

ChIP Sequencing

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— Sample to Insight –

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ChIP Sequencing

ChIP-Sequencing is used to analyze the interactions of proteins with genomic DNA. After a crosslinking step that covalently links proteins and DNA, ChIP-Seq uses chromatin immunoprecipitation (ChIP) to fish out the relevant pieces of genomic DNA. By subsequent massive parallel DNA sequencing and mapping to the reference genome it is possible to identify binding sites of DNA-associated proteins. It can be used to accurately map global binding sites for any protein of interest when specific antibodies are available. A natural next step bioinformatics analysis is to extract the binding regions and perform pattern discovery to learn about any conserved binding motif in the DNA. Usually a control experiment is performed where the immuno-precipitation step is left out. This control data is typically used to correct for sequencing biases, such as the ones occuring in genomic regions that are less accessible, repeated regions or copy number aberrations.

This tutorial takes you through a complete ChIP sequencing workflow using CLC Genomics Workbench. We will focus on how to run the analysis but we will not go through the technical details of how the Transcription Factor ChIP-Seq analysis is implemented. Click the **Help** button in the tool dialogs (see below) to read more about the tools used in the tutorial.

The workflow consists of five parts:

- Importing the raw sequencing data.
- Mapping the reads to a reference genome.
- Calling peaks.
- Visualizing the results.

We will look at a subset of a ChIP-Seq dataset for the transcription factor NRSF (Neural Restrictive Silencer Factor) on the human cell line Gm12878. Also known as REST (RE1-Silencing Transcription factor), NRSF is a transcription factor involved in the repression of neural genes in non-neuronal cells, such as the lymphoblastoid cell line Gm12878. We therefore expect NRSF ChIP-Seq peaks to be associated with genes involved in neural activity. The data was collected by the Myers Lab at the HudsonAlpha Institute for Biotechnology. This dataset is well studied and has been often used to evaluate the performances of ChIP-Seq algorithms [Rye et al., 2011]. In addition to the NRSF ChIP-Seq dataset, we will also use a control experiment where the immuno-precipitation step is left out.

In this tutorial, we will look at only a subset of the data, namely only the reads of the NRSF and control experiments mapping to human chromosome 21. The complete datasets can be found at the UCSC website.

Importing the raw sequencing data

- First, download the data set from our website: http://resources.giagenbioinformatics. com/testdata/raw_data/ChIP-seq_NRSF_chr21_bx.zip. Unzip the file somewhere on your computer (on the Desktop for example).
- 2. Start the workbench and import the sequencing data:

File | Import ((2) | Illumina... ((2)

This will bring up the dialog shown in figure 1:



 Choose where to run Import files and options 	Set parameters Look in: ChIP-seq_NRSF_chr21
	Recent Items
	File name: "control-chr21.fastq" "nrsf-chr21.fastq"
	Desktop Files of type: Illumina files (.txt/.fastq/.fq/.qseq)
	General options Paired reads Discard read names Discard quality scores Minimum distance 180 Mate-pair (reverse-forward) Maximum distance 250
	Illumina options Illumina protons Quality scores NCBI/Sanger or Illumina Pipeline 1.8 and later MISeq de-multiplexing Trim reads

Figure 1: Import raw reads. When analyzing your own data, you should select the sequencing technology appropriate for your data. This dataset consists of two fastq files obtained using an Illumina sequencer, so the Illumina importer should be chosen.

- 3. Select the nrsf-chr21.fastq and control-chr21.fastq files. Make sure the **Paired** reads checkbox is not checked, as the options to discard read names and quality scores (These last options are not significant in this context because of the relatively small amount of reads). Click Next, Save the imported reads list in a folder created for this tutorial and click **Finish**.
- 4. Next, import the reference genome sequence that was also included in the zip file in clc format: drag and drop in the Navigation Area the files NC_000021 (Genome).clc and NC_000021 (Gene).clc, which are the genomic chromosome 21 reference sequence and the gene annotation track for chromosome 21, respectively. You can also use the Standard Import tool with the option Automatic import:

Import (凸) | Standard Import (凸)

Mapping the reads to the reference genome

Once the data has been imported, the next step in the analysis is to map the reads to the reference genome:

1. Go to:

Toolbox | Resequencing Analysis () | Map Reads to Reference ()

- The first wizard window allows you to choose the files containing the raw reads. Since we want to map two lists, we check the **Batch** option to enable the batch mode and select the folder where the sequence lists are stored ChIP-Seq (see figure 2). Click on the button labeled **Next**
- 3. In the next window, check that only the two lists control-chr21 (I) and nrsf-chr21 (I) are selected (figure 3). Click Next.
- You can now select the reference sequence NC_000021 (Genome) \icon{clcobjects/sequence as shown in figure 4. Click Next.



Gx Map Reads to Reference	Select conversion roads				X
1. Choose where to run	Navigation Area			Selected elements (1)	
2. Select sequencing reads	CLC_Data CLC_DATA CL_DATA CL CLC_DATA CL_DATA CLC_DATA CL_DATA CL_DATA CL_DATA CL_DATA CLC_DATA CLC_DATA CL_DATA CLC_DATA	E E		ChIP-Seq	
?		+ Previous	→ Next	Finish	X Cancel

Figure 2: Select sequence list containing the reads. Since we want to map two lists, we choose the batch mode.

Gx Map Reads to Reference		X
1. Choose where to run	Batch overview Units	Contents
2. Select sequencing reads	control-chr21	i nrsf-dr21
3. Batch overview	Only use elements containing:	
RE IN THE AVERAGE CONTRACTOR	-	2 elements in total
?		← Previous → Next ✓ Finish X Cancel

Figure 3: Check that all reads are used as input for the mapping.

Gx Map Reads to Reference		×
1. Choose where to run	References	
2. Select sequencing reads	References	
3. Batch overview	References Ar NC_000021 (Genome)	Ø
4. References	Reference masking	
() e C	No masking	
6	Exclude annotated	
Stran Washington	Include annotated only	
Contraction of the second	Masking track	Ø
LOT Provide		
A CO TON AND A CONTRACT		
ALL IN		
?	← Previous → Next ✓ Finish X Cano	el

Figure 4: Specifying the reference sequences and masking parameters.

- 5. Leave mapping options as default (figure 5). You can use the Reset button if you are not sure whether you have previously changed the parameters for the tool. Then select to ignore the non-specific matches and click **Next**.
- 6. The dialog shown in figure 6 appears in the next window. Select **Create reads track** to create track-based results. Check **Create report** to obtain a detailed report about the read mapping and leave **Collect un-mapped reads** unchecked since we are not interested in those reads.

You can follow the progress of the mapping both in the status bar at the bottom left corner and under the **Processes** tab. There is also a log showing the progress. Because of the quite big reference sequence (Human chromosome 21, with a size of 47 Mbp), it may take a few minutes to map the data.



Gx Map Reads to Reference		×
1. Choose where to run	Mapping options	
2 Select sequencing reads	Read alignment	
2. Select sequencing reads	Match score 1	
3. Batch overview	Mismatch cost 2	
4. References	 Linear gap cost 	
5. Mapping options	Affine gap cost	
	Insertion cost 3	
	Deletion cost 3	
	Insertion open cost 6	
	Insertion extend cost 1	
	Deletion open cost 6	
	Deletion extend cost 1	
	Length fraction 0.5	
	Similarity fraction 0.8	
	Global alignment	
Contraction of the second	☑ Auto-detect paired distances	
1. C. J.	Non-specific match handling	
Man Jung	Map randomly	
TO TO ATTENTO LIVER	Ignore	
?	← Previous → Next ✓ Finish X Ca	ncel

Figure 5: A stringent read matching is desired for ChIP-Seq.

Gx Map Reads to Reference	
1. Choose where to run	Result handling
2. Select sequencing reads	Output options
3. Batch overview	Create stand-alone read mappings
4. References	☑ Create report
5. Mapping options	Collect un-mapped reads
6. Result handling	Result handling Open Save Into separate folders Log handling Open log
? 🔊	← Previous → Next ✓ Finish X Cancel

Figure 6: Select Create reads track, Create report, and Save.

Calling peaks

The results of the read mapping are now used as input to the Transcription Factor ChIP-Seq tool to detect significant peaks.

1. Go to:

Toolbox | Epigenomics Analysis (👼) | Transcription Factor ChIP-Seq (🛖)

- 2. This opens a dialog where you select the nrsf-chr21 (Reads) (=) and click Next (see figure 7).
- 3. You can now choose control-chr21 (Reads) (ﷺ) as control data (see figure 8). You can leave the Maximum P-value for peak calling to the default value of 0.1. A smaller



1. Choose where to run	Select one or more read mapping.	Selected elements (1)
2. Select one or more read mapping.	QtC Data ChIP-Seq Control-drr21 batch Strontor-drr21 (Reads) Image: Strontore (Reads) </th <th></th>	

Figure 7: Select the input reads.

P-value can be specified to obtain a smaller number of high-quality peaks, while a higher P-value threshold can be set to obtain a higher number of peaks.

Gx Transcription Factor ChIP	-Seq	×
1. Choose where to run	Peak shape parameters	
 Select one or more read mapping. Peak shape parameters Peak shape parameters 	Controls Control data 🚟 control-chr21 (Reads)	
?	← Previous	

Figure 8: Choose control data.

4. You can now choose the output data to be generated (see figure 9). In this tutorial, we select all the output which the Transcription Factor ChIP-Seq tool can generate.

Gx Transcription Factor ChIP	-Seq
1. Choose where to run	Result handling
 Select one or more read mapping. 	Output options
3. Peak shape parameters	Save peak shape filter
4. Result handling	Save peak shape score graph track
Coo of to of	Result handling Open Save
A CONTRACT OF CONTRACTON OF CONTRACTON OF CONTRACT OF CONTRACTON O	Log handling
?	← Previous → Next ✓ Finish X Cancel

Figure 9: Select the output data to be generated.

After a few minutes, the analysis will complete and the following results will appear:



- nrsf-chr21 (Reads) (Peaks) (>) the list of all called peaks.
- nrsf-chr21 (Reads) (QC Report) () The quality control reports. The QC report contains metrics about the quality of the ChIP-Seq experiment.
- nrsf-chr21 (Reads) (Peak shape filter) ([] The peak shape filter contains the peak shape that was learned during the ChIP-Seq analysis.
- nrsf-chr21 (Reads) (Peak shape score) () A graph track containing the peak shape score. The track shows the peak shape score for each genomic position.

Before continuing the analysis or looking at the results, we recommend to look at the quality control report. The most important sections of the report are the tables containing **Quality measures**. For each of the 3 quality measures, the table provides the name, the value, notes to better understand the meaning of the measure and a status. The status will be **OK** if the quality value is sufficient, or **Low** (or **Very Low**) if the value is lower than the quality threshold. For more details on how the quality thresholds were determined, see Landt et al., 2012 and Marinov et al., 2014.

In figure 10, the values for the relative strand correlation and the normalized strand coefficient are OK, while the number of reads is classified as **Very Low**. This should not be surprising or worrisome because the data used in this tutorial is a small subset of a ChIP-Seq experiment. In fact, the full datasets consists of about 16 millions reads, which is significantly higher than the threshold value. However, in normal circumstances, a small number of reads would be a strong indicator that the ChIP-Seq experiment is of low quality.

Measure	Value	Status	Notes
Number of reads	486,301	Very low	For mammalian cells, this value should be at least 10 million reads. For organisms with smaller genomes (e.g. worm and fly), this value should be at least 2 million reads
Relative strand correlation	1.009	ок	The relative strand correlation describes the ratio between the fragment-length peak and the read-length peak in the cross- correlation plot. This value should be greater than 0.8 for transcription factor binding sites, but can be lower for ChIP-seg input or for histone marks
Normalized strand coefficient	2.483	ок	The normalized strand coefficient describes the ratio between the fragment-length peak and the background cross-correlation values. This value should be greater than 1.05 for ChIP-seq experiments



Figure 10: Table of quality measures for the NRSF ChIP-Seq dataset.

The quality measures table for the control experiment (figure 11) can be interpreted in a similar fashion. We note that, since this is a control experiment, the value of the relative strand correlation is not important and the status would be OK also for low values. As for NRSF, the fact that the number of reads is very low is due to the fact that only a small subset of the data was used.

The quality report contains additional information that could be used for troubleshooting. For example, if the relative strand correlation or the normalized strand coefficient were classified as low, the cross-correlation plots should be examined in more details. More information regarding the cross-correlation plots and the Transcription Factor ChIP-Seq tool can be found in the user manual.



2.1 Quality measures

Measure	Value	Status	Notes
Number of reads	307,932	Very low	For mammalian cells, this value should be at least 10 million reads. For organisms with smaller genomes (e.g. worm and fly), this value should be at least 2 million reads
Relative strand correlation	1.191	ок	The relative strand correlation describes the ratio between the fragment-length peak and the read-length peak in the cross- correlation plot. This value should be greater than 0.8 for transcription factor binding sites, but can be lower for ChIP-seg input or for histone marks
Normalized strand coefficient	2.376	ок	The normalized strand coefficient describes the ratio between the fragment-length peak and the background cross-correlation values. This value should be greater than 1.05 for ChIP-seq experiments

Figure 11: Table of quality measures for the control ChIP-Seq dataset.

After having verified that the quality of the ChIP-Seq datasets is acceptable, the next step is to annotate them with information about their nearest upstream and downstream genes. This can be done using the Annotate with Nearby Gene Information tool:

- 1. Go to: Toolbox | Epigenomics Analysis (👼) | Annotate with Nearby Gene Information (😩)
- 2. Select first the track to annotate (nrsf-chr21 (Reads) (Peaks) () (figure 12), and click Next.

Gx Annotate with Nearby	Gene Information	
1. Choose where to run	Select an annotation track	
	Navigation Area	Selected elements (1)
2. Select an annotation track	CLC_Data ChIP-Seq P; NC_00021 (Gene) Control-drr21 batch P; rrsf-drr21 batch P; CLC_References Q:< <enter search="" term=""> Batch</enter>	Image: Specific control of the speci
? 5	+ Previous	→ Next ✓ Finish X Cancel

Figure 12: Select the track to annotate.

3. In the dialog shown in figure 13 choose NC_000021 (Gene) (as the reference gene track and click **Next**.

Gx Annotate with Nearby	Sene Information		
1. Choose where to run	Parameters		
2. Select an annotation track 3. Parameters	Selecte gene track Gene track		
? 4	← Previous → Next ✓ Finish X Cancel		

Figure 13: Select the annotation track to be used as gene reference.



4. Save the result. The file nrsf-chr21 (Reads) (Peaks, Annotated) (

Visualizing the results

The best way to visualize the results is to create a Track List:

New | Track List (

Select the tracks we created so far as shown in figure 14 and then press **Finish**.



Figure 14: Create a Track List to visualize the results.

Once the Track list is created, the easiest way to explore peaks is to make a split view of the table and the peak annotation track by double-clicking on the label nrsf-chr21 (Reads) (Peaks, Annotated). Sort the table according to P-value so that we can look at the top peak. You will then be able to browse through the peaks by clicking in the table, jumping in the track list to the position of the peak selected in the table. Click on the 1:1 zoom highlighted in figure 15 to zoom in on the peak of interest, and zoom out as needed to see the closest gene. You can browse through all the 144 peaks found for this sample by selecting in the table.

The strongest peak is close to the gene SYNJ1 (synaptojanin 1). This gene encodes a phosphoinositide phosphatase that regulates levels of membrane phosphatidylinositol-4,5-bisphosphate. The expression of this enzyme affects synaptic transmission and thus it is not a surprise that this gene is inhibited by NRSF, whose function is to repress neural genes in non-neuronal cells. Note the nicely distributed green (forward) and red (reverse) reads for this peak, this is a typical shape for transcription factors.

Extracting the DNA sequences of the peak regions

A common step in the analysis of ChIP-Seq data is to extract the DNA sequences associated with peaks in the ChIP-Seq data. These sequences are typically enriched with respect to some DNA motif, especially when the protein under examination is a transcription factor such as NRSF. The motif discovery can be then performed using external applications such as TRANSFAC[®].

1. Use the Extract Annotations tool to extract the sequences related to the peak regions:

Utility Tools | Extract Annotations (🏟)

2. Select the peak file nrsf-chr21 (Reads) (Peaks, Annotated) (*) as input (figure 16) and click Next.



Figure 15: A very strong peak near the gene SYNJ1.

Gx Extract Annotations		×
1. Choose where to run	Select annotated sequences OR annotation or variant tracks Navigation Area	Selected elements (1)
2. Select annotated sequences OR annotation or variant tracks	GLC_Data GLC_Data GLP.Seq Seaddh	
? 4	η	← Previous → Next ✓ Finish X Cancel

Figure 16: Select the annotation track.

- 3. In the next window (figure 17), select the sequence track NC_000021 (Genome) (%) and choose the annotation type Peak by clicking the green plus icon (+). Choose Include annotation region and Include annotation chromosome to give informative names to the resulting sequences. Click Next.
- 4. Choose to **Save** the sequences and click **Finish**. After few seconds, the sequences will be exported to a file named Extracted Annotations ().
- 5. Most external sequence-based analysis tools require an input in fasta file format. To export the sequence as fasta, you can run the Export tool:



1. Choose where to run	Set parameters	
 Select annotated sequences OR annotation or variant tracks 	Select a reference sequence track Reference sequence track X INC_000021 (Genome)	Ø
3. Set parameters	Refine extracted annotations Search terms Annotation types Peak	÷
	Flanking Flanking upstream residues Flanking downstream residues Naming of result sequences Include annotation name	
	Include annotation type Include annotation region Include annotation chromosome Include sequence/track name	
0		

Figure 17: Options for the Extract Annotations tool.

File | Export (

6. Then choose the **fasta** format (figure 18), select the file Extracted Annotations () and finally select the output file name.

Name	Description	Extension	Supported format v	
EMBL	Export sequences in EMBL format	[emb, embl]	Yes	
Excel 2010	Export tabular information in Excel format	[xlsx]	Yes	
Excel 97-2007	Export tabular information in Excel format	[zlx]	Yes	
Fasta	Export sequences and sequence lists in fasta format	[fa, fsa, fasta]	Yes	
Fasta alignment	Export alignments in fasta format	[fa, fsa, fasta]	Yes	
Fastq	Export sequences and sequence lists in fastq format	[fastq]	Yes	
GCG alignment	Export alignments in MSF format as specified in the GCG Wiscon	[msf]	Yes	
GCG sequence	Export sequences in GCG Wisconsin Package format	[gcg]	Yes	
GFF	Export sequence annotations in General Feature Format	[gff]	Yes	
GVF	Export variant tracks in Genome Variation Format	[gvf]	Yes	
GenBank	Export sequences and sequence lists in GenBank format	[gbk, gb, gp]	Yes	

Figure 18: Options for the Export tool.

You have now performed a complete ChIP-Seq Analysis on a small dataset using the *CLC Genomics Workbench*, starting from raw sequencing data and ending with the inspection of the called peaks and the extraction of the sequences within peak regions. The ChIP-Seq workflow described here does not require any tweaking of parameters and can be readily applied to larger ChIP-Seq datasets.



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