Analysis of Breast-Predict Project

ChIP-Seq Experiment

Introduction

A ChIP-Seq experiment targeting a specific transcription factor (TF) was performed on a human breast cancer cell line from the Irish Cancer Society's Breast-Predict Project. The resultant data has been analysed here to provide genome-wide TF binding locations, to offer potential regulatory roles of the TF, and to perform *de novo* TF motif discovery and comparison.

Although this analysis was performed using a premade script with automated file naming, the following will follow the steps taken to provide these results by explaining all relevant commands one at a time using what would be the generated file names in the command line. The actual script used is provided for comparison. It should also be noted that this script and thus all commands shown were run on the NUIG campus high-powered computing cluster to facilitate reasonable computing times.

Quality Control

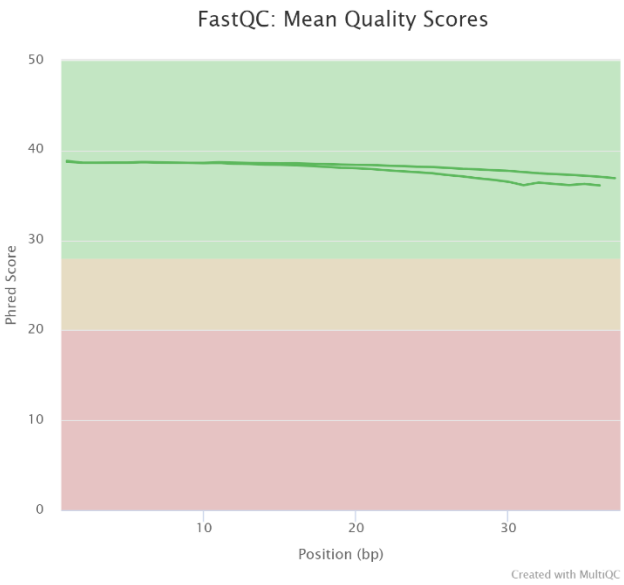
Two fastq sequence files were provided for analysis: chip.fastq, which contains the TF-targeted immunoprecipitated sequences; and input.fastq, which contains the experimental control sequences that were not immunoprecipitated. FastQC, followed by MultiQC for visualization, were used to first examine the quality of the sequence data:

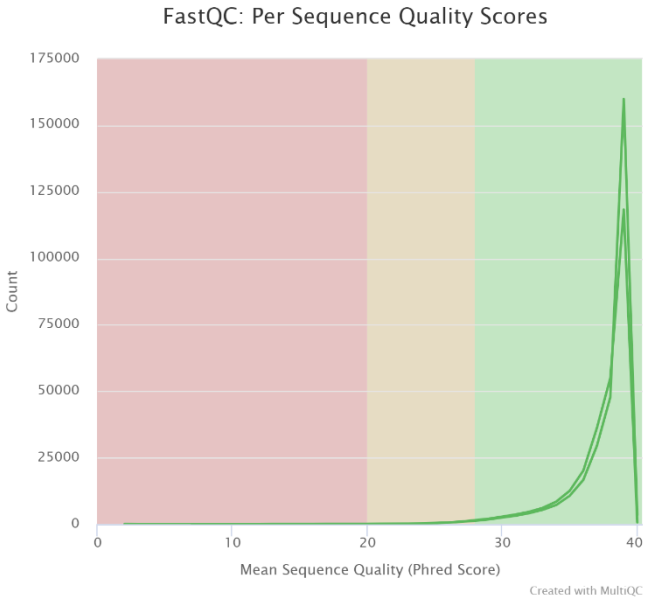
```
fastqc chip.fastq

fastqc input.fastq

multiqc .
```

Summary data are shown below:





Both sets of sequences show high quality. While mean quality drops towards the ends of the sequences, this is a normal feature of high-throughput sequencing. Furthermore, both sets of data show very few amounts of individual low-quality reads overall. Because of the high quality of reads, both data sets will be used as-is for the remainder of the analysis.

Alignment

To begin looking at binding site locations, both the ChIP and control sequences were first aligned to human reference genome hg19 using Bowtie2. For this analysis, only chromosome 21 was used for mapping, as the data provided correspond to a subset of the dataset that map to chromosome 21.

First, an index (named "ref_index" in the command) of the reference genome (chr21.fa) is built:

```
bowtie2-build chr21.fa ref_index
```

- bowtie2-build: specifies for bowtie2 to build a reference genome index
- chr21.fa: the name of the file containing the reference genome
- ref_index: the prefix name that will be given to the index files generated

Then, the alignment is run. The following is the code and parameters used to align the chip.fastq sequences:

```
bowtie2 -x ref_index -U chip.fastq -S chip.sam
```

- -x: specifies that ref_index is the prefix name of the genome index files to use
- -U: specifies that the file chip.fastq for alignment contains unpaired reads
- -S: specifies that the output should be written to a SAM (sequence alignment map) file with the name chip.sam

This produces a SAM file with alignment information for each read sequence. The same procedure was followed to produce a SAM file for the control sequences in input.fastq as well. An excerpt of 4 sequences from the chip.sam file is shown here, which besides sequence and alignment information also contains per-base alignment confidence scores:

```
@HD   VN:1.0     SO:unsorted
@SQ   SN:chr21   LN:48129895
@PG   ID:bowtie2 PN:bowtie2 VN:2.1.0
SRR540192.1580   0     chr21 20649661   42    37M   *     0     0
      CATCTTGGCCTCTGTGCAGCATTCCTTTCTCCATGGT
      IIIIIIHIIIIIIHIIIIIIIIIIIIIIIHIIHIHID AS:i:0     XN:i:0     XM:i:0
      XO:i:0     XG:i:0     NM:i:0     MD:Z:37    YT:Z:UU
SRR540192.1752   0     chr21 44763347   42    37M   *     0     0
      GCTCCCAGAAACCCAGGGCCACTGGCAGCTTCAGGGA
      GGGGGGGBG@GGGGB@>D<GGGF@<?<?9??;(?:2( AS:i:0     XN:i:0     XM:i:0
      XO:i:0     XG:i:0     NM:i:0     MD:Z:37    YT:Z:UU
SRR540192.1788   0     chr21 38025990   40    37M   *     0     0
      ATGGGCTTCCTCCGGCTTTCAGCCACCTGCGCCCTGC
      GG@G>G@E3<B=B;B<E>EDEAAAB:B.:=>A?;A8D AS:i:-5    XN:i:0     XM:i:1
      XO:i:0     XG:i:0     NM:i:1     MD:Z:26G10 YT:Z:UU
SRR540192.2271   0     chr21 41711175   42    37M   *     0     0
      TGATCATCTGGCTGATGCGGTGACTGCCACCCTTGAG
      IIGIIIIIIIIIIIIDIIIGIIIHGHHHIIIIHIHD AS:i:0     XN:i:0     XM:i:0
      XO:i:0     XG:i:0     NM:i:0     MD:Z:37    YT:Z:UU
```

File Post-Processing

The two SAM files produced were then processed with Samtools to:

- create faster, smaller, binary versions of the SAM files (BAM files)
- remove potential PCR duplicate reads
- sort each aligned sequence in the file by chromosome coordinate
- create an index of the BAM file
- create a summary of the mapping results

This was achieved for chip.sam with the following commands:

```
samtools view -Sb chip.sam > chip.bam
```

- outputs the data contained in the specified file
- -Sb: uses both the -S and -b options, which specify a SAM input file and produce a BAI index file, respectively
- chip.sam > chip.bam: the SAM file to view and the new file to write its contents to in binary format

```
samtools rmdup chip.bam chip.rmdup.bam
```

- removes duplicate alignments from chip.bam and outputs them to a new file chip.rmdup.bam

```
samtools sort chip.rmdup.bam chip.rmdup.sorted
```

- orders the contents of chip.rmdup.bam and writes them to a new bam file with the prefix chip.rmdup.sorted

```
samtools index chip.rmdup.sorted.bam
```

- creates a BAI index file of chip.rmdup.sorted.bam for quicker content access

```
samtools flagstat chip.rmdup.sorted.bam > chip_mappingstats.txt
```

- creates a text file containing summary data of the alignment

The same procedure was followed for input.sam. Mapping summary data are shown below. Note the high percentage of mapped reads, lack of QC-failed reads, and lack of duplicate reads.

From chipping_mappingstats.txt:

```
295896 + 0 in total (QC-passed reads + QC-failed reads)
0 + 0 duplicates
291344 + 0 mapped (98.46%:nan%)
```

From input_mappingstats.txt:

```
275043 + 0 in total (QC-passed reads + QC-failed reads)
0 + 0 duplicates
270767 + 0 mapped (98.45%:nan%)
```

Peak Calling

After the ChIP-Seq reads were aligned, peaks in the alignment were located and scored using MACS2. The control data in input.rmdup.sorted.bam served to control for noise in chip.rmdup.sorted.bam, which helped to identify significantly enriched alignment locations in the genome.

The following command was executed to run the MACS2 software:

```
macs2 callpeak -t chip.rmdup.sorted.bam -c input.rmdup.sorted.bam
     -f BAM -g hs -n macs_out --call-summits -B
```

- -t and -c: specify the treatment (ChIP) and control (input) files to use
- -f: specifies that BAM file format is being used
- -g: specifies that hs (homo sapiens) is the species whose genome is being used, so that statistical calculations include an accurate genome size
- -n: specifies that macs_out will be the prefix name for the output files produced
- --call-summits: specifies that subpeaks within peaks should try to be deconvolved to provide peak scores and positions for each subpeak
- -B: directs MACS2 to store additional information in bedGraph files for use with a genome browser

Following this peak calling, the MACS2 output file macs_out_peaks.xls provides information on alignment pile-ups, which hypothetically correspond to TF binding locations. From this file, the chromosome number, start position, and stop position were extracted to create a BED file for further use.

The command line language AWK and Unix utility sed were used for this purpose:

```
awk '!/^#|^$/ {print $1"\t"$2"\t"$3}' macs_out_peaks.xls | sed
'1d' > peaks.bed
```

- The AWK command above searches (/ /) for lines in the macs_out_peaks.xls file that do NOT (!) begin with (^) a hash sign (#) or (|) newline character ($)
    - This selects lines that do not begin with a comment and are not blank, removing the header region of this MACS2 output file
    - All that remains at this point are the data columns and their column names
- AWK is then commanded ({ }) to print columns 1 through 3 ($1, $2, $3) of each line separated by tab characters (“\t”)
    - This ensures that the file is tab separated and only contains the chromosome, start, and stop columns
- This output is then piped to sed, which deletes (d) the first (1) line of the text
    - This removes the line containing the column headings, leaving only the data in a BED format
- The output is then ultimately written to a file named peaks.bed

The complete list of peak locations, indicating possible TF binding sites, is provided at the end of this report.

Motif Search

To begin searching for motifs, the sequences under the peak regions were first obtained using peaks.bed as the coordinates to extract the sequences from a reference using bedtools' getfasta function:

```
bedtools getfasta -fi chr21.fa -bed peaks.bed -fo peaks.fasta
```

- -fi: specifies that chr21.fa is the reference genome from which to extract sequences
- -bed: specifies that peaks.bed is the BED file containing the sequence coordinates
- -fo: specifies that the extracted sequence should be written to peaks.fasta

An example sequence written to peaks.fasta is shown below:

```
>chr21:9478966-9479334
agtgcaagagtgaacacacactttgtttcggctttaaagaacccagcacgaagccagtctgcatggc
ctagagacatattttgctggataatgattacttgtttttctttttgtgtttgttgtatttgccggtt
tgcttagttcctgacatacaagaaaatcactgtcaaaacattagcttaacatttgtcaaggaaacaa
aaagacttcggtgaccacaccttataaagcaaacagttttgtaaatcactttggaaatttcagtaaa
aaaaaaaatccttaacaatataataagtaaagaaaatttaaaaccccaaaacattactgtgtttggg
ggggggggggttctgatttacagagtaaccaca
```

Once these sequences were obtained, a *de novo* motif search was performed across all peak sequences contained in peaks.fasta using MEME:

```
meme peaks.fasta -dna -mod zoops -minw 6 -maxw 26 -nmotifs 5
   -maxsize 750000 -o meme_out
```

- -dna: specifies that this is a DNA motif search using "ACGT"
- -mod zoops: specifies that Zero Or One (motif) Per Sequence should be searched for, rather than only one or multiple motifs per sequence
- -minw and -maxw: specifies that MEME should search for motifs of length 6 to 26 bases long
- -nmotifs: specifies that the search should stop once 5 motifs have been found
- -maxsize: sets the maximum allowable number of characters in the data to 750,000
    - Used because the default value of 100,000 is much too small for this ChIP dataset of ~650,000 characters
- -o: specifies the name of the directory to create that will hold the MEME output files

The 5 motif logos discovered by MEME are shown on the following page in order of descending E-value, which indicates the motifs' probability of random occurrence. While all 5 motifs show low E-values, motif 5 appears to have low conservation at each base overall, likely indicating less significance and confidence in the motif.

Motif 1

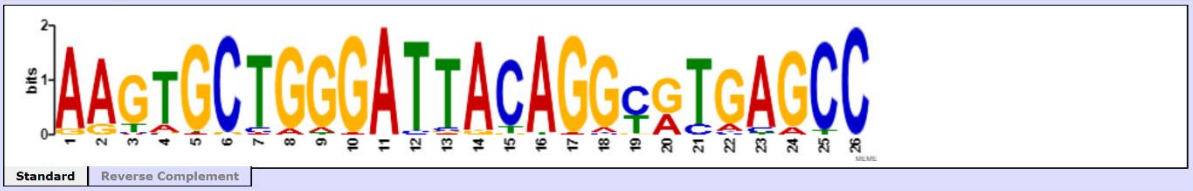
Summary	
E-value	9.5e-319
Width	26
Sites	52
show more	

Sequence Logo

Motif 2

Summary	
E-value	1.3e-253
Width	26
Sites	43
show more	

Sequence Logo



Motif 3

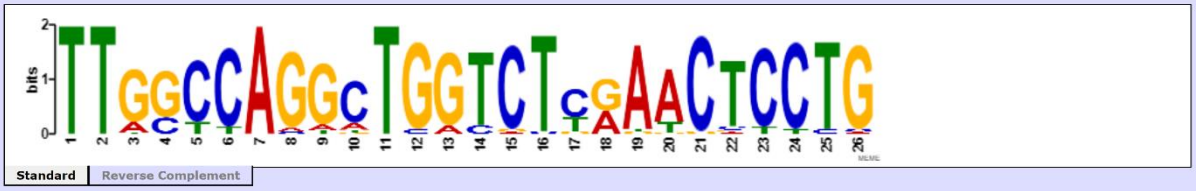
Summary	
E-value	2.2e-238
Width	26
Sites	43
show more	

Sequence Logo

Motif 4

Summary	
E-value	1.7e-215
Width	26
Sites	38
show more	

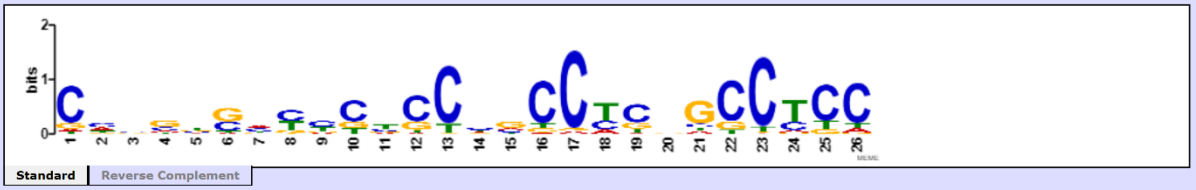
Sequence Logo



Motif 5

Summary	
E-value	3.4e-189
Width	26
Sites	252
show more	

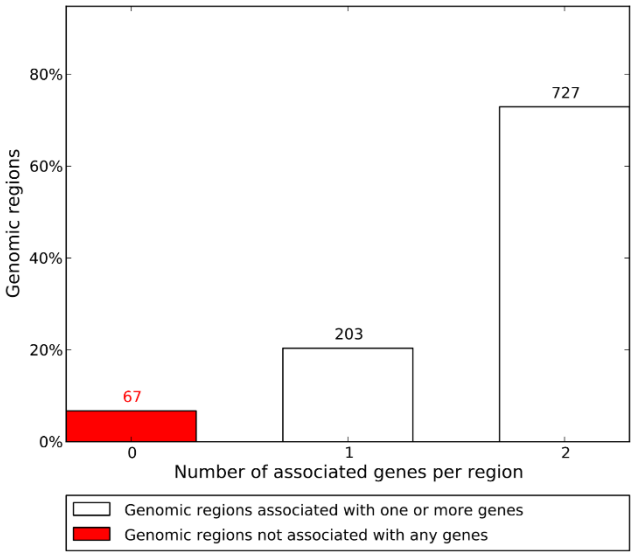
Sequence Logo



Peak Annotations

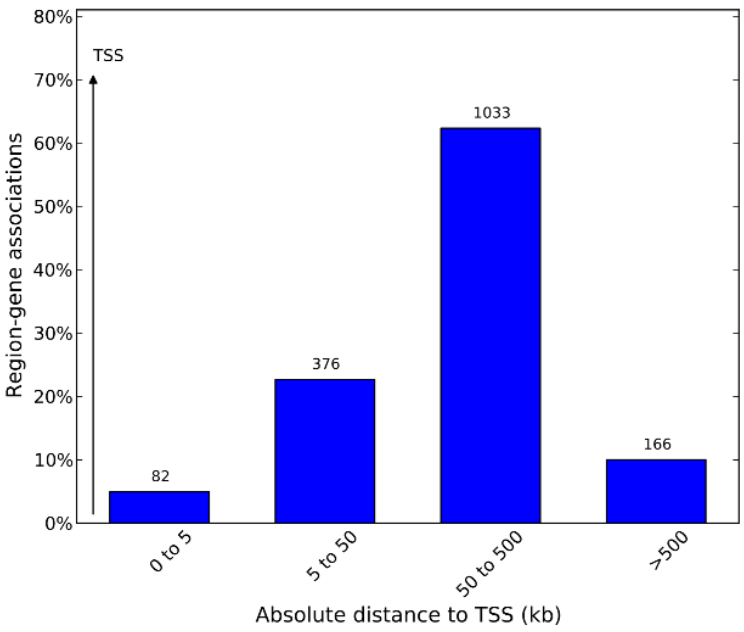
To explore the actual meaning and possible significance of the software outputs generated, the online GREAT tool was used to compile known associations between the 997 regions highlighted by this ChIP-Seq (now stored in peaks.bed) and annotated gene in online databases. In this way, cis-regulatory information can be gained by observing relationships between TF binding locations and the genes they may regulate.

The GREAT tool returns a summary of discovered associations across the human genome, also noting the distances from the submitted ChIP peak regions and the resultant associated annotated transcription start sites (TSS).



The graph at left shows the number of peak regions that have zero, one, or two found gene associations. Note that over 70% of peaks found in the ChIP-Seq experiment show two associated genes, while only ~6% of peaks found no associations. These sequences without gene associations may be indicative of non-specific TF binding, non-specific antibody binding, or may be novel associations that warrant further investigation.

The graph below shows the numbers of found gene associations grouped by distance from the peak region. Over 60% of found associations occurred from 50 to 500 kb away from the peak regions, while roughly 5% and 10% were found under 5 kb away and over 500 kb away, respectively. This shows that the bulk of gene associations were found to be within the expected range for a cis-regulatory element. In other words, the targeted transcription factor in this ChIP-Seq experiment appears to have high potential for gene regulatory function.



Motif Comparison

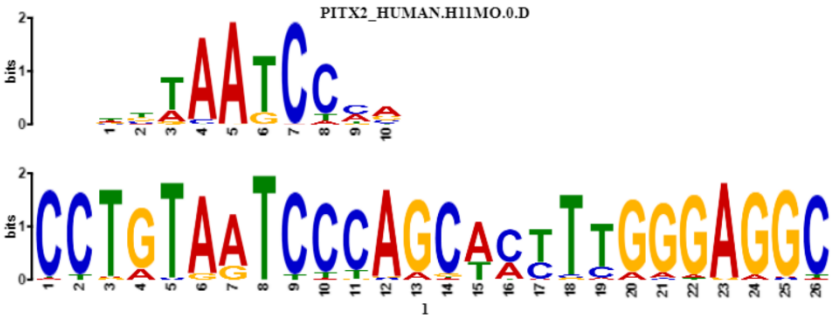
Finally, the online MEME-Suite tool Tomtom was used to compare the MEME motif results for this experiment with the HOCOMOCOv11 human and mouse motif databases. The general results for motifs 1 through 5 are shown below:

Database	ID	Matches	List
meme	1	10	PITX2_HUMAN.H11MO.0.D, PITX2_MOUSE.H11MO.0.D, IKZF1_HUMAN.H11MO.0.C, IKZF1_MOUSE.H11MO.0.C, ZN250_HUMAN.H11MO.0.C, TEAD4_MOUSE.H11MO.0.A, TEAD2_HUMAN.H11MO.0.D, TEAD2_MOUSE.H11MO.0.C,
meme	2	6	PITX2_HUMAN.H11MO.0.D, PITX2_MOUSE.H11MO.0.D, TBX5_HUMAN.H11MO.0.D, TBX5_MOUSE.H11MO.0.D, GFI1B_MOUSE.H11MO.0.A, TGIF2_HUMAN.H11MO.0.D
meme	3	47	RXRA_MOUSE.H11MO.0.A, RARA_MOUSE.H11MO.0.A, RXRG_MOUSE.H11MO.0.B, RARB_HUMAN.H11MO.0.D, RARB_MOUSE.H11MO.0.D, RXRA_MOUSE.H11MO.1.A, ERR2_MOUSE.H11MO.0.A, RARA_HUMAN.H11MO.0.A,
meme	4	24	ERR2_MOUSE.H11MO.0.A, GLI3_MOUSE.H11MO.0.D, GLI1_HUMAN.H11MO.0.D, GLI1_MOUSE.H11MO.0.C, RARA_MOUSE.H11MO.0.A, RXRA_MOUSE.H11MO.0.A, RXRG_MOUSE.H11MO.0.B, ZKSC3_HUMAN.H11MO.0.D,
meme	5	114	ZN770_HUMAN.H11MO.0.C, SP1_HUMAN.H11MO.0.A, ZN770_HUMAN.H11MO.1.C, ZFX_HUMAN.H11MO.1.A, MAZ_HUMAN.H11MO.0.A, MAZ_MOUSE.H11MO.0.A, SALL1_MOUSE.H11MO.0.D, PATZ1_HUMAN.H11MO.0.C,

From the results above, the PITX2 transcription factor shows matching motifs with both motif 1 in the reverse complement and with motif 2 in the forward direction, shown below. Without any information about the chosen antibody, its specificity, or the experimental conditions, this at least lends some credence to the idea that PITX2 could have been bound to this motif in the ChIP-Seq experiment.

Name	PITX2_HUMAN.H11MO.0.D
Database	HOCOMOCOv11 full HUMAN mono meme format
***p*-value**	1.21e-04
***E*-value**	1.57e-01
***q*-value**	1.57e-01
Overlap	10
Offset	-2
Orientation	Reverse Complement

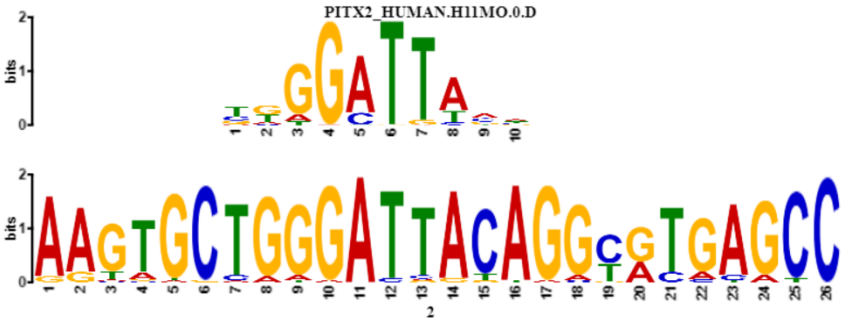
Show logo download options



1

Name	PITX2_HUMAN.H11MO.0.D
Database	HOCOMOCOv11 full HUMAN mono meme format
***p*-value**	1.03e-04
***E*-value**	1.34e-01
***q*-value**	1.34e-01
Overlap	10
Offset	-6
Orientation	Normal

Show logo download options



2

PITX2 may have implications for breast cancer, as it is involved in the production of prolactin, a hormone that is in turn involved in mammalian milk production and therefore breast function. However, PITX2 function occurs predominantly in the pituitary gland, and so any gene connection with actual breast cancer cell lines may not be causal, though it may warrant further investigation to identify connections with hormonal breast cancers.

Script Used

```
#!/bin/bash

# Job name:
#$ -N ChIPper

# The job should be placed into the queue 'all.q'
#$ -q all.q

# Running in the current directory
#$ -cwd

# Export some necessary environment variables
#$ -v PATH
#$ -v LD_LIBRARY_PATH
#$ -v PYTHONPATH
#$ -S /bin/bash

#Commands----------------------------

#Command-line arguments: qsub script.sh referencegenome chip_fastqfile input_fastqfile


#FastQC
for f in *.fastq;
do
            fastqc $f;
done


#MultiQC
multiqc .;


#Make Indexes from Reference Genome
bowtie2-build $1 ref_index
touch IndexingDone.txt

#Alignment and Post-Processing
for file in *.fastq;
do
      sam=${file//.fastq/.sam}
      bam=${file//.fastq/.bam}
      dups_out=${bam//.bam/.rmdup.bam}
      sorted=${dups_out//.rmdup.bam/.rmdup.sorted.bam}
      stats=${file//.fastq/_mappingstats.txt}


      bowtie2 -x ref_index -U $file -S $sam
      samtools view -Sb $sam > $bam
      samtools rmdup $bam $dups_out
      samtools sort $dups_out ${sorted//.bam}
      samtools index $sorted
      samtools flagstat $sorted > $stats

      touch ${file//.fastq/IsDone.txt}
done
```

```
#ChIP Peak Calling
macs2 callpeak -t ${2//.fastq/.rmdup.sorted.bam} -c ${3//.fastq/.rmdup.sorted.bam} -f BAM
-g hs -n macs_out --call-summits -B
touch MacsIsDone.txt

#MACS XLS Trimmer
awk '!/^#|^$/ {print $1"\t"$2"\t"$3}' macs_out_peaks.xls | sed '1d' > peaks.bed

#Extract ChIP Sequences
bedtools getfasta -fi $1 -bed peaks.bed -fo peaks.fasta
touch GetFastaIsDone.txt

#Motif Analysis
meme peaks.fasta -dna -mod zoops -minw 6 -maxw 26 -nmotifs 5 -maxsize 750000 -o meme_out
touch MemeIsDone.txt
```

Complete List of Called Peaks

Chrom.	Start	Stop						
chr21	9478966	9479334	chr21	16575731	16577694	chr21	18639918	18640461
chr21	9488140	9488479	chr21	16575731	16577694	chr21	18766256	18766644
chr21	9825338	9827051	chr21	16580008	16580812	chr21	18873626	18874207
chr21	9882928	9883227	chr21	16581191	16583209	chr21	18879800	18880330
chr21	10576105	10576614	chr21	16581191	16583209	chr21	18895076	18895811
chr21	10924251	10924815	chr21	16581191	16583209	chr21	18899458	18899880
chr21	11026478	11027107	chr21	16583796	16584411	chr21	18909528	18910222
chr21	11144289	11144781	chr21	16595649	16595983	chr21	18914568	18914972
chr21	14724142	14724644	chr21	16614146	16614735	chr21	18922015	18922674
chr21	14765889	14766285	chr21	16617123	16617693	chr21	18922886	18923615
chr21	14892260	14892828	chr21	16628157	16628800	chr21	19080671	19081191
chr21	14897414	14897810	chr21	16644462	16644914	chr21	19104561	19104944
chr21	14994757	14995125	chr21	16663099	16663599	chr21	19118285	19118790
chr21	15000171	15000534	chr21	16665462	16666001	chr21	19131500	19132563
chr21	15056140	15056531	chr21	16714586	16715837	chr21	19131500	19132563
chr21	15057037	15057801	chr21	16743092	16743787	chr21	20088933	20089484
chr21	15061144	15061820	chr21	16744927	16745826	chr21	20089949	20090568
chr21	15077114	15077870	chr21	16773584	16774204	chr21	20128490	20128829
chr21	15229316	15229800	chr21	16779056	16779831	chr21	20144014	20144899
chr21	15229943	15230480	chr21	16805250	16805828	chr21	20722489	20723313
chr21	15249176	15249828	chr21	16816381	16817076	chr21	20855099	20855558
chr21	15268764	15269356	chr21	16841350	16841940	chr21	20859581	20859897
chr21	15342255	15342582	chr21	16854666	16855157	chr21	20898090	20898389
chr21	15359675	15360343	chr21	16862408	16862712	chr21	20899963	20901179
chr21	15426954	15427494	chr21	16904172	16904576	chr21	20899963	20901179
chr21	15430644	15431447	chr21	16905872	16906429	chr21	20901229	20901918
chr21	15599037	15599773	chr21	16919580	16920706	chr21	20913687	20914433
chr21	15635425	15635927	chr21	16922056	16922582	chr21	20916800	20917099
chr21	15639394	15639864	chr21	16958985	16959896	chr21	20938661	20940018
chr21	15642778	15643481	chr21	16965215	16965923	chr21	20945614	20945964
chr21	15646038	15646486	chr21	16990291	16990822	chr21	20965867	20966508
chr21	15676269	15676951	chr21	17000774	17001788	chr21	20975382	20975836
chr21	15678285	15679069	chr21	17000774	17001788	chr21	20990359	20990950
chr21	15682861	15683319	chr21	17011123	17011863	chr21	20992973	20993634
chr21	15688582	15689393	chr21	17078957	17079426	chr21	20994109	20994936
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chr21	15699832	15700191	chr21	17102651	17102950	chr21	21084815	21085436
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