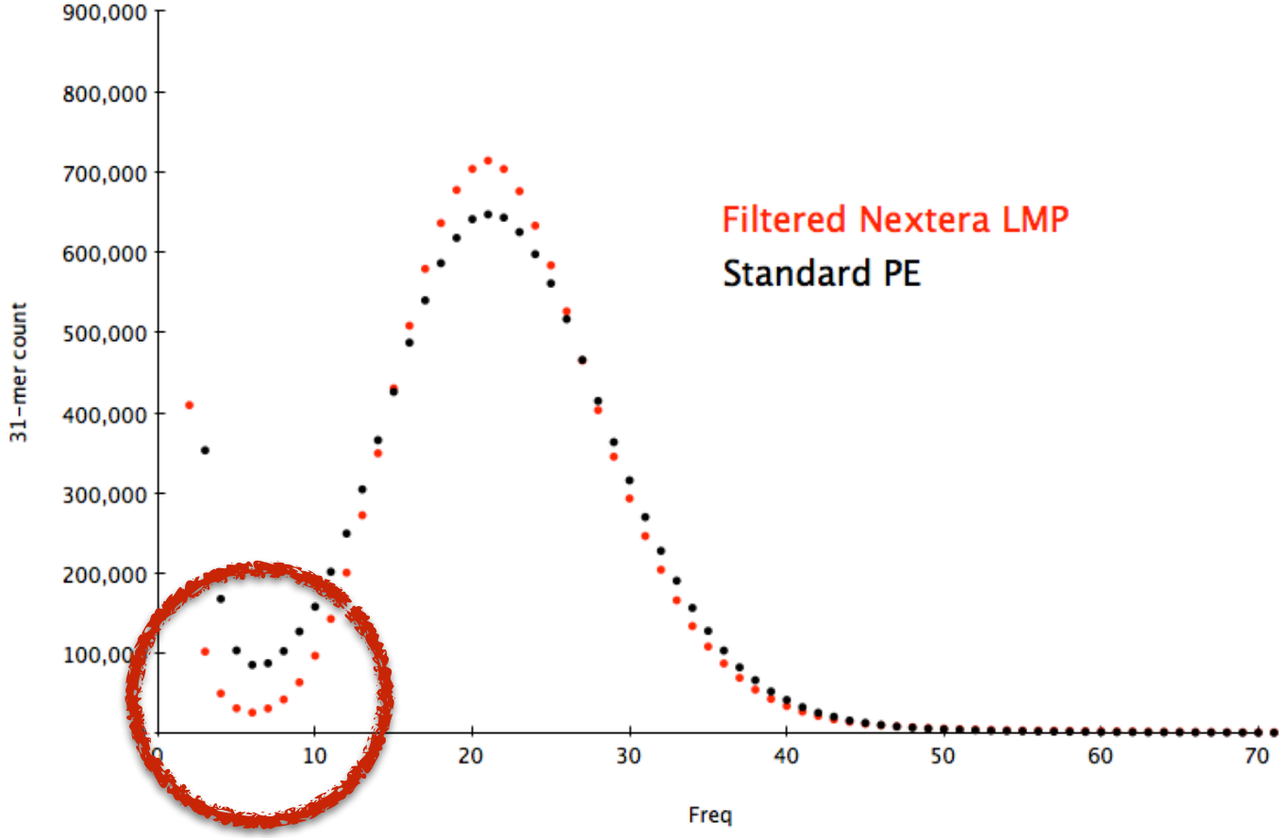
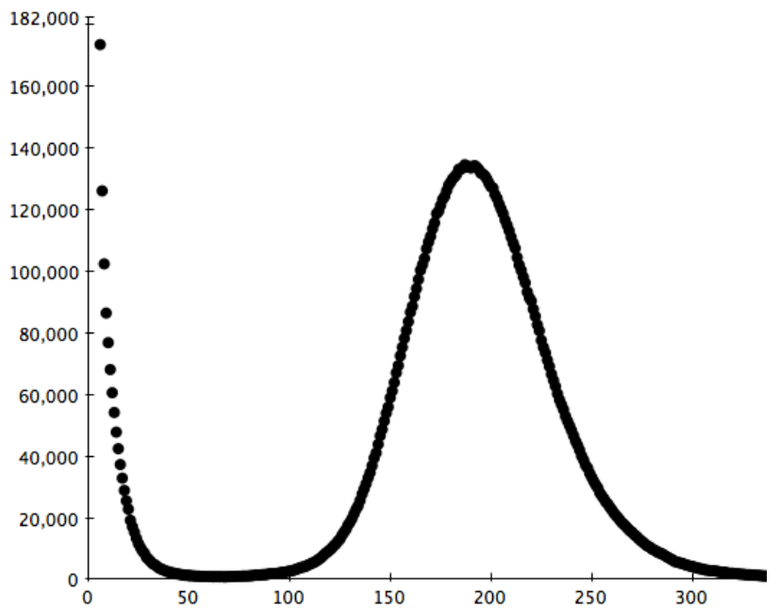
What? Why?

- Unitigs are longer: better coverage*, fewer errors (!)
- Contigs are longer: unitigs are longer, reads map better (!!)
- Scaffolds are longer: contigs are longer, reads map better (!!!)





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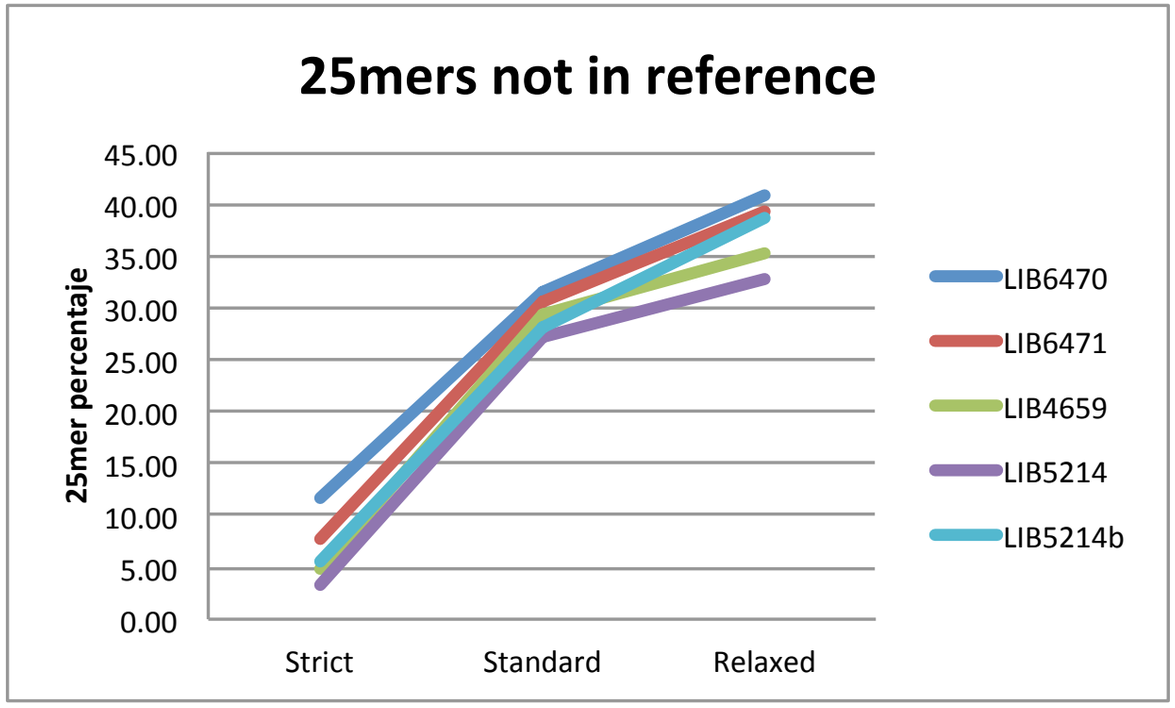
Getting even more from **our** Nextera LMP

- R1 and R2 usually overlap:
  - Because we need to maximise chances of finding the linker
  - We can join the overlapping pairs but then we need to

- PCR-free, and very few errors (more later).
  - Kmer spectra is clean (after Nextclip!).
  - Error correction dream dataset!!!

Fewer errors on Nextclip processed pairs?

Library	Strict			Standard				Relaxed			
	Total	Errors	Error %	Total	Errors	Error %	Error % (added)	Total	Errors	Error %	Error % (added)
LIB6470	2920076520	293879473	**10.06**	2960504161	304398051	10.28	**26.02**	2986028154	313188860	10.49	**34.44**
LIB6471	2975748804	198581925	**6.67**	3015112296	208350937	6.91	**24.82**	3040585503	216702885	7.13	**32.79**
LIB4659	2792344679	83943676	**3.01**	2814342003	88507030	3.14	**20.75**	2830348648	92654651	3.27	**25.91**
LIB5214	975451317	18809284	**1.93**	980158466	19687701	2.01	**18.66**	983874173	20564616	2.09	**23.60**
LIB5214b	2645386158	91401099	**3.46**	2677814948	97861650	3.65	**19.92**	2697174451	103550645	3.84	**29.39**



- Better linker match -> fewer errors.
- The worse reads won't match a linker.
- We can control strictness on matching.

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How would you ideally process a PAIR?

TGACTTGGAAGCLLLLMMMTTTGGGAAACCC

CCCCCAAAAAGGGGTTTTCCCCAAAAGGGTT
AACCCTTTTGGGGAAAACCCCTTTTTGGGGG

How would you ideally process a PAIR?

TGACTTGGAAGCLLLLMMMTTTGGGAAACCC

CCCCCAAAAAGGGGTTTTCCCCAAAAGGGTT

AACCCTTTTGGGGAAAACCCCTTTTTGGGGG

TGACTTGGAGCCLLLLMMMTTTGGGAAACCC

How would you ideally process a PAIR?

TGACTTGGAAGCLLLLMMMTTTGGGAAACCC

CCCCCAAAAAGGGGTTTTCCCCAAAAGGGTT

TGACTTGGAGCCLLLLMMMTTTGGGAAACCCTTTTGGGGAAAACCCCTTTTTGGGGG

How would you ideally process a PAIR?

TGACTTGGAAGCLLLLMMMTTTGGGAAACCC

CCCCCAAAAAGGGGTTTTCCCCAAAAGGGTT

TGACTTGGAGCCLLLLMMMTTTGGGAAACCCTTTTGGGGAAAACCCCTTTTTGGGGG

TGACTTGGAGCC

TTTGGGAAACCCTTTTGGGGAAAACCCCTTTTTGGGGG

CCCCCAAAAGGGGTTTTCCCCAAAAGGGTTCCCAAA

How would you ideally process a PAIR?

TGACTTGGAAGCLLLLMMMTTTGGGAAACCC CCCCCAAAAAGGGGTTTTCCCCAAAAGGGTT

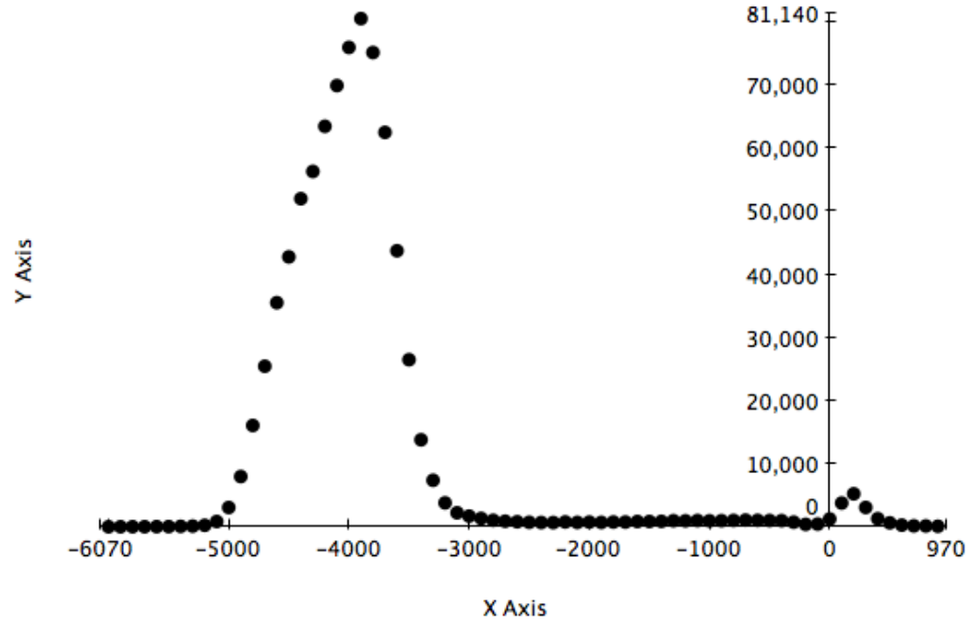
TGACTTGGAGCCLLLLMMMTTTGGGAAACCCTTTTGGGGAAAACCCCTTTTTGGGGG

TGACTTGGAGCC TTTGGGAAACCCTTTTGGGGAAAACCCCTTTTTGGGGG

TGACTTGGAGCC CCCCCAAAAAGGGGTTTTCCCCAAAAGGGTTTCCCAAA

TGACTTGGAACC CCCCCAAAAAGGGGTTTTCCCCAAAAGGGTTTCCCAAA

A. thaliana col-0 assembly




```
[clavijob@UV1 04-assembly]$ abyss-fac */*.scafSeq
n       n:500   n:N50   min     N80     N50     N20     max     sum
91954   3820    56      500     145714  504481  1225625 3219505 109.2e6 soap_4659/ath_4659.k71.scafSeq
88231   2206    26      500     232953  1090267 2540849 6126667 110.8e6 soap_5214b/ath_5214b.k71-101.scafSeq
110330  2189    28      500     233256  867462  2716797 6127137 110.8e6 soap_5214b/ath_5214b.k71.scafSeq
110260  2575    24      500     291558  1256637 3008384 6210307 112.7e6 soap_both/ath_both.k71-101.scafSeq
134053  2653    24      500     290498  1255489 3008402 6210370 112.6e6 soap_both/ath_both.k71.scafSeq
```


TGAC

Was it expected to work so well?

De novo fragment assembly with short mate-paired reads: Does the read length matter?

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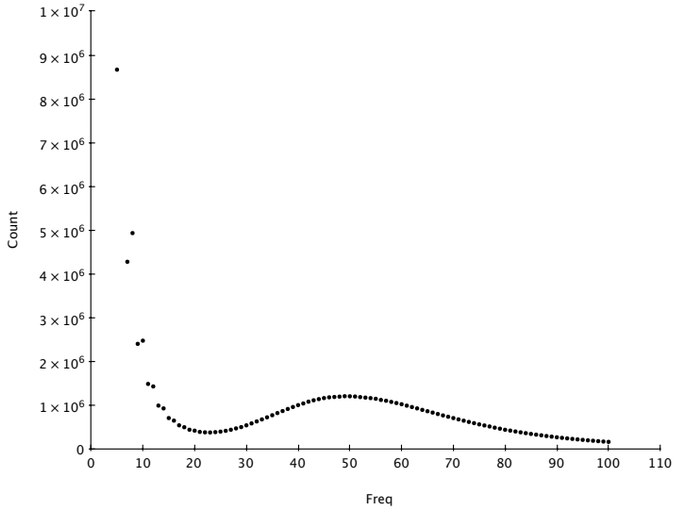
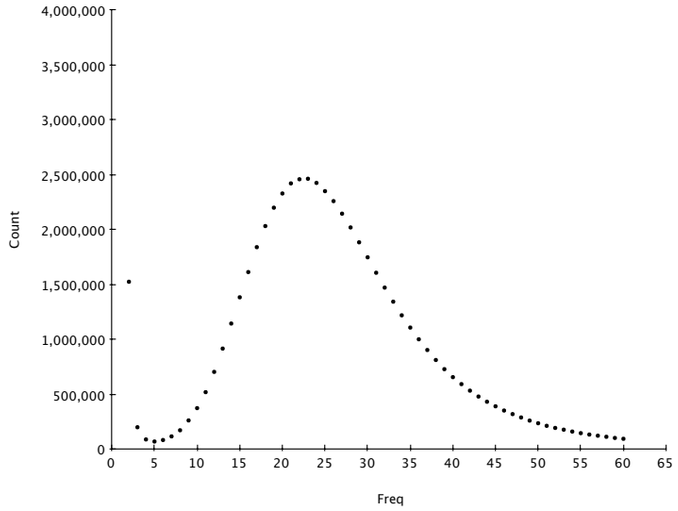
Increasing read length is currently viewed as the crucial condition for fragment assembly with next-generation sequencing technologies. However, introducing mate-paired reads (separated by a gap of length, GapLength) opens a possibility to transform short mate-pairs into long mate-reads of length ≈ GapLength, and thus raises the question as to whether the read length (as opposed to GapLength) even matters. We describe a new tool, EULER-USR, for assembling mate-paired short reads and use it to analyze the question of whether the read length matters. We further complement the ongoing experimental efforts to maximize read length by a new computational approach for increasing the effective read length. While the common practice is to trim the error-prone tails of the reads, we present an approach that substitutes trimming with error correction using repeat graphs. An important and counterintuitive implication of this result is that one may extend sequencing reactions that degrade with length "past their prime" to where the error rate grows above what is normally acceptable for fragment assembly.

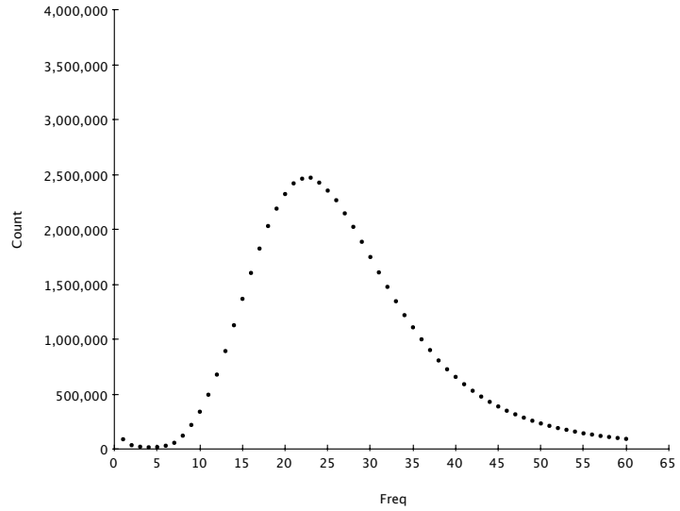
TGAC

C. fraxinea assembly

n	n:200	n:N50	min	N80	N50	N20	max
5418	2583	306	200	27049	61813	115341	363023








```
[clavijob@UV1 soap_k111r_nok]$ abyss-fac cha_soap_ope_lmp_k111r.contig
n       n:500   n:N50   min     N80     N50     N20     max     sum
18093   6620    781     500     8359    22816   48113   130784  62.84e6 cha_soap_ope_lmp_k111r.contig
[clavijob@UV1 soap_k111r_nok]$ abyss-fac -s 10000 cha_soap_ope_lmp_k111r.scafSeq
n       n:10000 n:N50   min     N80     N50     N20     max     sum
11058   23      9       189181  2611331 2982393 4115367 4495304 62.08e6 cha_soap_ope_lmp_k111r.scafSeq
```


TGAC

Questions?