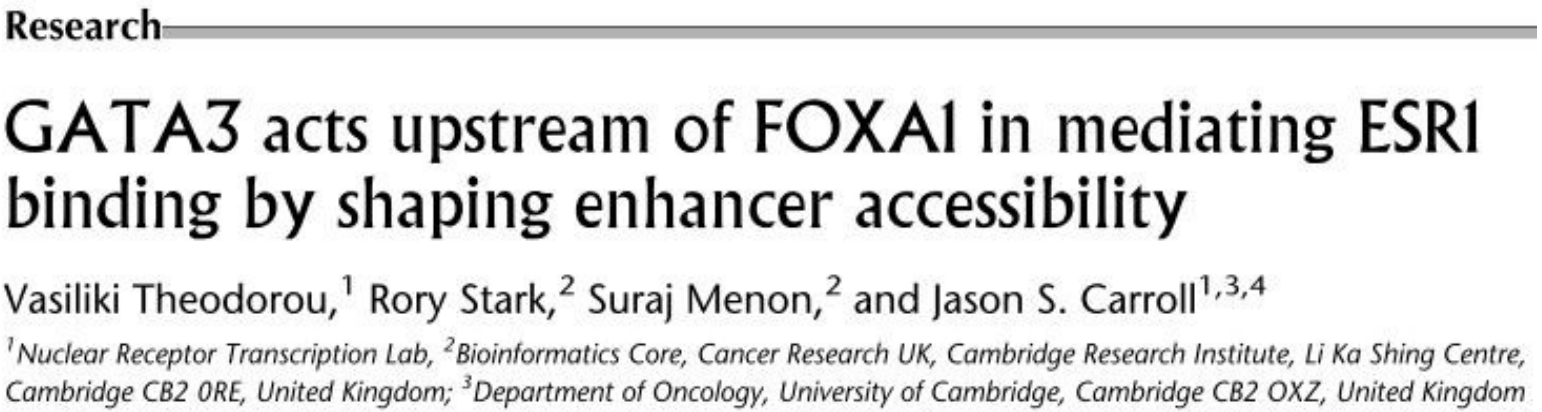
ChIP-seq analysis

M. Defrance, C. Herrmann, S. Le Gras, D. Puthier, M. Thomas.Chollier

- **Visualization, quality, normalization & peak-calling**
  - Presentation (Carl Herrmann)
  - Practical session
- **Peak annotation**
  - Presentation (Matthieu Defrance)
  - Practical session
- **Motif discovery in peaks**
  - Presentation (Jacques van Helden)
  - Practical session

Datasets used



Research

GATA3 acts upstream of FOXA1 in mediating ESR1 binding by shaping enhancer accessibility

Vasiliki Theodorou,[1] Rory Stark,[2] Suraj Menon,[2] and Jason S. Carroll[1,3,4]

[1]Nuclear Receptor Transcription Lab, [2]Bioinformatics Core, Cancer Research UK, Cambridge Research Institute, Li Ka Shing Centre, Cambridge CB2 0RE, United Kingdom; [3]Department of Oncology, University of Cambridge, Cambridge CB2 OXZ, United Kingdom

- estrogen-receptor (ESR1) is a key factor in **breast cancer developement**
- goal of the study: understand the dependency of ESR1 binding on presence of co-factors, in particular GATA3, which is mutated in breast cancers
- approaches: GATA3 silencing (siRNA), ChIP-seq on ESR1 in wt vs. siGATA3 conditions, chromatin profiling

Datasets used

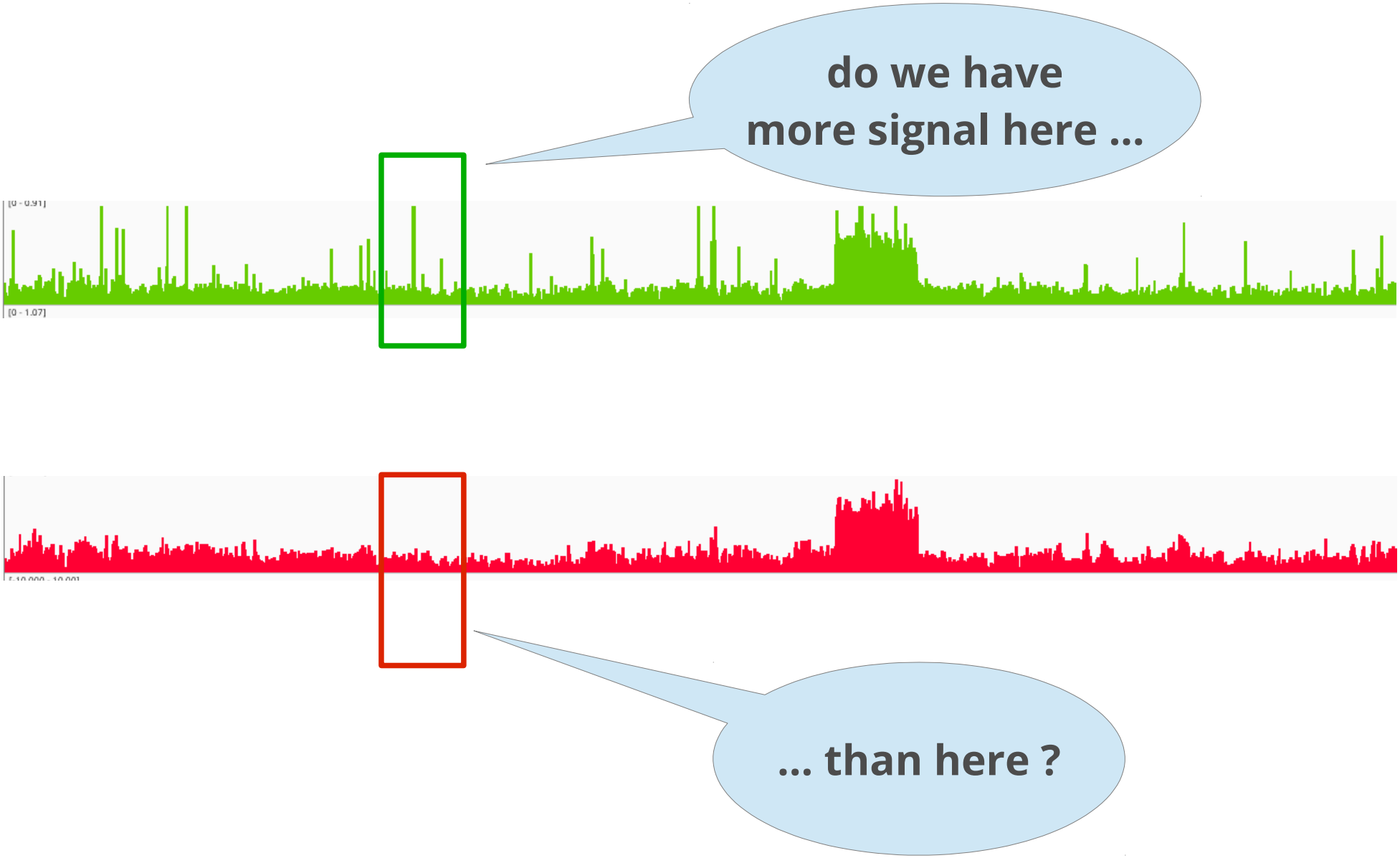
ExpName	CellLine	Replicate	SampleID	SRAExpID	Selected
siNT_ER_E2_r1	MCF-7	r1	GSM986059	SRX176856	X
siGATA_ER_E2_r1	MCF-7	r1	GSM986060	SRX176857	X
siNT_ER_E2_r2	MCF-7	r2	GSM986061	SRX176858	X
siGATA_ER_E2_r2	MCF-7	r2	GSM986062	SRX176859	X
siNT_ER_E2_r3	MCF-7	r3	GSM986063	SRX176860	X
siGATA_ER_E2_r3	MCF-7	r3	GSM986064	SRX176861	X
siNT_FOXA1_Veh_r1	MCF-7	r1	GSM986065	SRX176862	
siGATA_FOXA1_Veh_r1	MCF-7	r1	GSM986066	SRX176863	
GATA3_E2_r1	MCF-7	r1	GSM986067	SRX176864	
GATA3_Veh_r1	MCF-7	r1	GSM986068	SRX176865	
GATA3_E2_r2	MCF-7	r2	GSM986069	SRX176866	
GATA3_Veh_r2	MCF-7	r2	GSM986070	SRX176867	
GATA3_E2_r3	MCF-7	r3	GSM986071	SRX176868	
GATA3_Veh_r3	MCF-7	r3	GSM986072	SRX176869	
GATA3_E2_r4	MCF-7	r4	GSM986073	SRX176870	
GATA3_Veh_r4	MCF-7	r4	GSM986074	SRX176871	
GATA3_E2_r5	MCF-7	r5	GSM986075	SRX176872	
GATA3_Veh_r5	MCF-7	r5	GSM986076	SRX176873	
siNT_H3K27ac_E2_r1	MCF-7	r1	GSM986077	SRX176874	
siGATA_H3K27ac_E2_r1	MCF-7	r1	GSM986078	SRX176875	
siNT_H3K27ac_Veh_r1	MCF-7	r1	GSM986079	SRX176876	
siGATA_H3K27ac_Veh_r1	MCF-7	r1	GSM986080	SRX176877	
siNT_H3K4me1_E2_r1	MCF-7	r1	GSM986081	SRX176878	X
siGATA_H3K4me1_E2_r1	MCF-7	r1	GSM986082	SRX176879	X
siNT_H3K4me1_Veh_r1	MCF-7	r1	GSM986083	SRX176880	
siGATA_H3K4me1_Veh_r1	MCF-7	r1	GSM986084	SRX176881	
siNT_p300_E2_r2	MCF-7	r2	GSM986085	SRX176882	
siGATA_p300_E2_r2	MCF-7	r2	GSM986086	SRX176883	
siNT_p300_Veh_r2	MCF-7	r2	GSM986087	SRX176884	
siGATA_p300_Veh_r2	MCF-7	r2	GSM986088	SRX176885	
ZR751_siNT_ER_E2_r1	ZR751	r1	GSM986089	SRX176886	
ZR751_siGATA_ER_E2_r1	ZR751	r1	GSM986090	SRX176887	
MCF-7_input_r3	MCF-7	r3	GSM986091	SRX176888	X
ZR751_input_r1	ZR751	r1	GSM986092	SRX176889	
ZR751_input_r1	ZR751	r1	GSM986092	SRX176889	

- **ESR1 ChIP-seq in WT & siGATA3 conditions (3 replicates = 6 datasets)**
- **H3K4me1 in WT & siGATA3 conditions (1 replicate = 2 datasets)**
- **Input dataset in MCF-7 (1 replicate = 1 dataset)**
- p300 before estrogen stimulation
- GATA3/FOXA1 ChIP-seq before/after estrogen stimulation
- microarray expression data, etc ...

Hands on !!

Let's have a look at the data

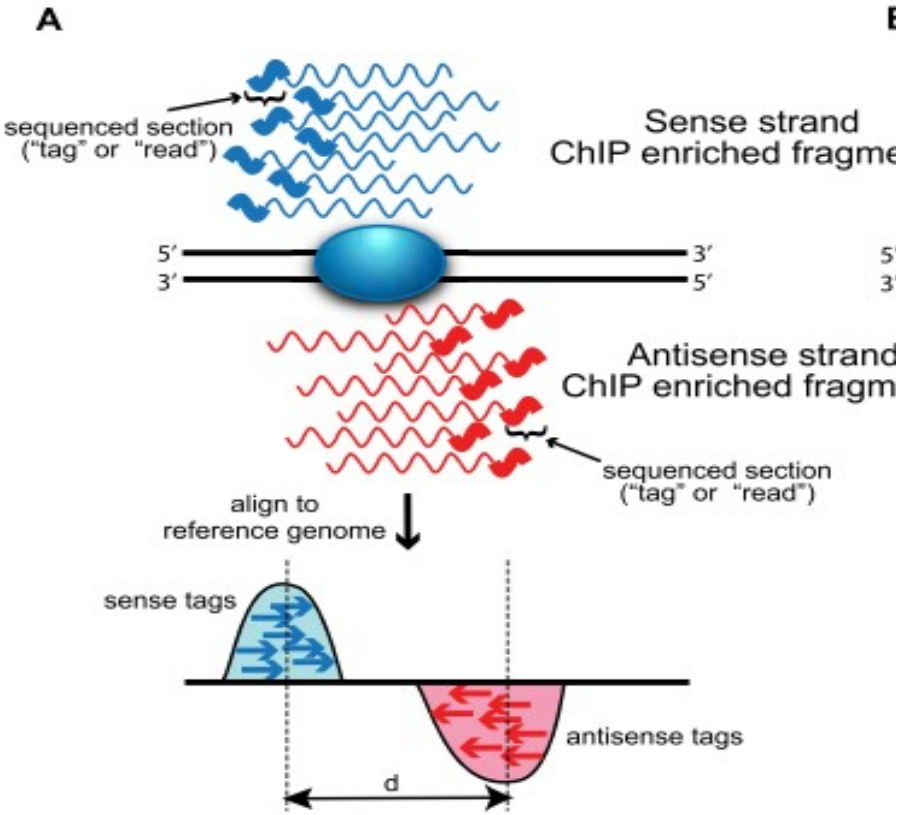
What we want to do



Keys aspects of ChIP-seq analysis

(1) Quality Control : do I have signal ?
(2) Determine signal **coverage**
(3) Modelling **noise** levels
(4) Scaling/**normalizing** datasets
(5) Detecting enriched **peak** regions
(6) Performing **differential** analysis

Principle of ChIP-seq

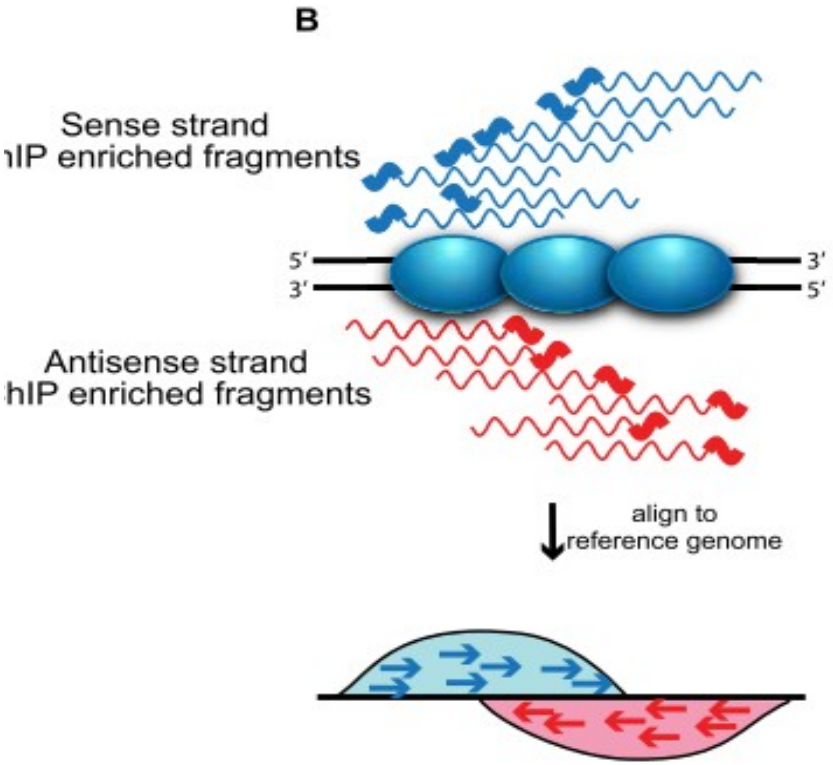


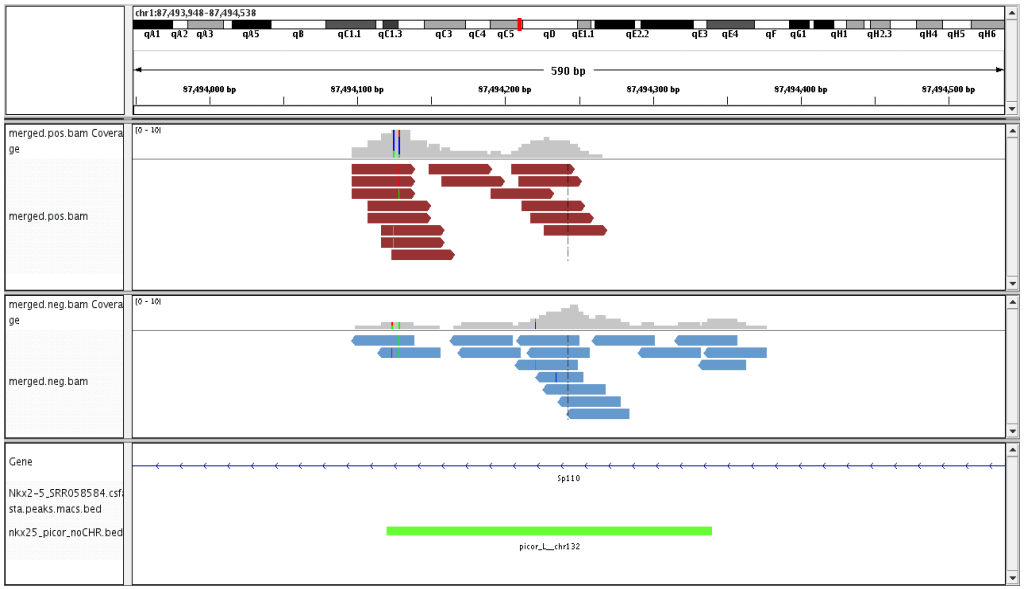
The binding site itself is generally not sequenced !

We expect to see a typical strand asymetry in read densities
→ ChIP peak recognition pattern

[Wilbanks & Faccioti PLoS One (2010)]

Principle of ChIP-seq





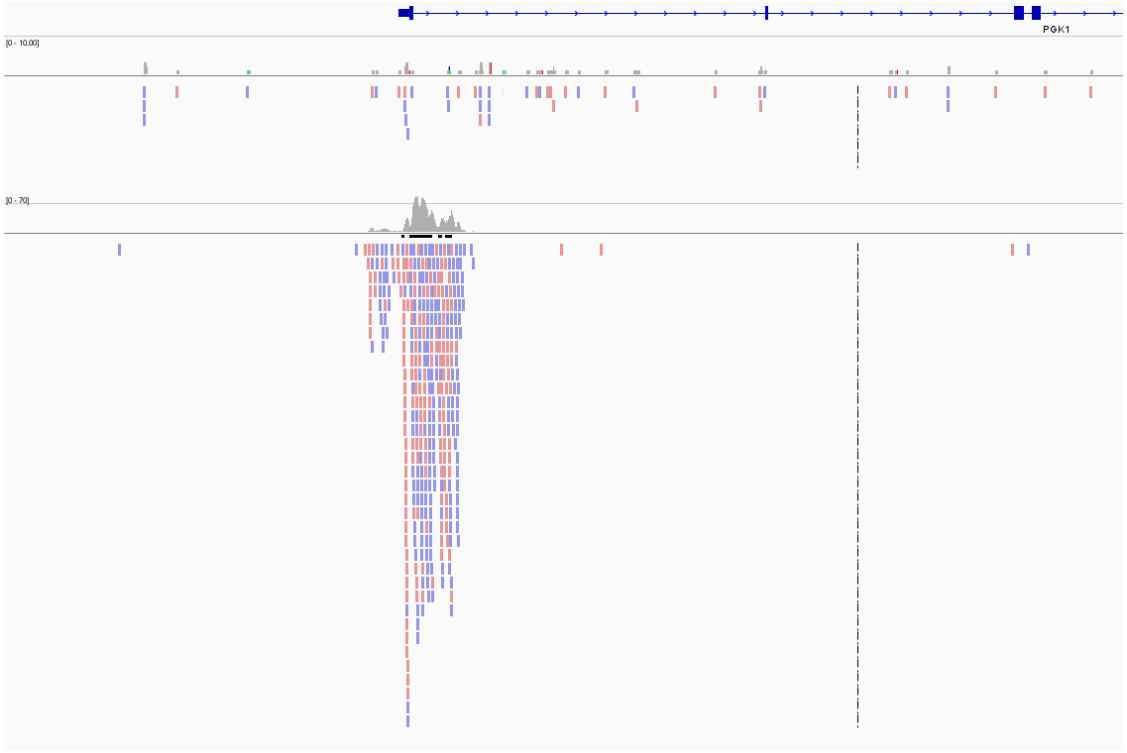
Strand asymetry is blurred when multiple proteins bind or in case of histone modifications ChIP

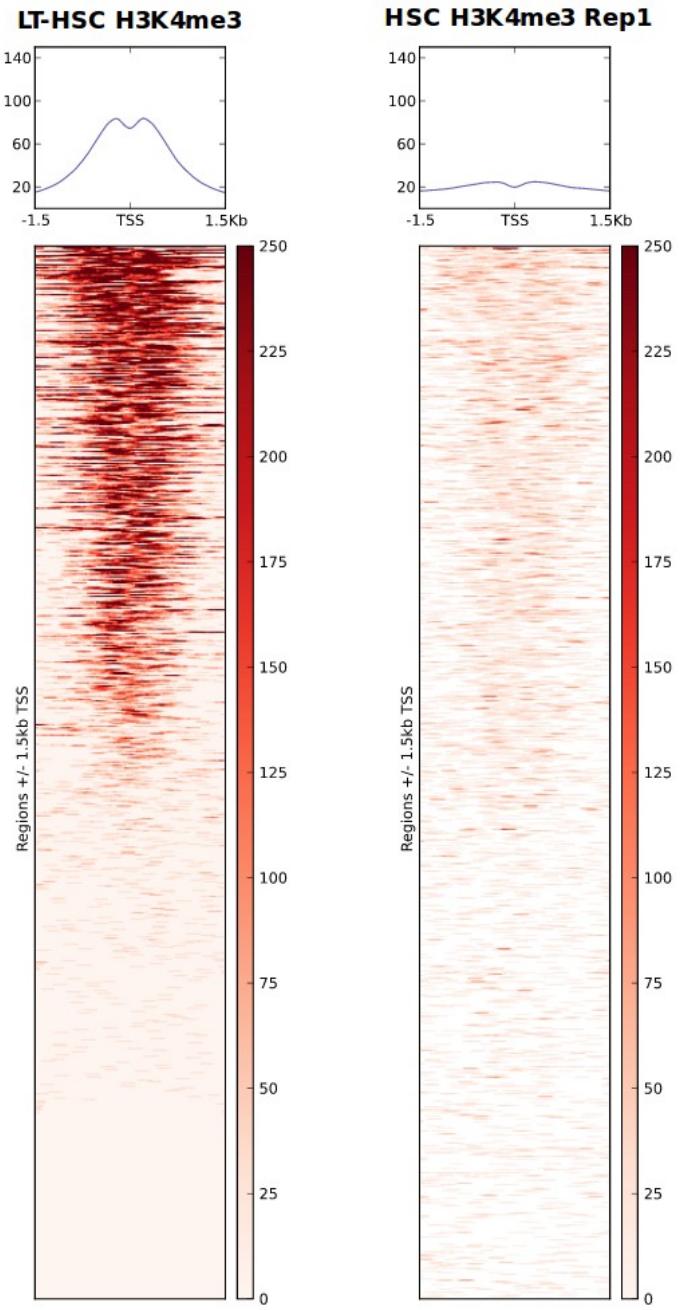
[Wilbanks & Facciotti PLoS One (2010)]

Principle of ChIP-seq

1. Quality control

- **Qualitative**
  - **Look at your favorite gene/locus in IGV !**
  - Heatmap of signal
    → e.g. H3K4me3 at promoters





1. Quality control

- **Quantitative**
  - *Fraction of reads in peaks* (FRiP)

$$FRiP = \frac{reads \in peaks}{total\ reads}$$

→ depends on type of ChIP (TF/histone)

  - *PCR Bottleneck coefficient* (PBC) : measure of library complexity

$$PBC = \frac{N_1}{N_d}$$

Genomic positions with 1 read aligned

Genomic positions with ≥ 1 read aligned

PBC < 0.5
0.5 < PBC < 0.8
0.8 < PBC

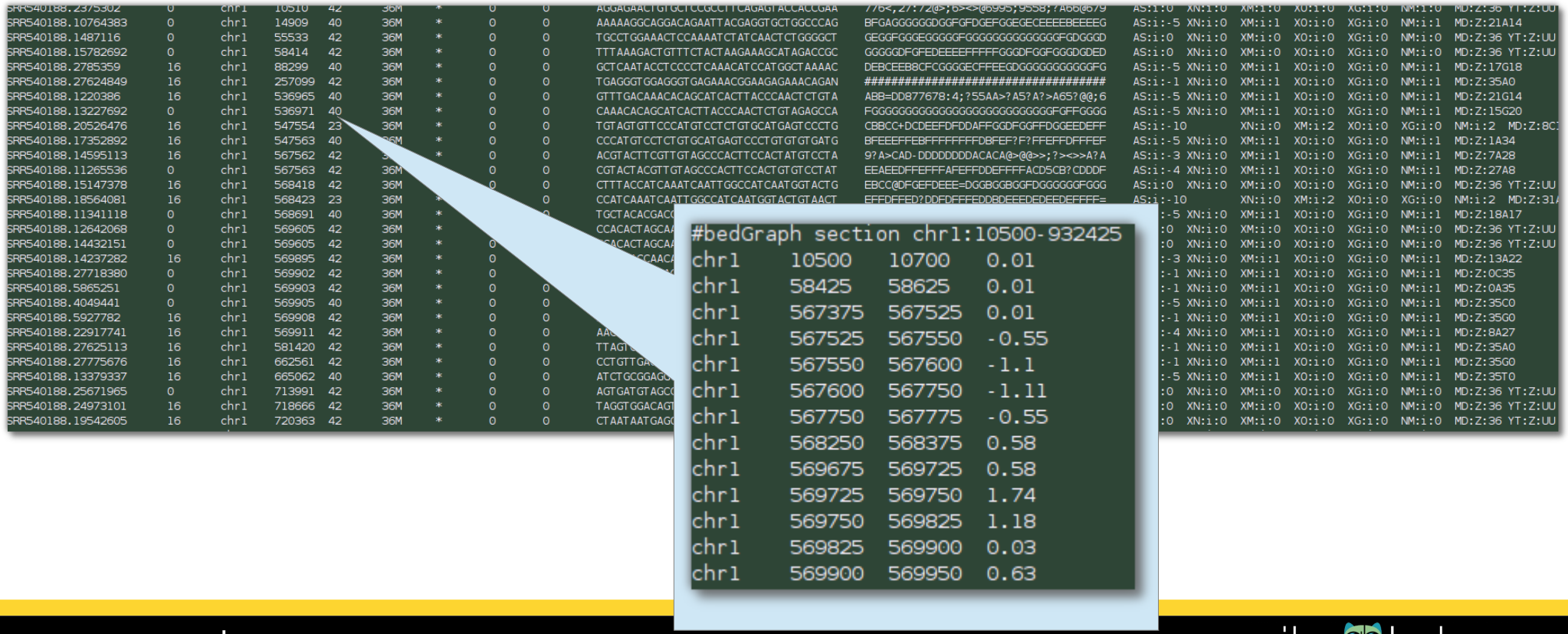
	A	B	C	D	F	J	K
1	Assay	Cell	Target	Treatment	N_uniq map reads	SPOT	PBC
2	TF-ChIP-seq	A549	CTCF	DEX_100nM	24,281,189	0.2361	0.71
3	TF-ChIP-seq	A549	CTCF	DEX_100nM	15,453,361	0.1249	0.41
4	TF-ChIP-seq	A549	GR	DEX_100nM	16,608,102	0.0754	0.91
5	TF-ChIP-seq	A549	GR	DEX_100nM	28,467,922	0.0723	0.44
6	TF-ChIP-seq	A549	POL2	DEX_100nM	19,005,470	0.6166	0.86
7	TF-ChIP-seq	A549	POL2	DEX_100nM	23,115,884	0.5388	0.86
8	TF-ChIP-seq	A549	USF1	DEX_100nM	22,289,881	0.0791	0.87
9	TF-ChIP-seq	A549	USF1	DEX_100nM	12,364,820	0.0517	0.82
10	TF-ChIP-seq	A549	GR	DEX_500pM	19,646,503	0.0105	0.96
11	TF-ChIP-seq	A549	GR	DEX_500pM	15,095,316	0.0109	0.94
12	TF-ChIP-seq	A549	GR	DEX_50nM	19,291,260	0.1289	0.96
13	TF-ChIP-seq	A549	GR	DEX_50nM	16,754,796	0.1426	0.95
14	TF-ChIP-seq	A549	GR	DEX_5nM	20,120,740	0.0343	0.98
15	TF-ChIP-seq	A549	GR	DEX_5nM	20,559,786	0.0641	0.96
16	TF-ChIP-seq	A549	CTCF	EtOH_0.02p	22,672,467	0.1601	0.75
17	TF-ChIP-seq	A549	CTCF	EtOH_0.02p	14,351,615	0.2040	0.42

Target	Treatment	N_uniq map reads	SPOT	PBC
H3K4ME3	None	23,262,787	0.7548	0.85
H3K4ME3	None	24,258,921	0.7129	0.87
H3K4ME3	None	25,830,582	0.7734	0.83
H3K4ME3	None	24,999,787	0.7708	0.83
H3K4ME3	None	27,183,786	0.841	0.75
H3K4ME3	None	18,723,894	0.7507	0.82
H3K4ME3	None	27,941,205	0.6917	0.79
H3K4ME3	None	20,608,672	0.8515	0.82
H3K4ME3	None	26,921,405	0.7402	0.84
H3K4ME3	None	27,322,283	0.7315	0.85
H3K4ME3	None	25,331,375	0.7984	0.82
H3K4ME3	None	21,265,457	0.7222	0.86
H3K27ME3	None	10,992,065	0.2188	0.97
H3K27ME3	None	14,241,301	0.2238	0.97
H3K36ME3	None	14,371,730	0.2897	0.96
H3K36ME3	None	14,363,395	0.2608	0.96
H3K4ME3	None	12,020,401	0.7748	0.9
H3K4ME3	None	16,286,127	0.7362	0.86
H3K27ME3	None	15,677,477	0.1573	0.95
H3K27ME3	None	13,552,847	0.1529	0.97
H3K36ME3	None	12,224,320	0.1934	0.98

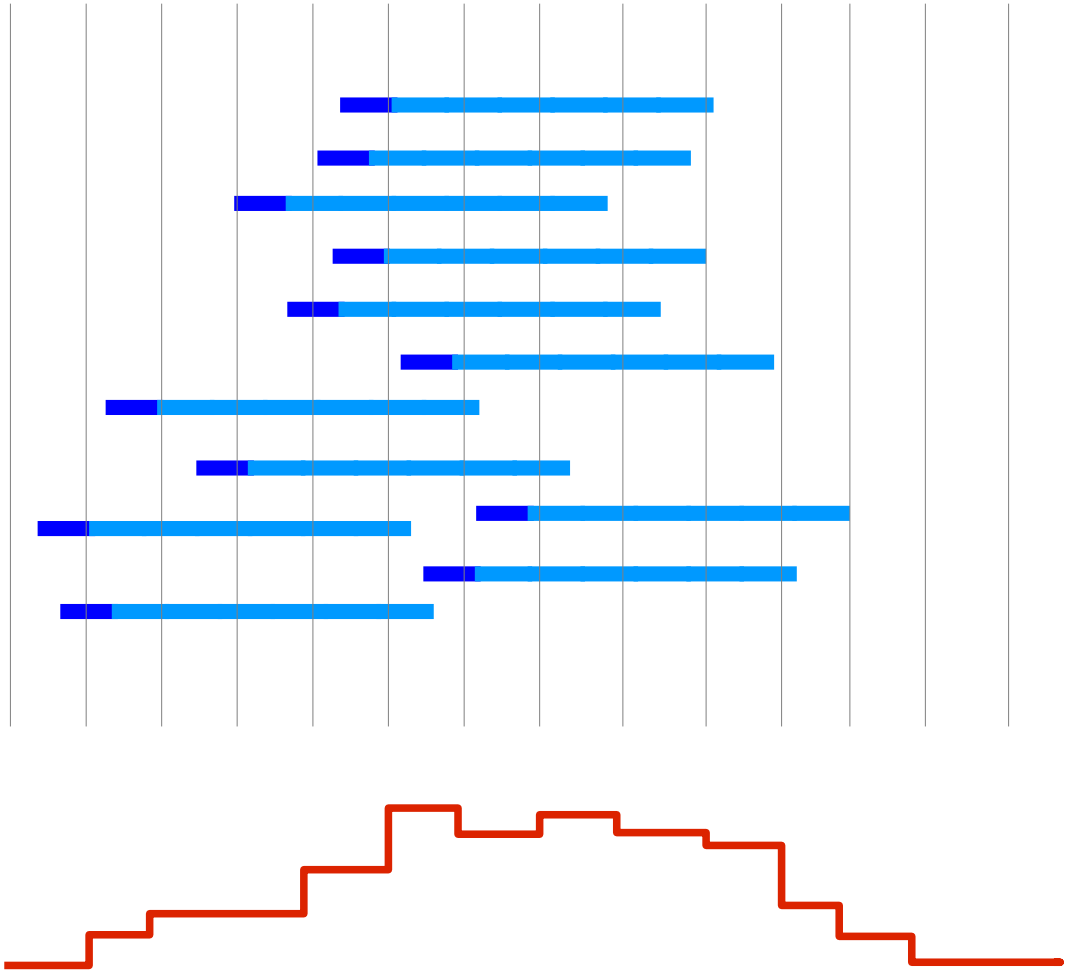
https://www.encodeproject.org/data-standards/2012-quality-metrics/

2. from reads to coverage

- to visualize the data, we use **coverage plots** (=density of fragments per genomic region)
- need to reduce BAM file to more compact format
  → **bigWig/bedGraph**



2. from reads to coverage



- Reads are extended to 3' to fragment length
- Read counts are computed for each bin
- Counts are normalized
  - reads per genomic content → normalize to 1x coverage

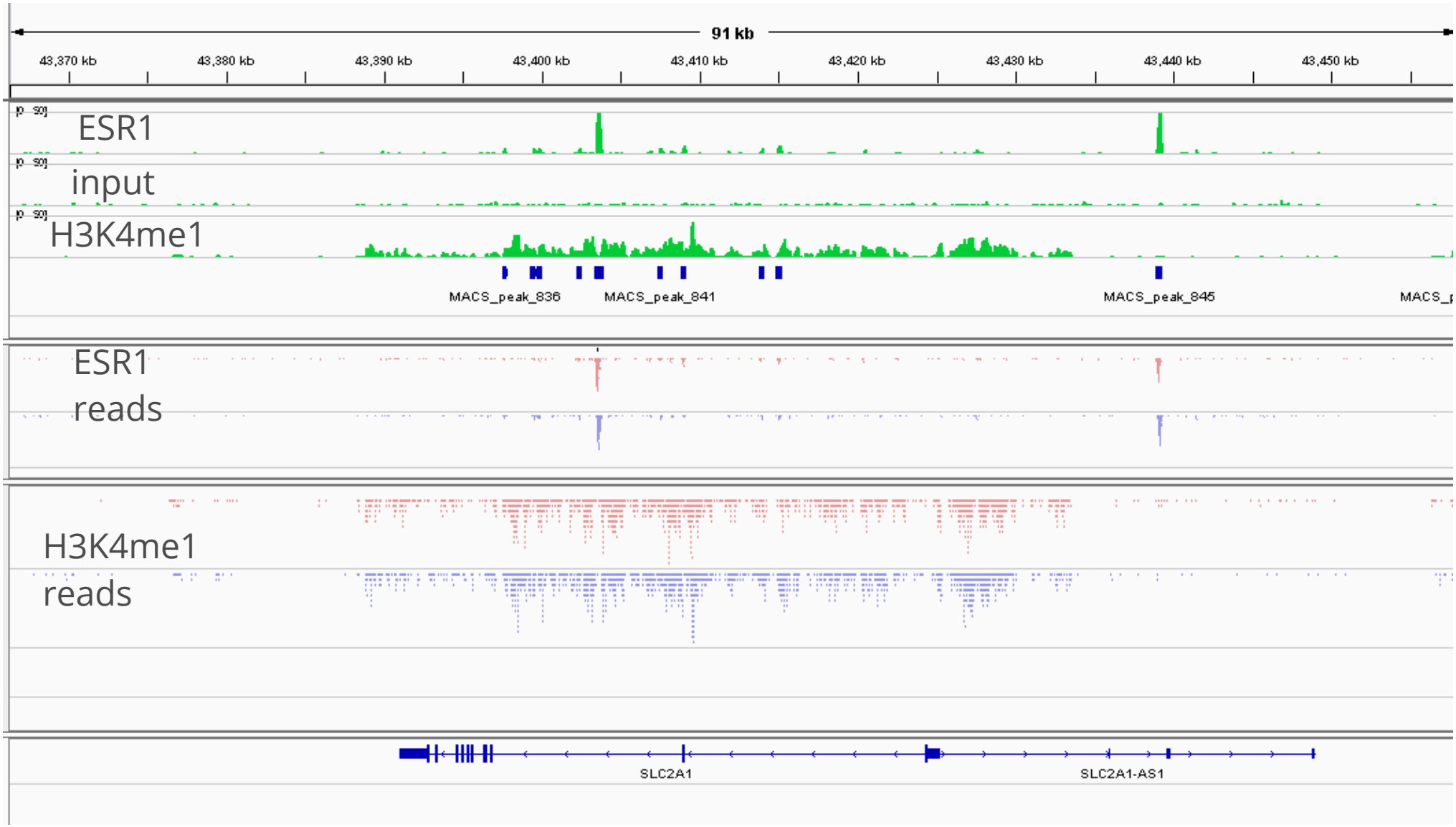
$$SD = \frac{n_{mapped\ reads} \times L}{G_{eff}}$$

  - reads per kilobase per million reads per bin

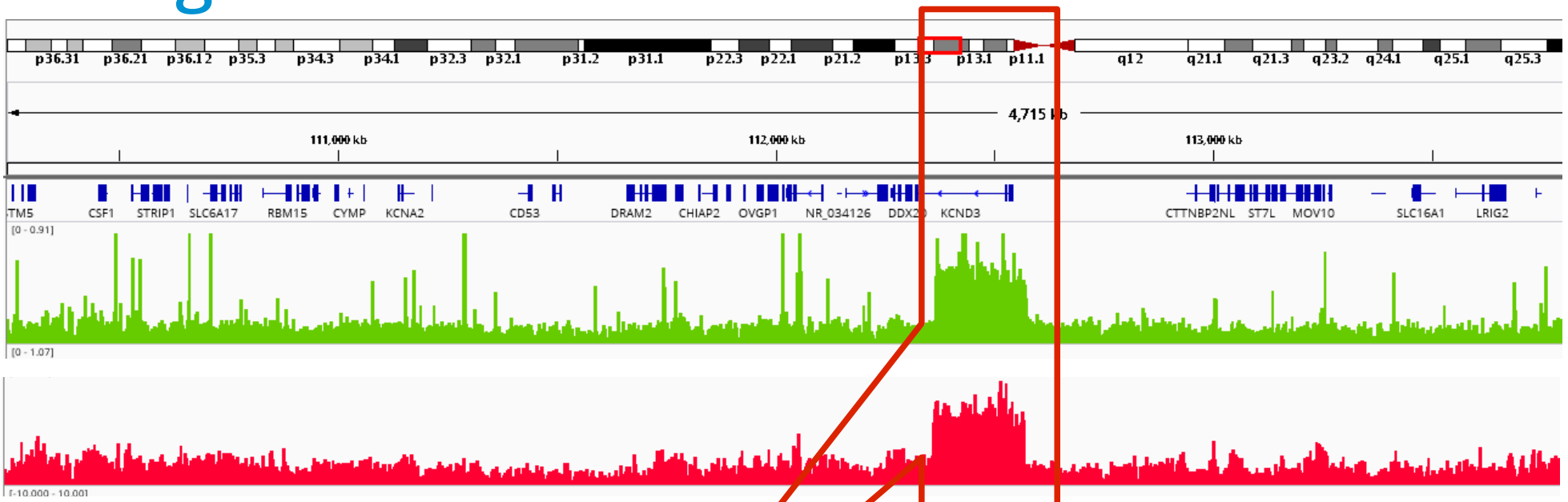
$$RPKM = \frac{n_{reads/bin} \times W_{bin}}{n_{mapped\ reads}}$$

→ deepTools : **bamCoverage**

2. from reads to coverage

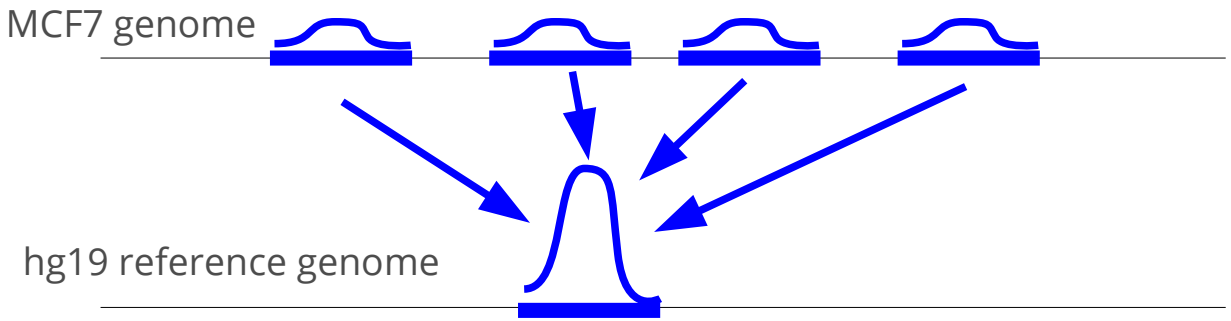


3. signal and noise



MCF-7 genome

The MCF-7 genome harbors 21 high-level CNAs, summarized in Table 1. Remarkably, many of the previously reported regions of genetic alteration split into multiple segments upon tiling resolution analysis. The 1p13 amplification described previously [40] in fact divides into three distinct segments of high-level amplifications: a 1,300 kb segment at 1p13.3, containing only two genes, those encoding arginine N-methyltransferase-6 (*PMRT6*) and netrin G1 (*NTNG1*);



3. signal to noise

- **Mappability issue** : alignability track shows, how many times a read from a given position of the genome would align
  - a=1 → read from this position ONLY aligns to this position
  - a=1/n → read from this position could align to n locations

  → we usually only keep uniquely aligned reads : **positions with a < 1 have no reads left**

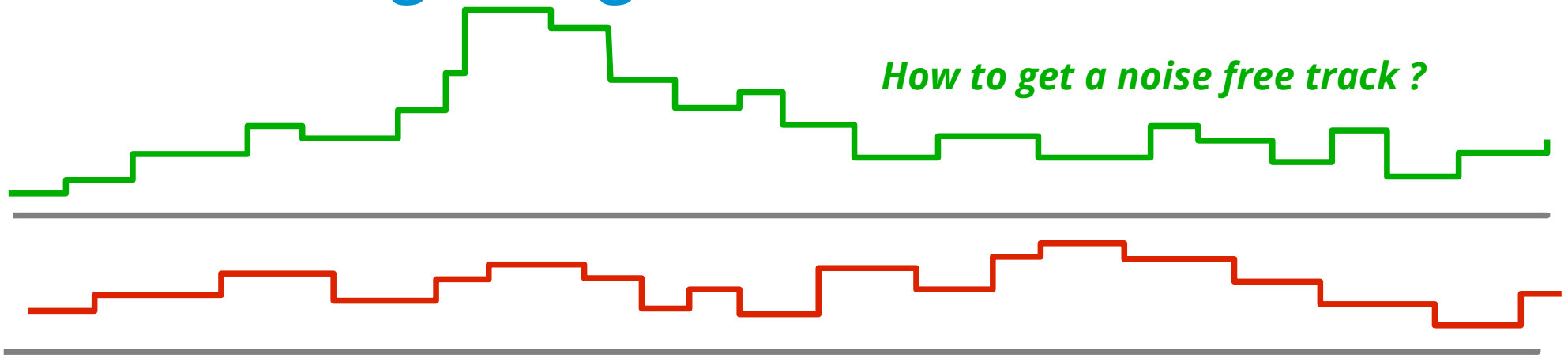
3. signal to noise

The availability of a control sample in mandatory !

→ mock IP with unspecific antibody

→ sequencing of input (=naked) DNA

4. modelling background level



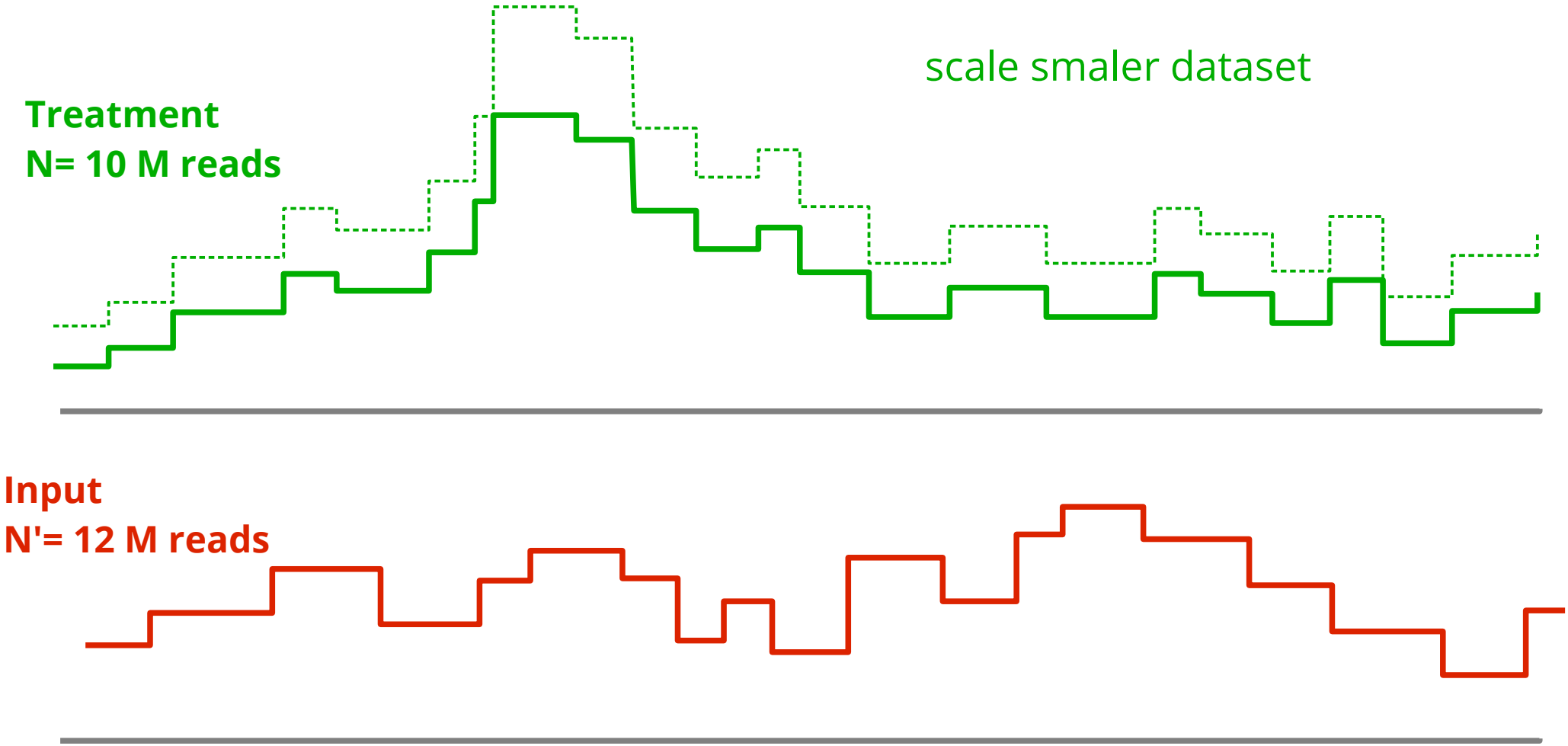
- **naïve subtraction** treatment – input is not possible, because both libraries have different sequencing depth !

- **Solution 1** : before subtraction, scale both libraries by total number of reads (library size)
  - RPGC
  - RPKM

$$SD = \frac{n_{mapped\ reads} \times L}{G_{eff}}$$

$$RPKM = \frac{n_{reads/bin} \times W_{bin}}{n_{mapped\ reads}}$$

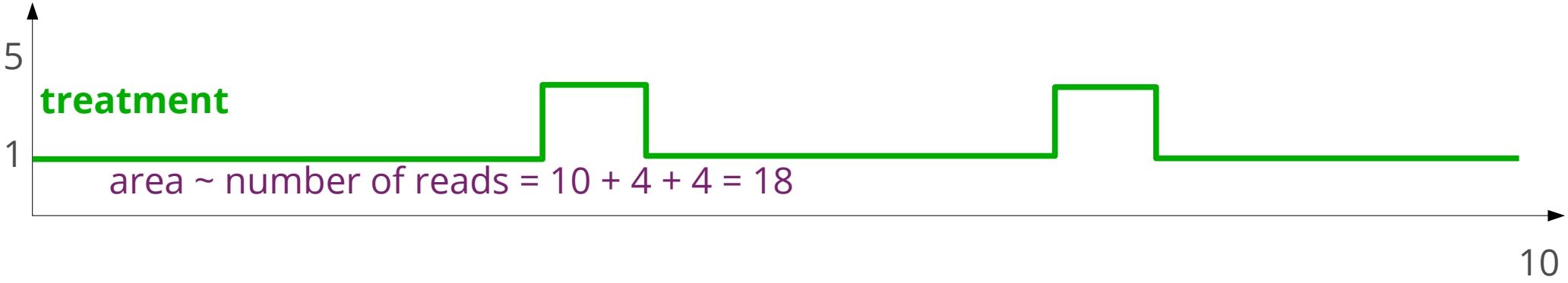
4. modelling background level



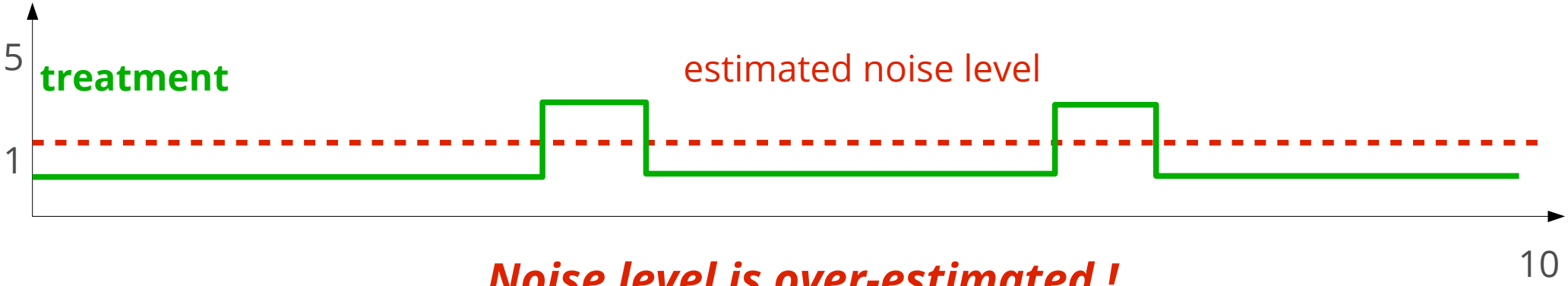
Problem : signal influences scaling factor

More signal (but equal noise) → artificial noise over-estimation

4. modelling background level

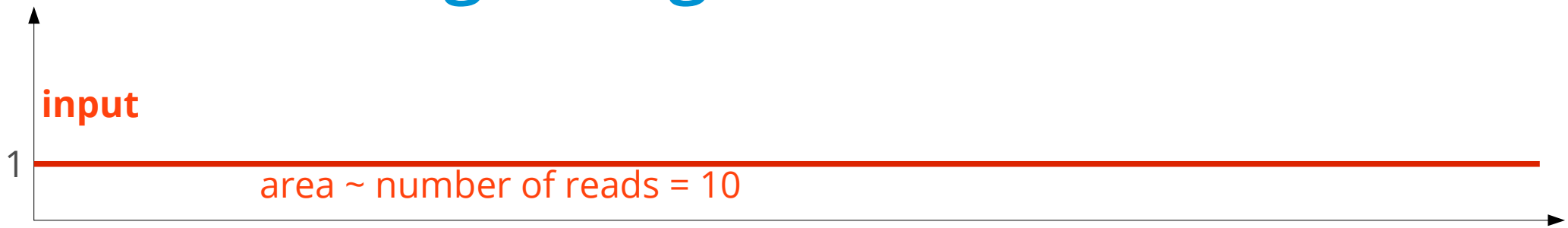


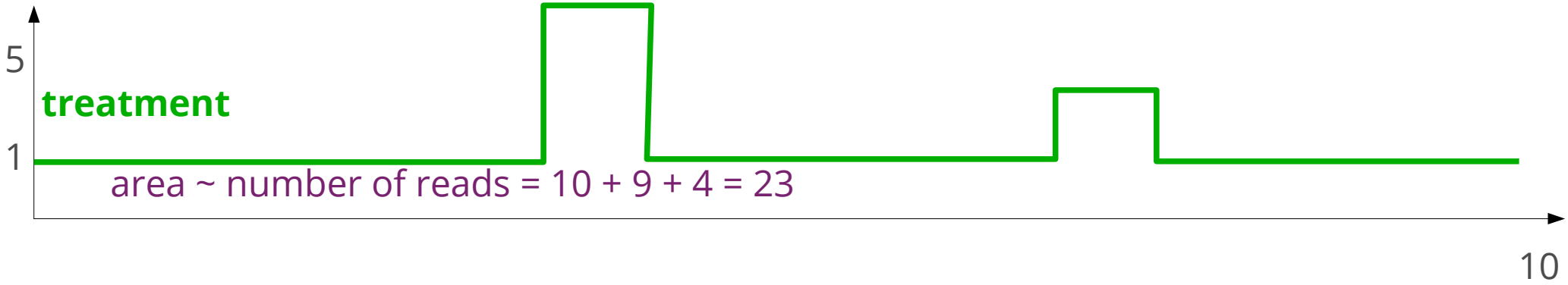
Scaling by library size : upscale input by 18/10 = 1.8



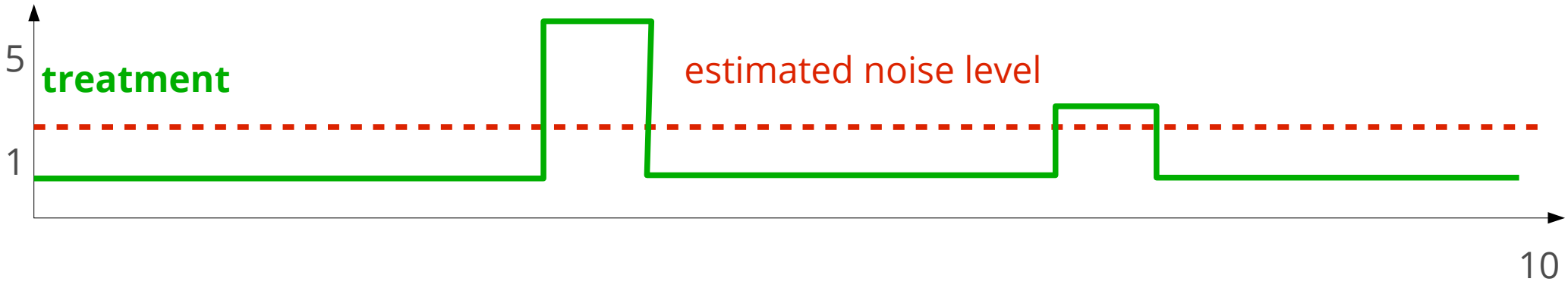
Noise level is over-estimated !

4. modelling background level

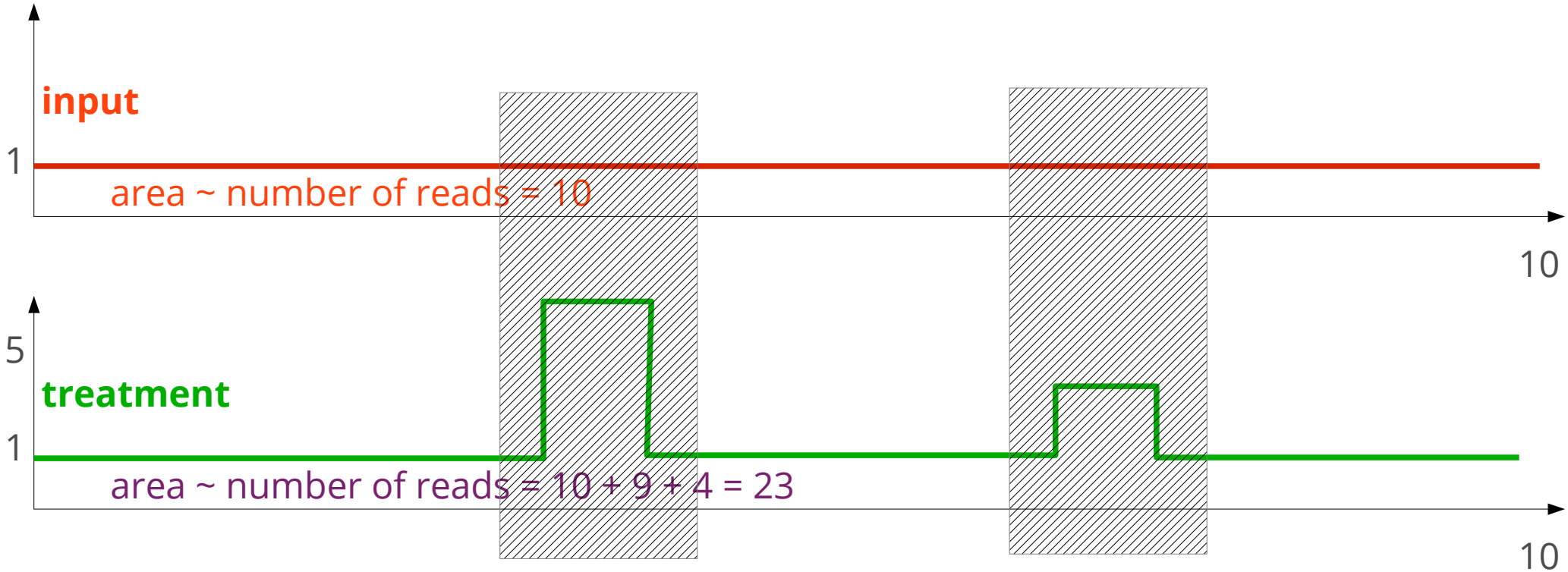




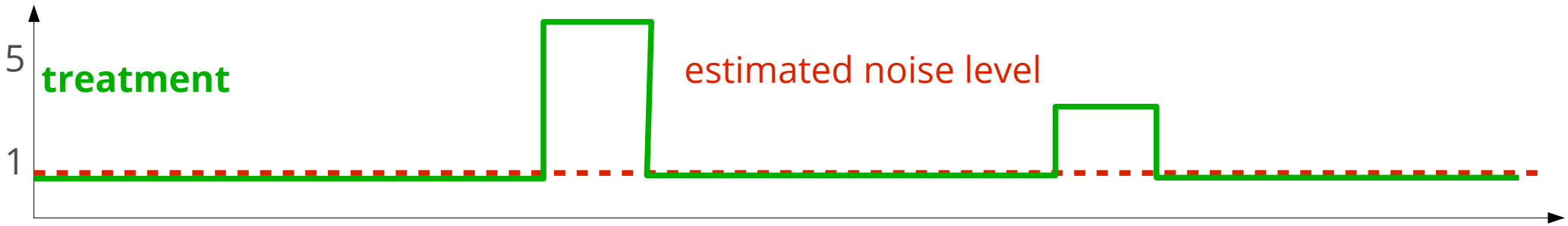
Scaling by library size : upscale input by 23/10 = 2.3



4. modelling background level

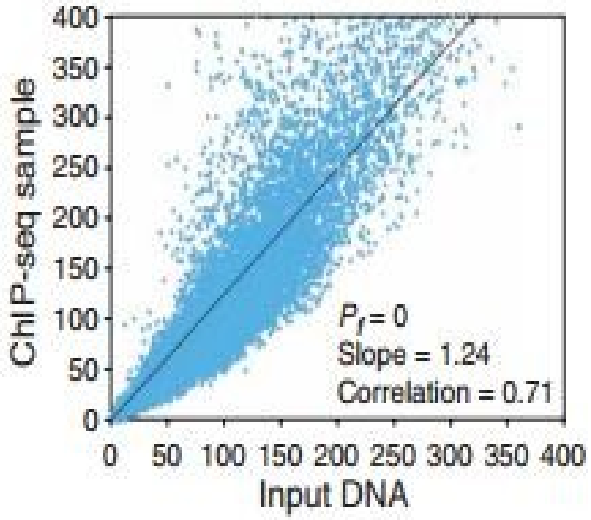


Scaling by library size : upscale input by 23/10 = 2.3

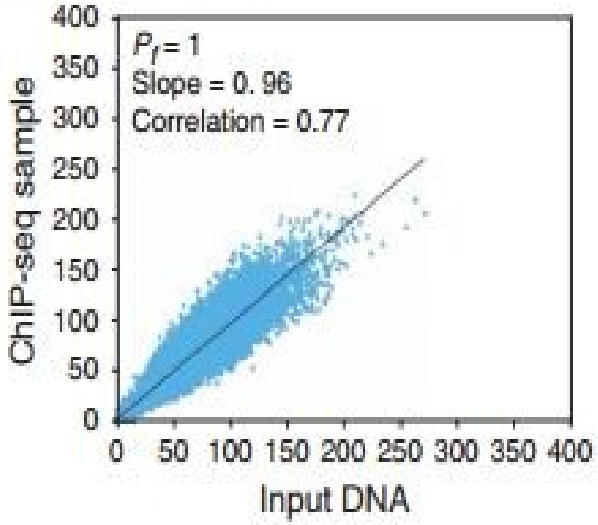


4. modelling background level

- **more advanced** : linear regression by exclusing peak regions (PeakSeq)
- read counts in 1Mb regions in input and treatment



all regions



excluding enriched (=signal) regions

PeakSeq enables systematic scoring of ChIP-seq experiments relative to controls

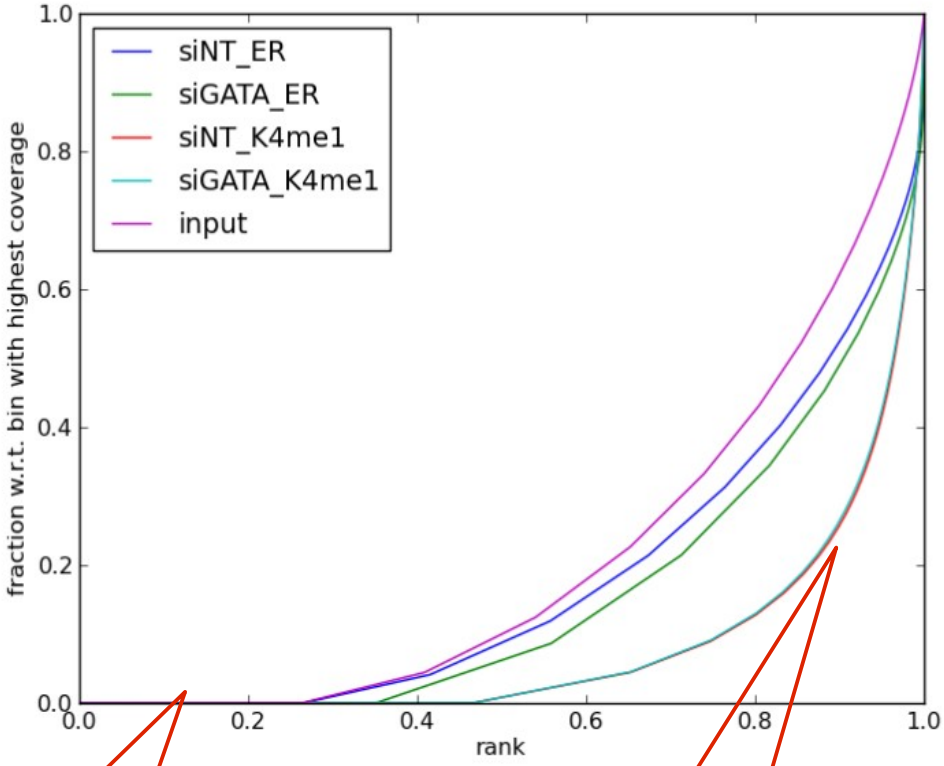
Joel Rozowsky[1], Ghia Euskirchen[2], Raymond K Auerbach[3], Zhengdong D Zhang[1], Theodore Gibson Robert Bjornson[4], Nicholas Carriero[4], Michael Snyder[1,2] & Mark B Gerstein[1,3,4]

4. modelling background level

- **Alternative strategy**
  (deepTools → Diaz et al.)
  1. bin genome into *n* 10 kb windows
  2. count reads in each window for input (X_i) and treatment (Y_i)
  3. total number of reads is N_X and N_Y
  4. order Y_i from less to most enriched → $Y_{(i)}$
  5. define and plot

$$p_j = \sum_{i=1}^{j} Y_{(i)} / M_Y \; ; \; q_j = \sum_{i=1}^{j} X_{(i)} / M_X$$

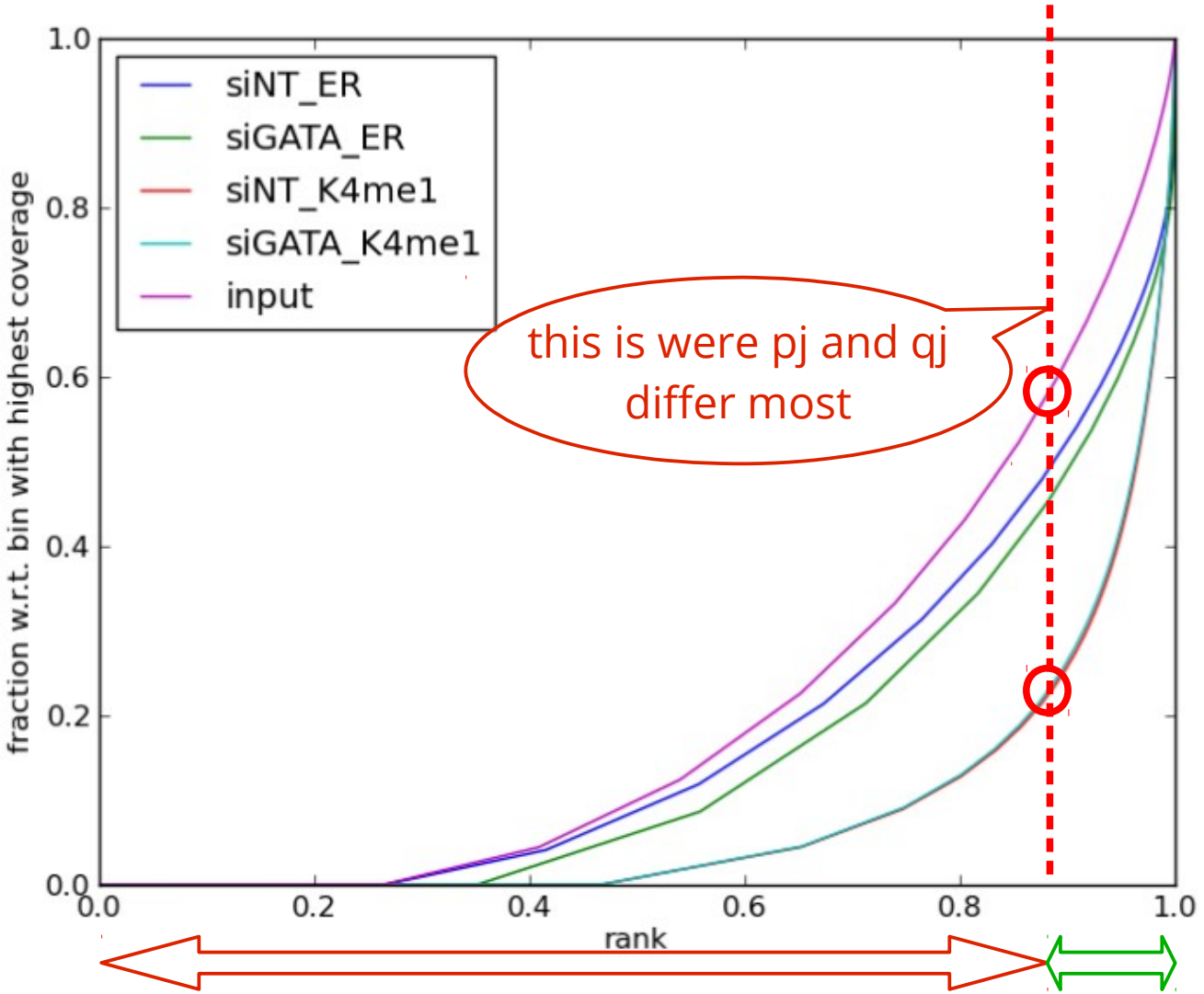
  - p_j = proportion of reads in the j less enriched windows



25% of the genome contains no reads !

90% of the genome contain ~ 25% of reads

4. modelling background level



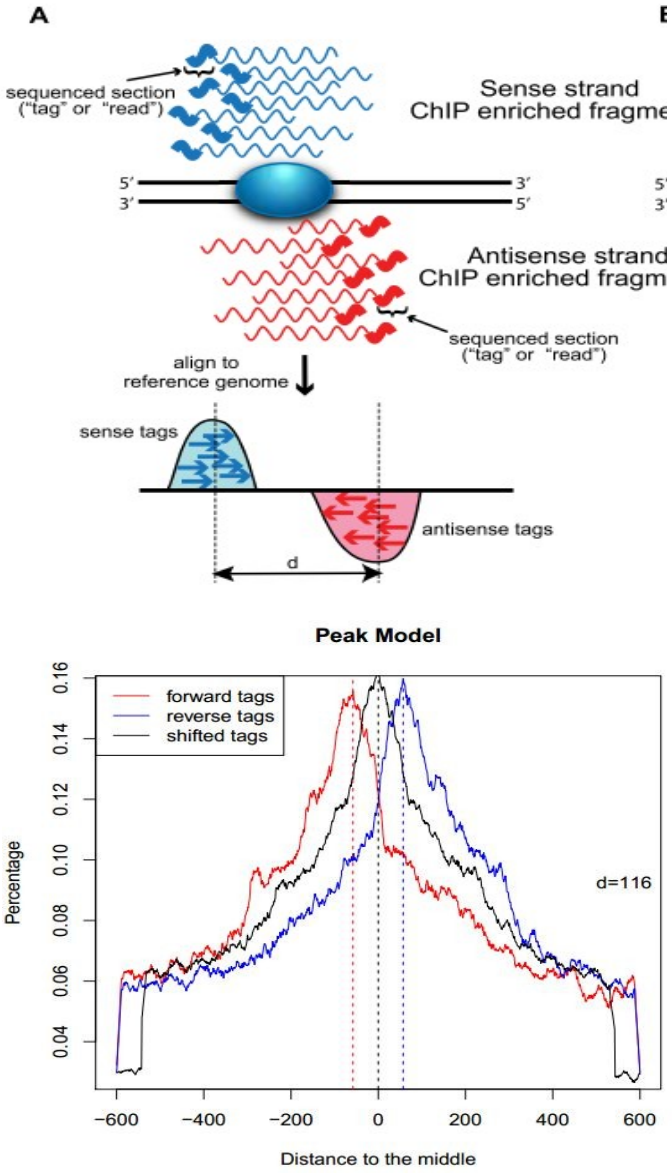
both datasets contain only noise in this range

→ scale according to number of reads in this range

treatment dataset contains signal

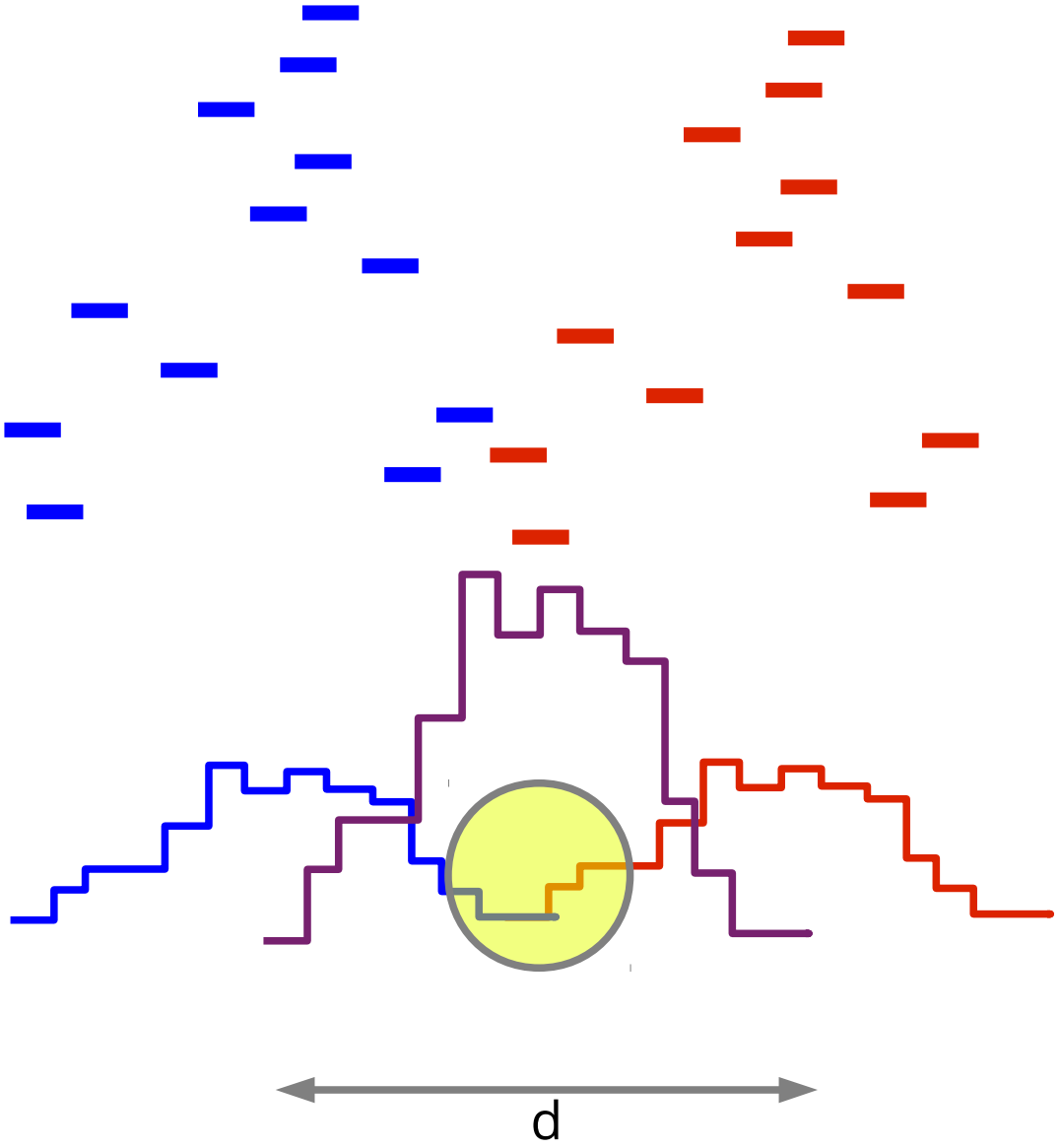
5. from reads to peaks

- **Tag shifting vs. extension**
  - positive/negative strand read peaks do not represent the true location of the binding site
  - fragment length is *d* and can be estimated from strand asymmetry
  - reads can be **elongated** to a size of *d*
  - reads can be **shifted** by *d/2* → increased resolution



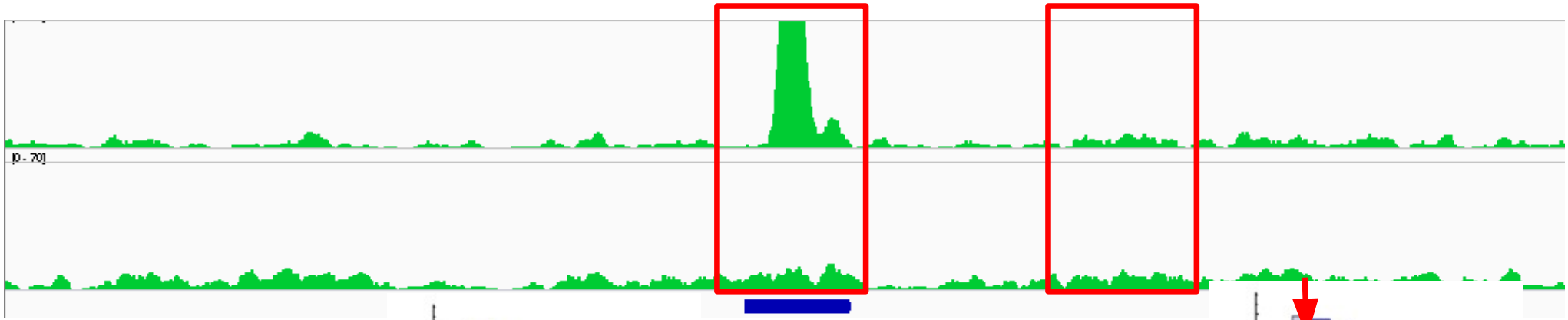
example of MACS model building using top enriched regions

5. from reads to peaks

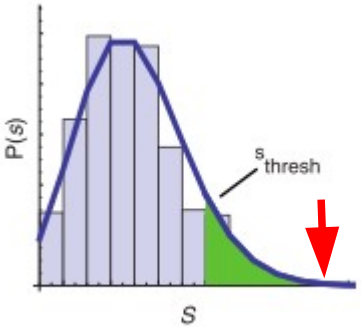


5. from reads to peaks

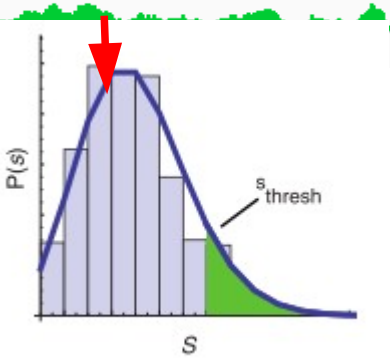
- **Determining "enriched" regions**
  - sliding window across the genome
  - at each location, evaluate the enrichement of the signal wrt. expected background based on the distribution
  - retain regions with P-values below threshold
  - evaluate FDR



Pval < 1e-20



Pval ~ 0.6



	Profile	Peak criteria[a]	Tag shift	Control data[b]	Rank by	FDR[c]	User input parameters[d]	Artifact filtering: strand-based duplicate[e]
CisGenome v1.1	Strand-specific window scan	1: Number of reads in window 2: Number of ChIP reads minus control reads in window	Average for highest ranking peak pairs	Conditional binomial used to estimate FDR	Number of reads under peak	1: Negative binomial 2: conditional binomial	Target FDR, optional window width, window interval	Yes / Yes
ERANGE v3.1	Tag aggregation	1: Height cutoff High quality peak estimate, per-region estimate, or input	High quality peak estimate, per-region estimate, or input	Used to calculate fold enrichment and optionally *P* values	*P* value	1: None 2: $\frac{\#\ \text{control}}{\#\ \text{ChIP}}$	Optional peak height, ratio to background	Yes / No
FindPeaks v3.1.9.2	Aggregation of overlapped tags	Height threshold	Input or estimated	NA	Number of reads under peak	1: Monte Carlo simulation 2: NA	Minimum peak height, subpeak valley depth	Yes / Yes
F-Seq v1.82	Kernel density estimation (KDE)	*s* s.d. above KDE for 1: random background, 2: control	Input or estimated	KDE for local background	Peak height	1: None 2: None	Threshold s.d. value, KDE bandwidth	No / No
GLITR	Aggregation of overlapped tags	Classification by height and relative enrichment	User input tag extension	Multiply sampled to estimate background class values	Peak height and fold enrichment	2: $\frac{\#\ \text{control}}{\#\ \text{ChIP}}$	Target FDR, number nearest neighbors for clustering	No / No
MACS v1.3.5	Tags shifted then window scan	Local region Poisson *P* value	Estimate from high quality peak pairs	Used for Poisson fit when available	*P* value	1: None 2: $\frac{\#\ \text{control}}{\#\ \text{ChIP}}$	*P*-value threshold, tag length, mfold for shift estimate	No / Yes
PeakSeq	Extended tag aggregation	Local region binomial *P* value	Input tag extension length	Used for significance of sample enrichment with binomial distribution	*q* value	1: Poisson background assumption 2: From binomial for sample plus control	Target FDR	No / No
QuEST v2.3	Kernel density estimation	2: Height threshold, background ratio	Mode of local shifts that maximize strand cross-correlation	KDE for enrichment and empirical FDR estimation	*q* value	1: NA 2: $\frac{\#\ \text{control}}{\#\ \text{ChIP}}$ as a function of profile threshold	KDE bandwidth, peak height, subpeak valley depth, ratio to background	Yes / Yes
SICER v1.02	Window scan with gaps allowed	*P* value from random background model, enrichment relative to control	Input	Linearly rescaled for candidate peak rejection and *P* values	*q* value	1: None 2: From Poisson *P* values	Window length, gap size, FDR (with control) or *E*-value	No / Yes
SiSSRs v1.4	Window scan	$N_+ - N_-$ sign change, $N_+ + N_-$ threshold in region[f]	Average nearest paired tag distance					
spp v1.0	Strand specific window scan	Poisson *P* value (paired peaks only)	Maximal strand cross-correlation					

Computation for ChIP-seq and RNA-seq studies

Shirley Pepke[1], Barbara Wold[2] & Ali Mortazavi[2]

	Profile
CisGenome v1.1	Strand-specific window scan
ERANGE v3.1	Tag aggregation
FindPeaks v3.1.9.2	Aggregation of overlapped tags
F-Seq v1.82	Kernel density estimation (KDE)
GLITR	Aggregation of overlapped tags
MACS v1.3.5	Tags shifted then window scan
PeakSeq	Extended tag aggregation
QuEST v2.3	Kernel density estimation
SICER v1.02	Window scan with gaps allowed
SiSSRs v1.4	Window scan
spp v1.0	Strand specific window scan

Some methods separate the tag densities into different strands and take advantage of tag asymmetry

Most consider merged densities and look for enrichment

	Profile	Peak criteria[a]	Tag shift
CisGenome v1.1	Strand-specific window scan	1: Number of reads in window 2: Number of ChIP reads minus control reads in window	Average for highest ranking peak pairs
ERANGE v3.1	Tag aggregation	1: Height cutoff High quality peak estimate, per-region estimate, or input	High quality peak estimate, per-region estimate, or input
FindPeaks v3.1.9.2	Aggregation of overlapped tags	Height threshold	Input or estimated
F-Seq v1.82	Kernel density estimation (KDE)	*s* s.d. above KDE for 1: random background, 2: control	Input or estimated
GLITR	Aggregation of overlapped tags	Classification by height and relative enrichment	User input tag extension
MACS v1.3.5	Tags shifted then window scan	Local region Poisson *P* value	Estimate from high quality peak pairs
PeakSeq	Extended tag aggregation	Local region binomial *P* value	Input tag extension length
QuEST v2.3	Kernel density estimation	2: Height threshold, background ratio	Mode of local shifts that maximize strand cross-correlation
SICER v1.02	Window scan with gaps allowed	*P* value from random background model, enrichment relative to control	Input
SiSSRs v1.4	Window scan	$N_+ - N_-$ sign change, $N_+ + N_-$ threshold in region[f]	Average nearest paired tag distance
spp v1.0	Strand specific window scan	Poisson *P* value (paired peaks only)	Maximal strand cross-correlation

Tag shift

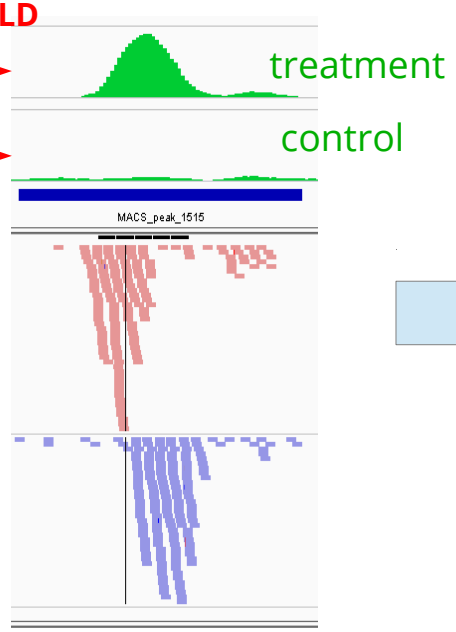
Tag extension

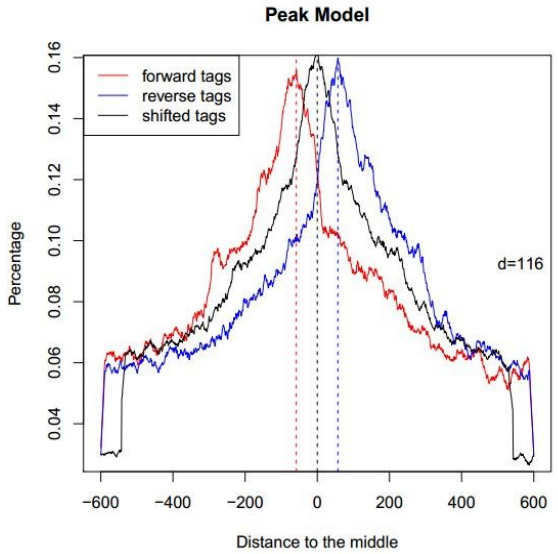
Tags unchanged

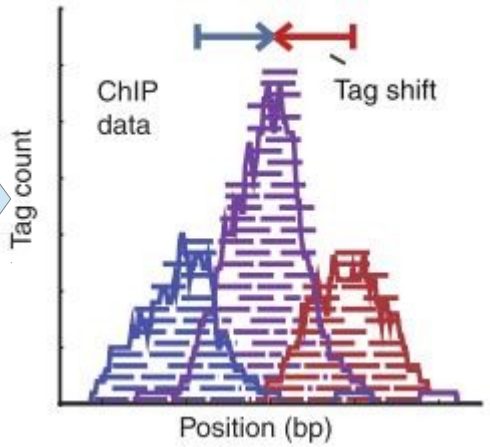
6. MACS [Zhang et al. Genome Biol. 2008]

- **Step 1 : estimating fragment length *d***
  - slide a window of size BANDWIDTH
  - retain top regions with MFOLD enrichment of treatment vs. input
  - plot average +/- strand read densities → estimate d

enrichment
> MFOLD

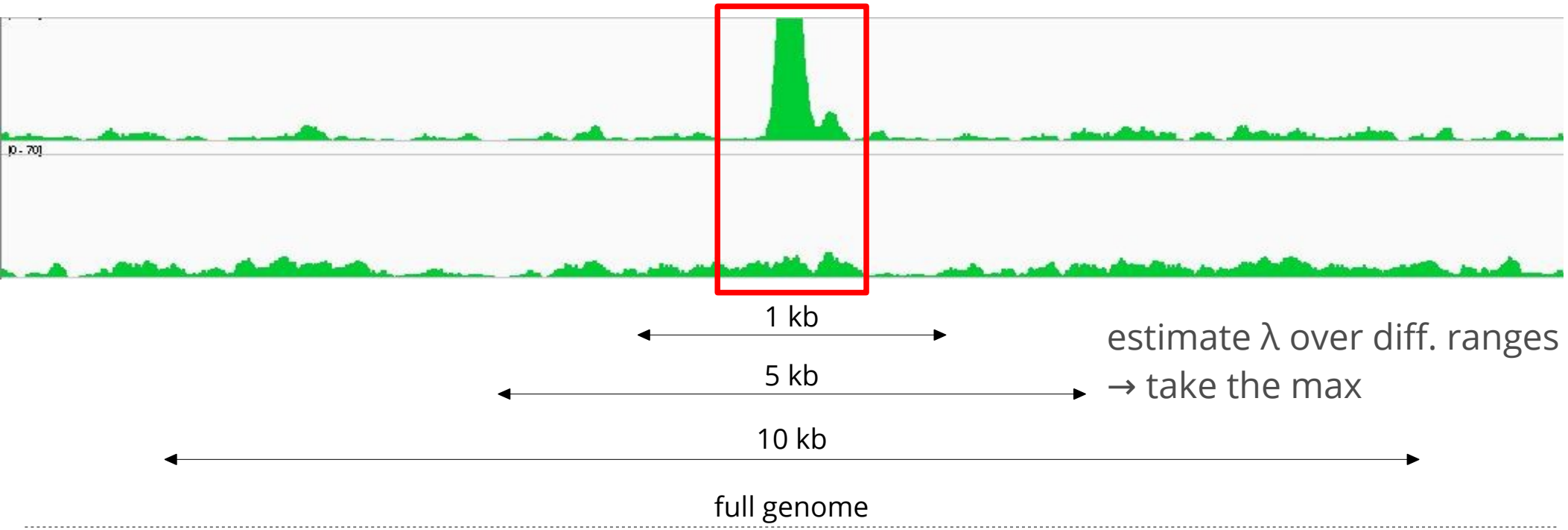






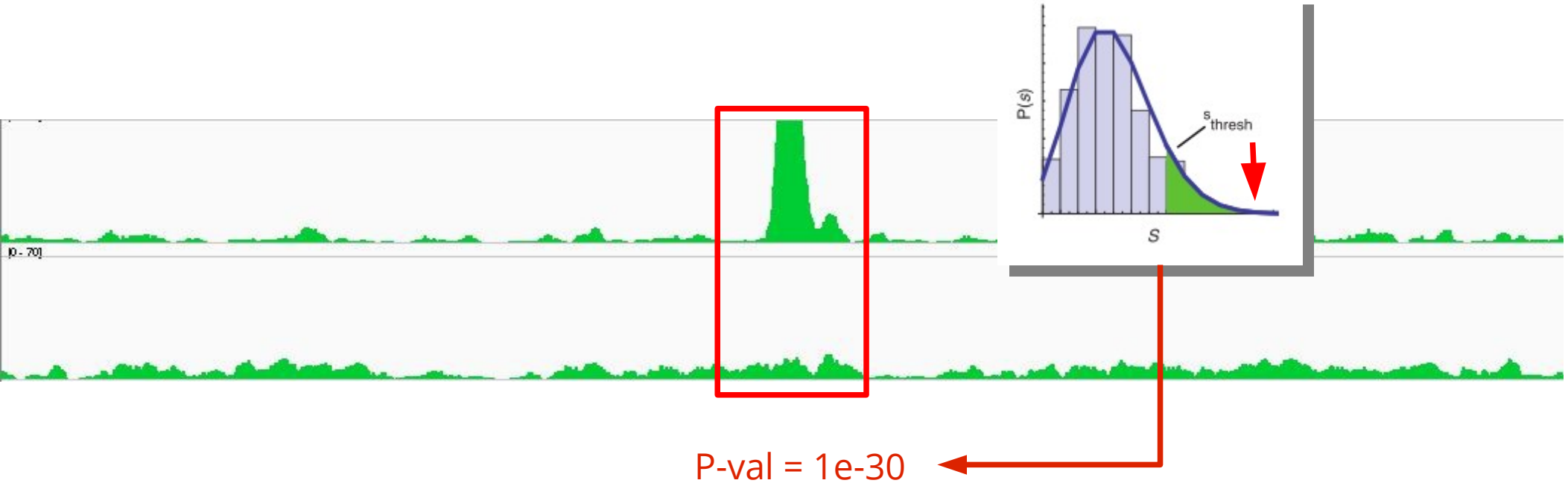
5. MACS [Zhang et al. Genome Biol. 2008]

- **Step 2 : identification of local noise parameter**
  - slide a window of size *2*d* across treatment and input
  - estimate parameter λ_{local} of Poisson distribution



5. MACS [Zhang et al. Genome Biol. 2008]

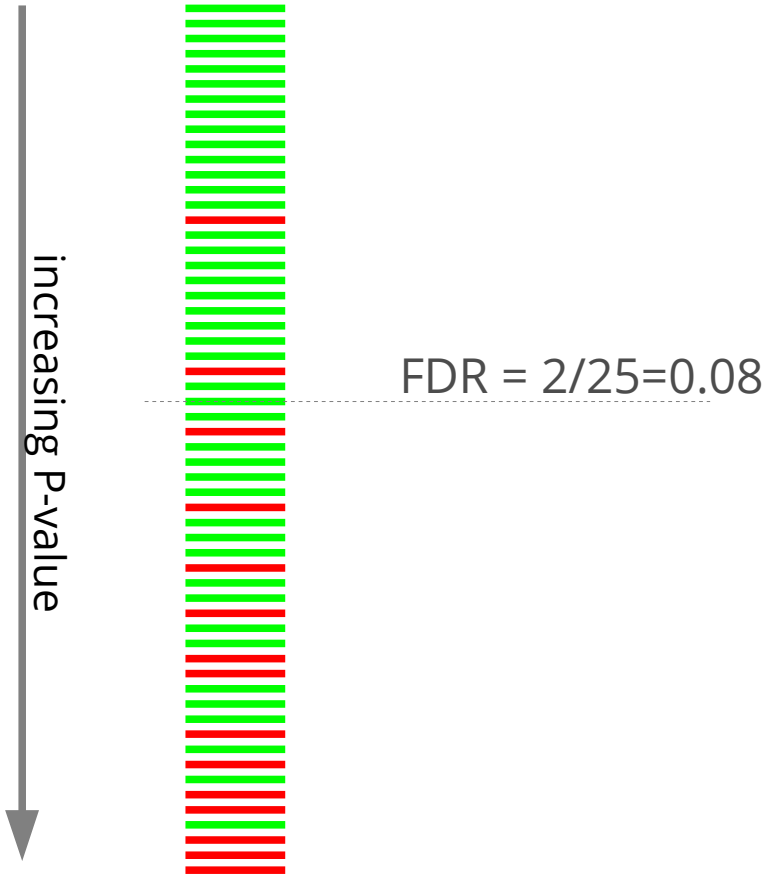
- **Step 3 : identification of enriched/peak regions**
  - determine regions with P-values < PVALUE
  - determine summit position inside enriched regions as max density



5. MACS [Zhang et al. Genome Biol. 2008]

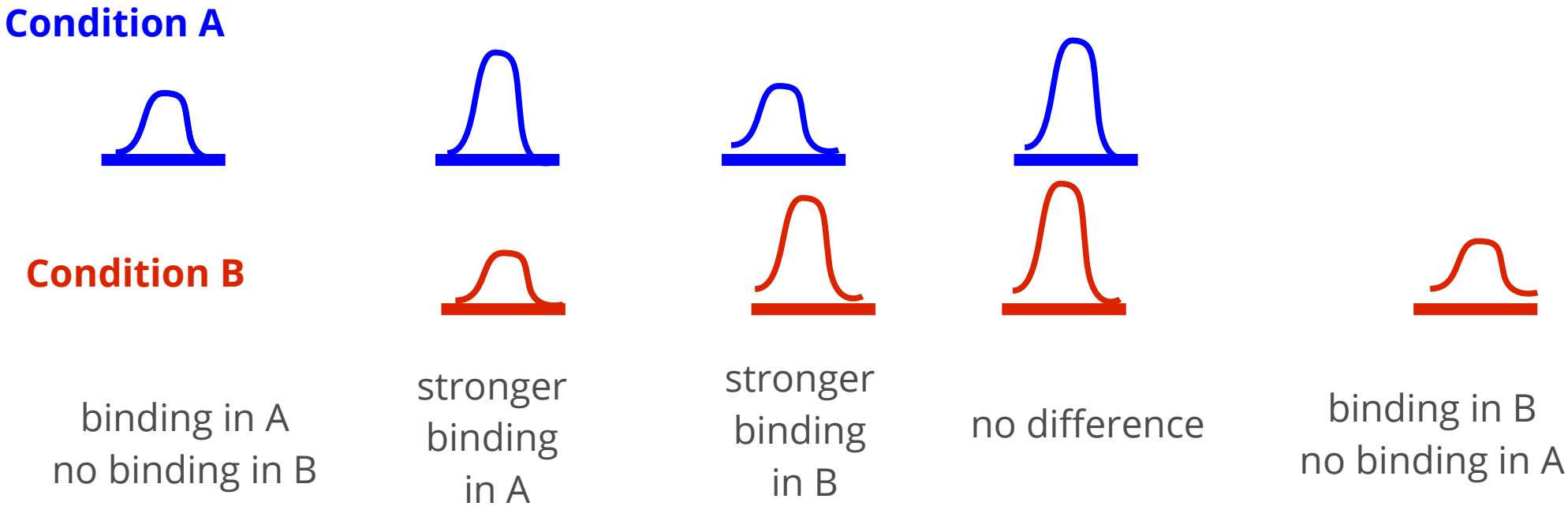
- **Step 4 : estimating FDR**
  - positive peaks (P-values)
  - swap treatment and input; call negative peaks (P-value)

$$FDR(p) = \frac{\#\ \textbf{negative}\ \text{peaks with Pval} < p}{\#\ \textbf{positive}\ \text{peaks with Pval} < p}$$



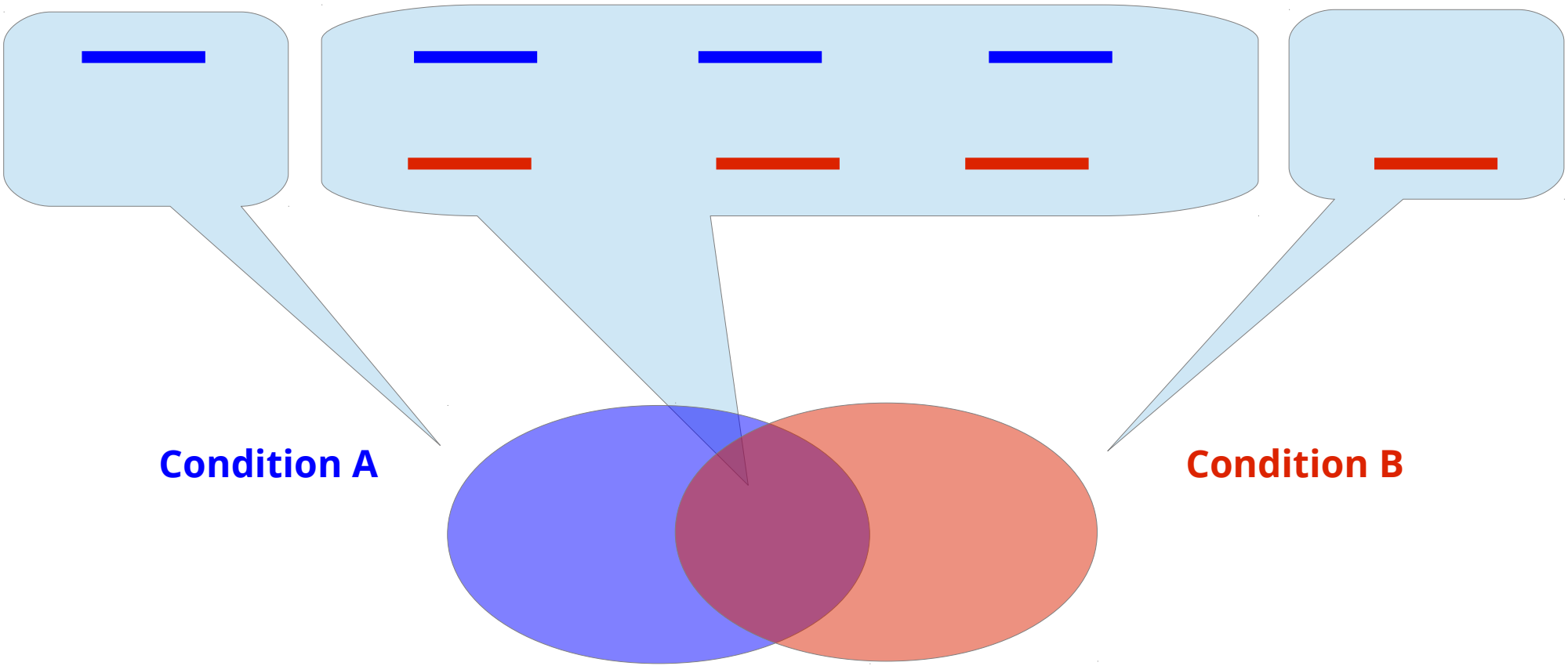
6. differential analysis

- given ChIP-set datasets in different conditions, we want to find **differential binding events** between 2 conditions
  - binding vs. no binding → qualitative analysis
  - weak binding vs. strong binding → quantitative analysis



6. differential analysis

- simple approach → compute common and specific peaks

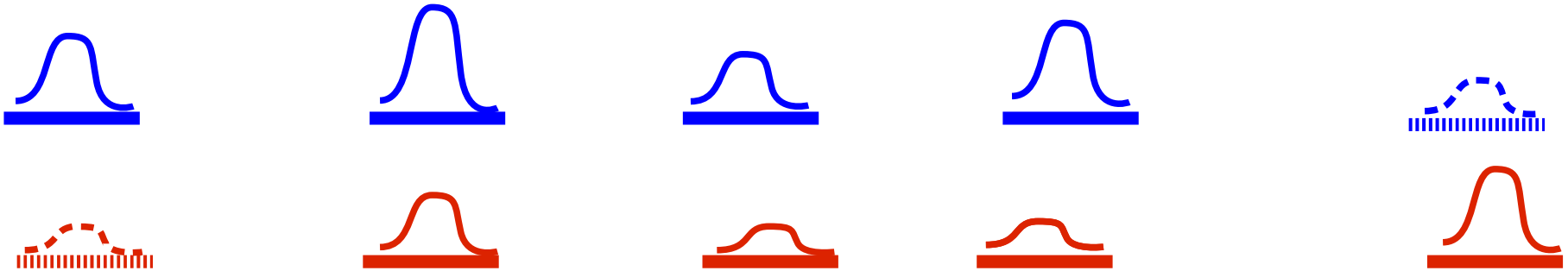


Drawback :

- common peaks can hide **differences in binding intensities**
- specific peaks can result from **threshold issues**

6. differential analysis

- **quantitative approach**
  - select regions which have signal (union of all peaks)
  - in these regions, perform quantitative analysis of differential binding based on **read counts**

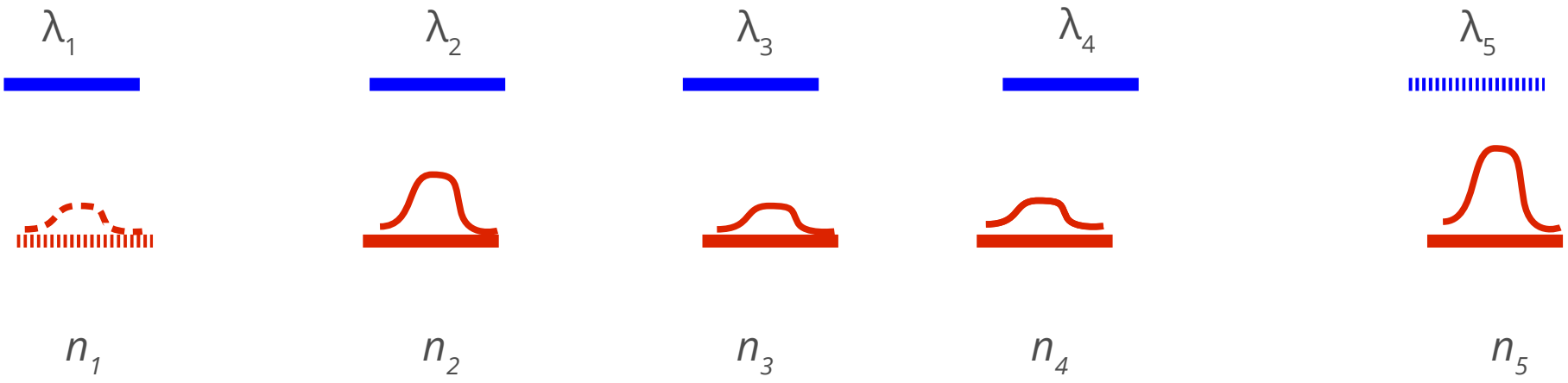


- statistical model
  - **without replicates** : assume simple Poisson model (→ SICER-df)
  - **with replicates** : perform differential test using DE tools from RNA-seq (diffBind using EdgeR, DESeq,...) based on read counts

6. differential analysis

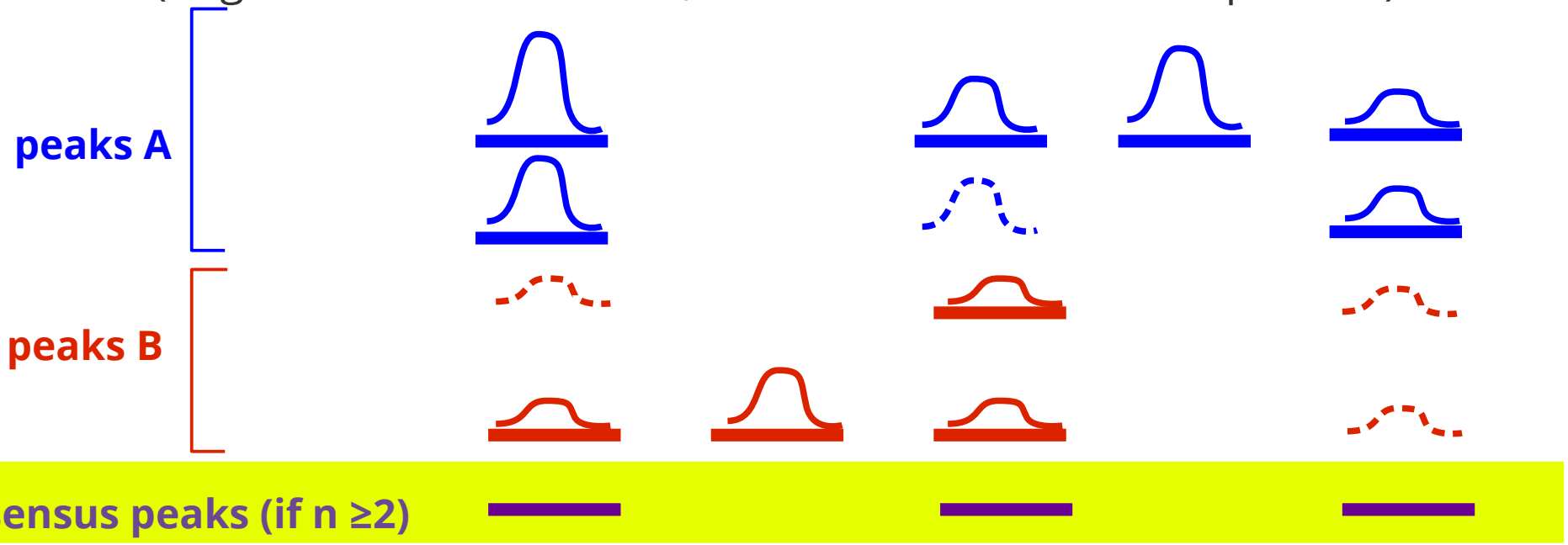
- **without replicates (sicer-df)**
  - consider one condition to be the reference (condition A)
  - call peaks on each condition independently
  - take union of peaks
  - assume Poisson model based on expected number of reads in region
  - compute P-value, log(fold-change)

$$\lambda_i = w_i N_A / L_{eff}$$



6. differential analysis

- **with replicates (diffBind)**
  - provide list of peaks for replicates A and replicates B
  - determine consensus peakset based on presence in at least *n* datasets
  - compute read counts in each consensus peak in each dataset
  - run DESeq / EdgeR to determine differential peaks between condition A and B (negative binomial model, variance estimated on replicates)



Program of the Practical Session

Step 0 : Find datasets on Gene Expression Omnibus

Step 1 : Import datasets into your Galaxy history

Step 2 : data inspection : coverage plots, correlation,...

Step 3 : peak calling using MACS

Step 5 : differential analysis

Step 6 : visualizing results in IGV