ChIP-Seq Analysis

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Babraham Bioinformatics

What this course covers

- The theory of ChIP-Seq
- ChIP-Seq library properties
- Sequencing, Data processing and QC
- Data visualisation and exploration
- Types of analysis
  - Peak Calling
  - Differential Binding

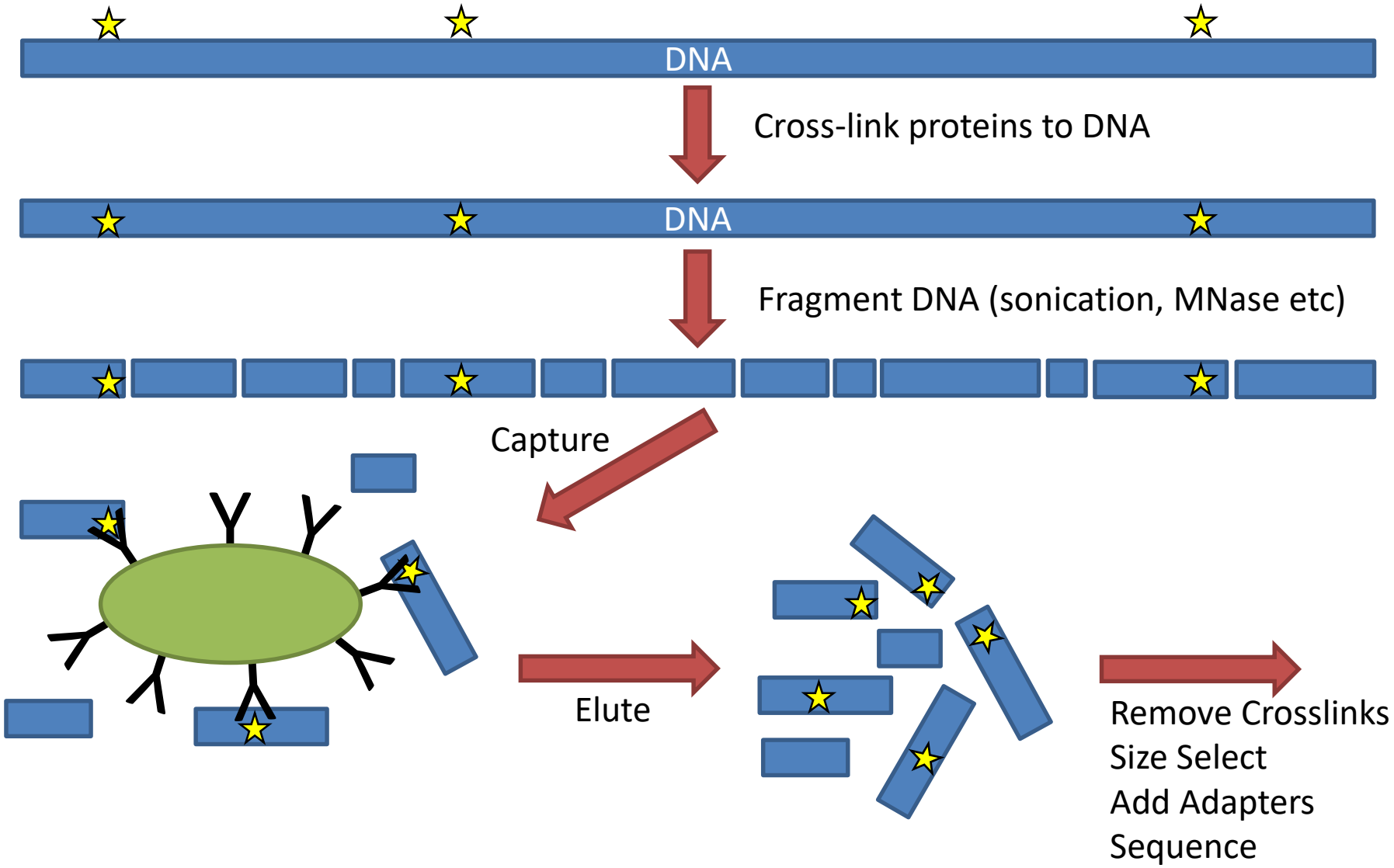
What is ChIP-Seq?

ChIP-Seq is a technology which uses high-throughput sequencing to infer the positions of any mark associated with DNA which can be captured by an antibody.

Types of antibody

- Transcription factors / repressors
  - nanog, CTCF
- Histones and histone modifications
  - H3, H3K4me3
- DNA modifications
  - Methyl-Cytosine, Formyl cytosine
- Chromatin remodelling proteins
  - BMI1, EZH2
- Transcription machinery
  - Pol2

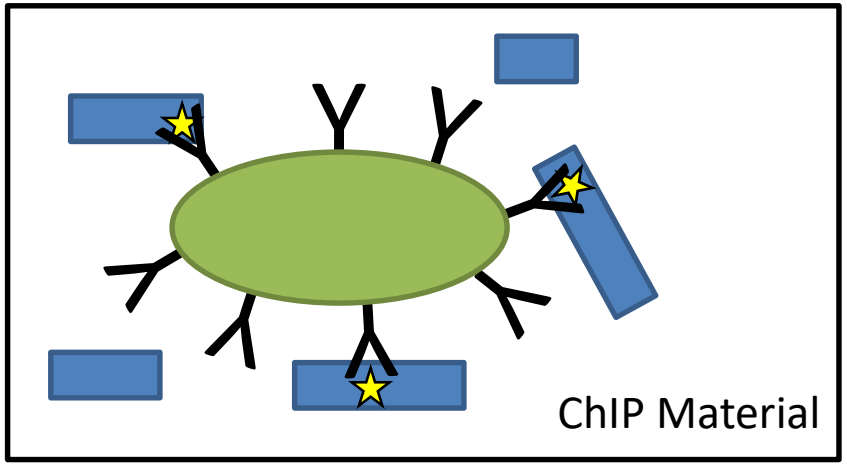
How Does ChIP-Seq work

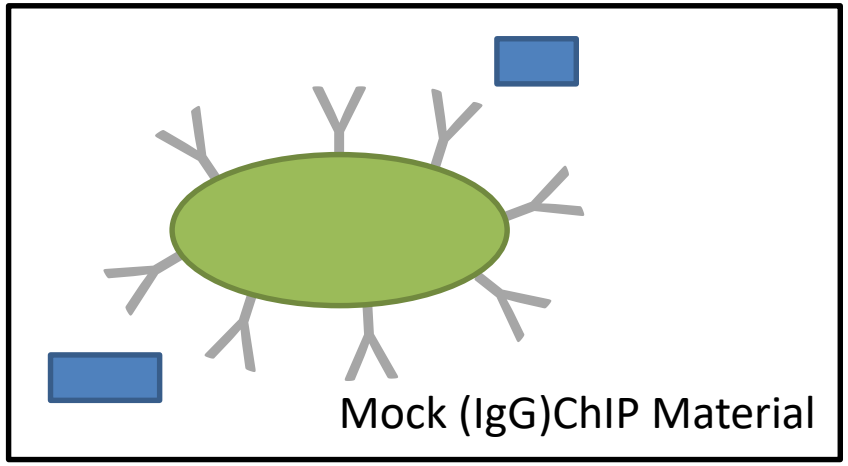


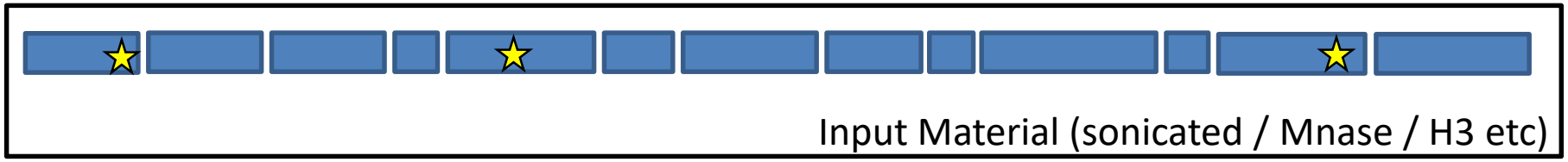
Related Techniques

- ATAC-Seq
  - Uses transposases to digest exposed DNA to enrich for accessible DNA.
- Cut and Tag
  - Uses transposases fused to antibodies to find marked, accessible chromatin

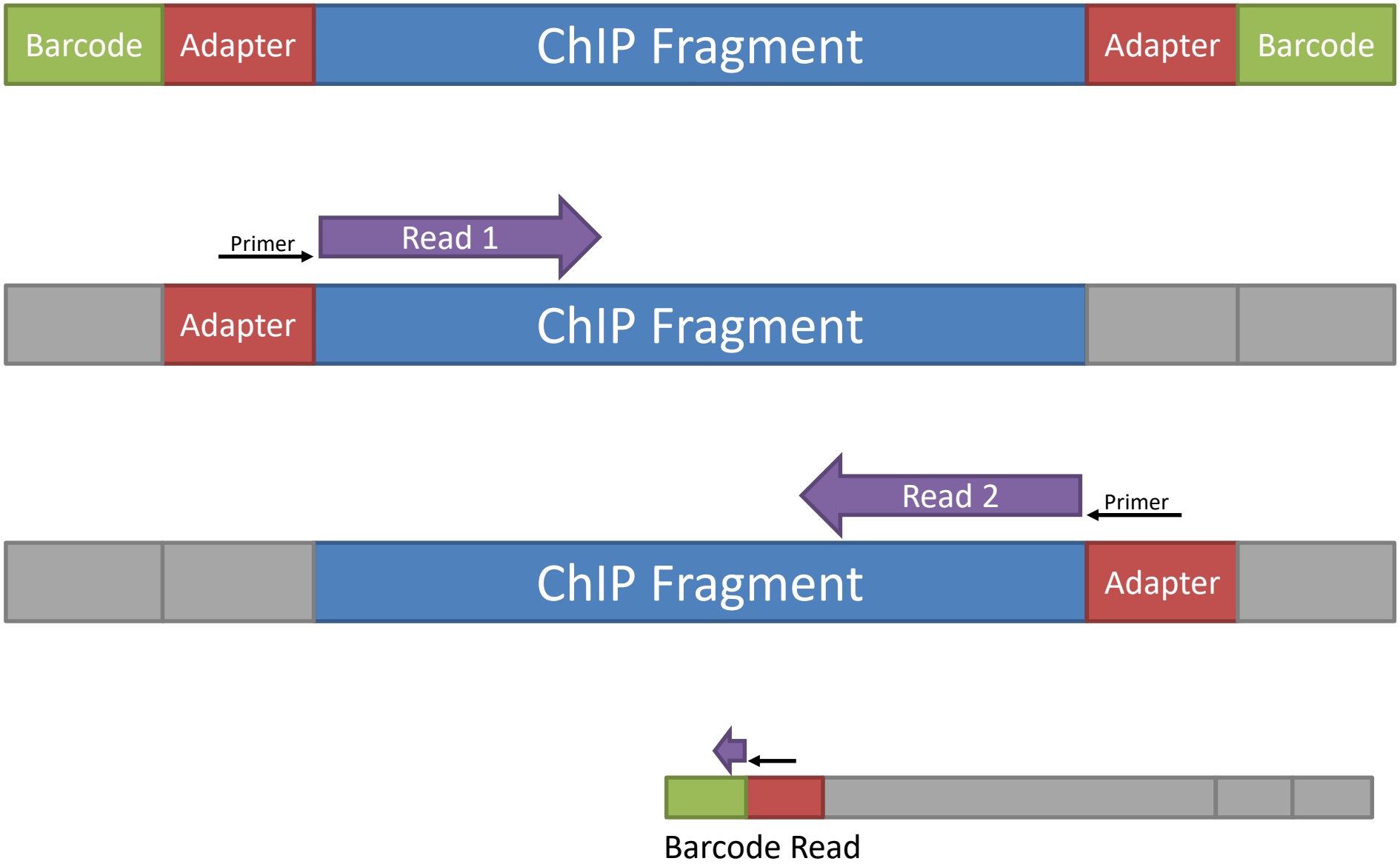
What can you sequence?



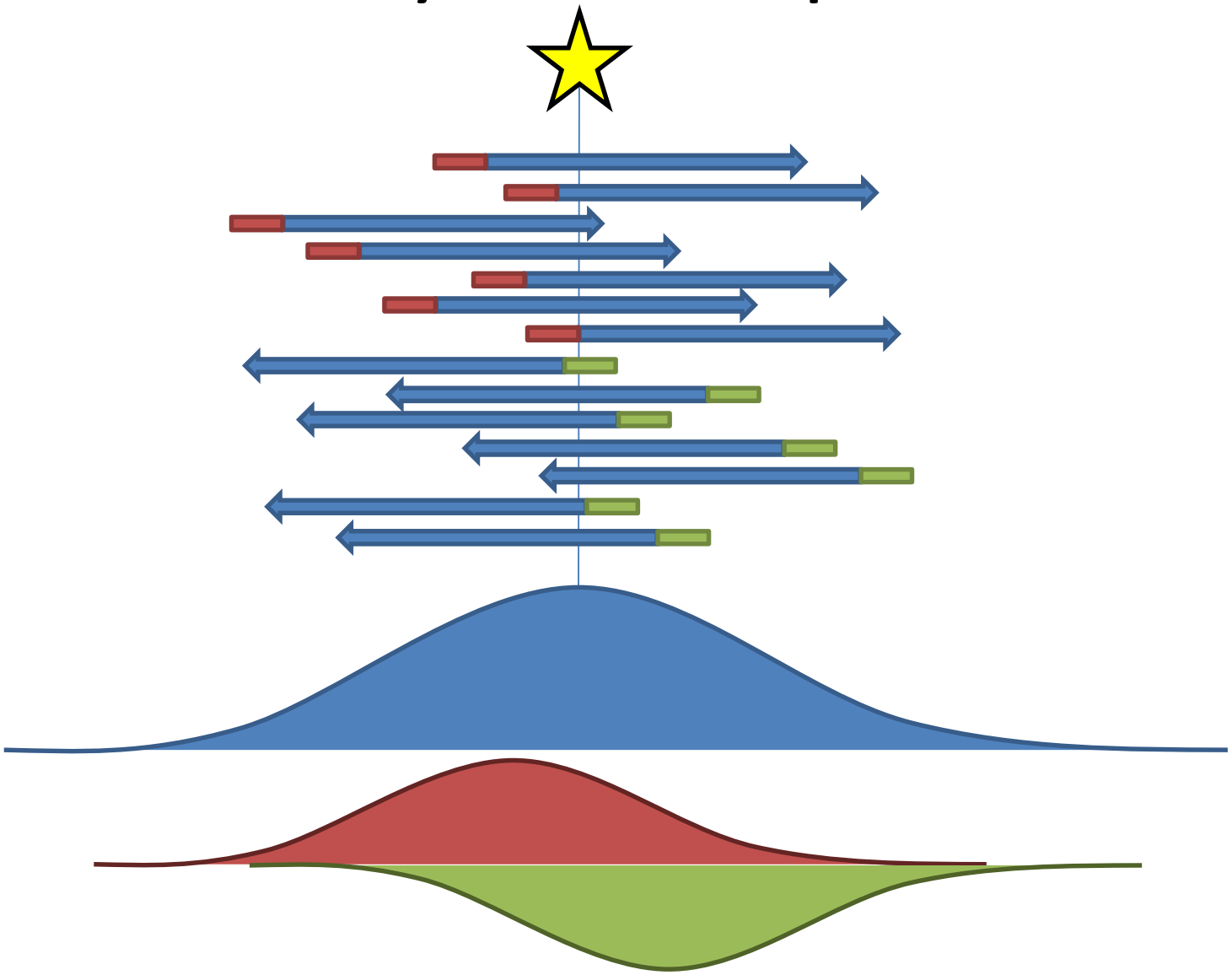




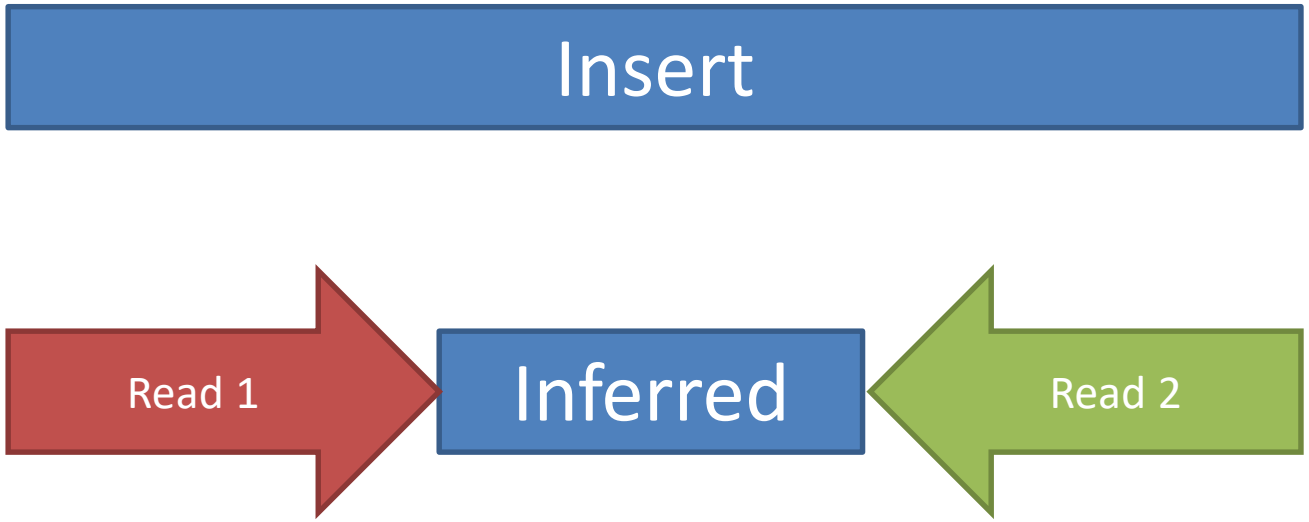
Sequencing for ChIP



What you end up with



Single End vs Paired End

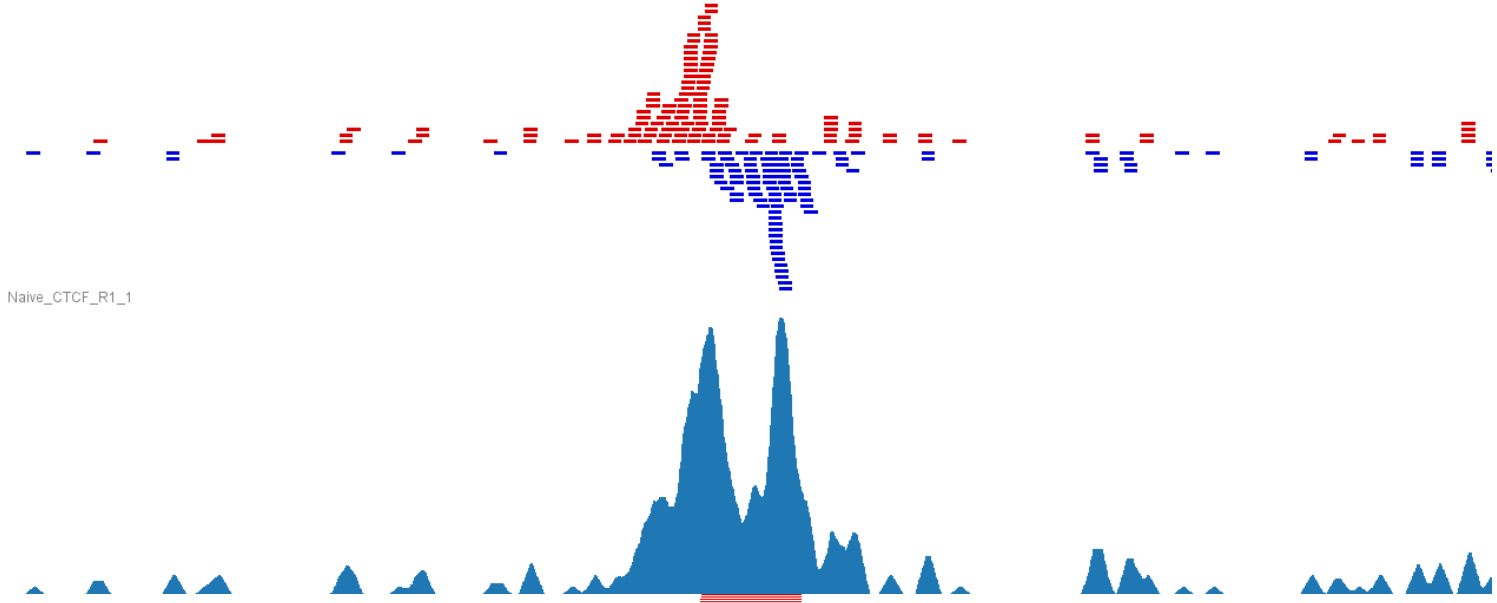


Paired
(= >$£€¥)

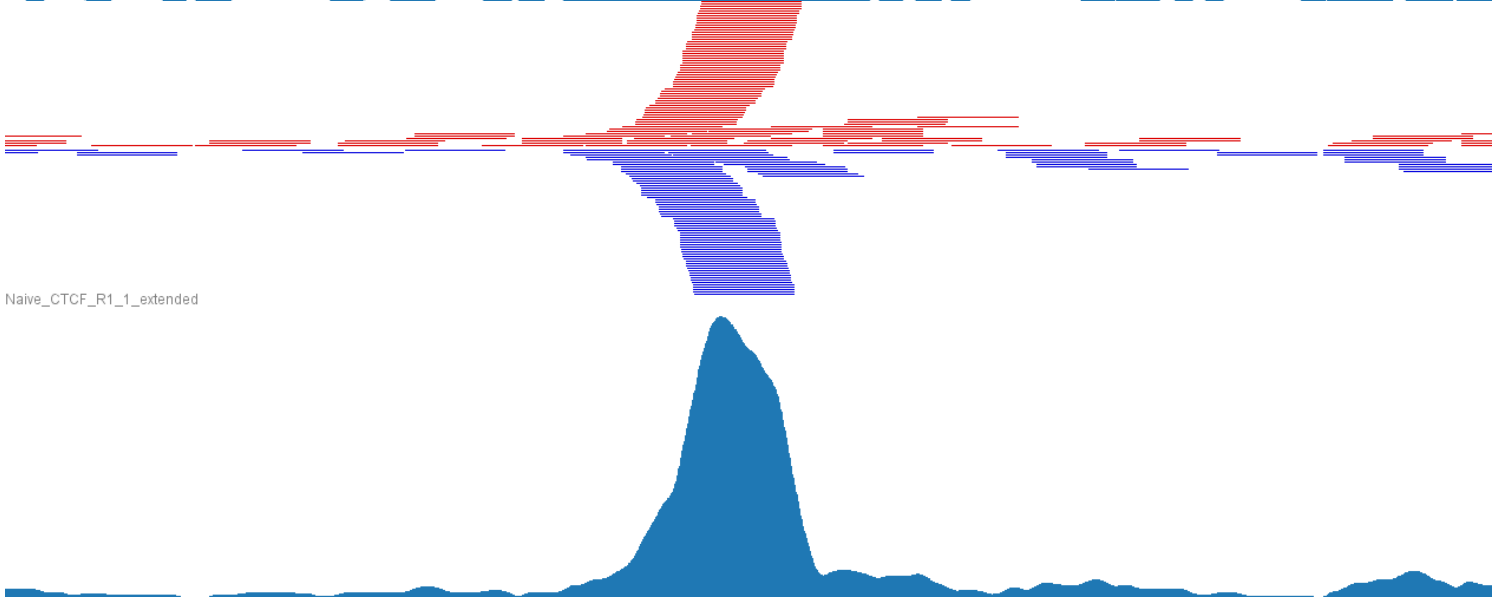
Single
(= Cheaper!)

What you end up with

Original 40bp Reads

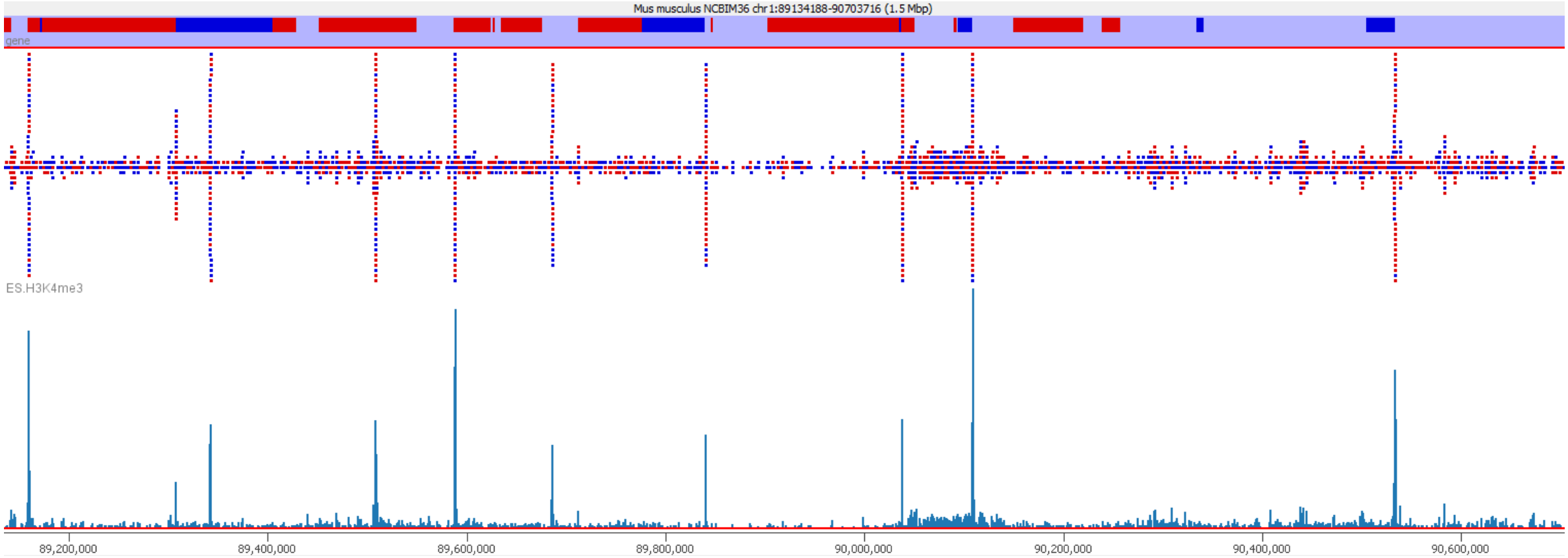


Extended by 250bp



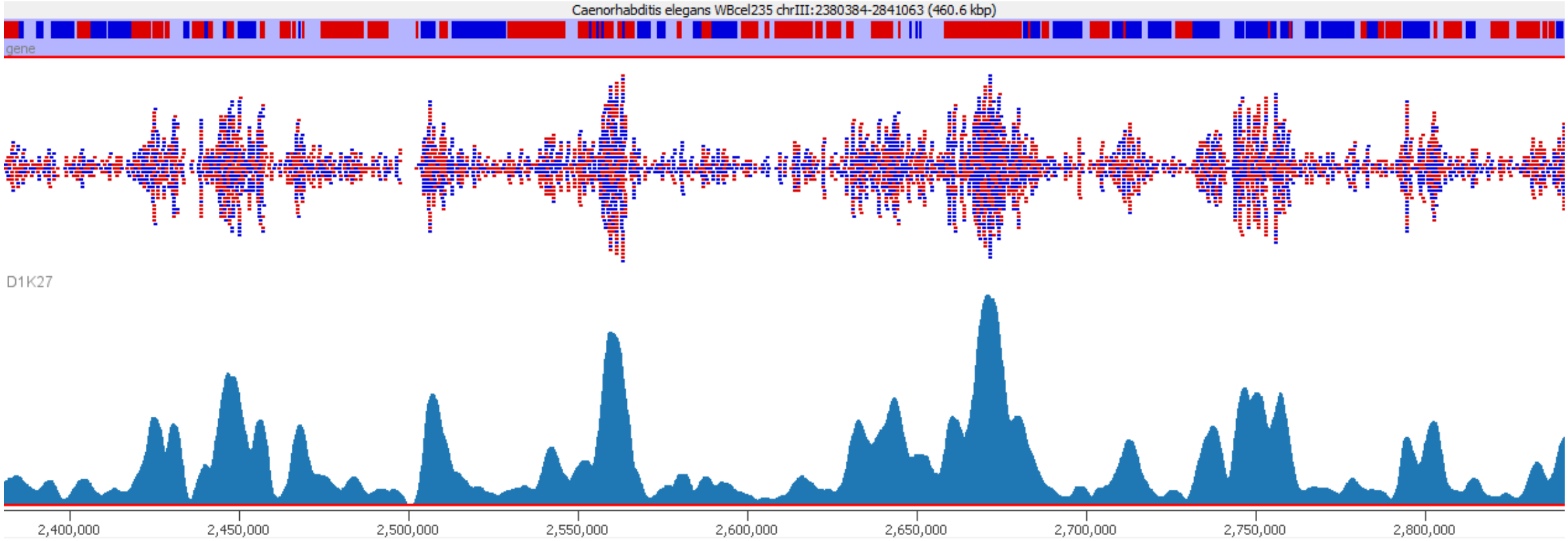
Types of Enrichment

- Single points (typical TF, some histone marks)



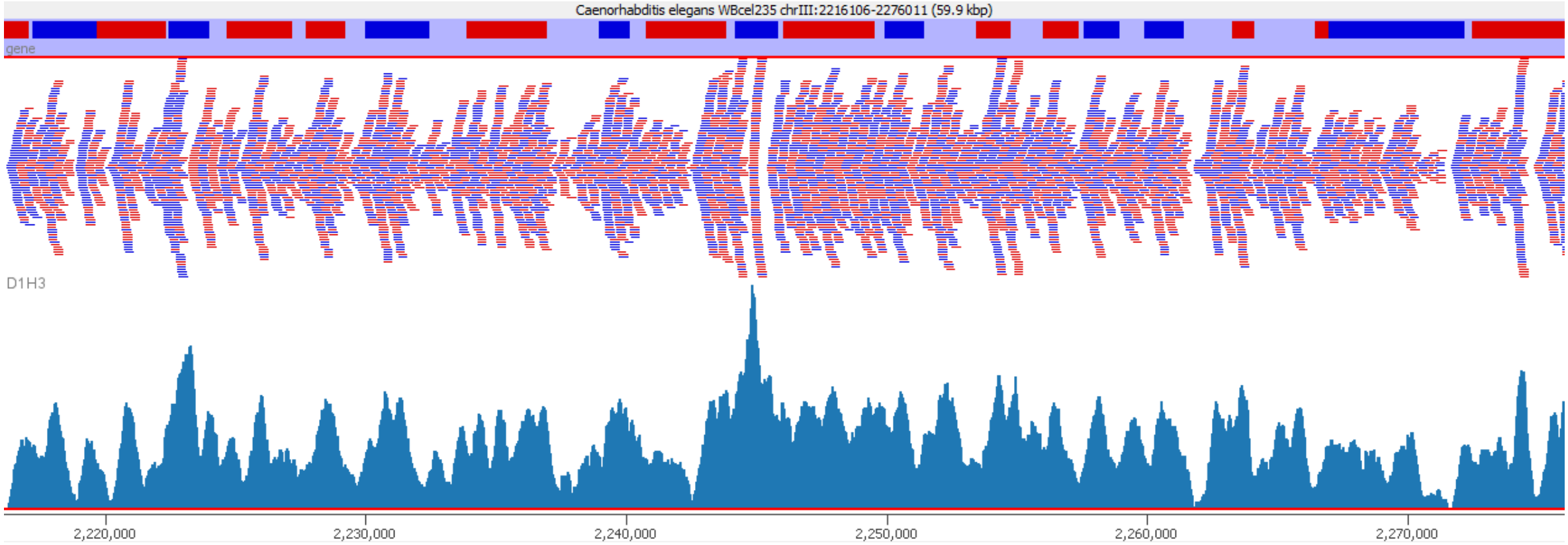
Types of Enrichment

- Broad Regions (some histone marks, PolII)



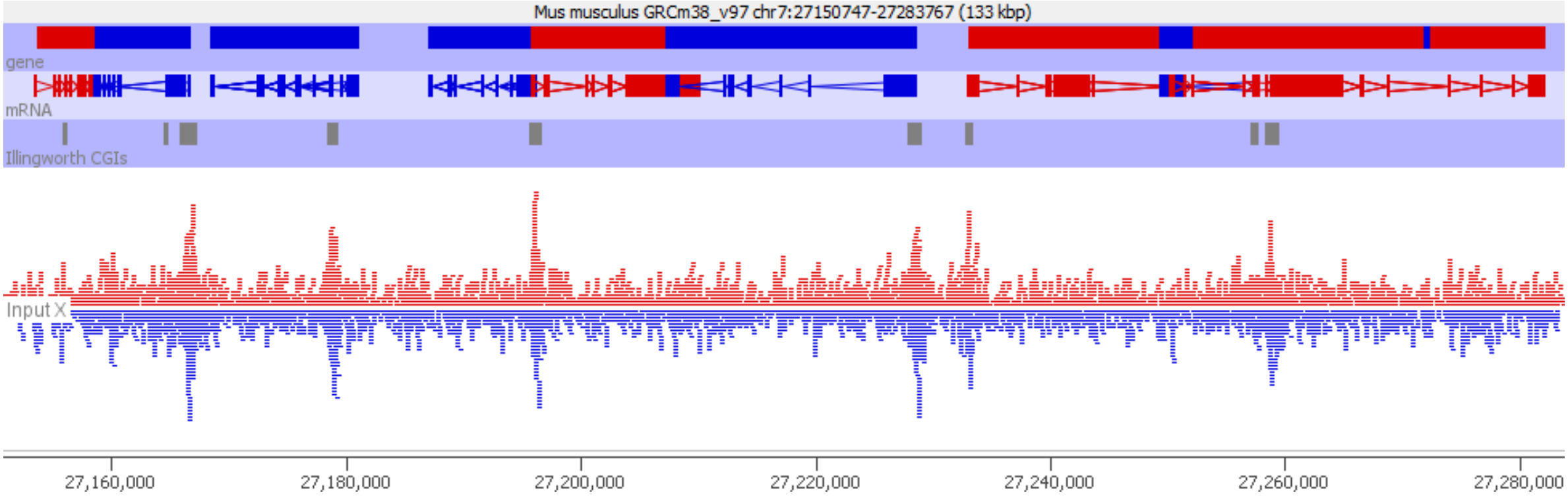
Types of Enrichment

- Virtually everywhere (h3)



Types of Enrichment

- Artefactual (GC in this case)



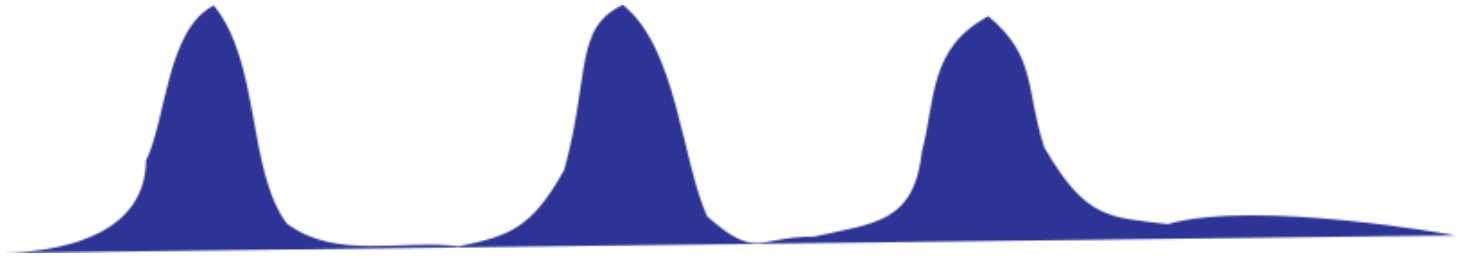
What are you actually measuring?

- ChIP Seq measures **RELATIVE** enrichment
  - Region A has twice as much signal as Region B

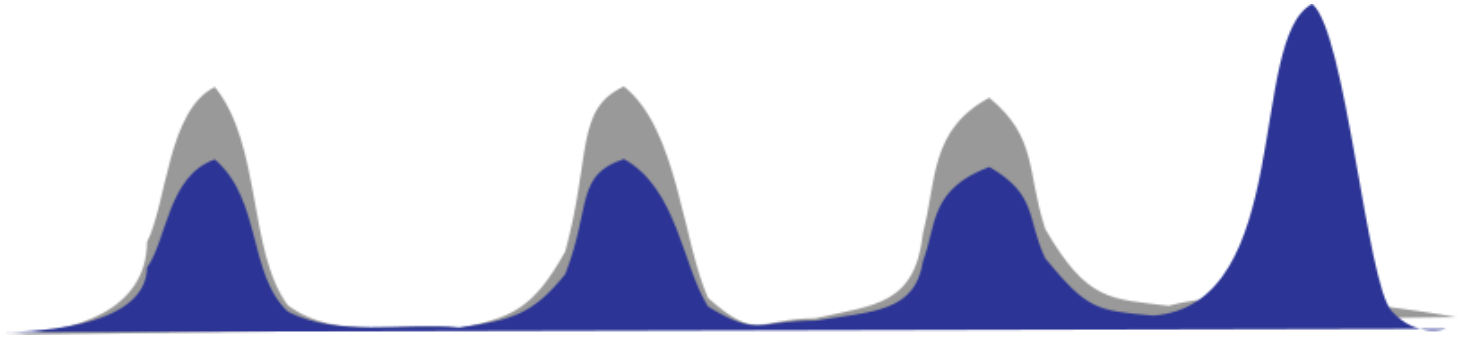
- Without some external calibration, **NOTHING** in ChIP-Seq gives an ABSOLUTE measure.

What can affect enrichment?

Starting



More Sites



Poorer Signal

What sort of questions can you answer?

- Where is this mark present?
  - General - it's in promoters, gene bodies etc.
  - Specific - it's at these loci
- How does this mark change when I do XXX?
  - Categorical: A peak disappears
  - Quantitative: The enrichment of a locus changes

ChIP-Seq Data Processing and QC

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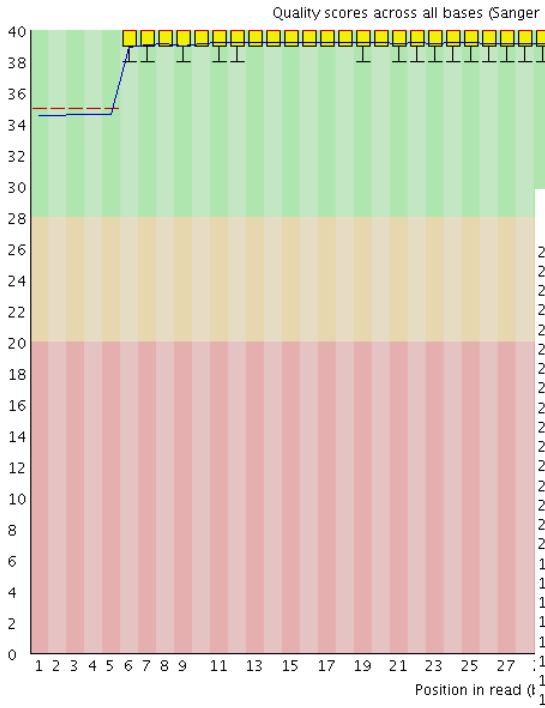
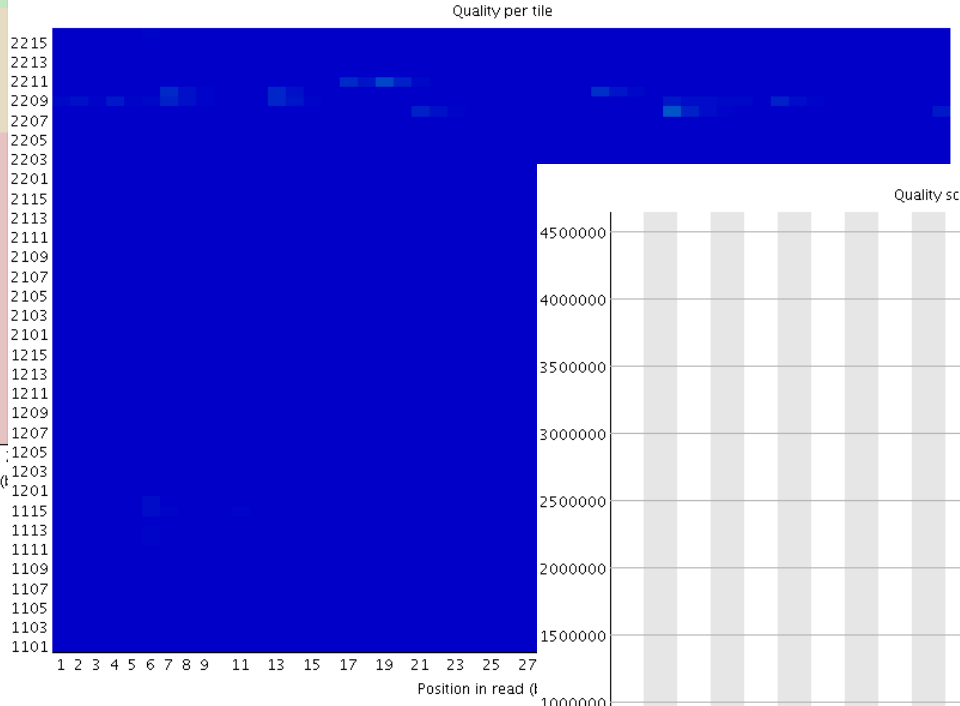
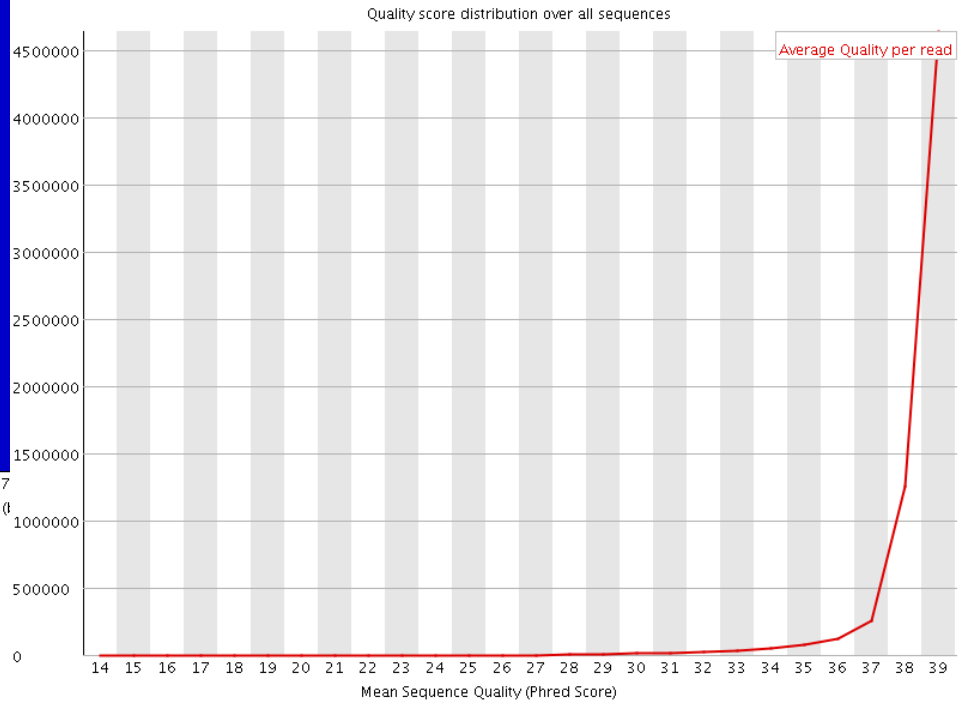
A typical ChIP Library

- Potential technical problems
  - Adapter contamination
  - PCR Duplication
- Potential biological problems
  - Lack of enrichment
  - Other selection biases

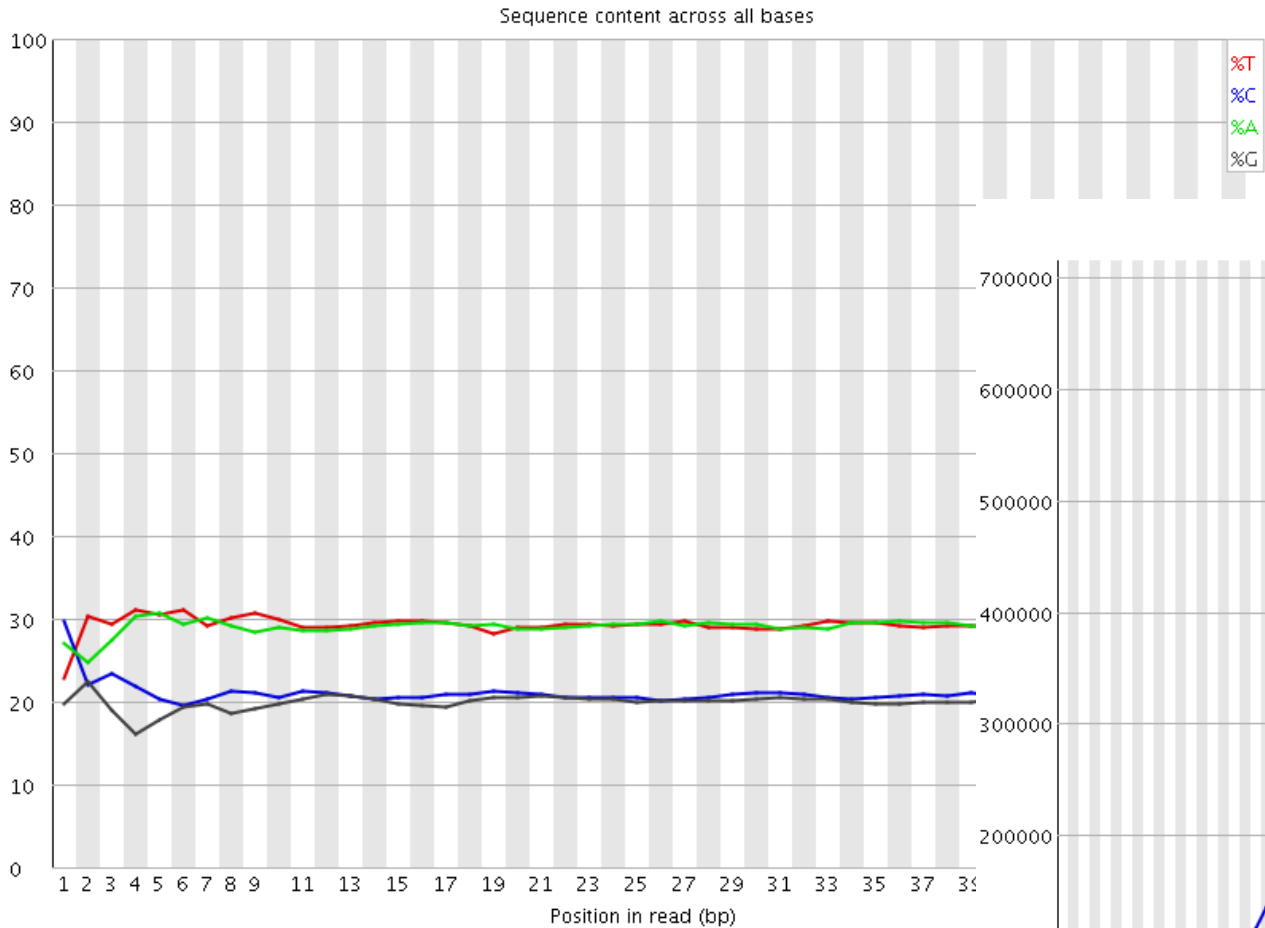
QC of raw sequence
Base Call Quality

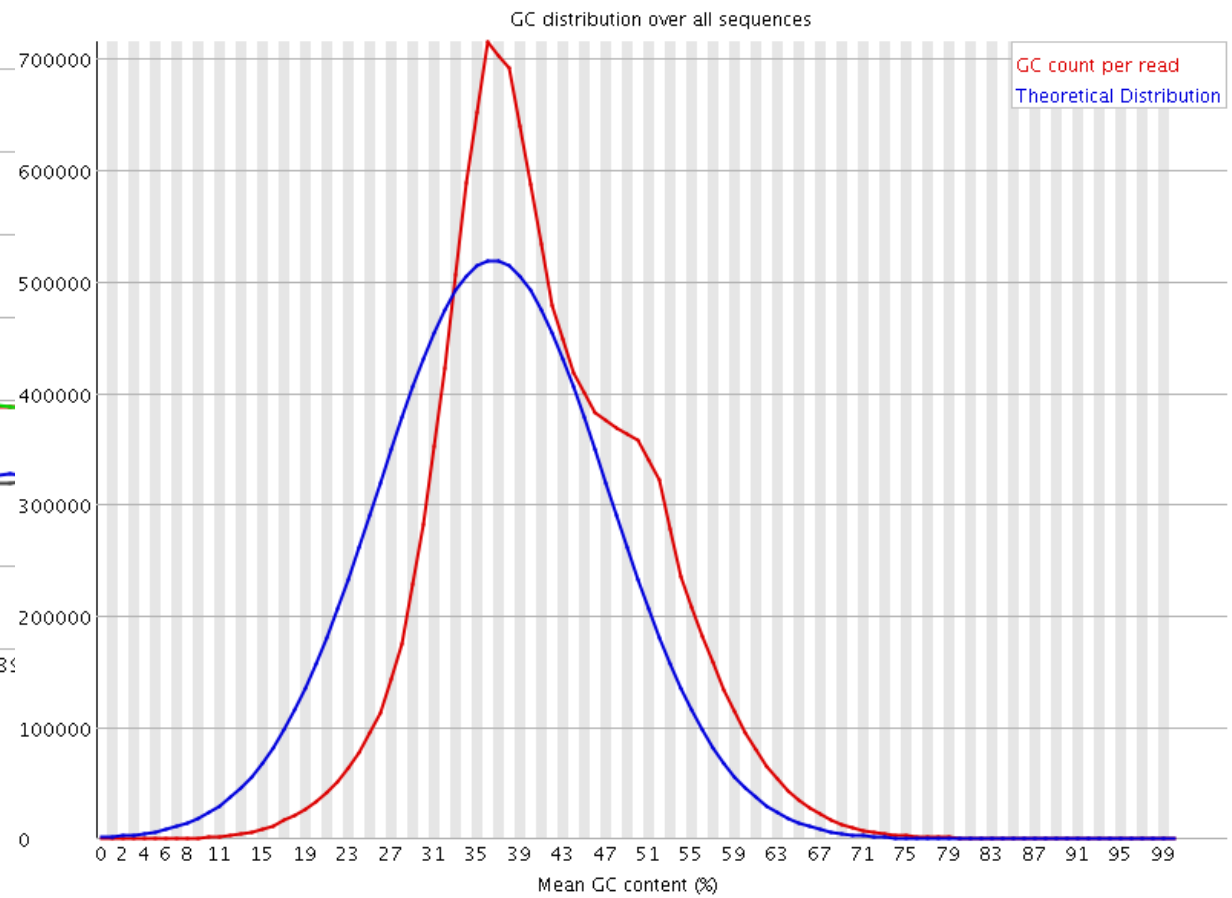






QC of raw sequence
Sequence Composition



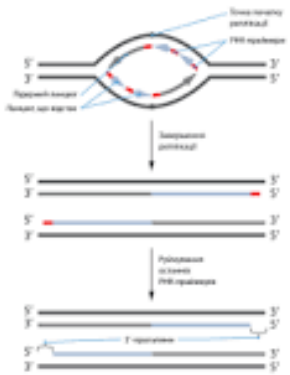


QC of raw sequence
Sequence Composition

Overrepresented sequences

Sequence	Count	Percentage	Possible Source
GGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGG	11933	0.17178556359211344	No Hit
CCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCC	8917	0.1283677089207136	No Hit
CCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACC	7298	0.10506084329969362	No Hit
CTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCT	7282	0.104830509853161	No Hit
GTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGT	7040	0.10134671647435503	No Hit

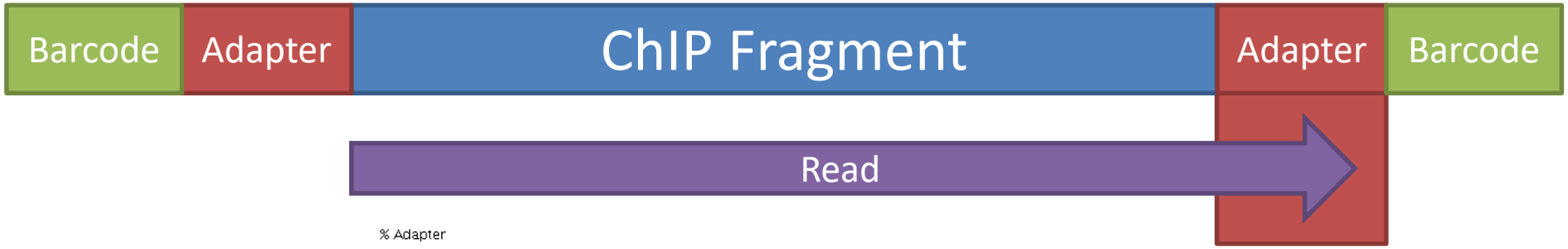
A **telomere** is a region of repetitive nucleotide **sequences** at each end of a chromosome, which protects the end of the chromosome from deterioration or from fusion with neighboring chromosomes. ... This **sequence** of TTAGGG is **repeated** approximately 2,500 times in humans.

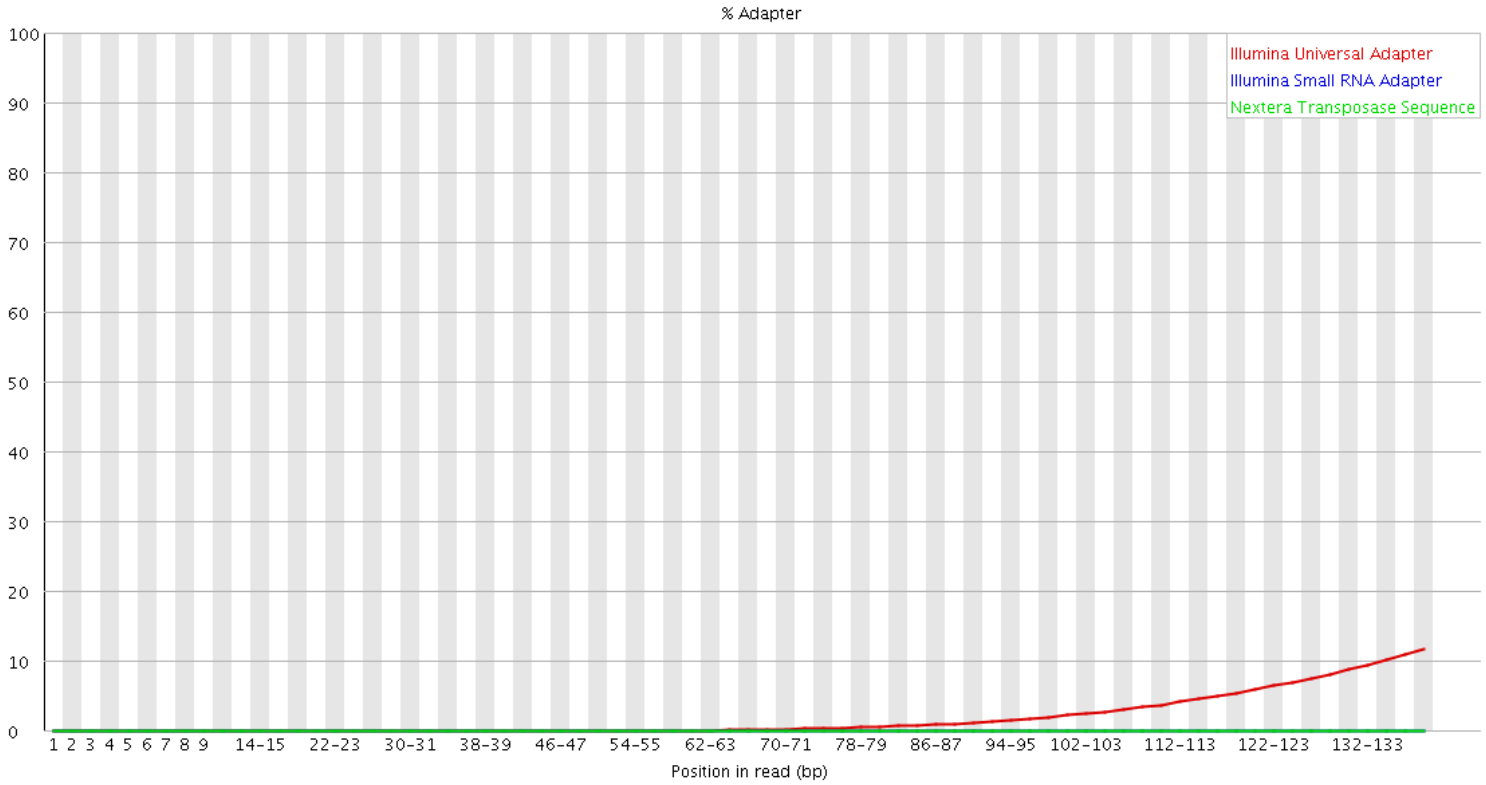


Telomere - Wikipedia
https://en.wikipedia.org/wiki/Telomere

About this result Feedback

QC of raw sequence
Adapter Contamination





Trim Galore!

Quality and Adapter Trimming

Mapping ChIP Data

- All regions should be linear genomic stretches
- Standard genomic aligners are fine
  - Bowtie2 http://bowtie-bio.sourceforge.net/bowtie2/
  - BWA http://bio-bwa.sourceforge.net/

Example Bowtie2 Mapping

- Create Genome Index (once - slow!)

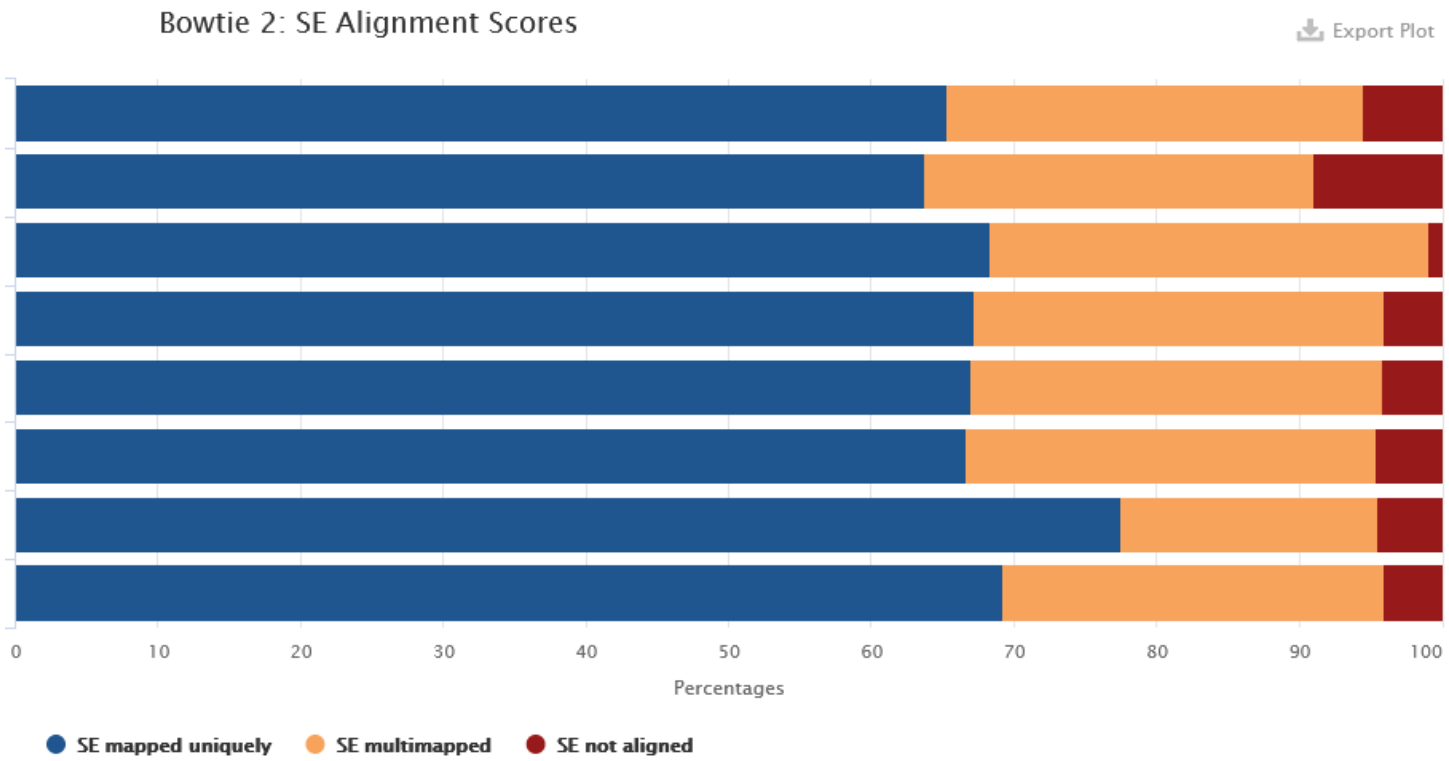
```
bowtie2-build yeast_genome.fa yeast_index
```

- Map a single FastQ file

```
bowtie2 \
-x yeast_index \
-U data.fastq.gz \
| samtools view \
-bS \
-o data.bam
```

Post Alignment QC
Mapping Statistics

```
41523294 reads; of these:
  41523294 (100.00%) were unpaired; of these:
    1851792 (4.46%) aligned 0 times
    32175322 (77.49%) aligned exactly 1 time
    7496180 (18.05%) aligned >1 times
95.54% overall alignment rate
```

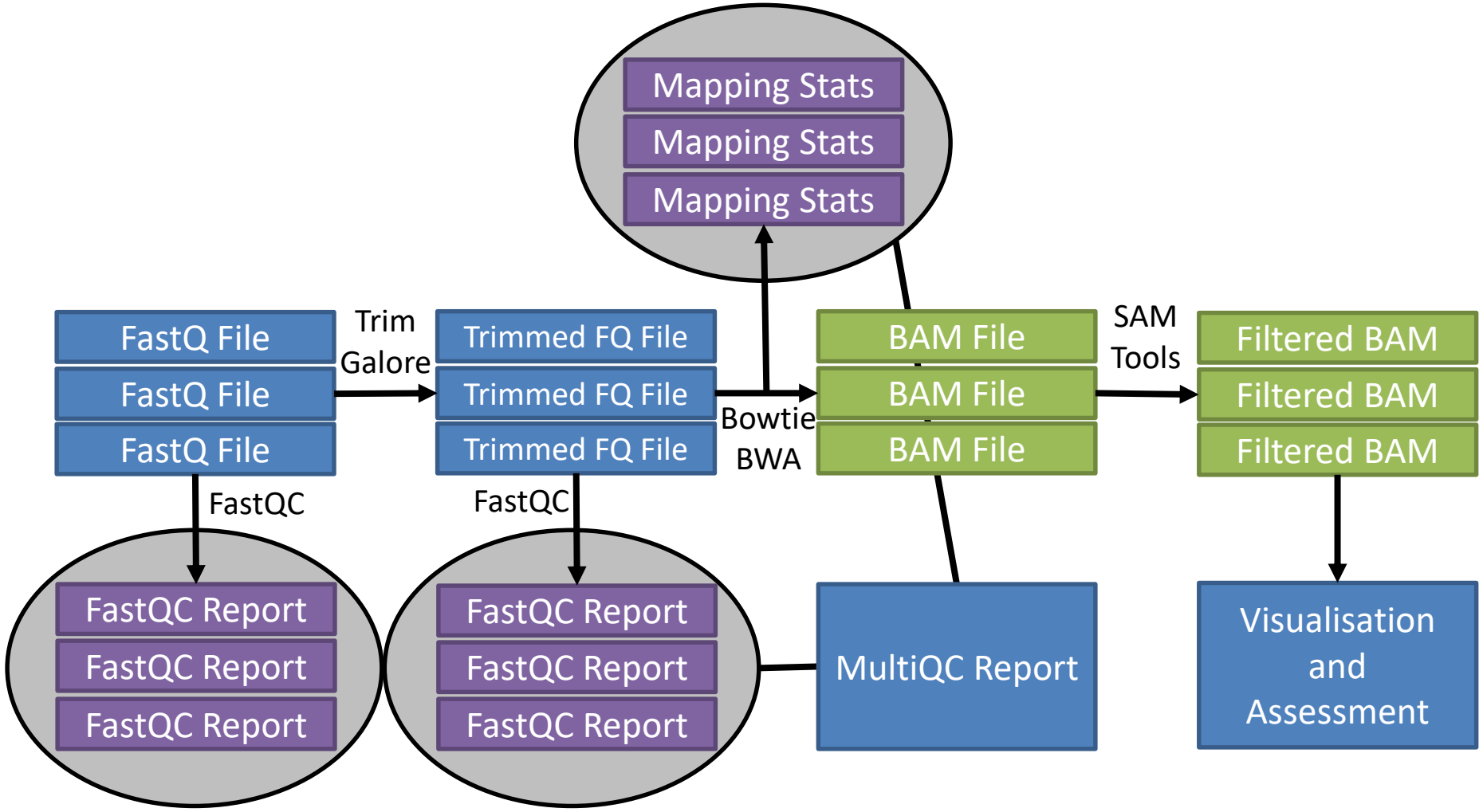


Post Alignment Processing
MAPQ Filtering

- ChIP-Seq relates sequences to positions in a reference genome
- You need to be confident that the reported position is correct
- Filtering on MAPQ value (likelihood of reported position being incorrect) is an easy way to do this
- MAPQ filtering should be performed in most cases

```
samtools view -q 20 -b -o filtered.bam data.bam
```

Standard Processing Workflow



Data Processing Exercise

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Running programs in Linux

- Open a shell (text based OS interface)

- Type the name of the program you want to run
  - Add on any options the program needs
  - Press return - the program will run
  - When the program ends control will return to the shell

- Run the next program!

Running programs

```
babraham@babraham-VirtualBox:~$ ls
Desktop  Documents  Downloads  examples.desktop
Music  Pictures  Public  Templates  Videos

babraham@babraham-VirtualBox:~$
```

- Command prompt - you can't enter a command unless you can see this
- The command we're going to run (`ls` in this case, to list files)
- The output of the command - just text in this case

The structure of a unix command

```
ls  -ltd --reverse Downloads/  Desktop/  Documents/
```

Program name

Switches

Data
(normally files)

Each option or section is separated by spaces. Options or files with spaces in must be put in quotes.

Command line switches

- Change the behaviour of the program
- Come in two flavours (each option often has both types available)
  - Minus plus single letter (eg `-x -c -z`)
  - Two minuses plus a word (eg `--extract --gzip`)
- Some take an additional value

  `-f somfile.txt` (specify a filename)

  `--width=30` (specify a value)

Specifying file paths

- Specify names from whichever directory you are currently in
  - If I'm in `/home/simon`
  - `Data/big_data.fq.gz`
    - is the same as `/home/simon/Data/big_data.fq.gz`

- Move to the directory with the data and just use file names
  - `cd Data`
  - `big_data.fq.gz`

Command line completion

- Most errors in commands are typing errors in either program names or file paths
- Shells (ie BASH) can help with this by offering to complete path names for you
- Command line completion is achieved by typing a partial path and then pressing the TAB key (to the left of Q)

Command line completion

List of files / folders:

```
Desktop
Documents
Downloads
Music
Public
Published
Templates
Videos
```

`T` [TAB] → `Templates`

`P` [TAB] → `Publi`

`Do` [TAB] → [beep]

`Do` [TAB] [TAB] → `Documents Downloads`

`Doc` [TAB] → `Documents`

You should ALWAYS use TAB completion to fill in paths for locations which exist so you can't make typing mistakes

(it obviously won't work for output files though)

Debugging Tips

- Be wary of anything which finishes suspiciously quickly!

- Look for errors before asking for help. They will either be
  - The last piece of text before the program exited
  - The first piece of text produced after it started (followed by the help file)

- Programs which are stuck can be cancelled with Control+C

Some useful commands

`cd mydir`	Change directory to `mydir`
`ls -ltrh`	List files in the current directory, show details and put the newest files at the bottom
`less x.txt`	View the `x.txt` text file Return = down one line Space = down one page q = quit

Data Processing Exercise

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Exploring and Understanding ChIP-Seq data

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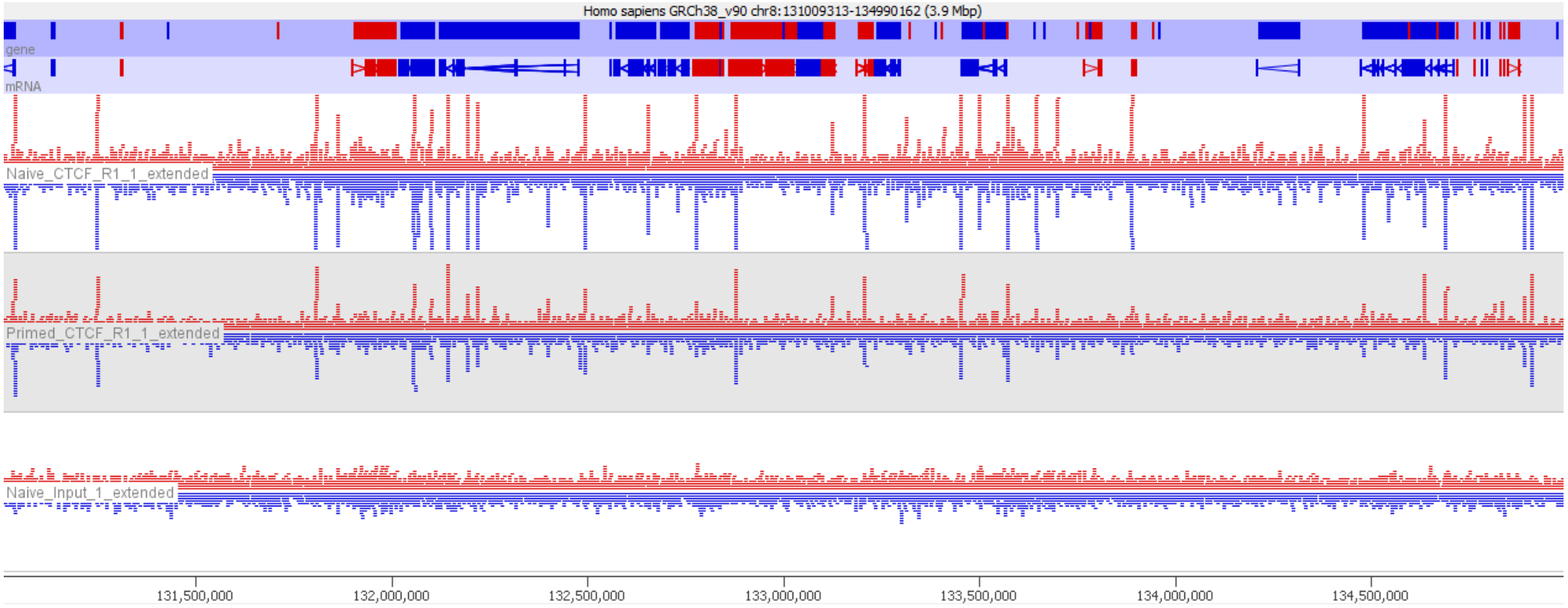
Some Basic Questions

- Is there any enrichment?
  - What is the size / patterning of enrichment?
- How well are my controls behaving?
- What is the best way to quantitate this data?
- Are there any technical artefacts?

Start with a visual inspection



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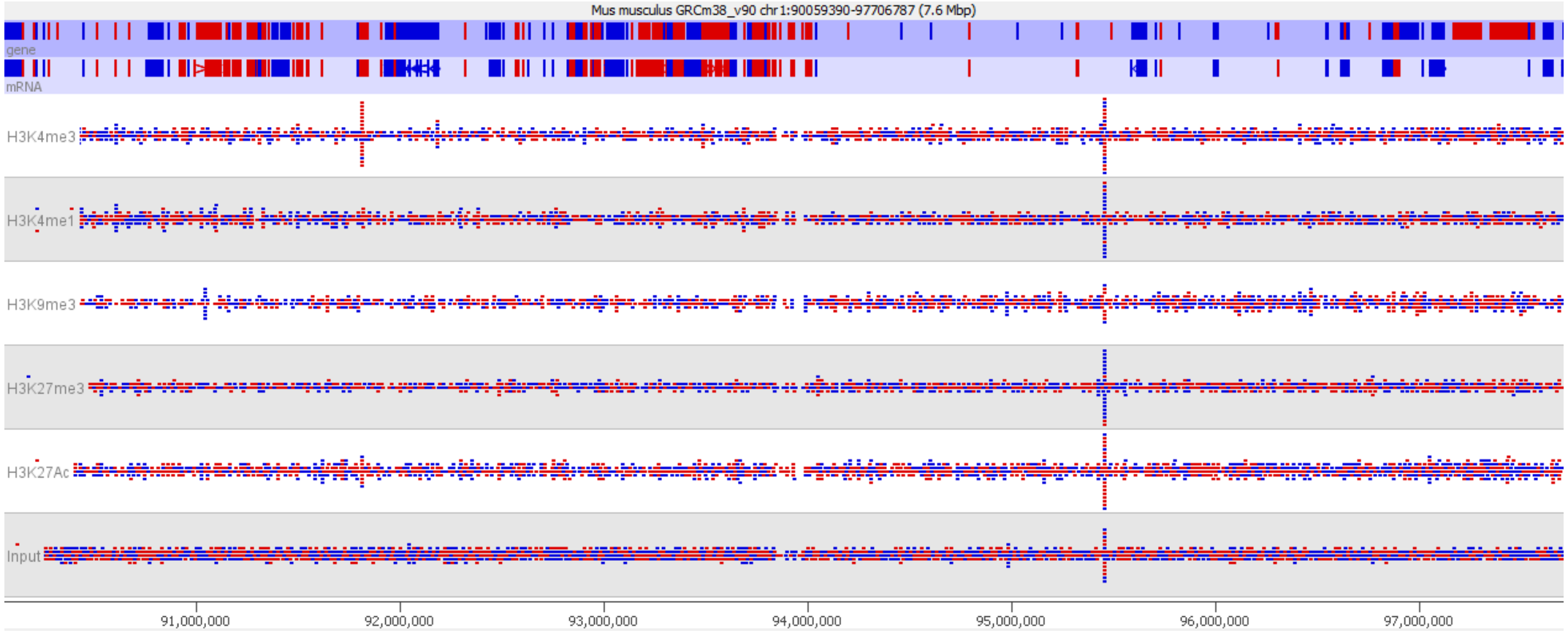
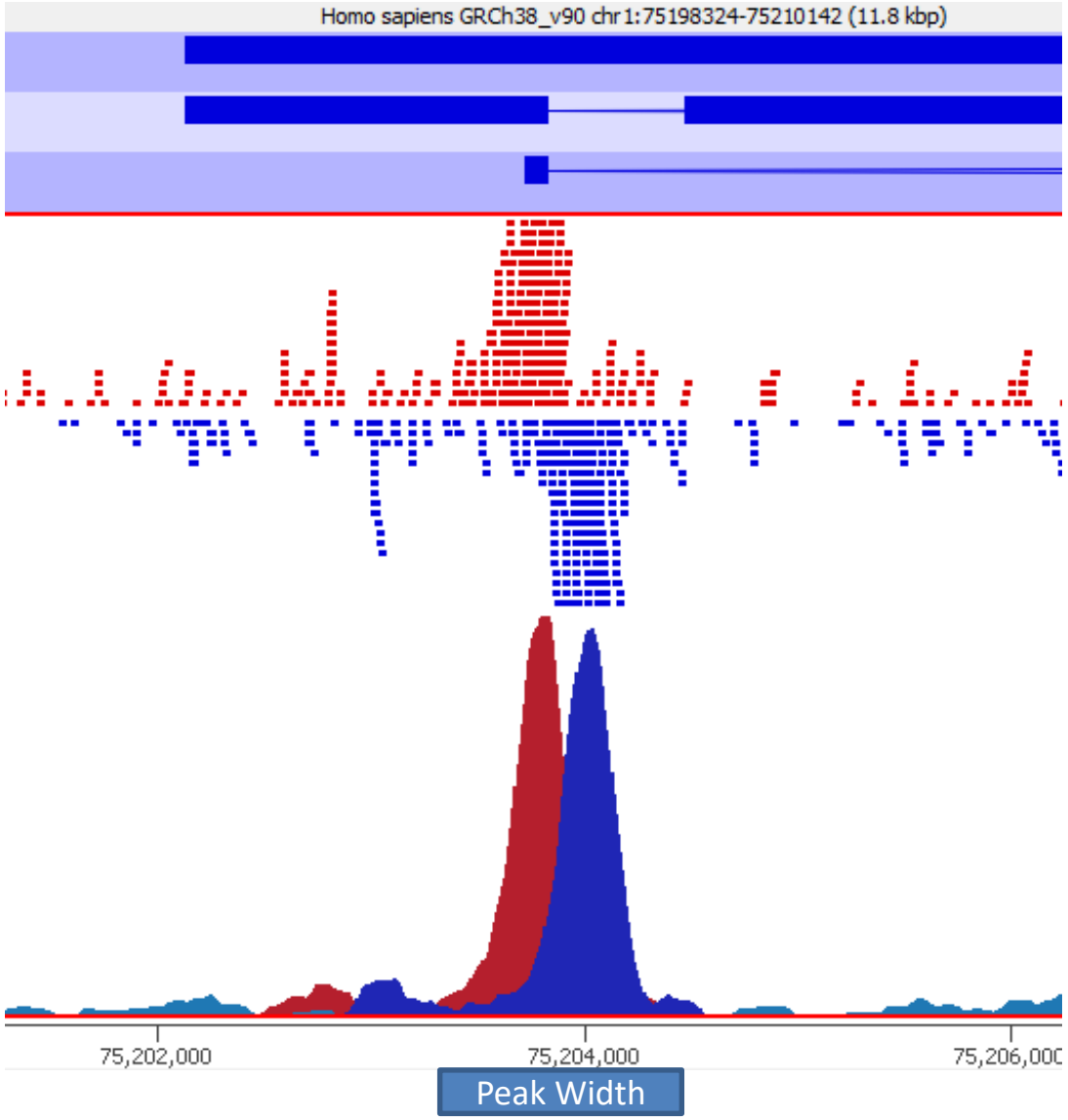


- Is there any enrichment?
- What is the size / patterning of enrichment?
- How well are my controls behaving?

Extending reads if necessary

For point enrichment, insert size is roughly peak width/2

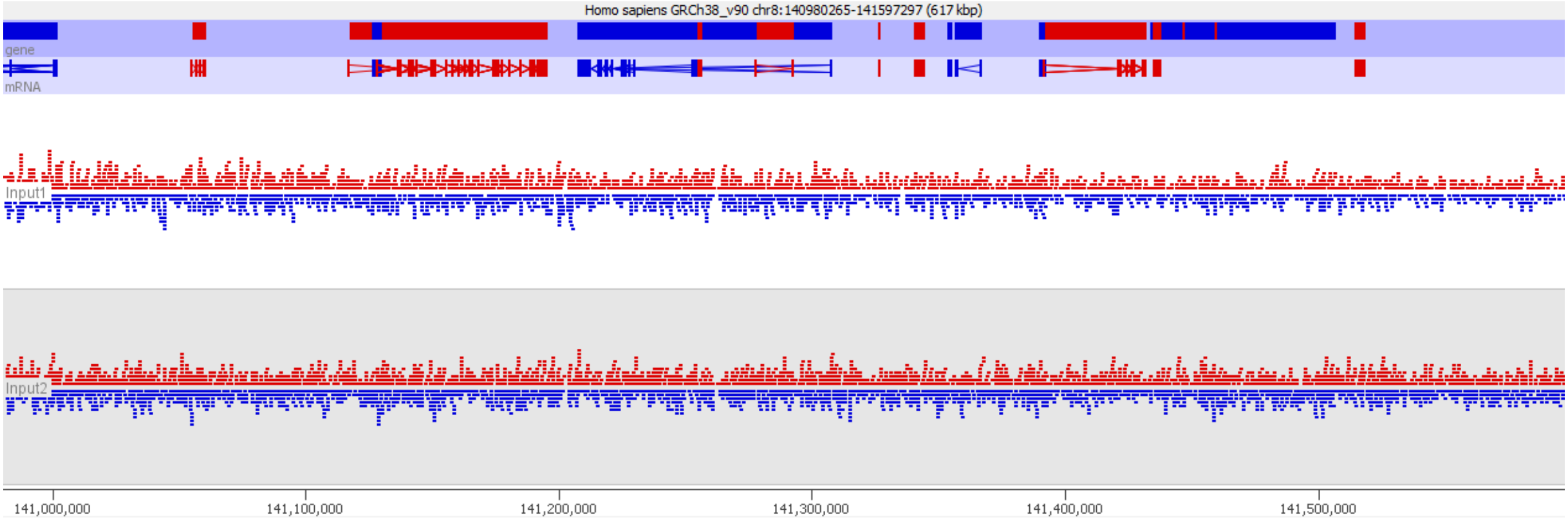


Examine Controls

- IgG or other Mock IP
  - Good result is no material at all
  - Not worth sequencing. Reads are only informative if the ChIP hasn't worked.
  - May be justified for Cut and Run where there is no real input

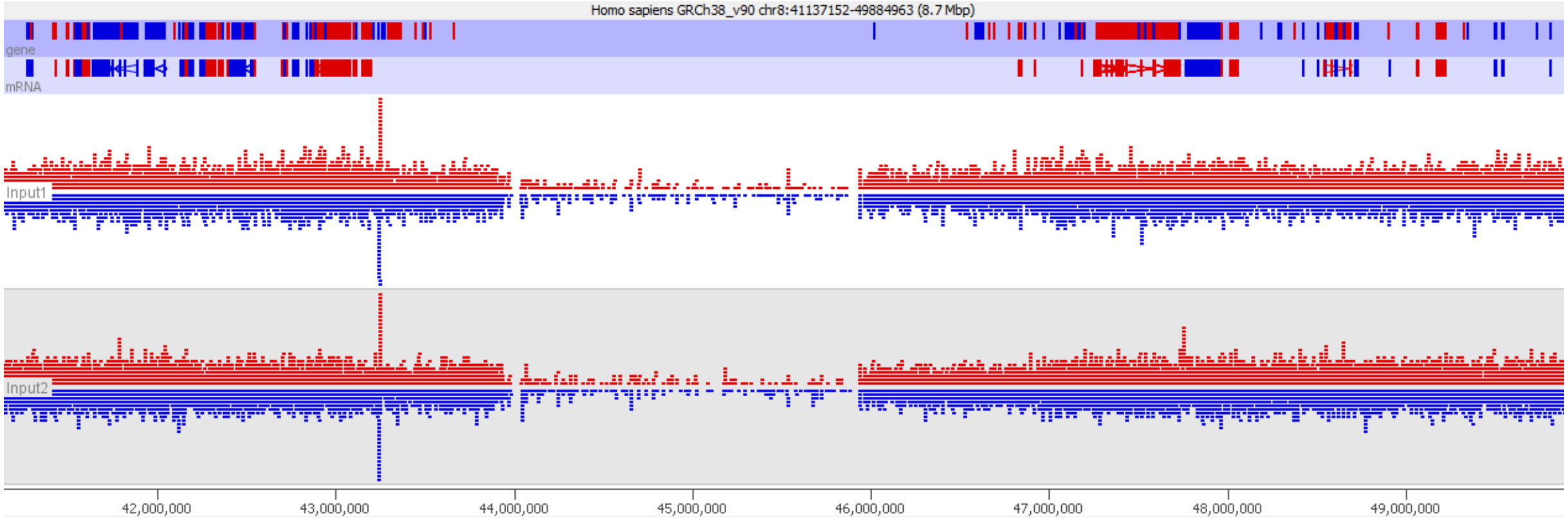
- Input material (sonicated / Mnase etc)
  - Genomic library - everywhere equally
  - Technical issues can cause variation

Examine Controls



- Does the coverage look even
- If there are multiple inputs to do they look similar

Examine Controls



Why do controls misbehave?

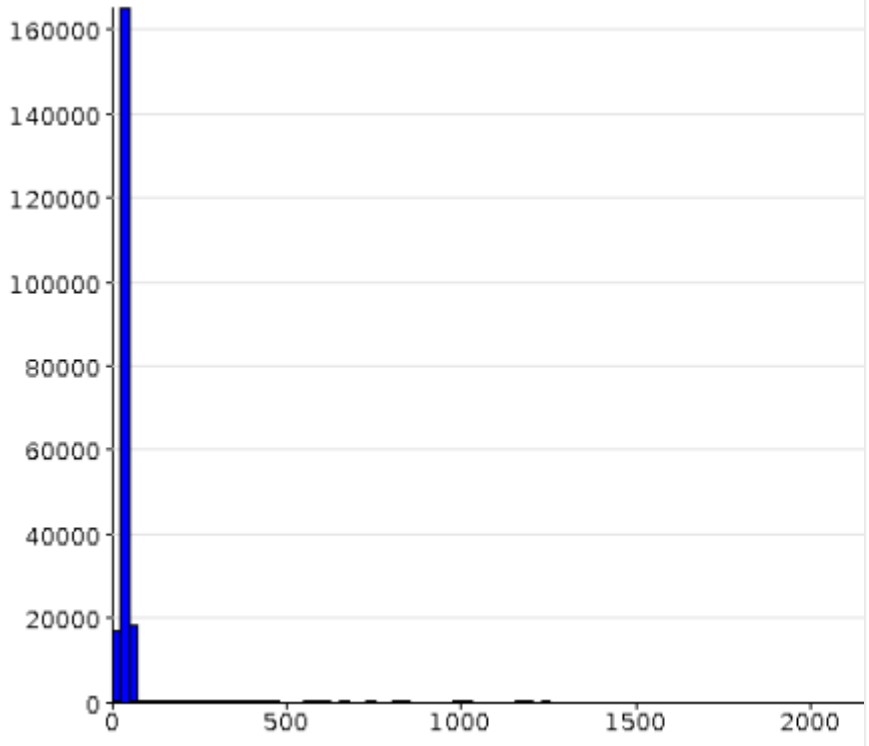
- Low coverage
  - Repetitive unmappable regions
  - Holes in the assembly
- High coverage
  - Mismapped reads from outside the assembly
- Biases
  - GC content
  - Segmental Duplication

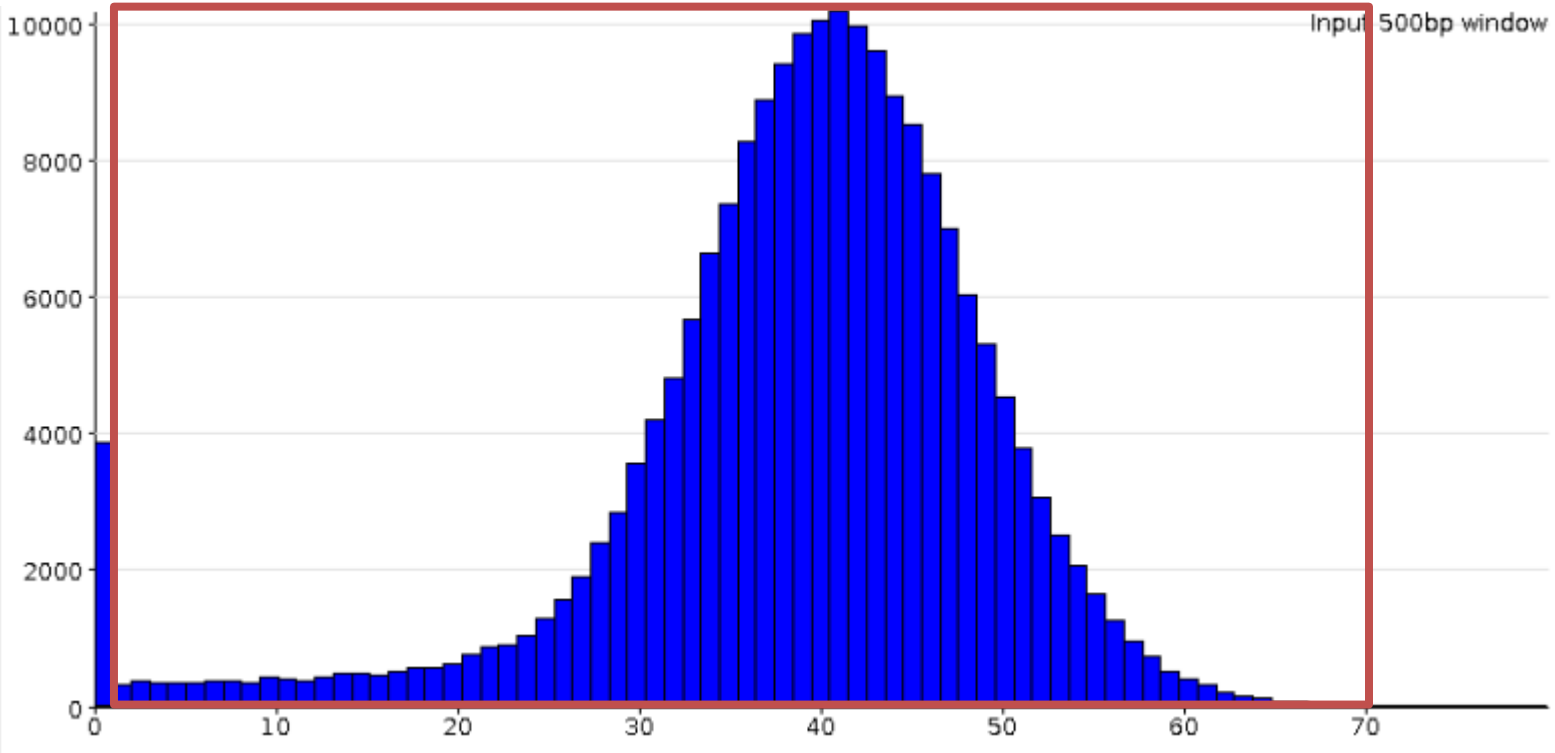
Blacklist these regions and remove them from the analysis (ignore hits within these regions)

Input normalisation might help, but requires further examination

Making Blacklists

- Look at distribution of Input counts
  - Set limits on unusually high/low values
  - Remove regions outside those limits





Comparison of samples

Initial Quantitation

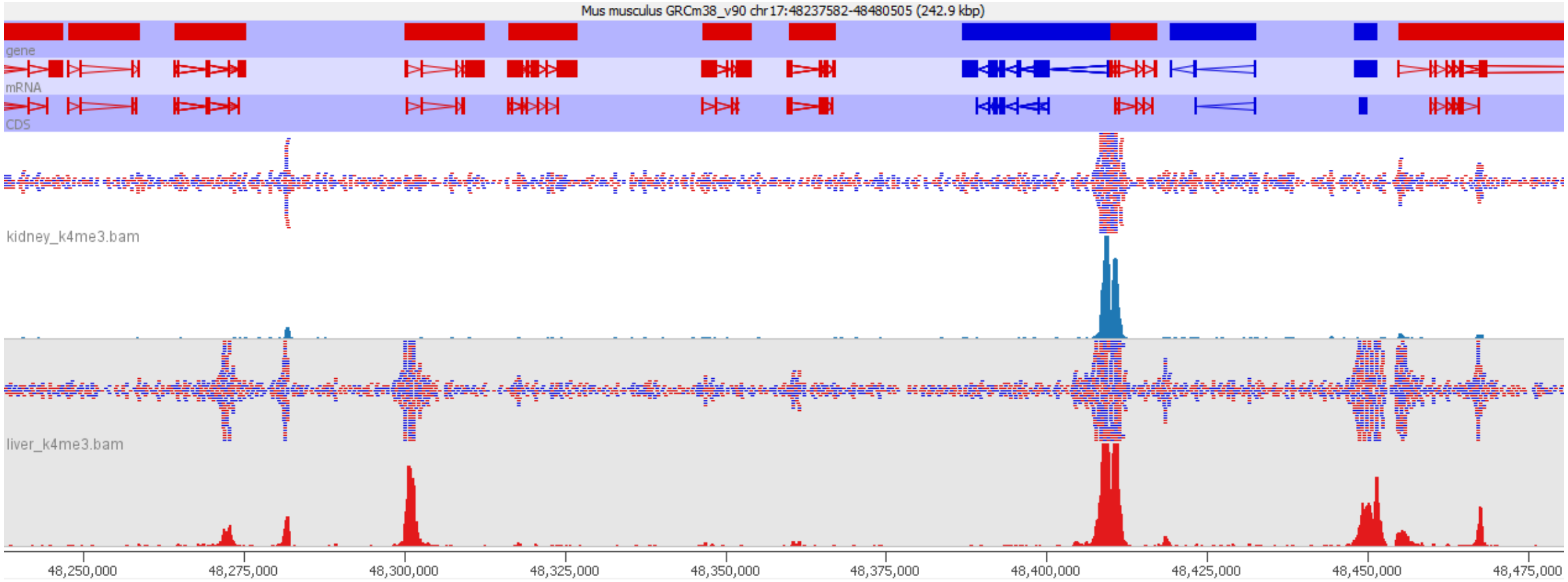
- Always start with a simple unbiased quantitation (not focussed on features/peaks)

- Tiled measures over the whole genome
  - Use approximate insert size as window size
  - Something around 500bp is normally sensible

- Linear read count quantitation corrected for total library size

Compare samples

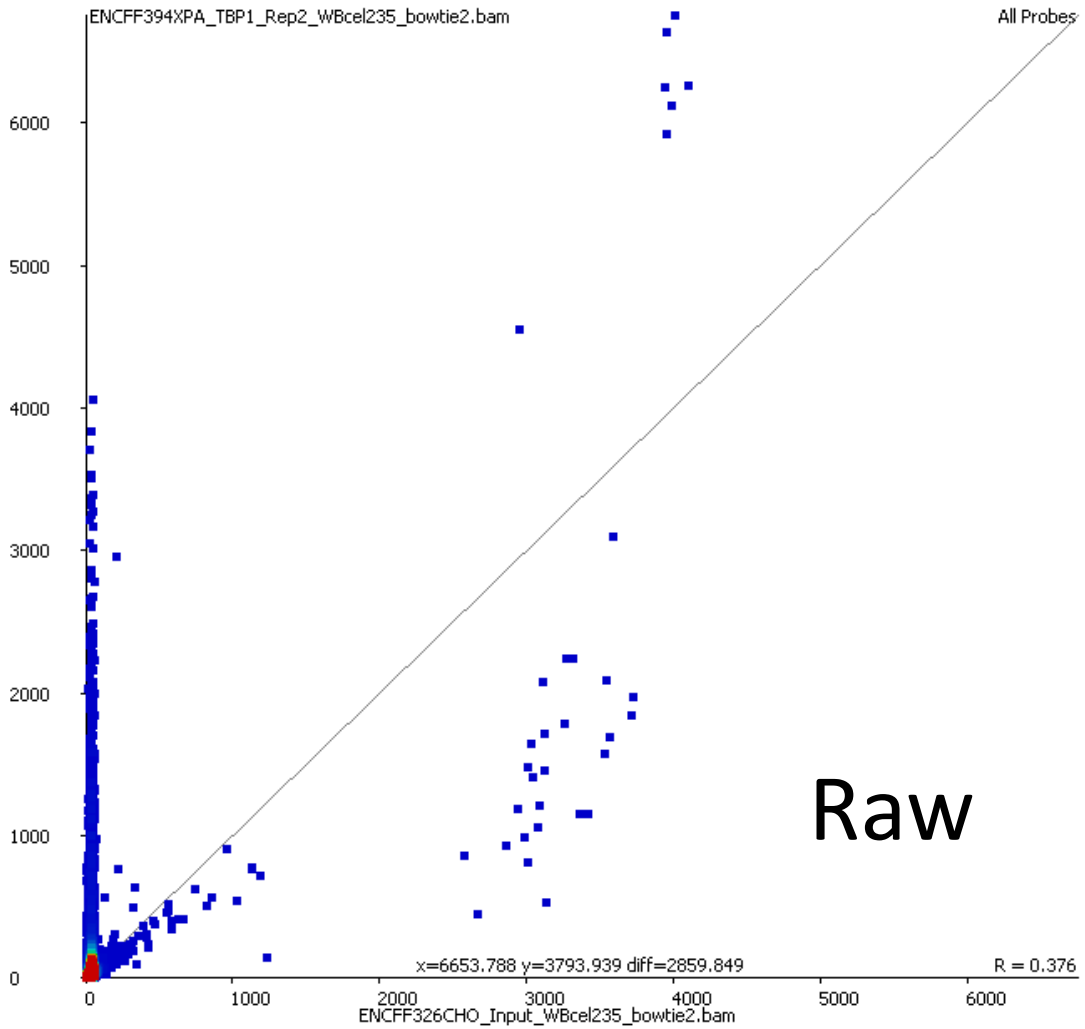
Visual comparison against raw data



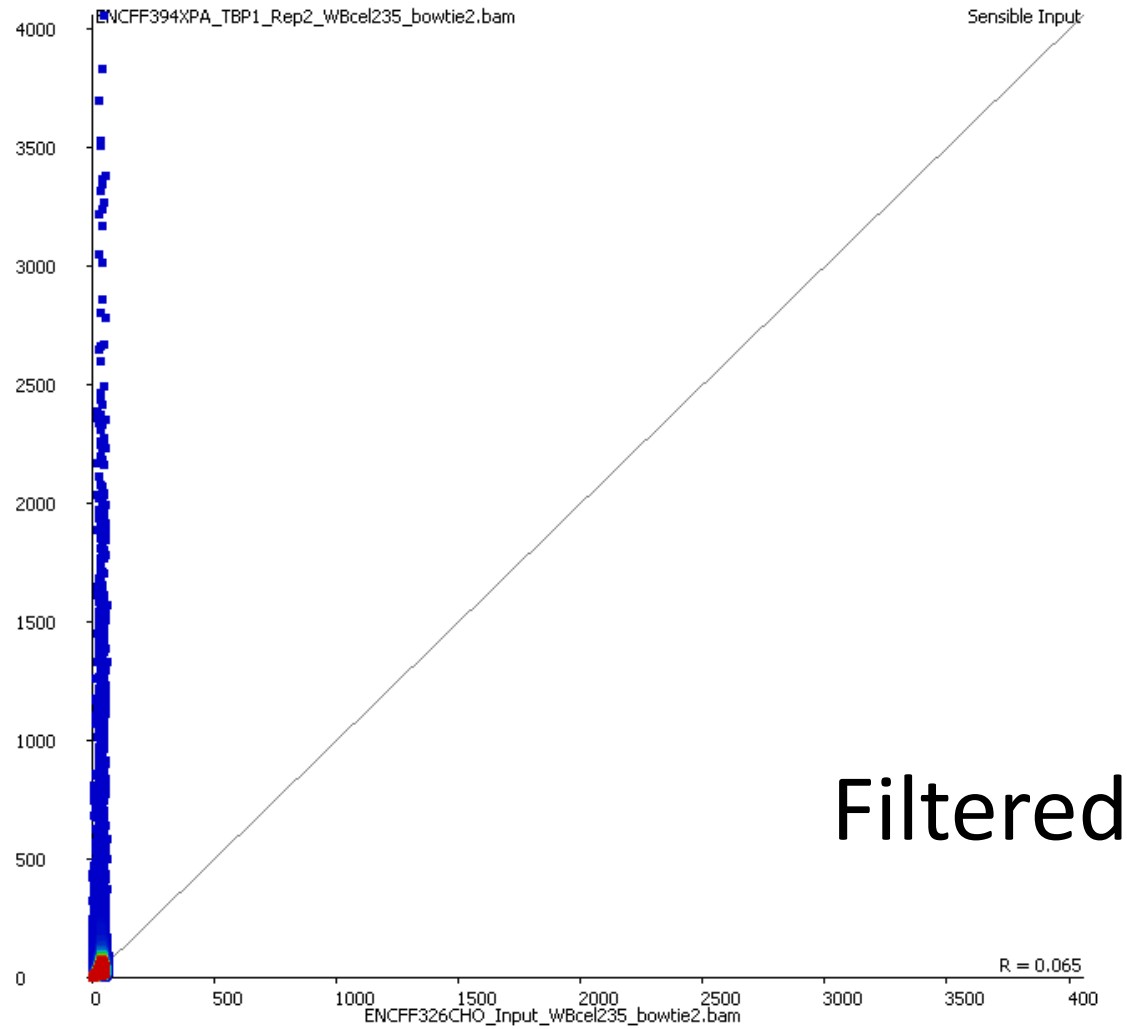
- Similar apparent overall enrichment
- Any obvious differences?

Compare samples

Scatterplot input vs ChIP



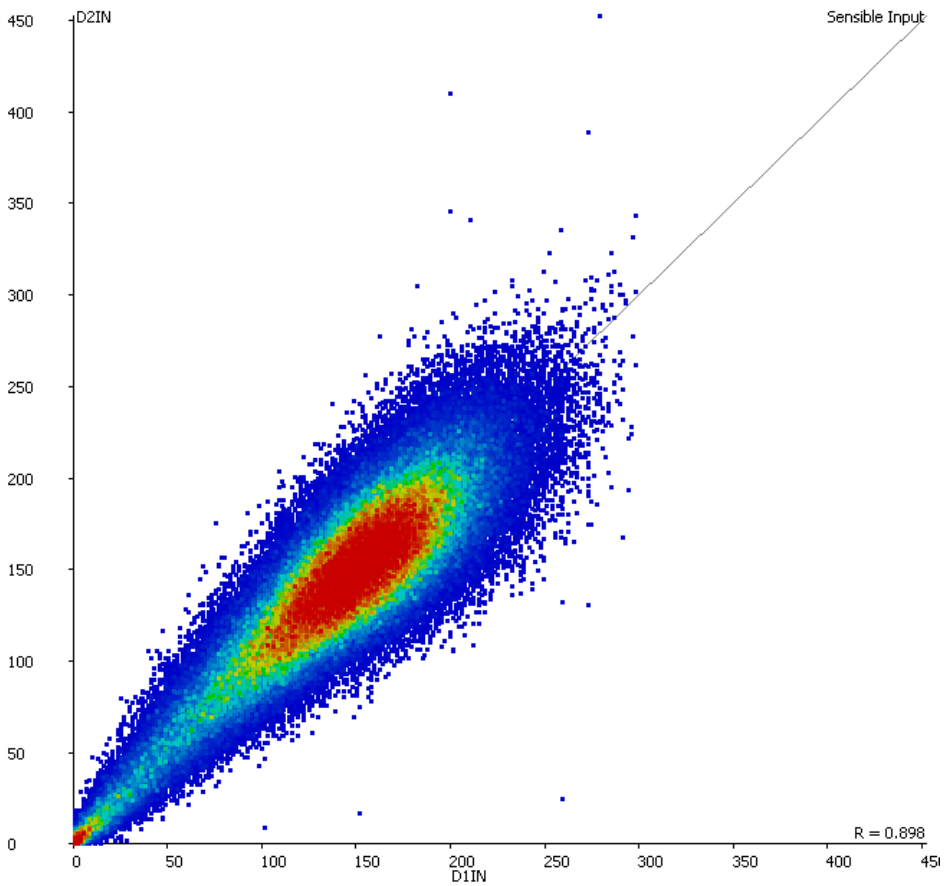
Raw



Filtered

Compare samples

Scatterplot input vs input



- Any suggestion of differential biases in inputs
- Can we merge them to use as a common input

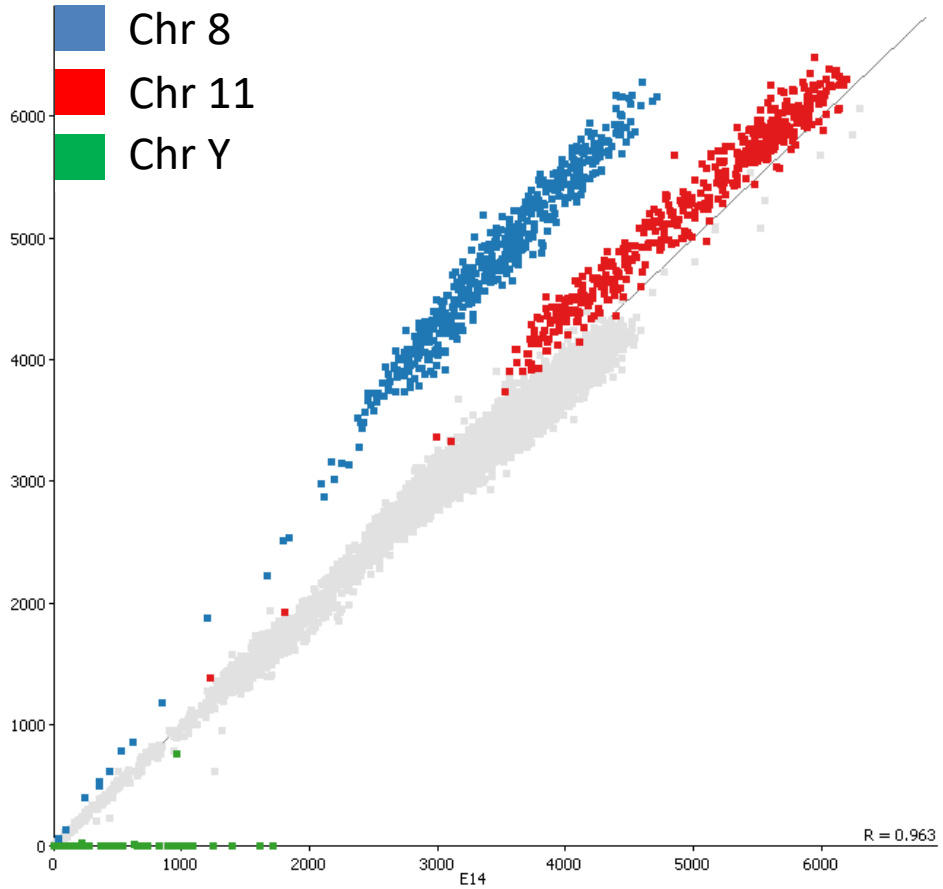
PNAS

Extensive genomic copy number variation in embryonic stem cells

Qi Liang, Nathalie Conte, William C. Skarnes, and Allan Bradley[1]

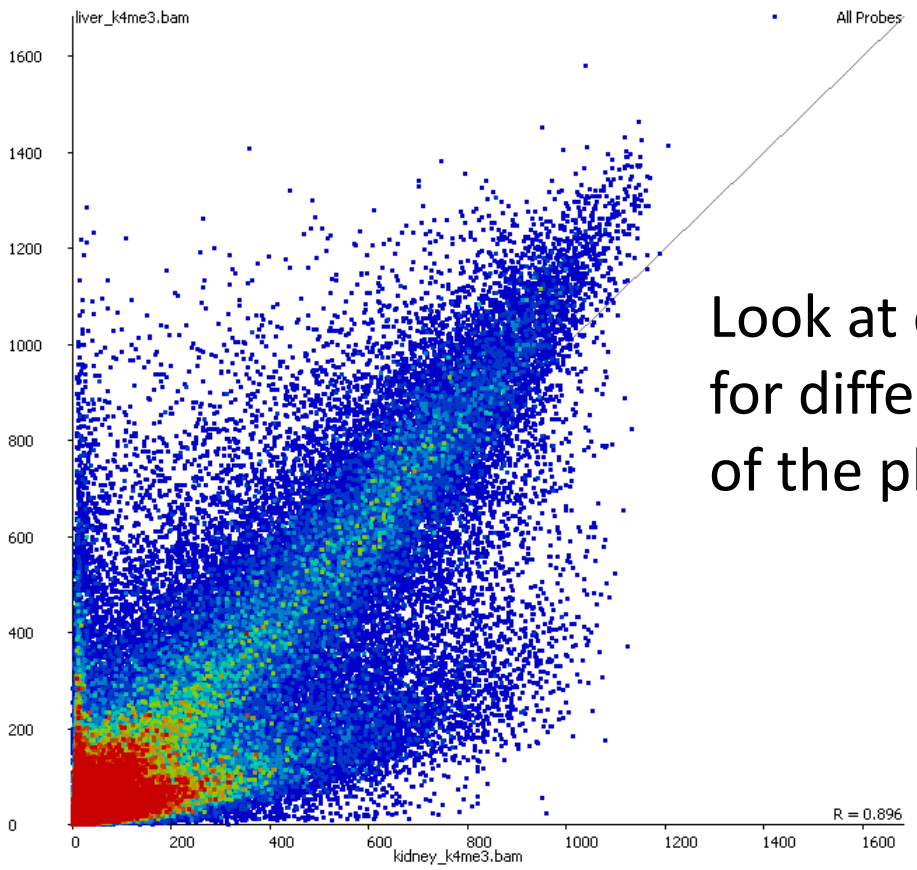
Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, United Kingdom

Of 26 clones that could not contribute to the mouse germ line, trisomies were detected in 7 which involved chromosomes 1, 6, 8, and 11. In 5 cases, loss of the Y chromosome was detected.

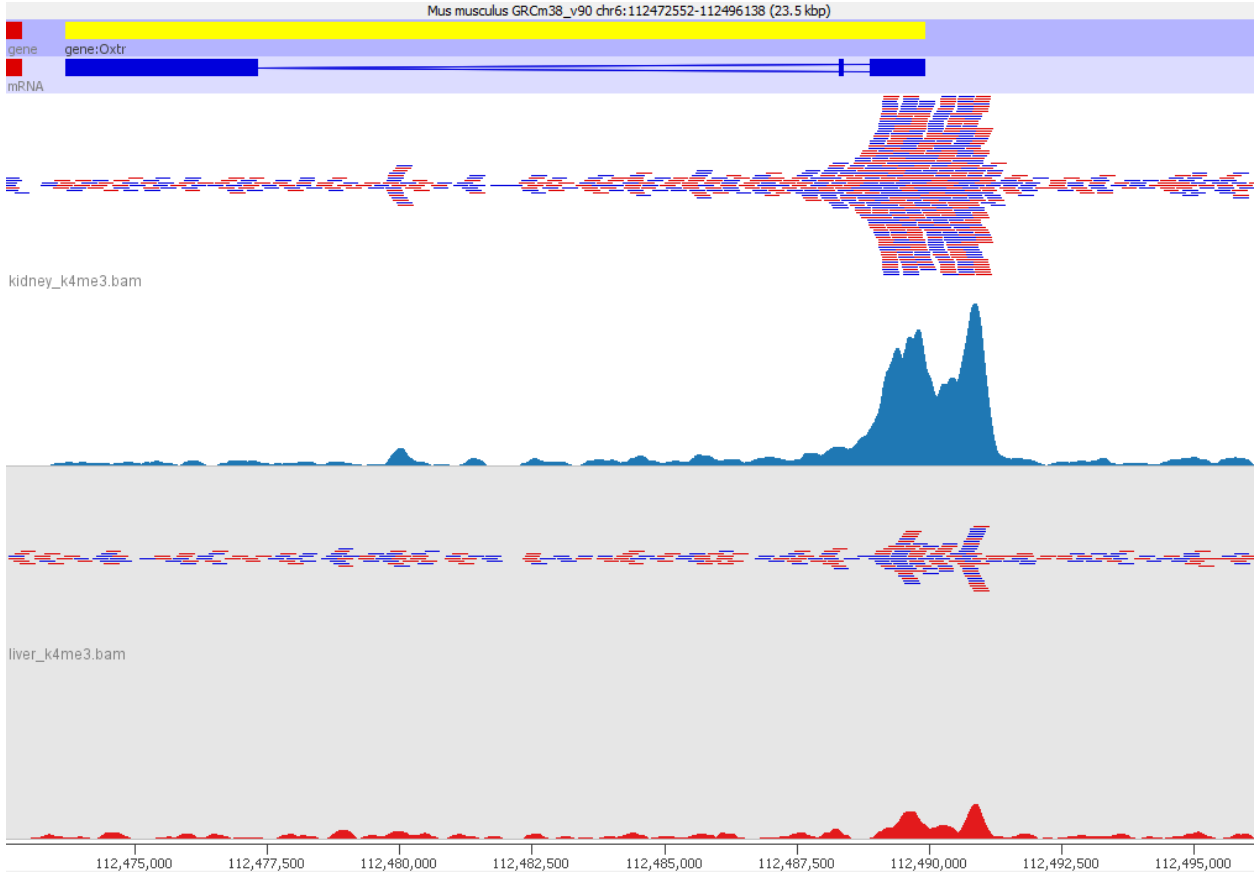


Compare samples

Scatterplot ChIP vs ChIP



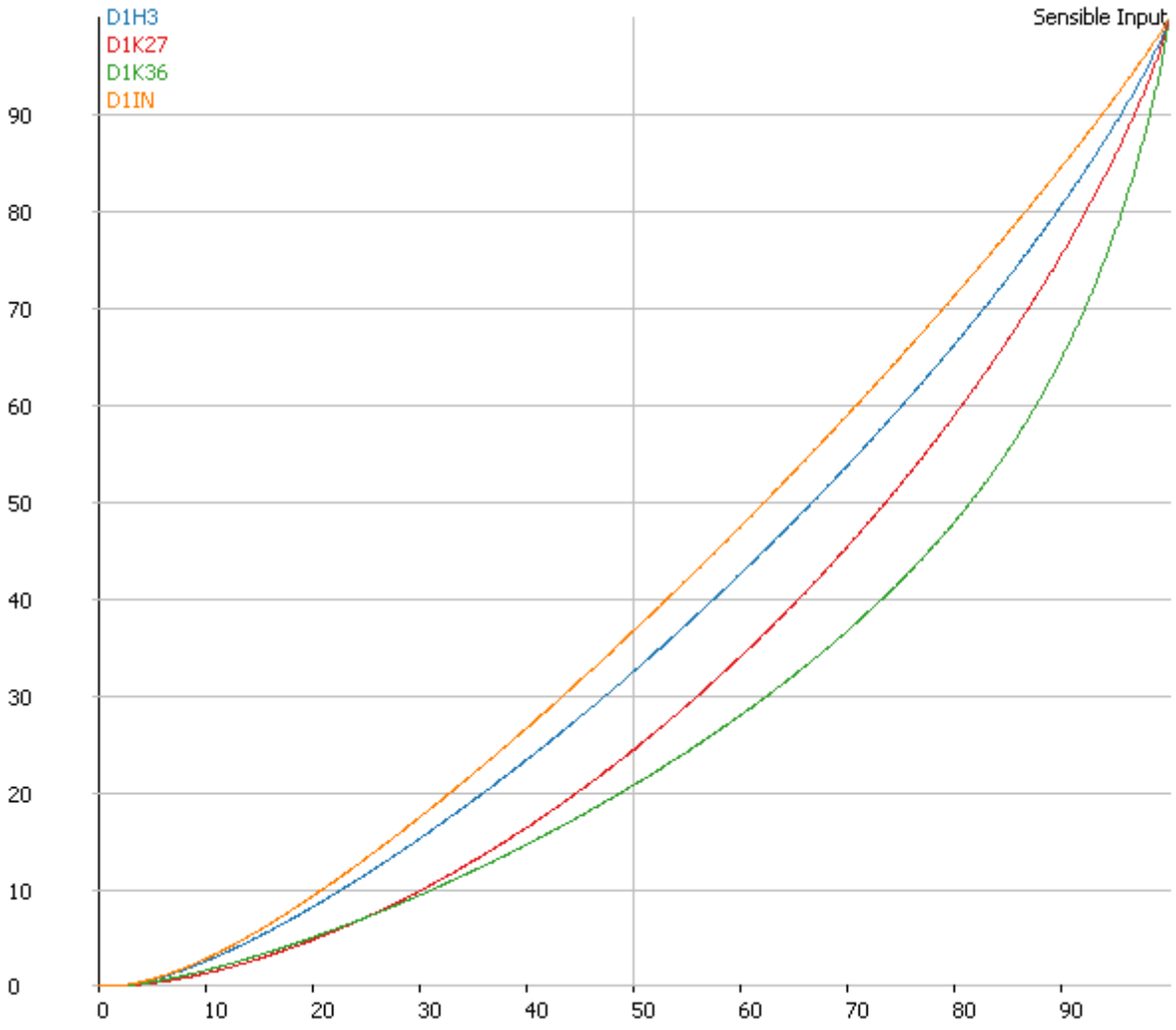
Look at examples for different parts of the plot



- Look for outgroups (differentially enriched)
- Compare level of enrichment (compare to diagonal)

Compare samples

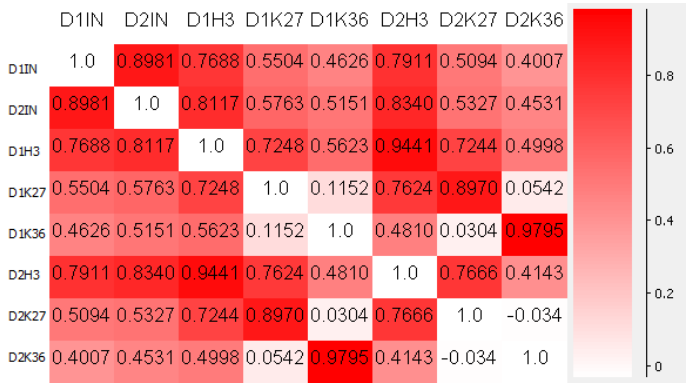
Summarise distributions



- QQPlot
  - Percentile though measures(x) vs Percentile through total quantitation (y)
  - Perfect input is on the diagonal
  - More enrichment moves the curve down and right
  - How flat is your input? How consistent are the ChIPs?

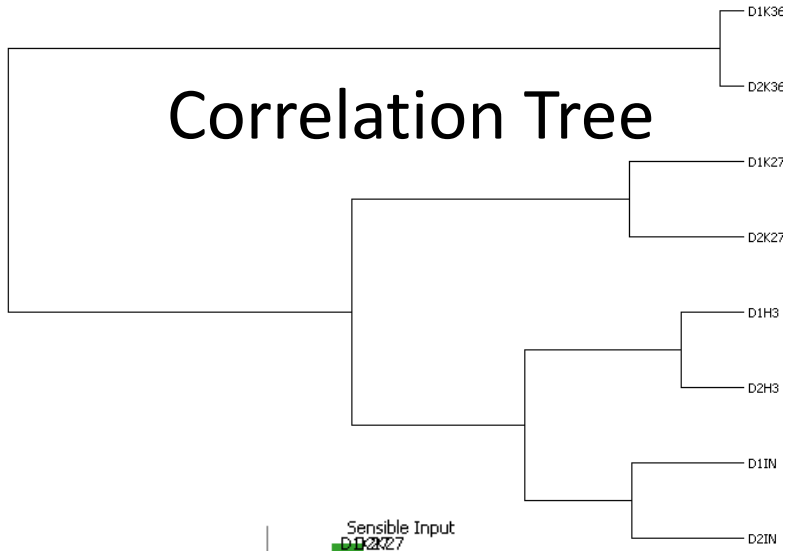
Compare samples

Higher level clustering

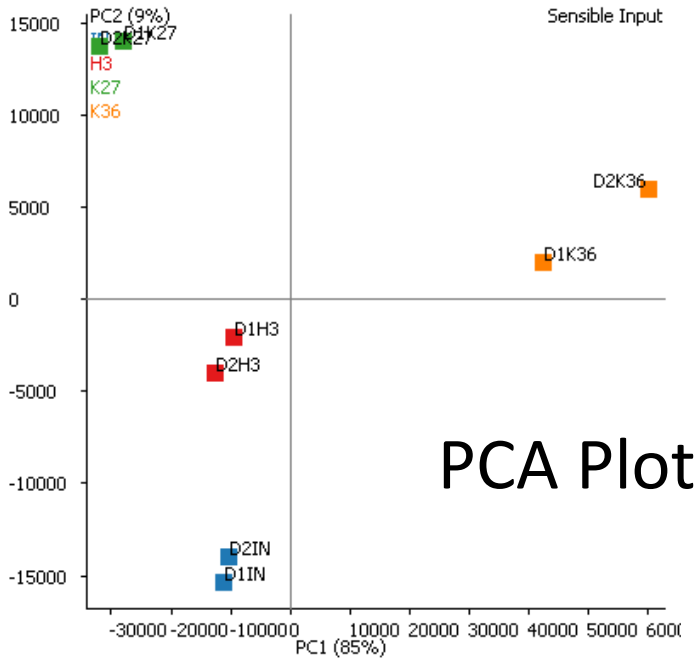


	D1IN	D2IN	D1H3	D1K27	D1K36	D2H3	D2K27	D2K36
D1IN	1.0	0.8981	0.7688	0.5504	0.4626	0.7911	0.5094	0.4007
D2IN	0.8981	1.0	0.8117	0.5763	0.5151	0.8340	0.5327	0.4531
D1H3	0.7688	0.8117	1.0	0.7248	0.5623	0.9441	0.7244	0.4998
D1K27	0.5504	0.5763	0.7248	1.0	0.1152	0.7624	0.8970	0.0542
D1K36	0.4626	0.5151	0.5623	0.1152	1.0	0.4810	0.0304	0.9795
D2H3	0.7911	0.8340	0.9441	0.7624	0.4810	1.0	0.7666	0.4143
D2K27	0.5094	0.5327	0.7244	0.8970	0.0304	0.7666	1.0	-0.034
D2K36	0.4007	0.4531	0.4998	0.0542	0.9795	0.4143	-0.034	1.0

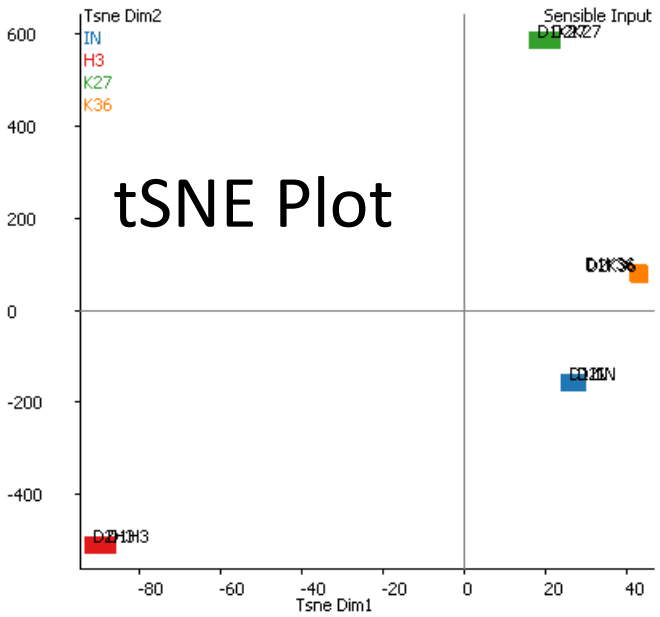
Correlation Matrix



Correlation Tree



PCA Plot



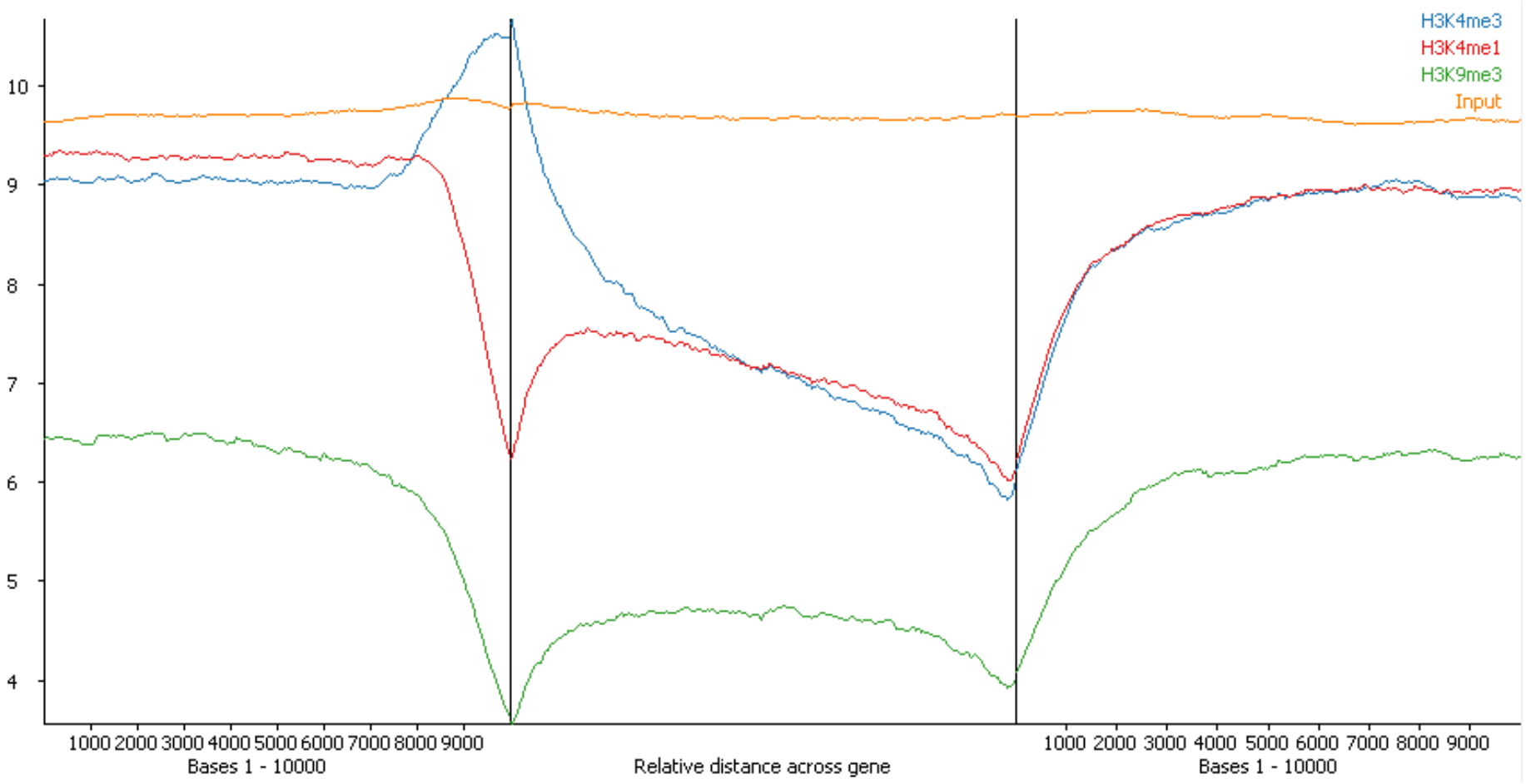
tSNE Plot

Associate enrichment with features

Trend Plots

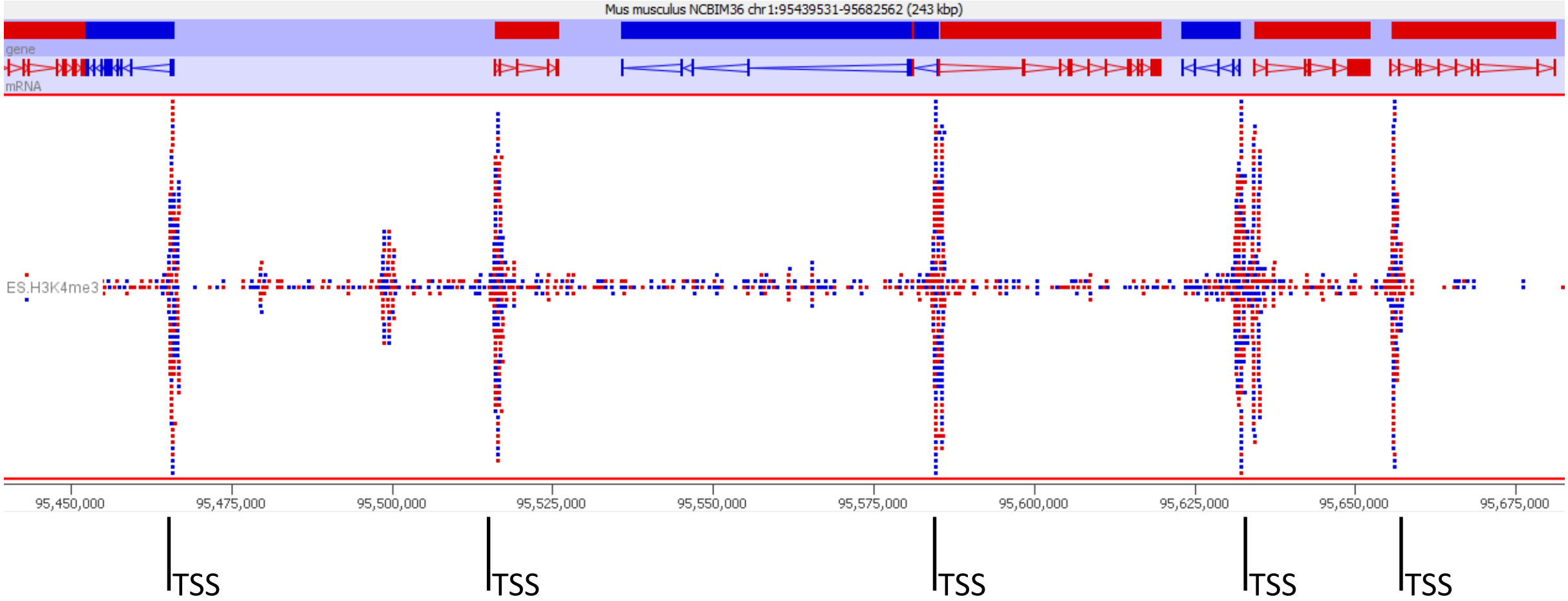
- Graphical way to look at overall enrichment relative to positions in features
  - Gene bodies
  - Promoters
  - CpG islands
- May influence how we later quantitate and analyse the data
  - Analyse per feature
  - Look for exceptions to the general rule

Trend Plot Example

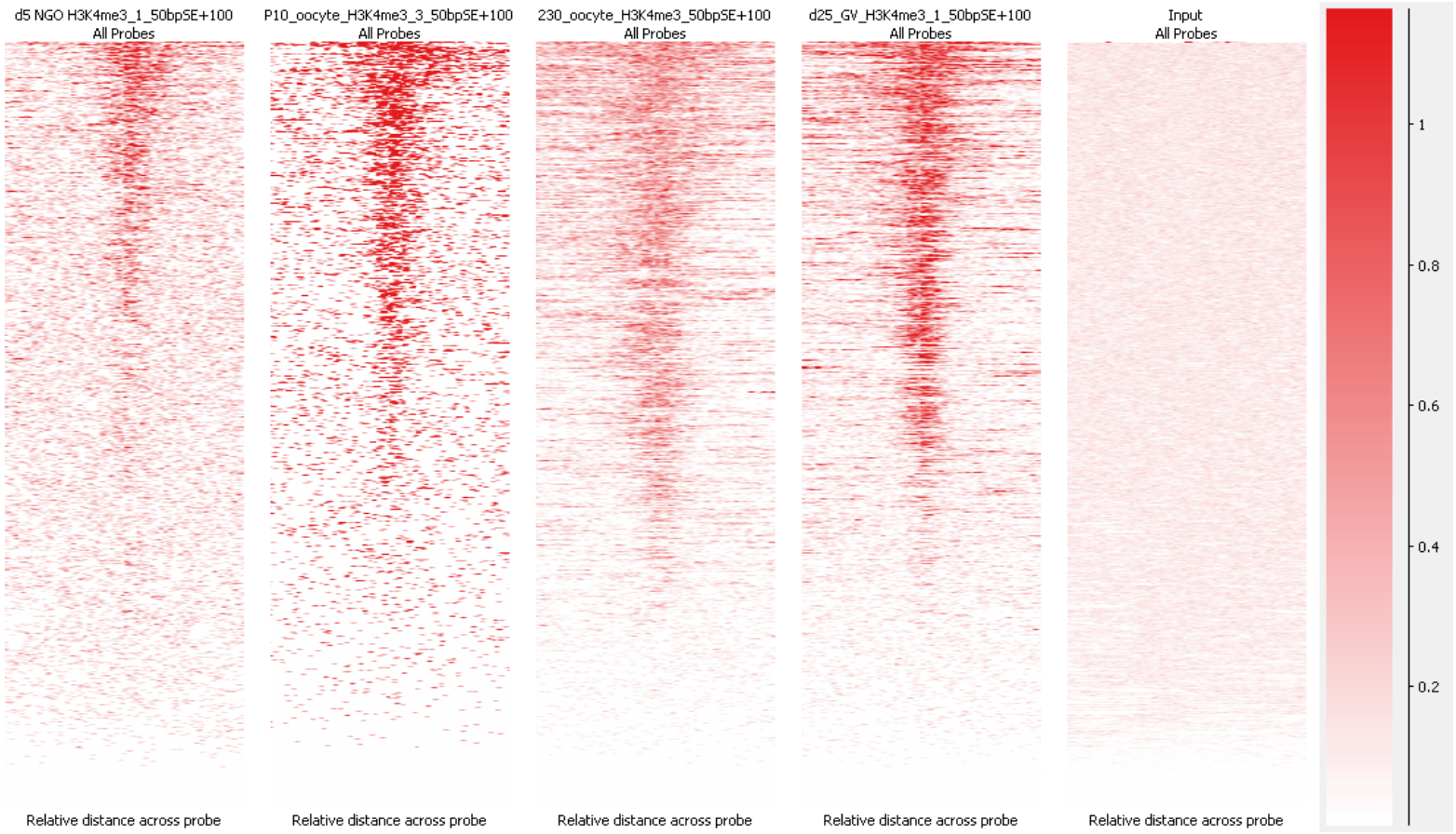


- Overall average
- Says nothing about the number / proportion of features affected

Check trend plot results against data



Aligned Probes Plots give more detail



- Information per feature instance
- Comparison of equivalent features in different marks/samples

After exploration you should…

- Know whether your ChIP is really enriched
- Know the nature / shape of the enrichment
- Know whether your controls behave well
- Know whether you're likely to have differential enrichment
- Know if you will need additional normalisation
- Know the best strategy to measure your data

Data Exploration Exercise

Babraham Bioinformatics

Analysing ChIP-Seq Data

Babraham Bioinformatics

Steps in Analysis

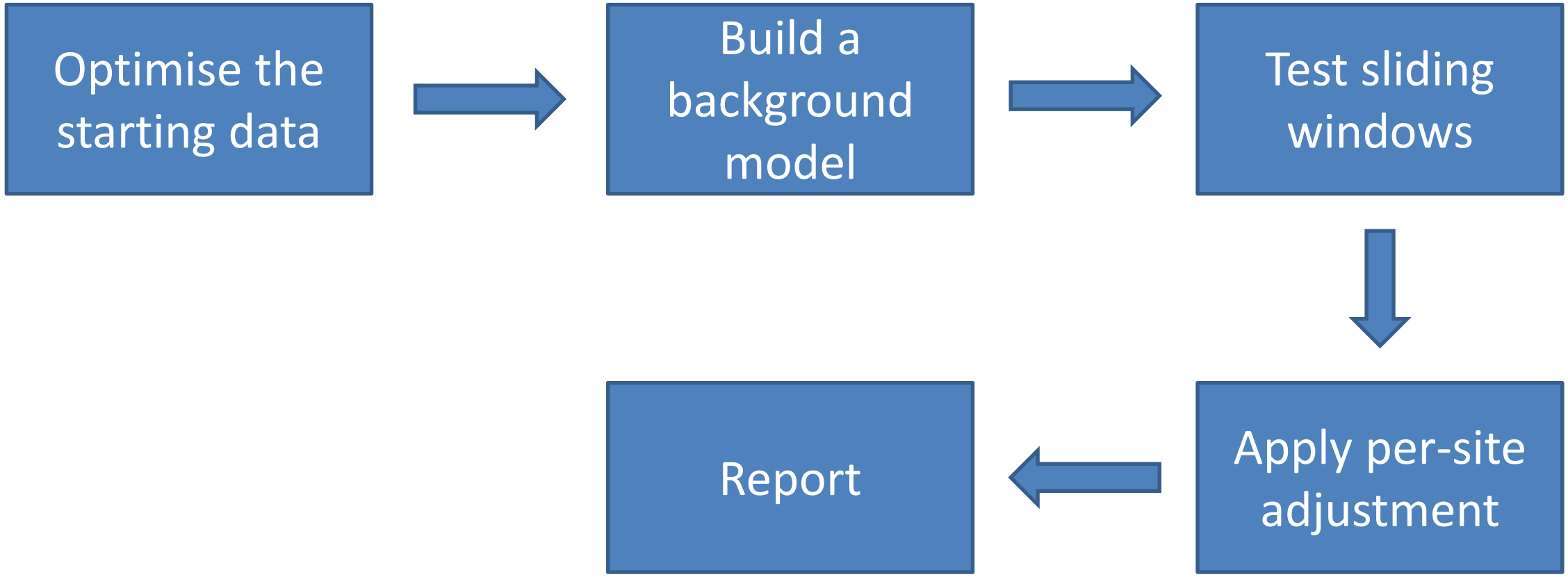
- Define enriched regions
  - Based around features
  - De-novo peak prediction
- Quantitate
  - Corrections and Normalisation
- Compare
  - Categorical
  - Quantitative

Defining Regions - Should I peak call?

- Choices
  - Make measurements around features (promoters / genes / CpG islands etc)
  - Make measurements around enriched regions (peaks)

- Can I use features?
  - Do you see a strong and complete linkage between enrichment and the type of feature you want to use?
  - If not, then you should peak call

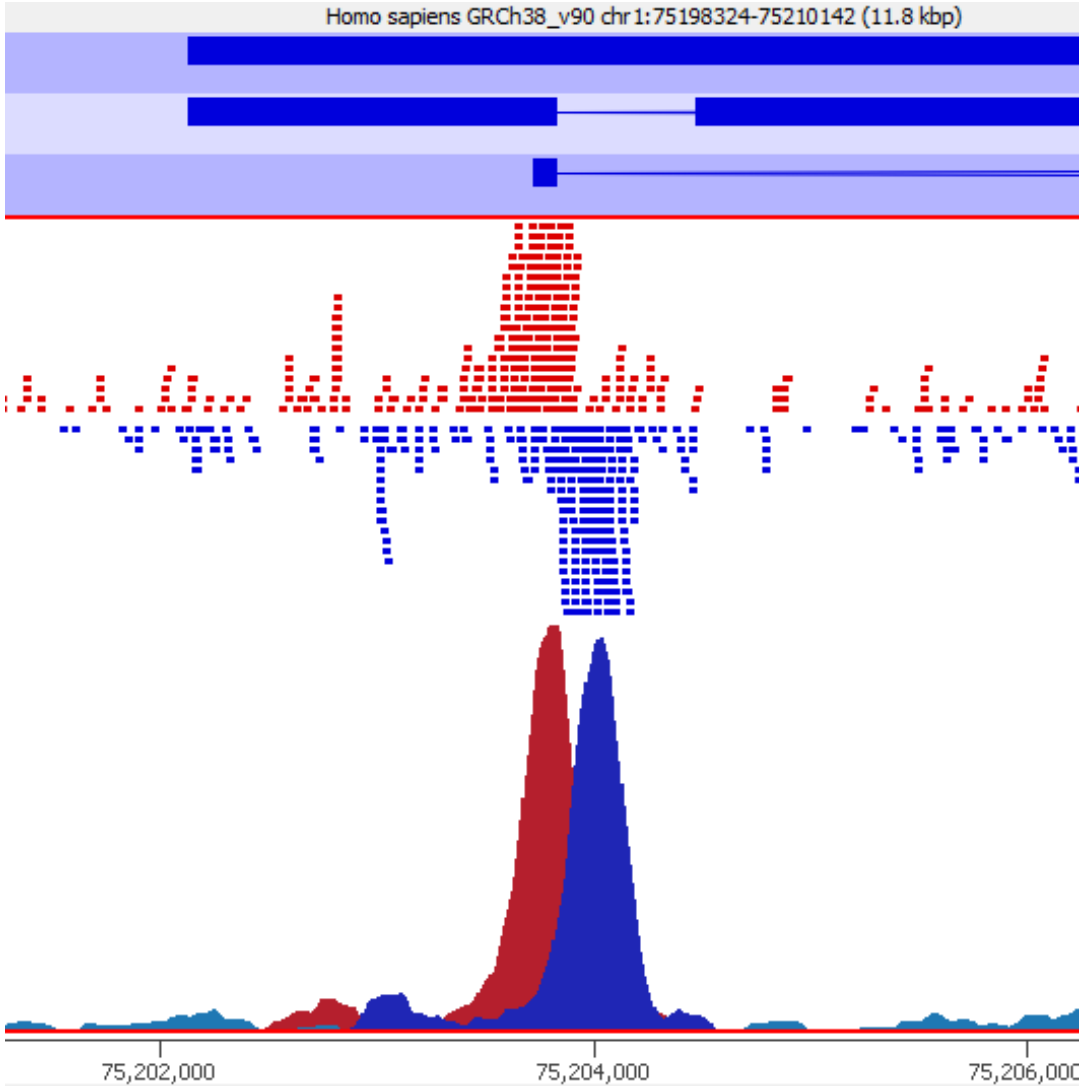
How MACS Works



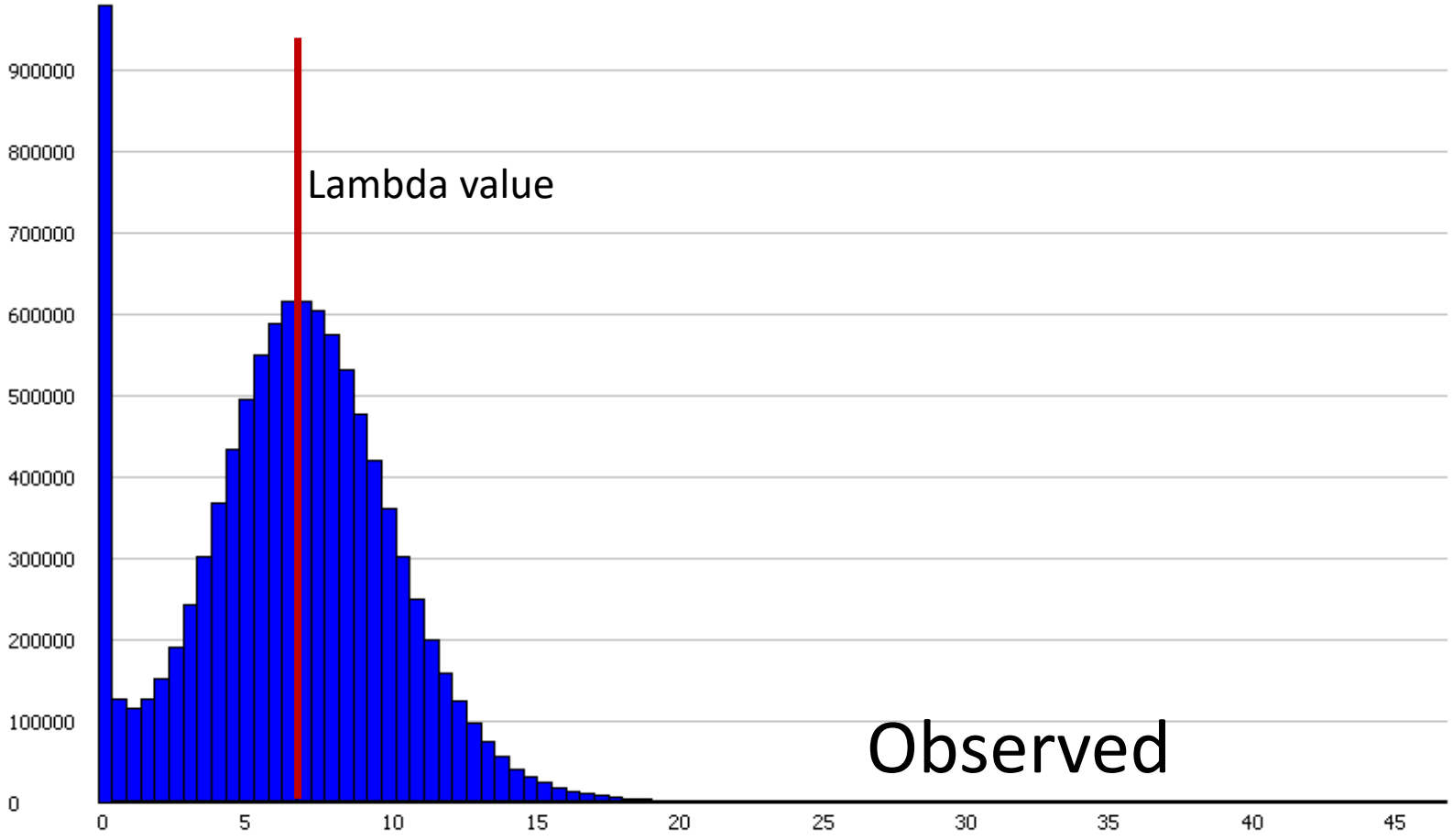

```
macs2 callpeak --broad -t chip.bam -c input.bam
```

Optimise the starting data

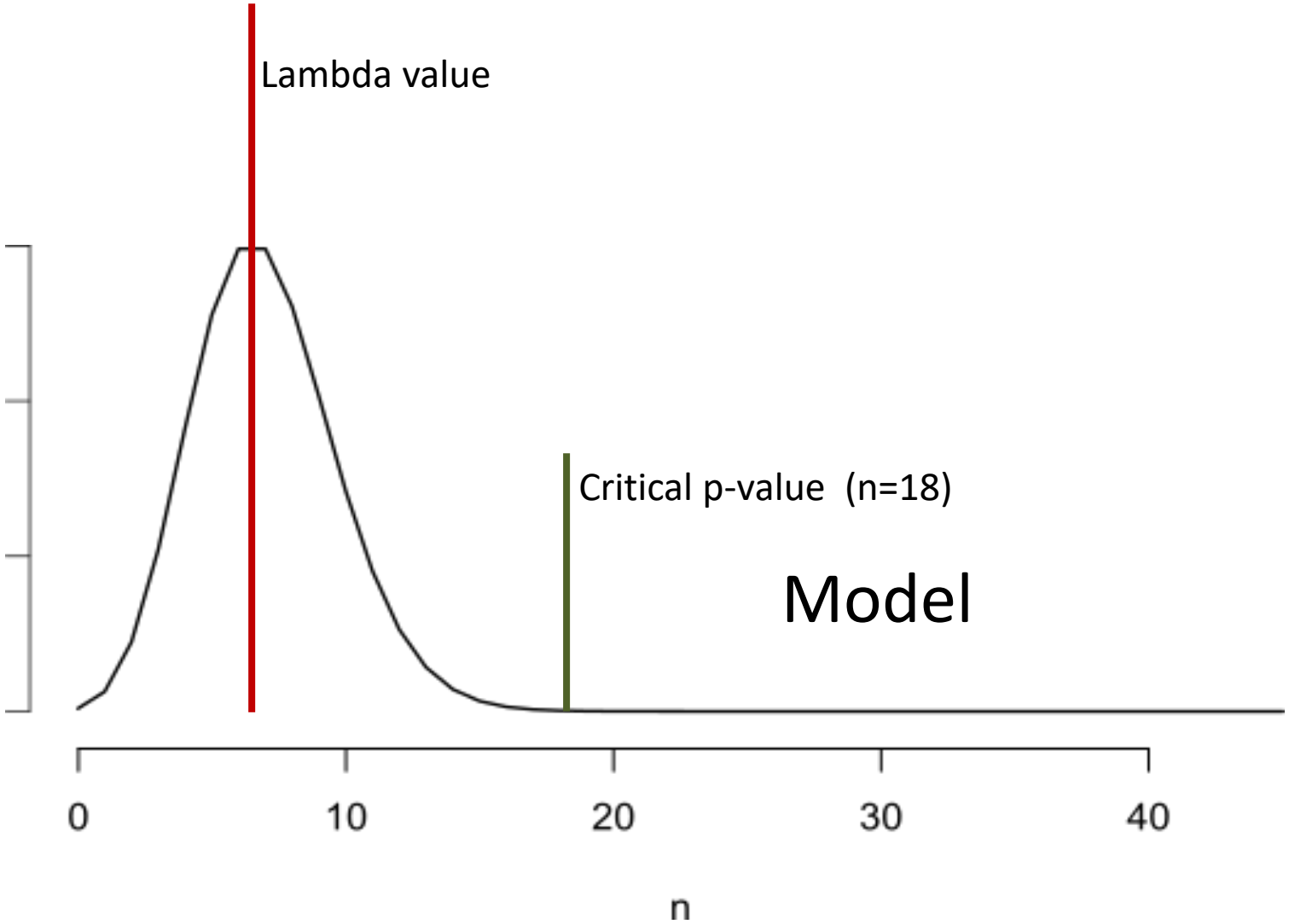
- Correct the for/rev offset
- Deduplicate



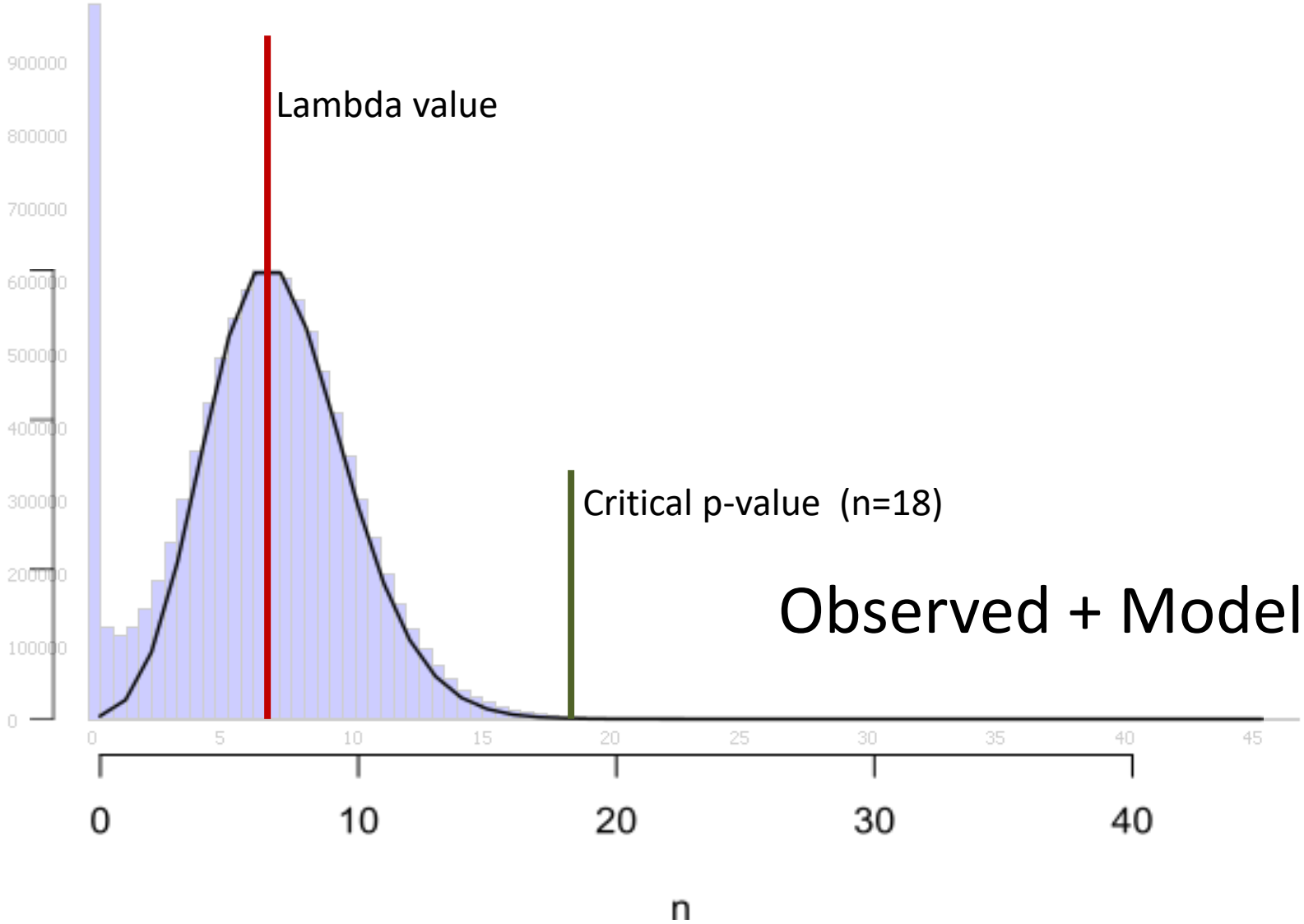
Build a background model



Build a background model



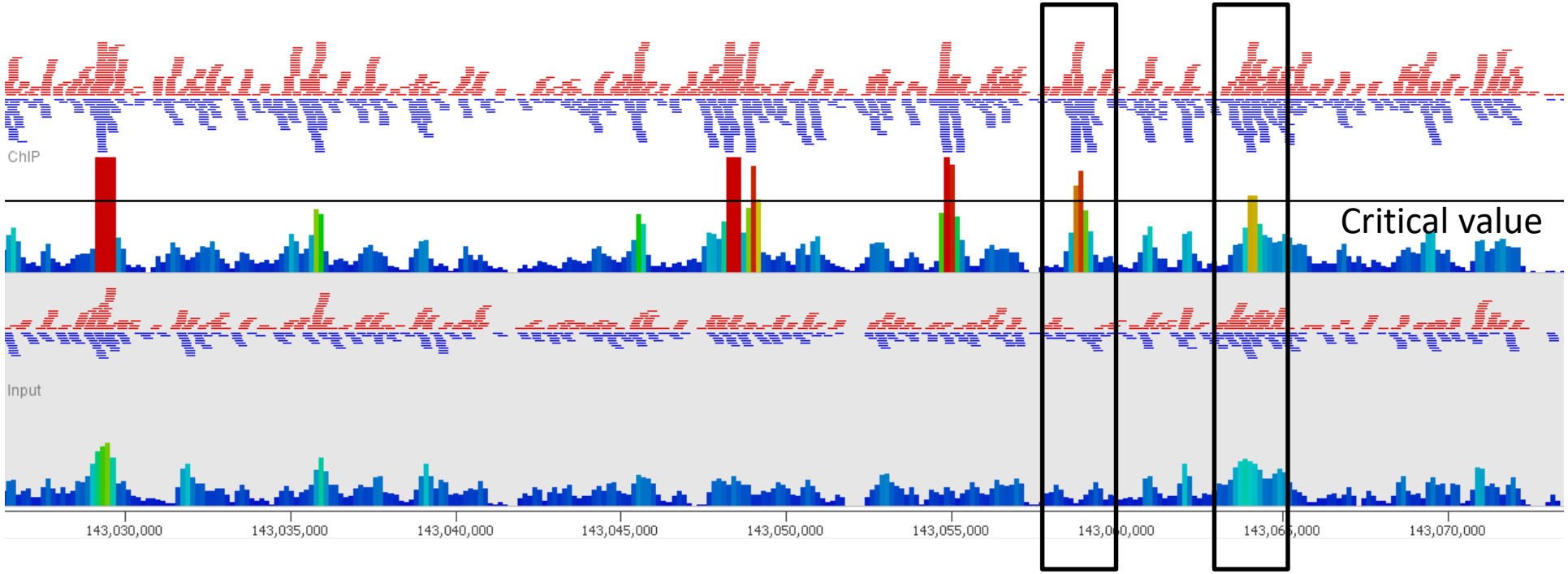
Build a background model



Test Sliding Windows

- Generally use half of the library fragment size
- Windows whose count exceeds the critical value are kept
- Merge adjacent windows over the critical value to form peaks
- Generates candidate (not final) peak set

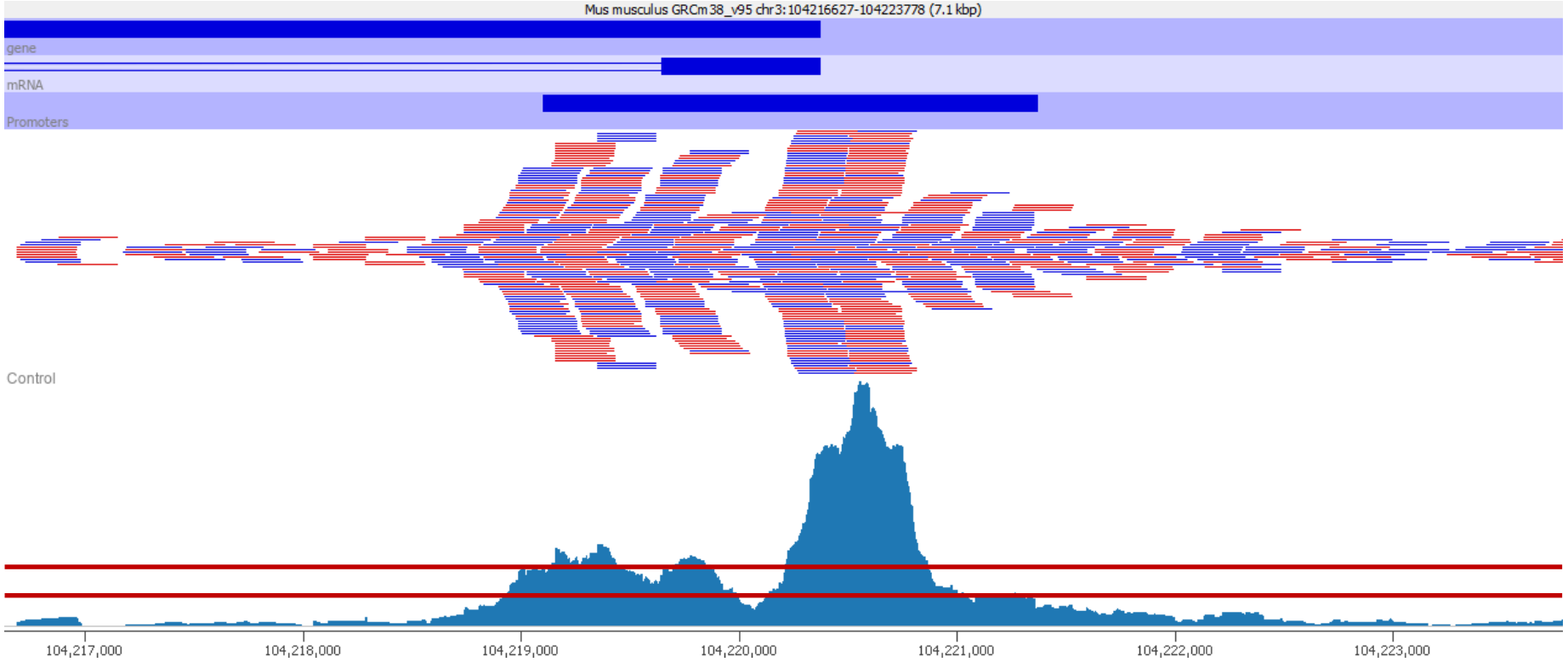
Correct for local variation



Generate localised model if input density
is higher than the global value
Most pessimistic p-value is kept

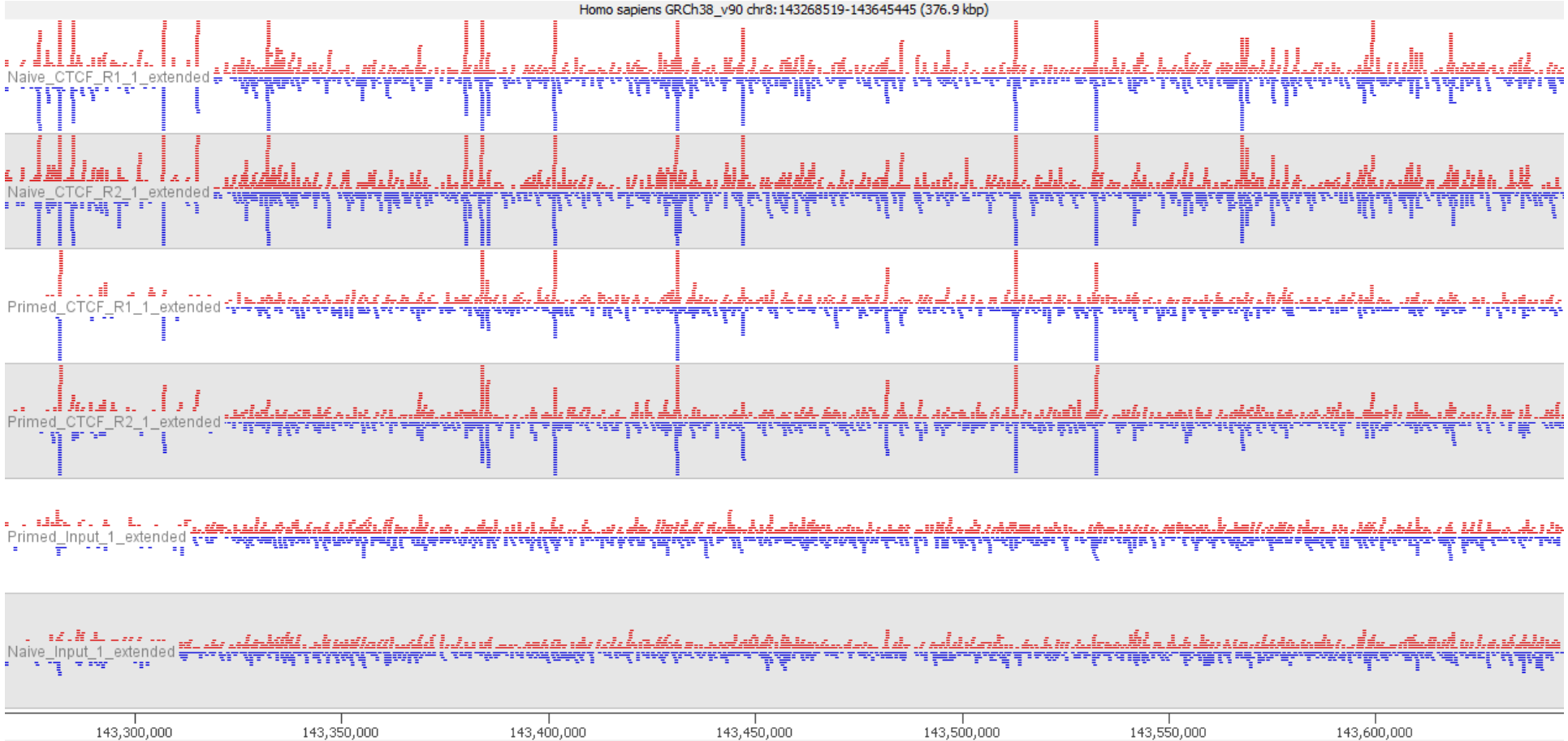
Broad Peaks

- Added in MACS2 – suitable where larger regions with variable enrichment exist
- Uses two thresholds for enrichment



How should you apply peak callers

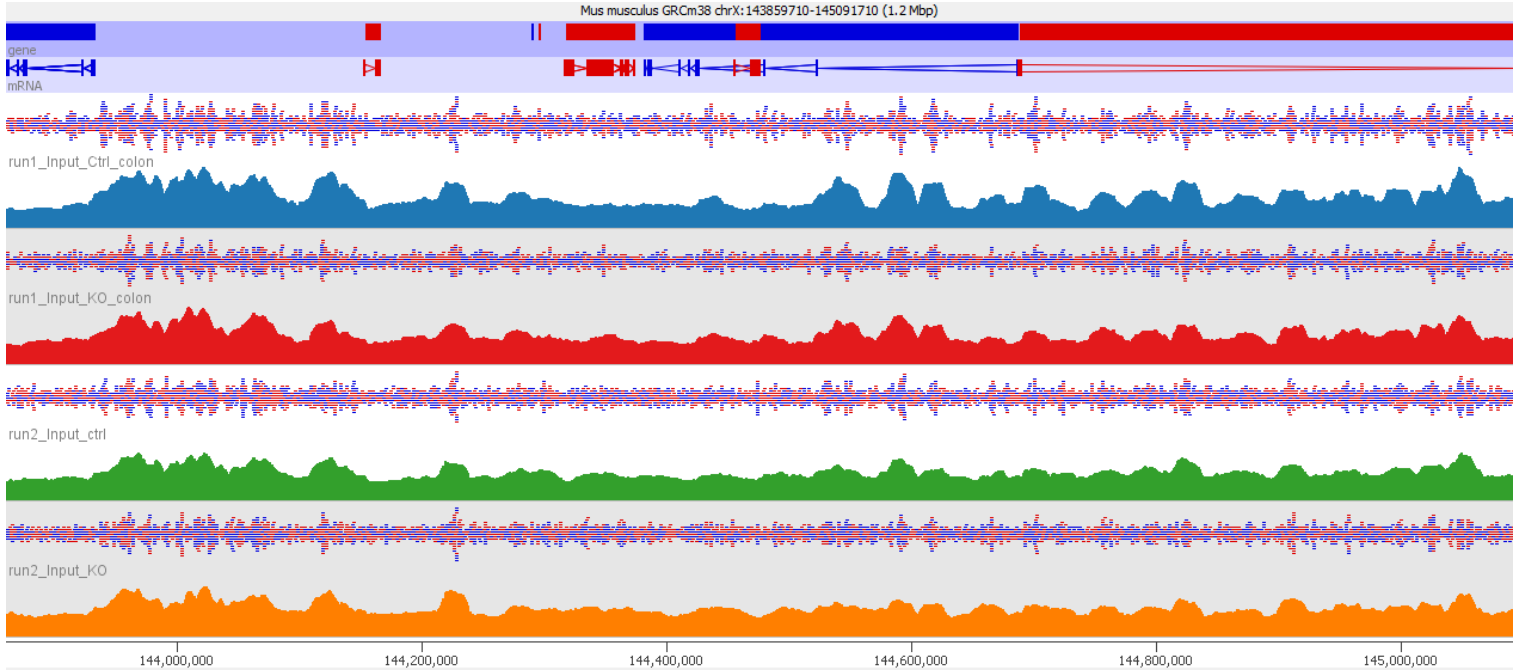
- Multiple ChIPs (over multiple conditions)
- Multiple Inputs



Multiple Inputs

Input variability is generally consistent

- Mapability
- Genome Assembly
- Fragmentation biases



Unless you see substantial variability between inputs it's better to combine them into a single reference input sample

Multiple ChIPs

BAM Files

WT ChIP 1

WT ChIP 2

KO ChIP 1

KO ChIP 2

WT ChIP 1
+
WT ChIP 2
+
KO ChIP 1
+
KO ChIP 2

Peak Sets

Peaks

WT ChIP 1
+
WT ChIP 2
+
KO ChIP 1
+
KO ChIP 2

Multiple ChIPs

BAM Files

WT ChIP 1

WT ChIP 2

KO ChIP 1

KO ChIP 2

Peak Sets

WT Peaks 1

WT Peaks 2

KO Peaks 1

KO Peaks 2

WT Peaks 1
And
WT Peaks 2

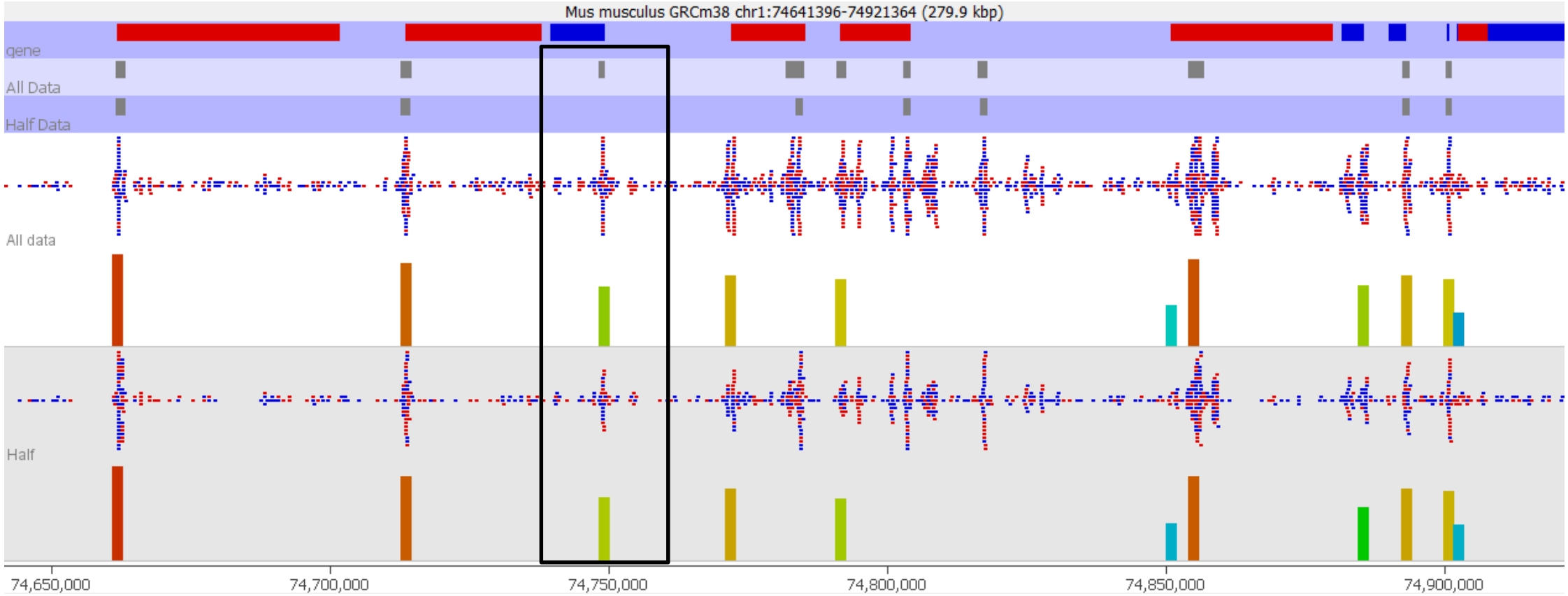
KO Peaks 1
And
KO Peaks 2

WT Peaks 1
And
WT Peaks 2

Or

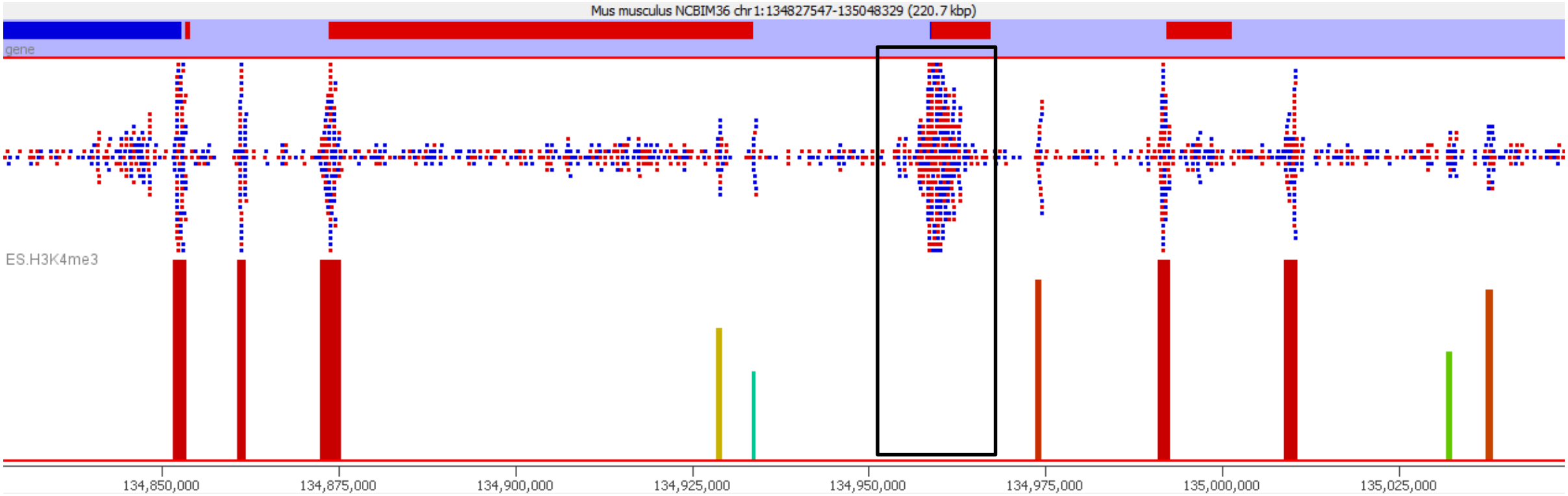
KO Peaks 1
And
KO Peaks 2

Why isn't a peak called



Fewer peaks are called by just sub-sampling the same data

Why isn't a peak called



With no input the region around the peak is used to model the background. Broader peaks can be missed

For ATAC data (no input) you should skip the rescoring step (**`--nomodel`**)

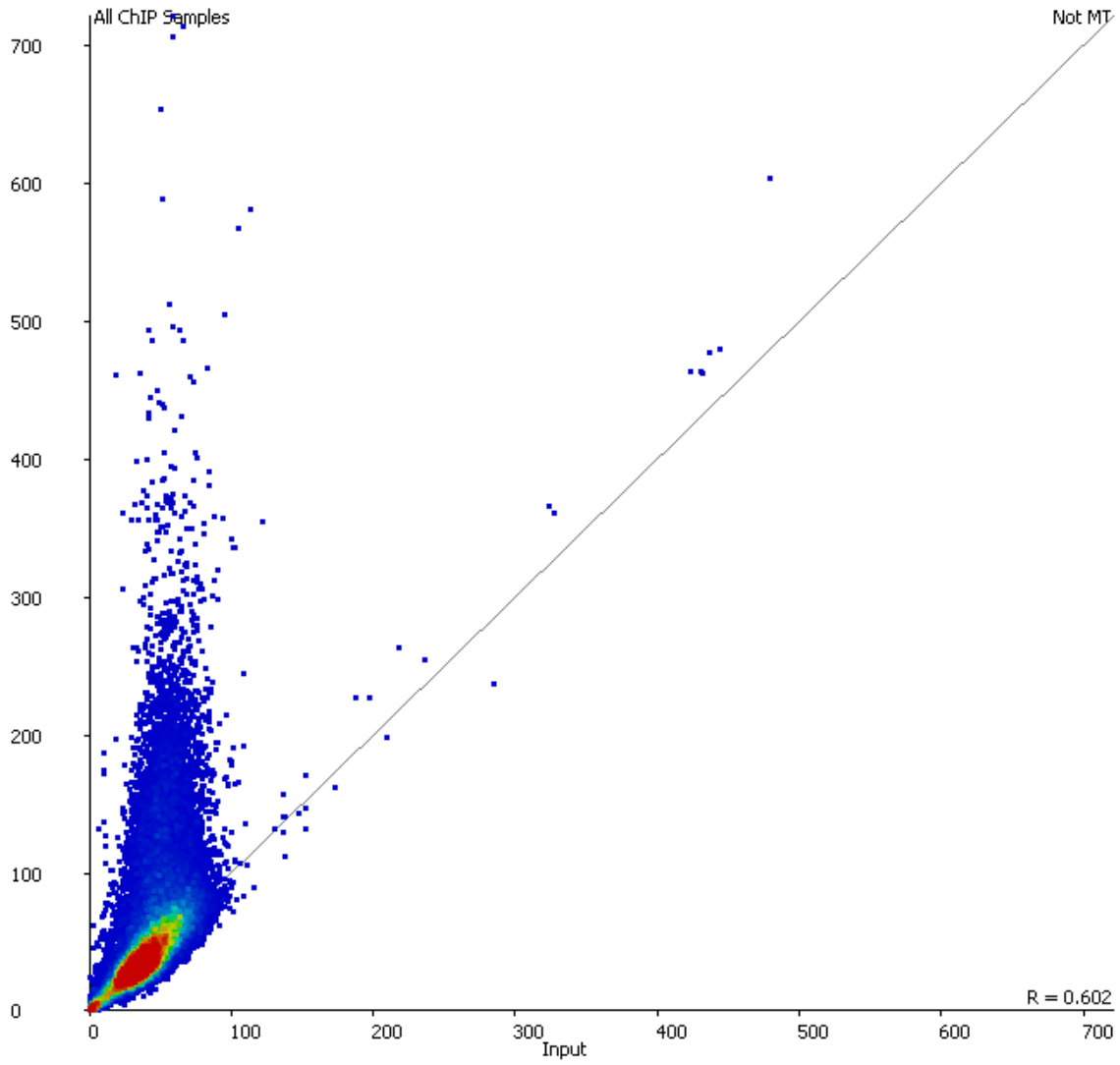
Reporting on Peak sets

- Don’t make claims based solely on the number of peaks (“there were more WT peaks than KO peaks” for example)
- Don’t make claims based on regions being peaks in 1 set but not another (there were 465 peaks which were specific to KO)
- It is OK to make statements about overlap (there were 794 peaks which were common to WT and KO)
- You have to address differential enrichment problems quantitatively

Quantitating ChIP data for analysis

- Quantitation of ChIP is **not** a simple problem
- Can start with something simple but in many cases you will need to refine this
- Globally corrected log counts are a good place to start

Should I normalise to input?



- Only consider input normalisation if:
  1. You have substantial variation in the coverage of your input (excluding outliers)
  2. Your ChIP signal is correlated with the input level

Why not just always do "fold over input"?

- Inputs are generally poorly measured
  - Poor coverage compared to ChIP

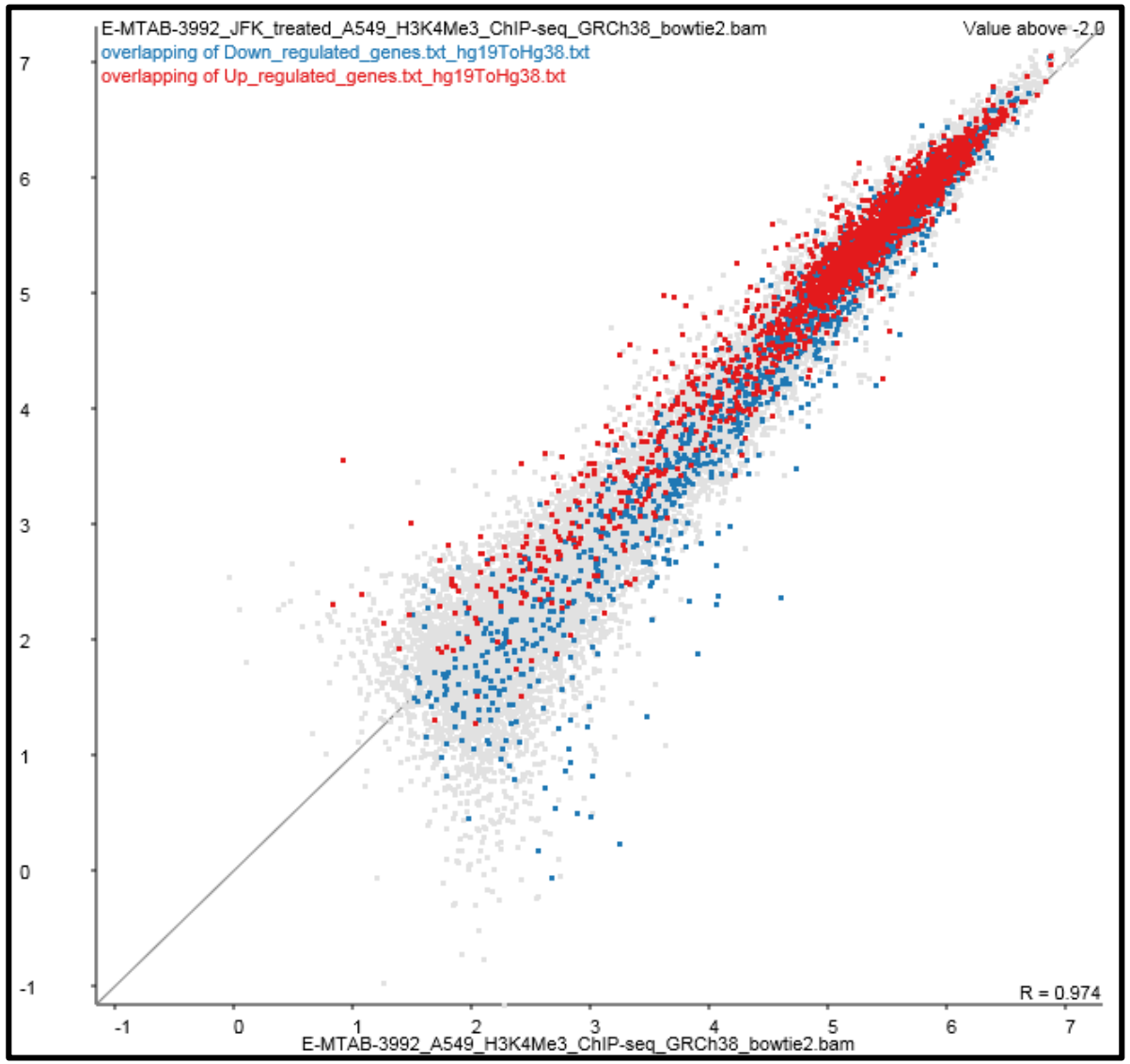
Region	Input	ChIP	ChIP/Input
Region A	5	200	40
Region B	2	200	100

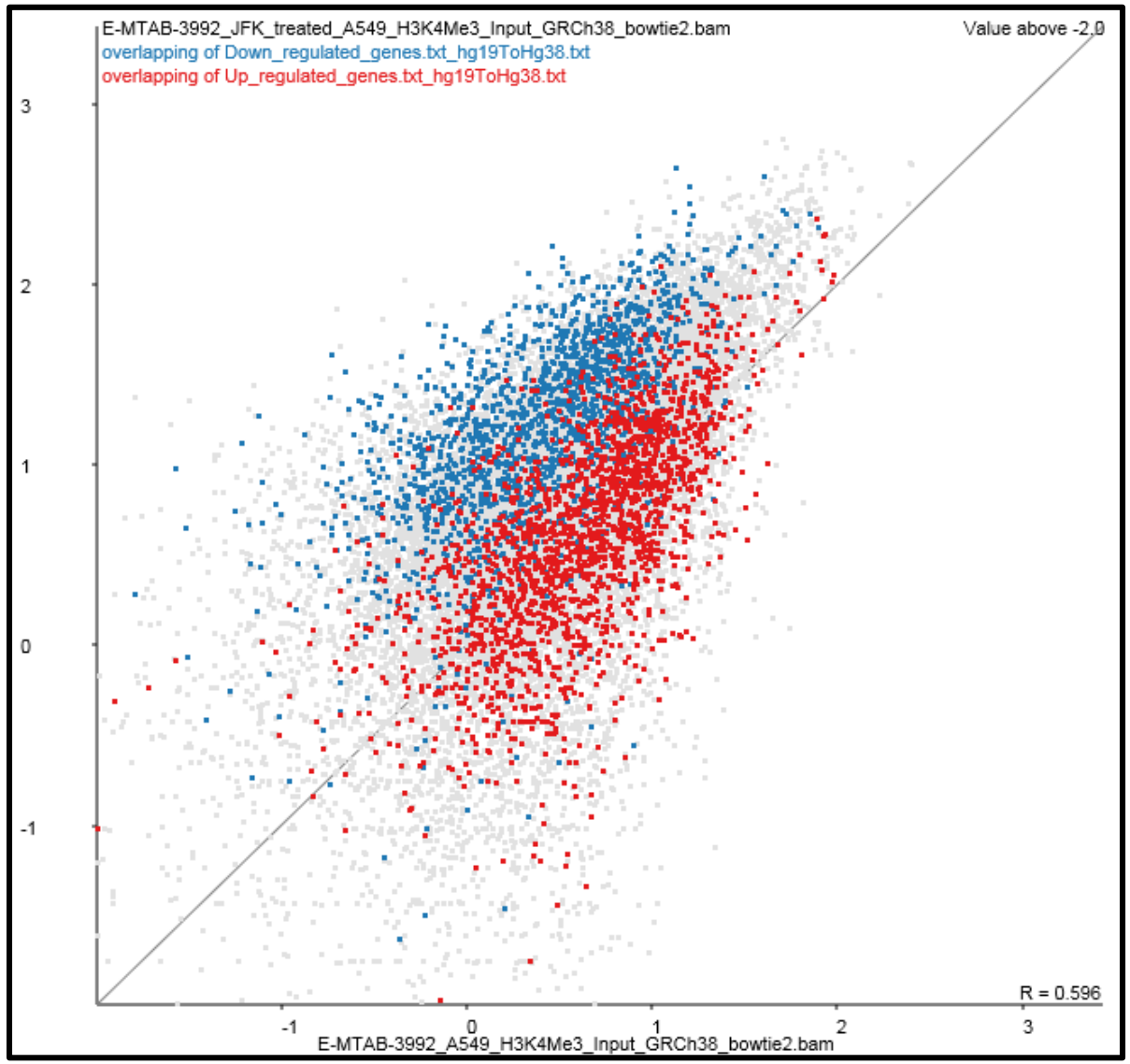
- Fold change values are more influenced by input than ChIP

- Biases in the input are smaller than enrichment power of the antibody

Hits with increased enrichment

Hits with decreased enrichment

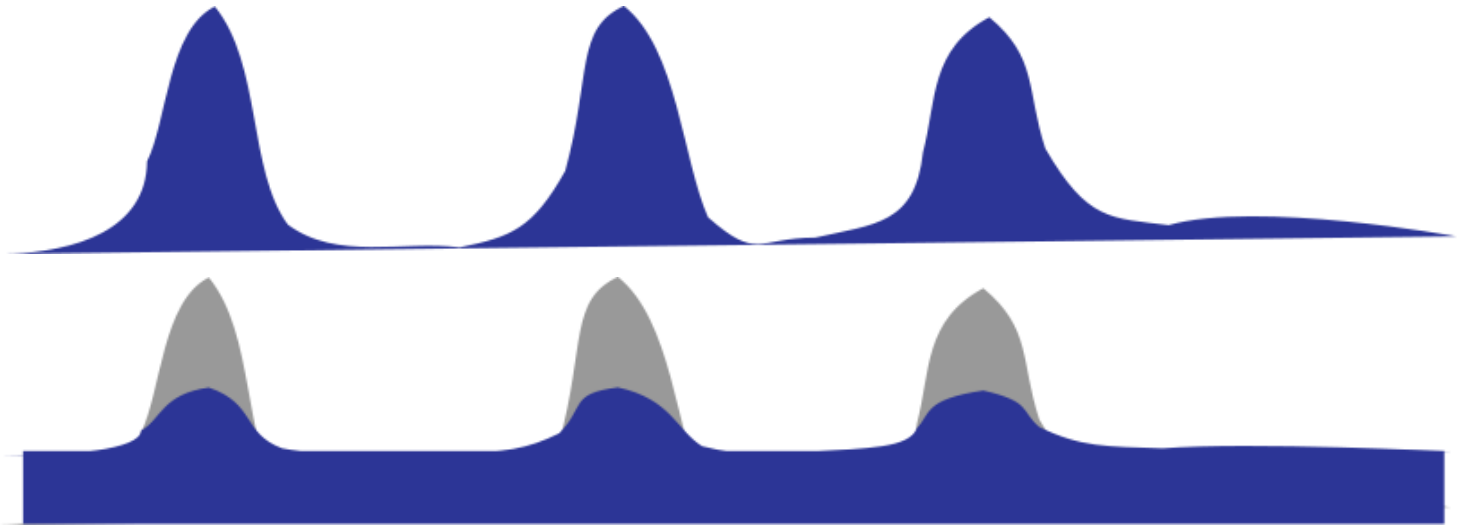


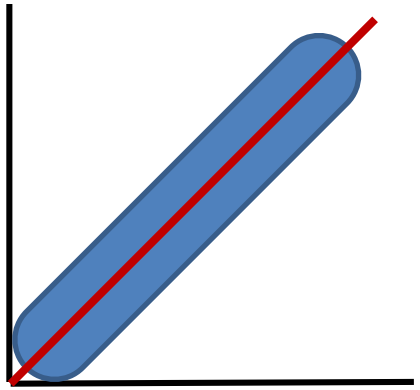


Evaluating and Normalising Enrichment

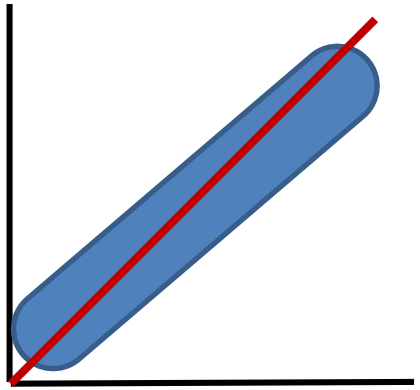
Good Enrichment

Worse Enrichment

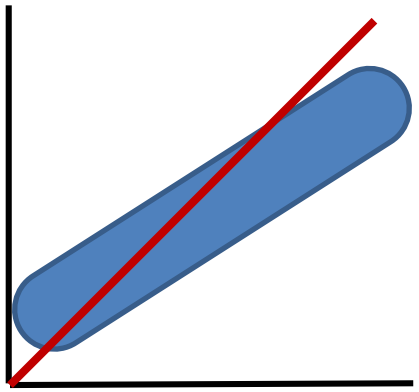




Similar Enrichment

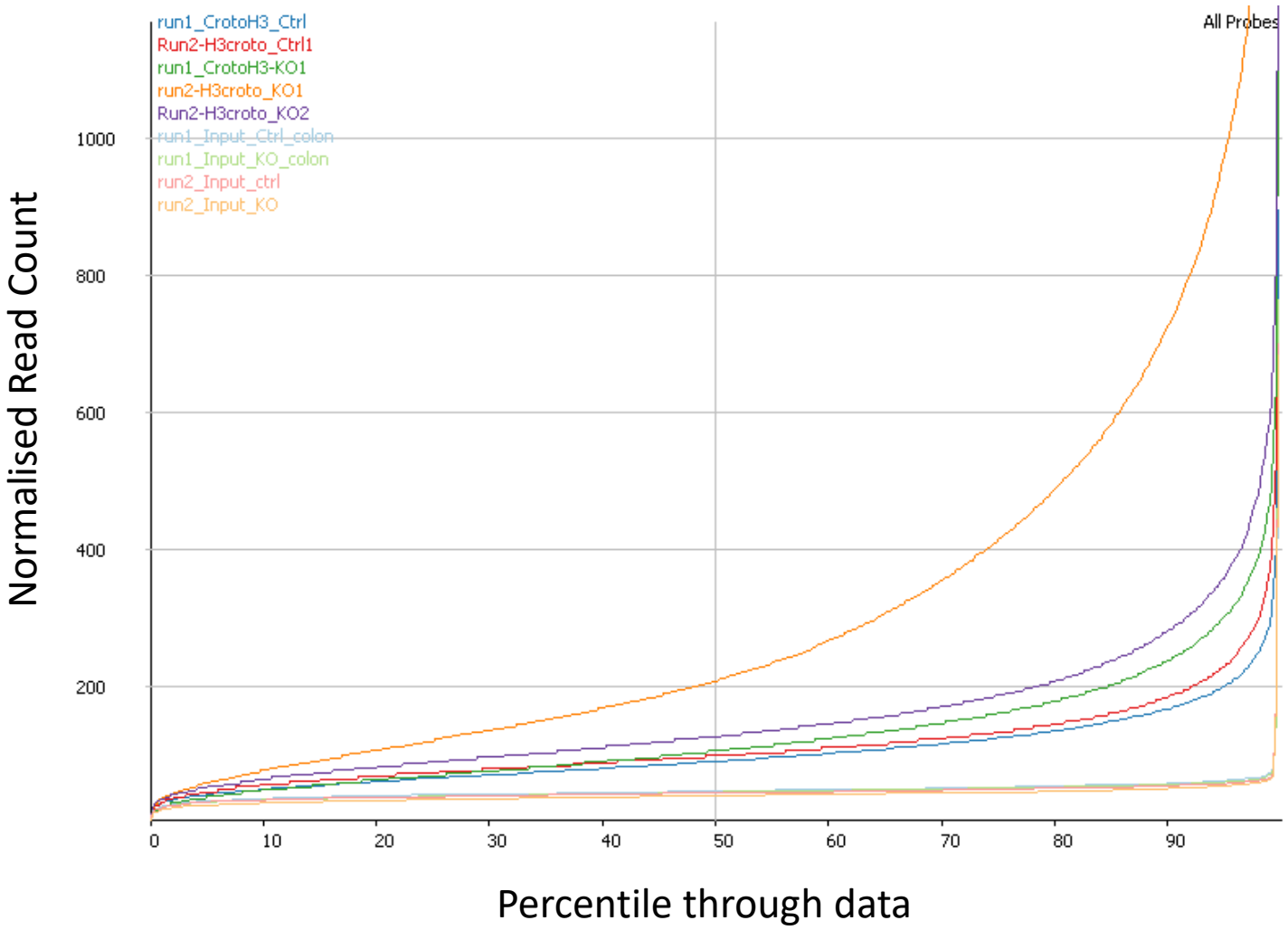


Small Difference



Large Difference

Evaluating and Normalising Enrichment

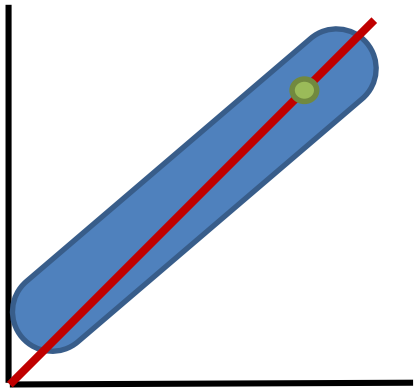


Normalising Enrichment

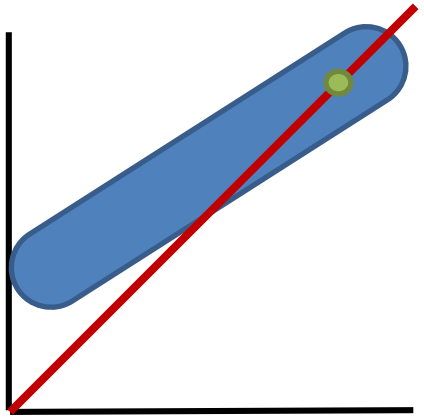
Size Factor

Single point of comparison

Works well for small differences
Insufficient for large differences
Allows the use of count based stats



Small Difference

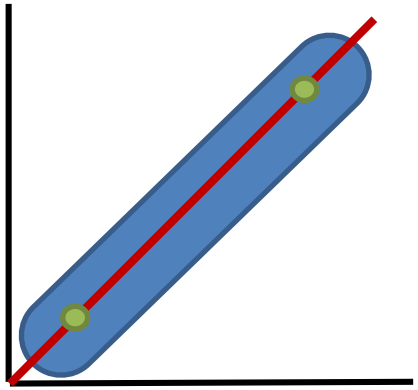


Large Difference

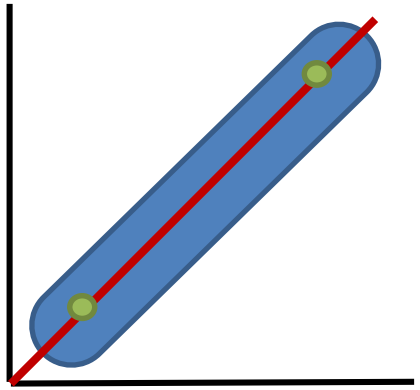
Enrichment

Two points of comparison

Corrects for larger differences
Not directly compatible with count based stats



Small Difference

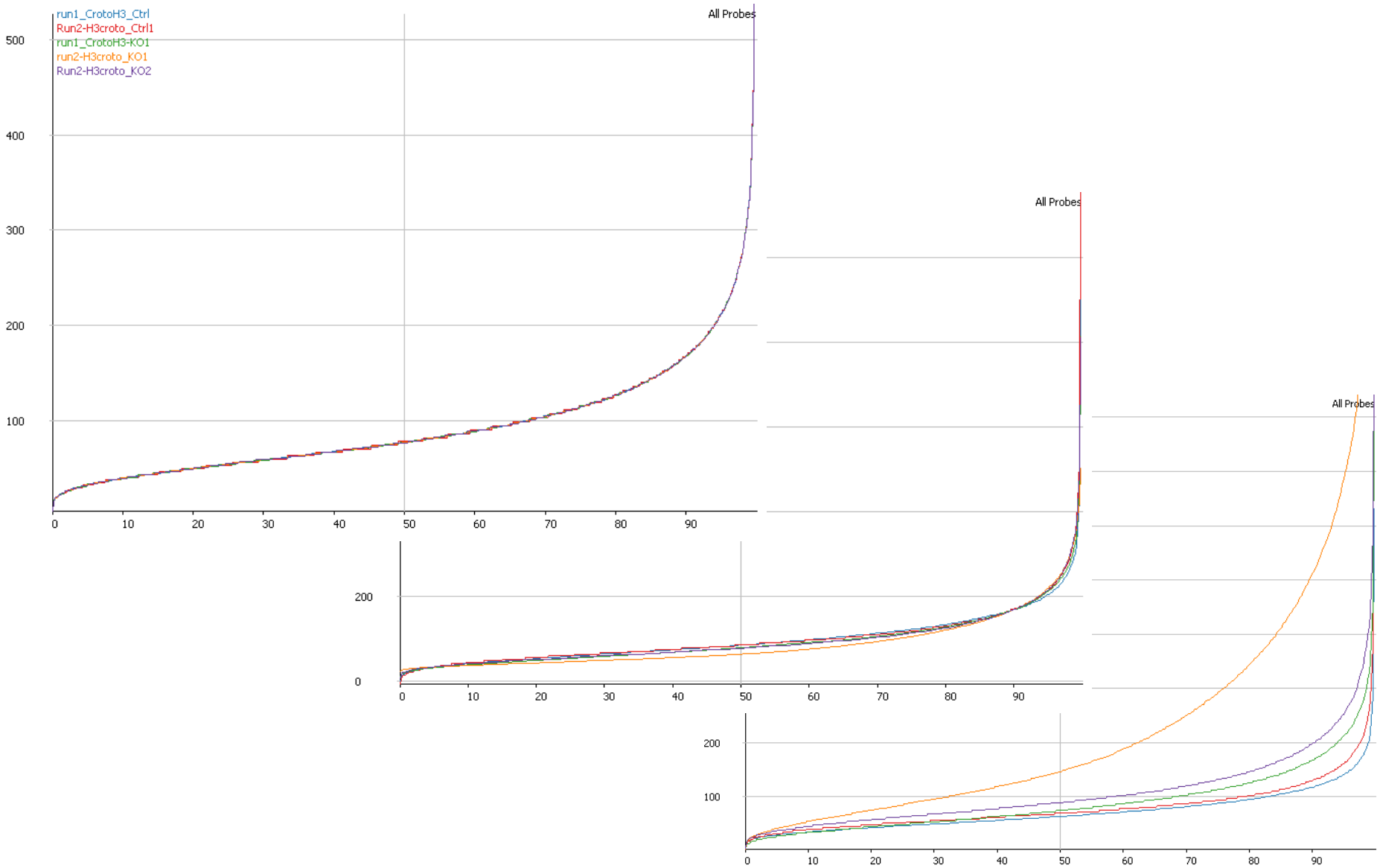


Large Difference

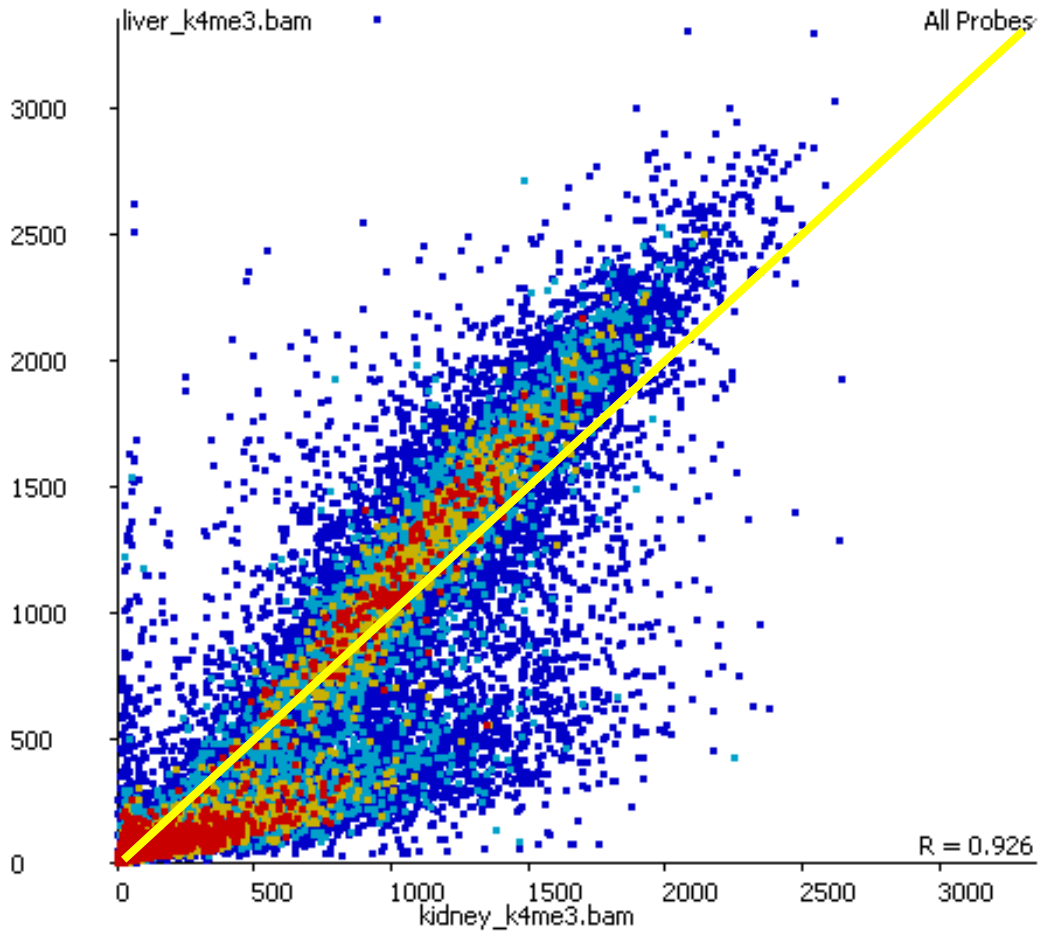
Quantile

Forces distributions to be identical
Corrects any differences, easy to apply

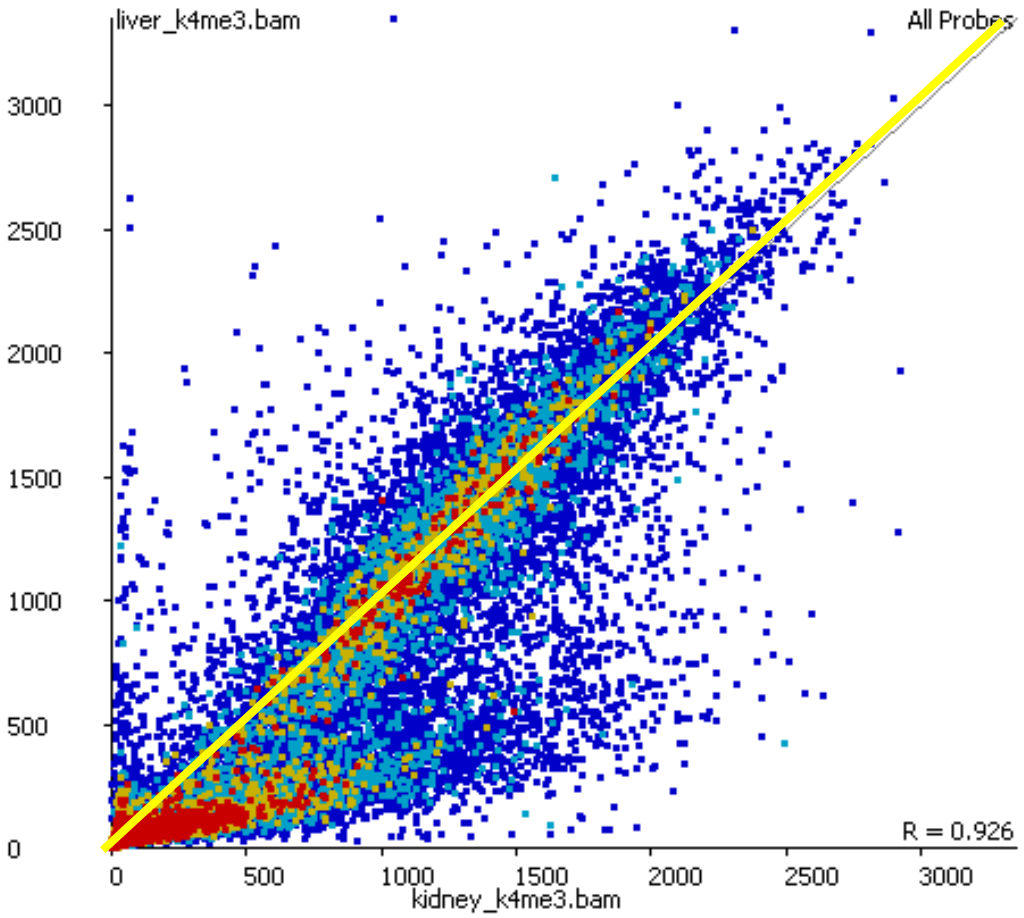
Normalising Enrichment



Checking Normalisation

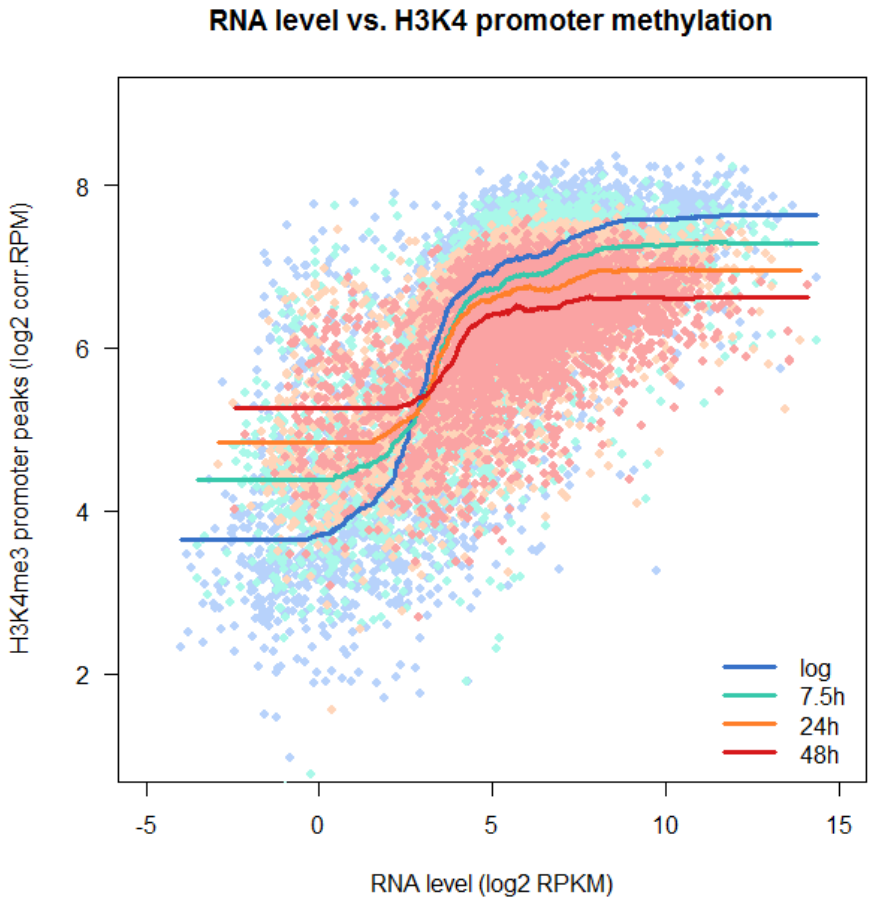


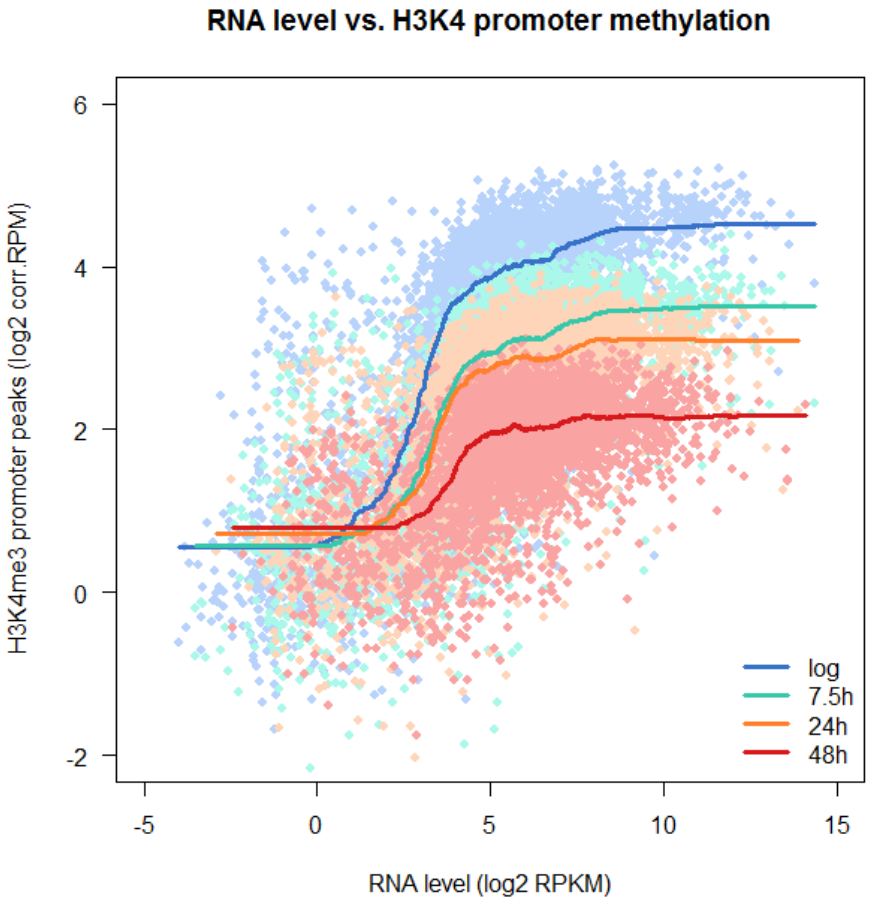
Before Normalisation



After Normalisation

Look for systematic enrichment changes (real biology!!)





Use replicates to build a case for a biological rather than technical difference

Differential enrichment analysis

- Needs to be quantitative
- Needs to operate on non-deduplicated data
- Two statistical options
  - Count based stats on raw uncorrected counts
    - DESeq
    - EdgeR
  - Continuous quantitation stats on normalised enrichment values
    - LIMMA

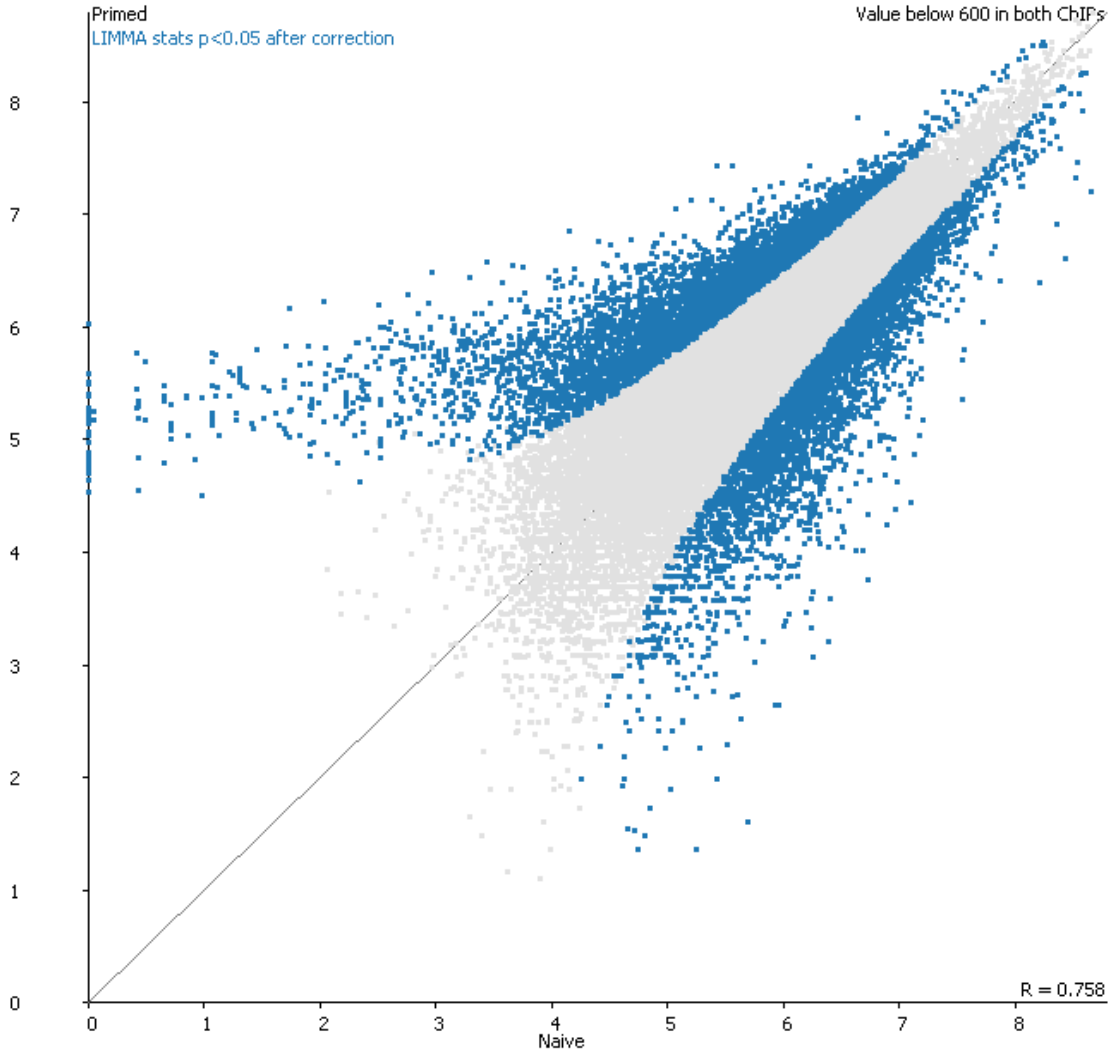
Which statistic to pick?

- If enrichment is roughly similar
  - Raw counts, then DESeq/EdgeR
- If there are large differences in enrichment
  - Enrichment normalisation
  - LIMMA statistics

Visualisation of hits

- Map onto scatterplot for simple verification
- Normally makes sense to use log transformed counts
- Look at the data underneath candidates you make specific claims about

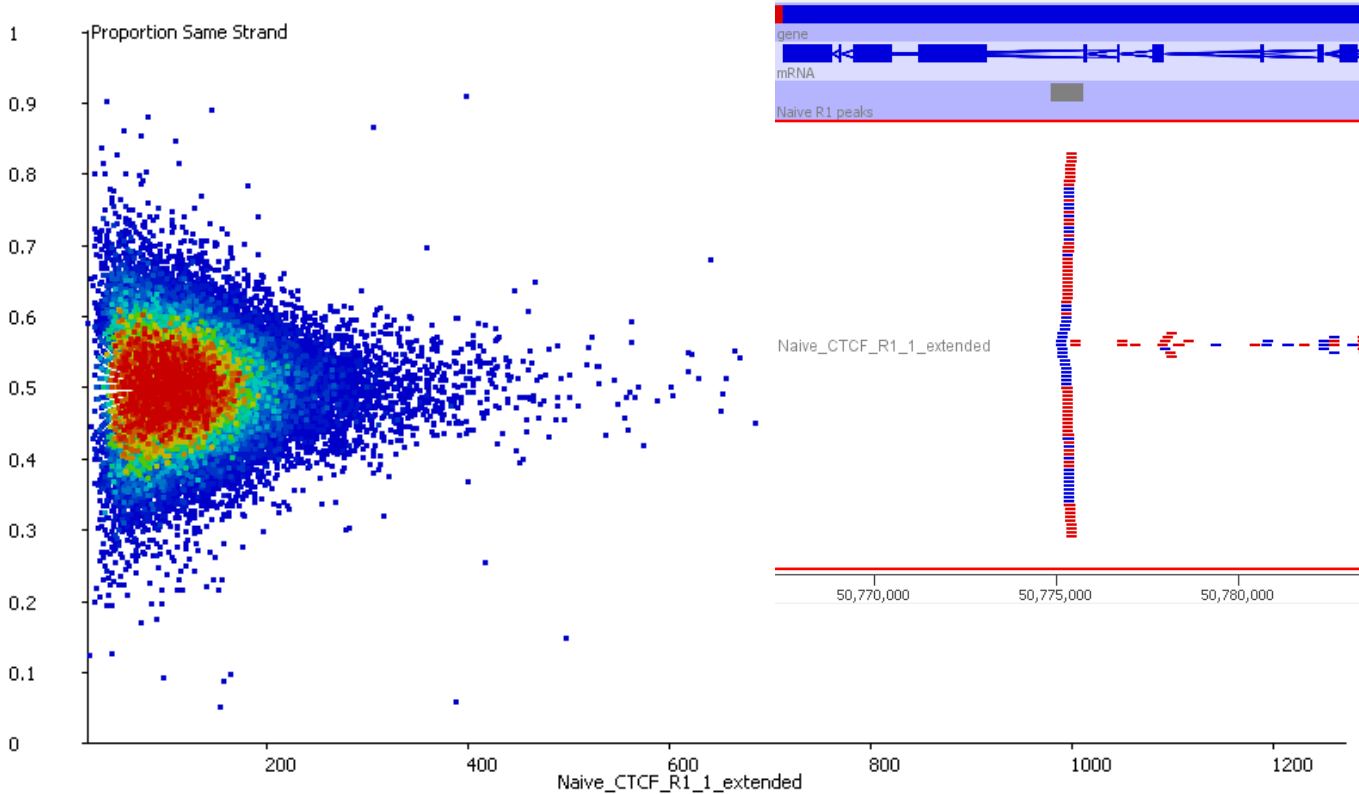
Hit validation

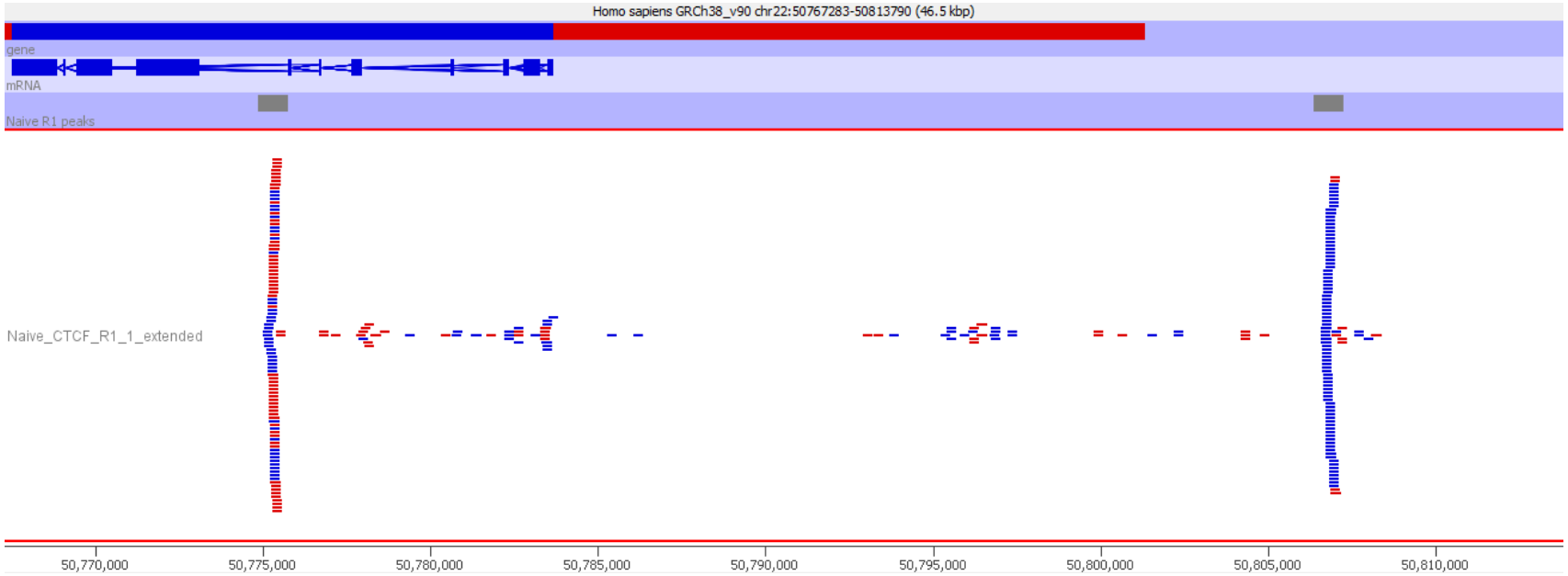


- Look whether hits make sense
- Look at points which change but were not selected
- Log scale should be used
- Keep the context of non-hits

Hit validation
Directionality

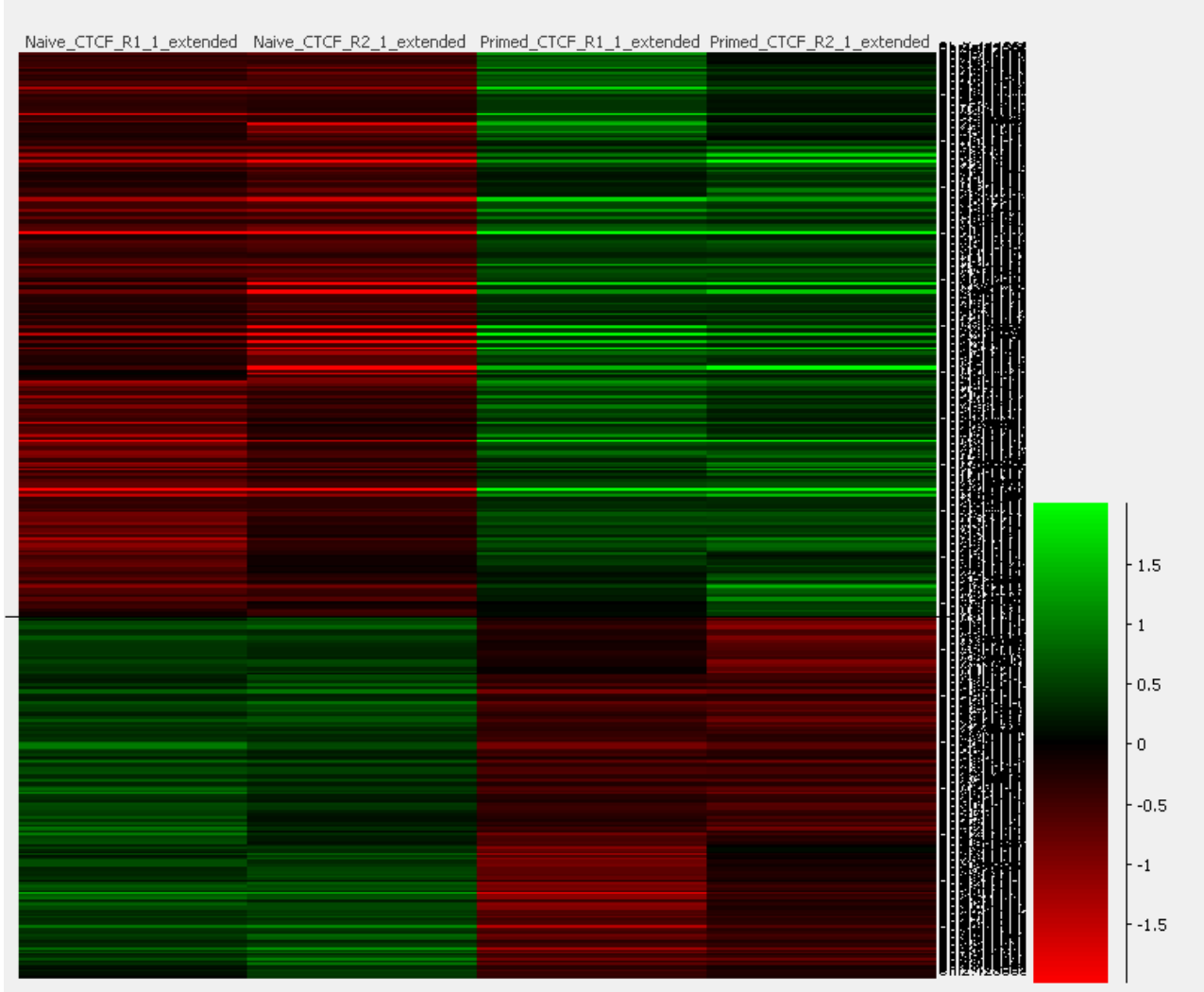
- Most ChIP enrichments are not strand-specific
- Should expect to see enrichment on both strands





Hit validation

Heatmap



- You should be able to see consistency between replicates

Data Analysis Exercise

Babraham Bioinformatics

Experimental Design

Experimental Design Considerations

- All normal rules apply
  - Think about sources of variation
  - Don't confound variables
  - Think about what batch effects might exist

- Test your antibody well before starting
  - By far the biggest factor in success
  - Good performance on Western / in-situ is not a guarantee, but it's a good start

Experimental Design Considerations

- Number of replicates
  - Lots of studies use 2 replicates
  - Fine for just finding binding sites (motif analysis)
  - Not really enough for differential binding
    - Huge reliance on 'information sharing'
    - No accurate measurement of variance per peak
    - Potentially over-predicts differential binding
  - Should think about likely levels of variability and make replicates to match

Experimental Design Considerations

- Amount of sequencing
  - Can be difficult to predict
  - Depends on
    - Genome size
    - Proportion of genome which is enriched
    - Efficiency of enrichment
  - ENCODE standard is ~20M reads per sample
    - Can get away with fewer (K4me3 for example)
    - Will need more for some marks (H3 for example)
    - Sequencing depth will affect ability to detect changes

Experimental Design Considerations

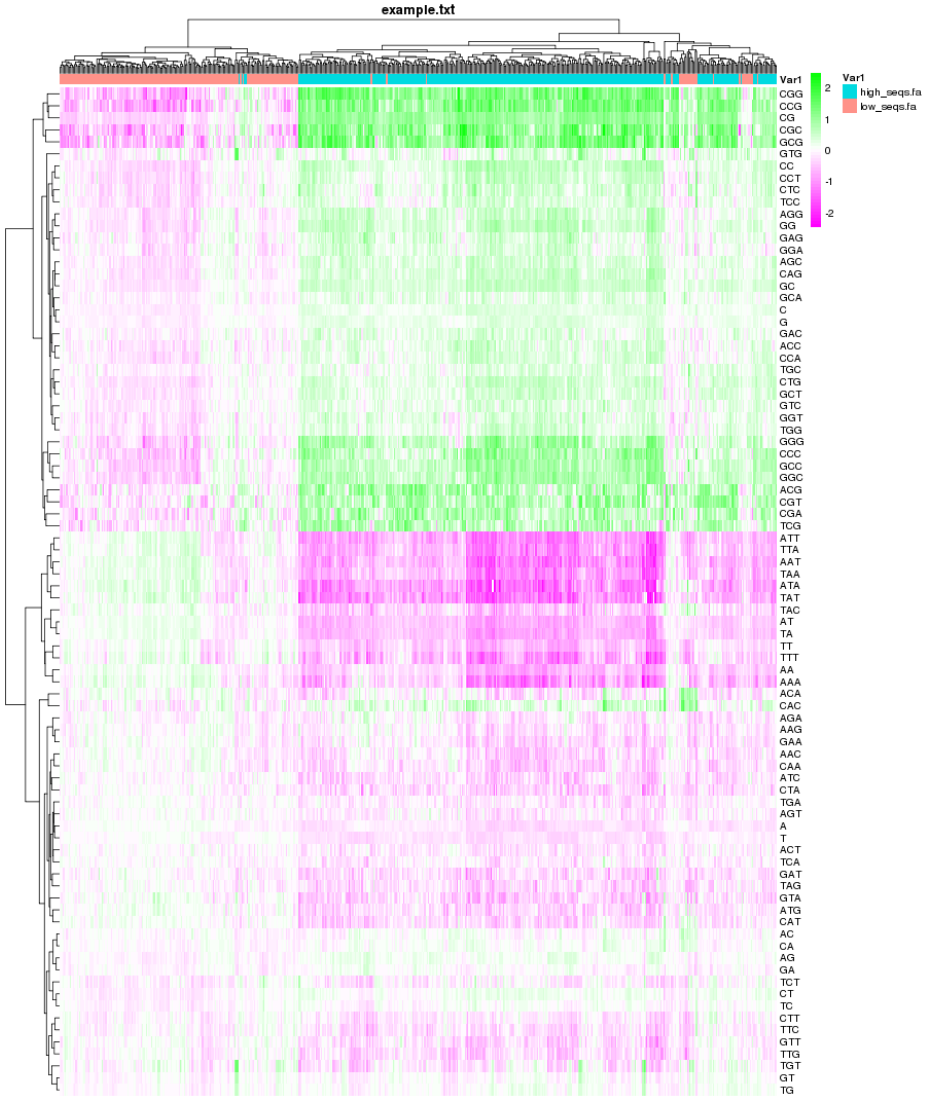
- Type of sequencing
  - Single end is fine for most applications
    - ATAC-Seq can require paired end for some analyses
  - Moderate read length is required
    - Can map anywhere in the genome
    - 50bp is probably OK. 100bp would be preferable

Downstream Analyses

Composition / Motif Analysis

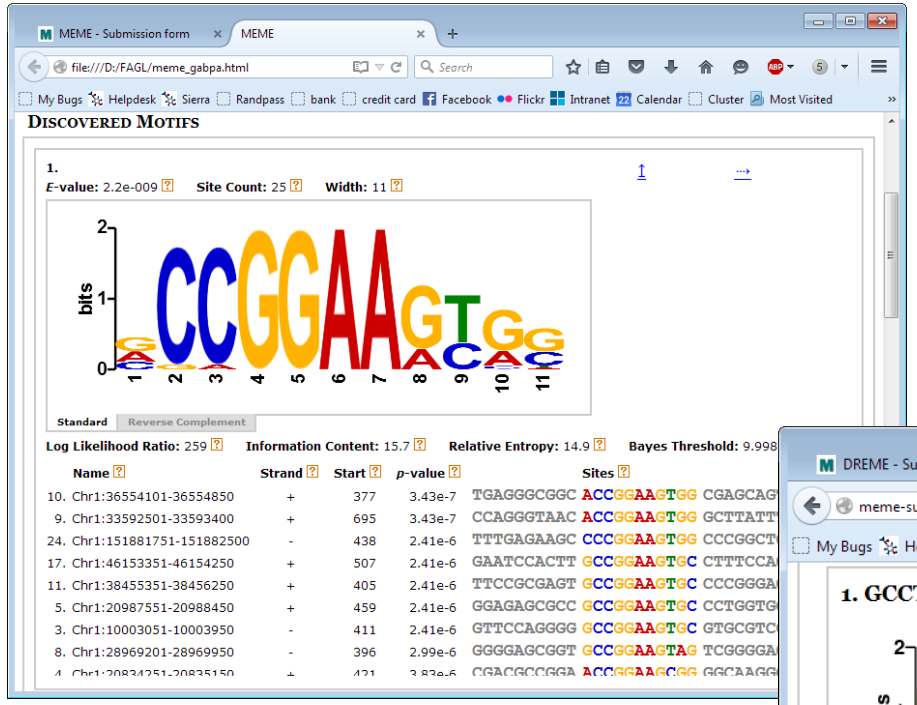
- Composition
  - Good place to start, can provide either biological or technical insight
  - See if hits (up vs down) cluster based on the underlying sequence composition
- Motifs
  - Great for defining putative binding sites
  - Interesting to do sensitivity check
  - Can do differential motif calling (for hit/non-hit)

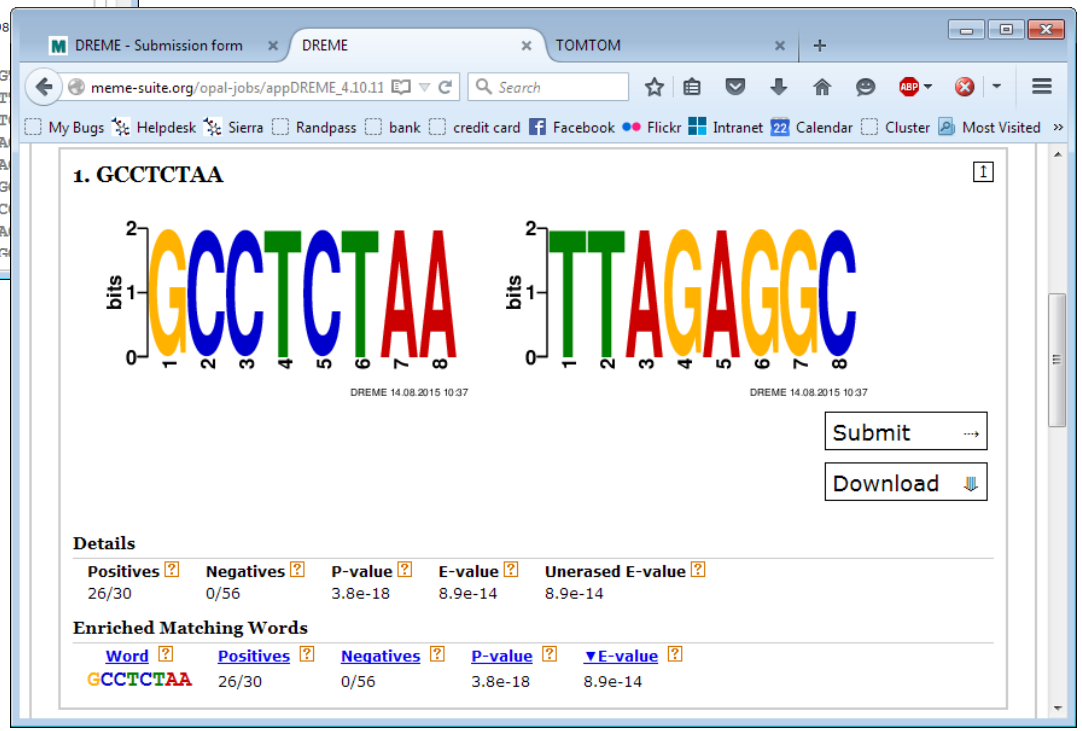
Compter - composition analysis



www.bioinformatics.babraham.ac.uk/projects/compter

MEME - Motif Analysis





Gene Ontology / Pathway

- Be careful how you relate hits to genes
  - Really need to have a global link between peak positions and genes
  - Random positions will give significant GO hits if you just use closest/overlapping genes