

TSS analysis in *Streptomyces venezuelae*

Time to get started! There are three main sections in this analysis: 1) TSS identification 2) RNA-seq analysis 3) integration of RNA-seq and TSS data

First up!

Differential RNA sequencing (dRNA-seq) is a technique designed to examine the architecture of bacterial operons and the precise position of transcription start sites (TSS).

The following analysis is designed to **i) identify, ii) annotate, and iii) analyze TSSs** in the model organism *Streptomyces venezuelae*. In addition to this, steps following identification of TSSs will aim to integrate these data with RNA-sequencing analyses of *S. venezuelae* mutant strains, where the genes encoding two different ribonucleases (RNases) are deleted.

The first step of the analysis is to **quality check** the illumina-sequenced dRNA-seq reads:

FastQC

```
#using the fastqr program to quality check the sequences
#the following are packages that need to be installed. For the sake of being able to properly
#knit the markdown the functions are commented out - simply remove the comment and run the
#functions.

#install.packages("fastqcr")
library(fastqcr)
fastqc_install(dest.dir = "~/Desktop/TSS_vnz/")

#run the fastQC tool on all .fastq files
#(it will automatically run on every .fastq file in the directory you call)

fastqc(fq.dir = "~/Desktop/TSS_vnz/raw/",
       qc.dir = "~/Desktop/TSS/FastQC/",
       fastqc.path = "~/Desktop/TSS/FastQC/fastqc")
```

You can go into the output folder on your local computer and read through the html FastQC outputs. Or, you can look here:

10h_control

10h_TSS

14h_control

14h_TSS

18h_control

18h_TSS

24h_control

24h_TSS

In this specific example you'll note that there are many sequences which are flagged as "over-represented". Given the nature of dRNA-sequencing this is expected, as the reads are generated to align precise transcription start sites and therefore overlap with each other exactly.

The next step is to **align the fastq files to the reference genome using Rbowtie2**:

RBowtie2

```
library(Rbowtie2)
library(Rsamtools)

bowtie2_build(references="ref/vnz_genome.fasta", bt2Index = "ref/venezuelae_bowtie", overwrite = TRUE)

bowtie2(bt2Index = "ref/venezuelae_bowtie",
        samOutput = "sam_out/10h_control",
        seq1 = "raw/10h_control.fastq",
        seq2 = NULL)
bowtie2(bt2Index = "ref/venezuelae_bowtie",
        samOutput = "sam_out/10h_TSS",
        seq1 = "raw/10h_TSS.fastq",
        seq2 = NULL)

bowtie2(bt2Index = "ref/venezuelae_bowtie",
        samOutput = "sam_out/14h_control",
        seq1 = "raw/14h_control.fastq",
        seq2 = NULL)
bowtie2(bt2Index = "ref/venezuelae_bowtie",
        samOutput = "sam_out/14h_TSS",
        seq1 = "raw/14h_TSS.fastq",
        seq2 = NULL)

bowtie2(bt2Index = "ref/venezuelae_bowtie",
        samOutput = "sam_out/18h_control",
        seq1 = "raw/18h_control.fastq",
        seq2 = NULL)
bowtie2(bt2Index = "ref/venezuelae_bowtie",
        samOutput = "sam_out/18h_TSS",
        seq1 = "raw/18h_TSS.fastq",
        seq2 = NULL)

bowtie2(bt2Index = "ref/venezuelae_bowtie",
        samOutput = "sam_out/24h_control",
        seq1 = "raw/24h_control.fastq",
        seq2 = NULL)
bowtie2(bt2Index = "ref/venezuelae_bowtie",
        samOutput = "sam_out/24h_TSS",
        seq1 = "raw/24h_TSS.fastq",
        seq2 = NULL)
```

The bowtie2 function outputs .sam files which need to be converted to .bam files for further analysis:

File conversion and pre-processing

```
asBam(file = "sam_out/10h_control", destination = "bam_out/10h_control")
asBam(file = "sam_out/14h_control", destination = "bam_out/14h_control")
asBam(file = "sam_out/18h_control", destination = "bam_out/18h_control")
asBam(file = "sam_out/24h_control", destination = "bam_out/24h_control")

asBam(file = "sam_out/10h_TSS", destination = "bam_out/10h_TSS")
asBam(file = "sam_out/14h_TSS", destination = "bam_out/14h_TSS")
asBam(file = "sam_out/18h_TSS", destination = "bam_out/18h_TSS")
asBam(file = "sam_out/24h_TSS", destination = "bam_out/24h_TSS")
```

The .bam files need to then be sorted, indexed, and filtered

#sort (by leftmost coordinates)

```
sortBam(file = "bam_out/10h_control.bam", destination = "sort_bam/10h_control")
sortBam(file = "bam_out/14h_control.bam", destination = "sort_bam/14h_control")
sortBam(file = "bam_out/18h_control.bam", destination = "sort_bam/18h_control")
sortBam(file = "bam_out/24h_control.bam", destination = "sort_bam/24h_control")

sortBam(file = "bam_out/10h_TSS.bam", destination = "sort_bam/10h_TSS")
sortBam(file = "bam_out/14h_TSS.bam", destination = "sort_bam/14h_TSS")
sortBam(file = "bam_out/18h_TSS.bam", destination = "sort_bam/18h_TSS")
sortBam(file = "bam_out/24h_TSS.bam", destination = "sort_bam/24h_TSS")
```

#index

```
indexBam("bam_out/10h_control.bam", "sort_bam/10h_control")
indexBam("bam_out/14h_control.bam", "sort_bam/14h_control")
indexBam("bam_out/18h_control.bam", "sort_bam/18h_control")
indexBam("bam_out/24h_control.bam", "sort_bam/24h_control")

indexBam("bam_out/10h_TSS.bam", "sort_bam/10h_TSS")
indexBam("bam_out/14h_TSS.bam", "sort_bam/14h_TSS")
indexBam("bam_out/18h_TSS.bam", "sort_bam/18h_TSS")
indexBam("bam_out/24h_TSS.bam", "sort_bam/24h_TSS")
```

#filter out unmapped sequences

```
filterBam(file = "sort_bam/10h_control.bam",
          index = "bam_out/10h_control.bam.bai",
          destination = "filter_bam/10h_control_subset.bam",
          param = ScanBamParam(flag=scanBamFlag(isUnmappedQuery=FALSE),
                                what="seq"))
filterBam(file = "sort_bam/14h_control.bam",
          index = "bam_out/14h_control.bam.bai",
          destination = "filter_bam/14h_control.bam",
          param = ScanBamParam(flag=scanBamFlag(isUnmappedQuery=FALSE),
                                what="seq"))
filterBam(file = "sort_bam/18h_control.bam",
          index = "bam_out/18h_control.bam.bai",
          destination = "filter_bam/18h_control.bam",
          param = ScanBamParam(flag=scanBamFlag(isUnmappedQuery=FALSE),
                                what="seq"))
```

```

                                what="seq"))
filterBam(file = "sort_bam/24h_control.bam",
          index = "bam_out/24h_control.bam.bai",
          destination = "filter_bam/24h_control.bam",
          param = ScanBamParam(flag=scanBamFlag(isUnmappedQuery=FALSE),
                                what="seq"))

filterBam(file = "sort_bam/10h_TSS.bam",
          index = "bam_out/10h_TSS.bam.bai",
          destination = "filter_bam/10h_TSS.bam",
          param = ScanBamParam(flag=scanBamFlag(isUnmappedQuery=FALSE),
                                what="seq"))

filterBam(file = "sort_bam/14h_TSS.bam",
          index = "bam_out/14h_TSS.bam.bai",
          destination = "filter_bam/14h_TSS.bam",
          param = ScanBamParam(flag=scanBamFlag(isUnmappedQuery=FALSE),
                                what="seq"))

filterBam(file = "sort_bam/18h_TSS.bam",
          index = "bam_out/18h_TSS.bam.bai",
          destination = "filter_bam/18h_TSS.bam",
          param = ScanBamParam(flag=scanBamFlag(isUnmappedQuery=FALSE),
                                what="seq"))

filterBam(file = "sort_bam/24h_TSS.bam",
          index = "bam_out/24h_TSS.bam.bai",
          destination = "filter_bam/24h_TSS.bam",
          param = ScanBamParam(flag=scanBamFlag(isUnmappedQuery=FALSE),
                                what="seq"))

```

TSSAR

The program we are going to use to identify TSS in *S. venezuelae* is called **TSSAR** (TSS annotation regime)

It required .sam input files and for the alignment file to only contain one chromosome. In *S. venezuelae* there is a plasmid in our typical .gff and .fasta files so we will have to filter out those sequences to proceed with the TSS identification.

To filter out the plasmid annotations/sequence we will use **samtools**. The programming for this is better suited to bash (accessing it from your terminal or a remote server). I've installed it on my local computer.

More pre-processing for TSSAR

```
#filtering out plasmid-aligned files (only keeping chromosome CP018074.1)
```

```
#conda install -c bioconda samtools (uncomment if you need to install this program)
```

```

samtools view -h -b ~/Desktop/TSS/bam_out/10h_TSS.bam CP018074.1 -o ~/Desktop/TSS/bam_chr1/10h_TSS_chr1
samtools view -h -b ~/Desktop/TSS/bam_out/14h_TSS.bam CP018074.1 -o ~/Desktop/TSS/bam_chr1/14h_TSS_chr1
samtools view -h -b ~/Desktop/TSS/bam_out/18h_TSS.bam CP018074.1 -o ~/Desktop/TSS/bam_chr1/18h_TSS_chr1
samtools view -h -b ~/Desktop/TSS/bam_out/24h_TSS.bam CP018074.1 -o ~/Desktop/TSS/bam_chr1/24h_TSS_chr1

```

```

samtools view -h -b ~/Desktop/TSS/bam_out/10h_control.bam CP018074.1 -o ~/Desktop/TSS/bam_chr1/10h_cont
samtools view -h -b ~/Desktop/TSS/bam_out/14h_control.bam CP018074.1 -o ~/Desktop/TSS/bam_chr1/14h_cont

```

```
samtools view -h -b ~/Desktop/TSS/bam_out/18h_control.bam CP018074.1 -o ~/Desktop/TSS/bam_chr1/18h_cont
samtools view -h -b ~/Desktop/TSS/bam_out/24h_control.bam CP018074.1 -o ~/Desktop/TSS/bam_chr1/24h_cont
```

#converting back into .sam

```
samtools view -h ~/Desktop/TSS/bam_chr1/10h_TSS_chr1.bam > ~/Desktop/TSS/sam_chr1/10h_TSS_chr1.sam
samtools view -h ~/Desktop/TSS/bam_chr1/14h_TSS_chr1.bam > ~/Desktop/TSS/sam_chr1/14h_TSS_chr1.sam
samtools view -h ~/Desktop/TSS/bam_chr1/18h_TSS_chr1.bam > ~/Desktop/TSS/sam_chr1/18h_TSS_chr1.sam
samtools view -h ~/Desktop/TSS/bam_chr1/24h_TSS_chr1.bam > ~/Desktop/TSS/sam_chr1/24h_TSS_chr1.sam
```

```
samtools view -h ~/Desktop/TSS/bam_chr1/10h_control_chr1.bam > ~/Desktop/TSS/sam_chr1/10h_control_chr1.
samtools view -h ~/Desktop/TSS/bam_chr1/14h_control_chr1.bam > ~/Desktop/TSS/sam_chr1/14h_control_chr1.
samtools view -h ~/Desktop/TSS/bam_chr1/18h_control_chr1.bam > ~/Desktop/TSS/sam_chr1/18h_control_chr1.
samtools view -h ~/Desktop/TSS/bam_chr1/24h_control_chr1.bam > ~/Desktop/TSS/sam_chr1/24h_control_chr1.
```

TSSAR is a Perl and R-based program. The program can be downloaded from [here](#) and stored either on your local computer or a remote server. The program takes input **.sam files**, writes coverage files, generates an R-script for the statistical analysis and prediction of TSS, runs the R. script, and writes .bed files where each predicted TSS is listed.

The layout of this function is quite simple. You must call the downloaded TSSAR program (which is within the downloaded TSSAR_1.0.1 folder). You then call the library which is enriched for triphosphate 5' ends (library plus; -libP), call the library which is NOT enriched for triphosphate 5' ends (library minus; -libM), and then a .fasta file for the genome to which these reads are aligned so that TSSAR can compute the effective genome size.

Note that these functions (particularly the R.script) takes a verrrrrry long to run. For the analysis I used infoserv (thank you Brian!)

TSSAR syntax

#library 18h

```
TSSAR_1.0.1/TSSAR --libP ~/TSSAR/TSSAR_input/sam_chr1/10h_TSS_chr1.sam
--libM ~/TSSAR/TSSAR_input/sam_chr1/10h_control_chr1.sam
--fasta ~/TSSAR/TSSAR_input/ref/vnz_genome.fasta > 10h_TSS.bed
```

```
TSSAR_1.0.1/TSSAR --libP ~/TSSAR/TSSAR_input/sam_chr1/14h_TSS_chr1.sam
--libM ~/TSSAR/TSSAR_input/sam_chr1/14h_control_chr1.sam
--fasta ~/TSSAR/TSSAR_input/ref/vnz_genome.fasta > 14h_TSS.bed
```

```
TSSAR_1.0.1/TSSAR --libP ~/TSSAR/TSSAR_input/sam_chr1/18h_TSS_chr1.sam
--libM ~/TSSAR/TSSAR_input/sam_chr1/18h_control_chr1.sam
--fasta ~/TSSAR/TSSAR_input/ref/vnz_genome.fasta > 18h_TSS.bed
```

```
TSSAR_1.0.1/TSSAR --libP ~/TSSAR/TSSAR_input/sam_chr1/24h_TSS_chr1.sam
--libM ~/TSSAR/TSSAR_input/sam_chr1/24h_control_chr1.sam
--fasta ~/TSSAR/TSSAR_input/ref/vnz_genome.fasta > 24h_TSS.bed
```

I found that when running this program, the function stores the entire output into a file. Before any analysis I opened the .bed files in a simple text editor to comment out the descriptive output (number of TSSAR outputs,etc.).

#13733 individual TSS were annotated (p-Value cut off 0.0001). 8230 remained after consecutive TSS were merged together. In total 1071 region(s) (with a total length of 335700 nt [2 % of the genome]) could not be modelled with our zero-inflated Poisson regression (see Dump.bed for details; manual inspection is advised)

CP018074.1	355	356	TSS_00001	22.000000	+
CP018074.1	2275	2276	TSS_00004	133.000000	+
CP018074.1	9293	9294	TSS_00007	35.000000	+
CP018074.1	11936	11937	TSS_00008	94.000000	+
CP018074.1	12779	12780	TSS_00009	16.000000	+
CP018074.1	14357	14358	TSS_00011	29.000000	+
CP018074.1	18649	18650	TSS_00014	53.000000	+
CP018074.1	37205	37206	TSS_00017	290.000000	+
CP018074.1	38110	38111	TSS_00018	30.000000	+
CP018074.1	46724	46725	TSS_00020	114.000000	+
CP018074.1	48248	48249	TSS_00021	987.000000	+
CP018074.1	56131	56132	TSS_00022	14.000000	+
CP018074.1	60310	60311	TSS_00025	146.000000	+
CP018074.1	85576	85577	TSS_00027	38.000000	+
CP018074.1	86569	86570	TSS_00029	364.000000	+
CP018074.1	99022	99023	TSS_00034	11.000000	+
CP018074.1	109341	109342	TSS_00036	78.000000	+
CP018074.1	128503	128504	TSS_00042	34.000000	+
CP018074.1	135434	135435	TSS_00045	79.000000	+
CP018074.1	140068	140069	TSS_00047	125.000000	+
CP018074.1	146032	146033	TSS_00050	79.000000	+
CP018074.1	148424	148425	TSS_00053	1501.000000	+
CP018074.1	151174	151175	TSS_00055	12.000000	+
CP018074.1	159988	159989	TSS_00056	387.000000	+
CP018074.1	162464	162465	TSS_00059	114.000000	+
CP018074.1	171420	171421	TSS_00060	304.000000	+
CP018074.1	184386	184387	TSS_00062	28.000000	+
CP018074.1	186191	186192	TSS_00065	3426.000000	+
CP018074.1	188857	188858	TSS_00066	88.000000	+
CP018074.1	190528	190529	TSS_00067	26.000000	+
CP018074.1	198764	198765	TSS_00072	17.000000	+
CP018074.1	217237	217238	TSS_00074	38.000000	+

Figure 1: Example of TSSAR output with commented first line

Filtering TSSAR output

We now want to subset the data to make the most meaningful comparisons. TSSAR outputs a **bedscore** associated with each TSS. As far as I can tell this is a non-linear arbitrary score (the values range from 1-278308 for 10h sample). There are many more TSSs with scores between 1-100 than those with scores >100. By manually inspecting the TSSs with some representative scores (1, 10, 50, 100, 1000, 10000) it seems that the higher the score the more “significant” the TSS is (*i.e.* the bigger the difference is between the enriched/+ library and the control/- library). Generally, it seems that the extremely low scores are not biologically significant, even though the program called them as statistically significant.

By the end of the analysis I’ll inspect the short-list of interesting TSS candidates manually to see how strong the enrichment is and what the counts are of reads that are aligning in the +library. As a preliminary filter it would be worthwhile to get rid of some poor quality TSSs. By manually inspecting the .bam files in IGV it seems that anything with a bed score under 30 isn’t terribly significant. So I will do a preliminary filtering of the TSSs by bedscore:

```
awk -F"\t" '$5>30' ~/Desktop/TSS_vnz/TSS_output/10h_TSS.txt > ~/Desktop/TSS_vnz/TSS_output/TSS_10h_new.bed
awk -F"\t" '$5>30' ~/Desktop/TSS_vnz/TSS_output/14h_TSS.txt > ~/Desktop/TSS_vnz/TSS_output/TSS_14h_new.bed
awk -F"\t" '$5>30' ~/Desktop/TSS_vnz/TSS_output/18h_TSS.txt > ~/Desktop/TSS_vnz/TSS_output/TSS_18h_new.bed
awk -F"\t" '$5>30' ~/Desktop/TSS_vnz/TSS_output/24h_TSS.txt > ~/Desktop/TSS_vnz/TSS_output/TSS_24h_new.bed
```

Sort bed files by chromosomal position

Now we can start cleaning up the .bed files. The output stores them by strand order first (all + strand TSSs and then all - strand TSSs). I want to sort them instead by chromosomal position:

```
bedtools sort -i ~/Desktop/TSS_vnz/TSS_output/TSS_10h_new.bed > ~/Desktop/TSS_vnz/TSS_sorted_bed/10h.bed
bedtools sort -i ~/Desktop/TSS_vnz/TSS_output/TSS_14h_new.bed > ~/Desktop/TSS_vnz/TSS_sorted_bed/14h.bed
bedtools sort -i ~/Desktop/TSS_vnz/TSS_output/TSS_18h_new.bed > ~/Desktop/TSS_vnz/TSS_sorted_bed/18h.bed
bedtools sort -i ~/Desktop/TSS_vnz/TSS_output/TSS_24h_new.bed > ~/Desktop/TSS_vnz/TSS_sorted_bed/24h.bed
```

This sorting also gets rid of the commented description:

Annotate TSSs based on their genomic context

We will be using several different **bedtools** functions to annotate all of the TSSs based on adjacent genes.

Internal TSSs

There are a few things we are interested. The easiest first thing to do is to find TSS that overlap directly with known genes (*i.e.* are internal to gene regions). These can be sense and anti-sense to the direction of the gene. To differentiate these I chose to use bedtools on my local computer (intersect function):

```
#the lowercase -s flag forces same-strandedness (looking for same directional internal
#promoters). The -wo flag asks it to write the original A and B entries (I want a table
#that contains both the TSS output and the corresponding gene info from the .gff file )
```

```
bedtools intersect -a ~/Desktop/TSS_vnz/TSS_sorted_bed/10h.bed -b ~/Desktop/TSS_vnz/ref/vnz_genome.gff
bedtools intersect -a ~/Desktop/TSS_vnz/TSS_sorted_bed/14h.bed -b ~/Desktop/TSS_vnz/ref/vnz_genome.gff
```


CP018074.1	630	631	TSS_00003	673.000000	-
CP018074.1	2275	2276	TSS_00004	133.000000	+
CP018074.1	4162	4163	TSS_00005	74.000000	-
CP018074.1	9293	9294	TSS_00007	35.000000	+
CP018074.1	11936	11937	TSS_00008	94.000000	+
CP018074.1	14058	14059	TSS_00010	247.000000	-
CP018074.1	17937	17938	TSS_00013	102.000000	-
CP018074.1	18649	18650	TSS_00014	53.000000	+
CP018074.1	37205	37206	TSS_00017	290.000000	+
CP018074.1	46563	46564	TSS_00019	181.000000	-
CP018074.1	46724	46725	TSS_00020	114.000000	+
CP018074.1	48248	48249	TSS_00021	987.000000	+
CP018074.1	56427	56428	TSS_00024	2292.000000	-
CP018074.1	60310	60311	TSS_00025	146.000000	+
CP018074.1	68425	68426	TSS_00026	223.000000	-
CP018074.1	85576	85577	TSS_00027	38.000000	+
CP018074.1	86569	86570	TSS_00029	364.000000	+
CP018074.1	89146	89147	TSS_00030	633.000000	-
CP018074.1	92385	92386	TSS_00032	42.000000	-
CP018074.1	97755	97756	TSS_00033	35.000000	-
CP018074.1	107366	107367	TSS_00035	40.000000	-
CP018074.1	109341	109342	TSS_00036	78.000000	+
CP018074.1	118775	118776	TSS_00038	48.000000	-
CP018074.1	128503	128504	TSS_00042	34.000000	+
CP018074.1	131774	131775	TSS_00043	665.000000	-
CP018074.1	135434	135435	TSS_00045	79.000000	+
CP018074.1	139948	139949	TSS_00046	154.000000	-
CP018074.1	140068	140069	TSS_00047	125.000000	+
CP018074.1	146032	146033	TSS_00050	79.000000	+
CP018074.1	148304	148305	TSS_00051	66.000000	-
CP018074.1	148424	148425	TSS_00053	1501.000000	+
CP018074.1	159988	159989	TSS_00056	387.000000	+
CP018074.1	162402	162403	TSS_00057	216.000000	-
CP018074.1	162464	162465	TSS_00059	114.000000	+
CP018074.1	171420	171421	TSS_00060	304.000000	+
CP018074.1	186191	186192	TSS_00065	3426.000000	+
CP018074.1	188857	188858	TSS_00066	88.000000	+

Figure 2: Example of .bed file sorted by chromosomal position


```

bedtools intersect -a ~/Desktop/TSS_vnz/TSS_sorted_bed/18h.bed -b ~/Desktop/TSS_vnz/ref/vnz_genome.gff
bedtools intersect -a ~/Desktop/TSS_vnz/TSS_sorted_bed/24h.bed -b ~/Desktop/TSS_vnz/ref/vnz_genome.gff

#the upercase -s flag forces opposite-strandedness (looking for anti-sense directional
#internal promoters). The -wo flag asks it to write the original A and B entries
#(I want a table that contains both the TSS output and the corresponding gene info from the
#.gff file )
bedtools intersect -a ~/Desktop/TSS_vnz/TSS_sorted_bed/10h.bed -b ~/Desktop/TSS_vnz/ref/vnz_genome.gff

bedtools intersect -a ~/Desktop/TSS_vnz/TSS_sorted_bed/14h.bed -b ~/Desktop/TSS_vnz/ref/vnz_genome.gff

bedtools intersect -a ~/Desktop/TSS_vnz/TSS_sorted_bed/18h.bed -b ~/Desktop/TSS_vnz/ref/vnz_genome.gff

bedtools intersect -a ~/Desktop/TSS_vnz/TSS_sorted_bed/24h.bed -b ~/Desktop/TSS_vnz/ref/vnz_genome.gff

```

Primary TSSs

We also want to identify primary TSS - that is, promoters upstream (directionally) of a gene. I will get bedtools to report the distance each TSS is from the closest gene (-D a), forcing same-strandedness (-s), only calling downstream features (ignoring upstream -iu), and only calling non-overlapping features (-io). Later, I will subset the output so that the “distance” is ≤ 250 bp.

```

#the -s forces same strandedness
#The -iu ignores genes which are upstream of each TSS
#(we only want genes which are downstream of TSS; i.e. TSS is upstream of given gene)
#-io ignores genes with which the TSS is directly overlapping
#-D a calls distance the gene is from TSS with respect to a (TSS)

bedtools closest -a ~/Desktop/TSS_vnz/TSS_sorted_bed/10h.bed -b ~/Desktop/TSS_vnz/ref/vnz_genome.gff -s
bedtools closest -a ~/Desktop/TSS_vnz/TSS_sorted_bed/14h.bed -b ~/Desktop/TSS_vnz/ref/vnz_genome.gff -s
bedtools closest -a ~/Desktop/TSS_vnz/TSS_sorted_bed/18h.bed -b ~/Desktop/TSS_vnz/ref/vnz_genome.gff -s
bedtools closest -a ~/Desktop/TSS_vnz/TSS_sorted_bed/24h.bed -b ~/Desktop/TSS_vnz/ref/vnz_genome.gff -s

```

Antisense downstream TSSs

Similarly, we are interested in identifying TSS which are antisense to a given gene, just downstream of it. I will again report the distance to the closest gene, forcing opposite-strandedness. Later, I will subset this so that the “distance” is ≤ 40 bp.

```

bedtools closest -a ~/Desktop/TSS_vnz/TSS_sorted_bed/10h.bed -b ~/Desktop/TSS_vnz/ref/vnz_genome.gff -S
bedtools closest -a ~/Desktop/TSS_vnz/TSS_sorted_bed/14h.bed -b ~/Desktop/TSS_vnz/ref/vnz_genome.gff -S
bedtools closest -a ~/Desktop/TSS_vnz/TSS_sorted_bed/18h.bed -b ~/Desktop/TSS_vnz/ref/vnz_genome.gff -S
bedtools closest -a ~/Desktop/TSS_vnz/TSS_sorted_bed/24h.bed -b ~/Desktop/TSS_vnz/ref/vnz_genome.gff -S

```

Orphan TSS will be called by filtering all of the TSS for ones that do not fall into any of the above lists.

Now we can read these files into R and start cleaning them up!

Processing TSSAR output in R

Read output into R-studio

```
#Read in internal antisense TSS files
internal_antisense_10h <- read.delim("~/Desktop/TSS_vnz/internal/10h_antisense.bed", sep = '\t', header = FALSE)
internal_antisense_14h <- read.delim("~/Desktop/TSS_vnz/internal/14h_antisense.bed", sep = '\t', header = FALSE)
internal_antisense_18h <- read.delim("~/Desktop/TSS_vnz/internal/18h_antisense.bed", sep = '\t', header = FALSE)
internal_antisense_24h <- read.delim("~/Desktop/TSS_vnz/internal/24h_antisense.bed", sep = '\t', header = FALSE)

#read in internal-sense TSS files
internal_10h <- read.delim("~/Desktop/TSS_vnz/internal/10h_internal.bed", sep = '\t', header = FALSE)
internal_14h <- read.delim("~/Desktop/TSS_vnz/internal/14h_internal.bed", sep = '\t', header = FALSE)
internal_18h <- read.delim("~/Desktop/TSS_vnz/internal/18h_internal.bed", sep = '\t', header = FALSE)
internal_24h <- read.delim("~/Desktop/TSS_vnz/internal/24h_internal.bed", sep = '\t', header = FALSE)

#read in primary TSS files
primary_10h <- read.delim("~/Desktop/TSS_vnz/primary/10h_upstream.bed", sep = '\t', header = FALSE)
primary_14h <- read.delim("~/Desktop/TSS_vnz/primary/14h_upstream.bed", sep = '\t', header = FALSE)
primary_18h <- read.delim("~/Desktop/TSS_vnz/primary/18h_upstream.bed", sep = '\t', header = FALSE)
primary_24h <- read.delim("~/Desktop/TSS_vnz/primary/24h_upstream.bed", sep = '\t', header = FALSE)

#read in downstream antisense TSS files
downstream_10h <- read.delim("~/Desktop/TSS_vnz/downstream_antisense/10h_downstream.bed", sep = '\t', header = FALSE)
downstream_14h <- read.delim("~/Desktop/TSS_vnz/downstream_antisense/14h_downstream.bed", sep = '\t', header = FALSE)
downstream_18h <- read.delim("~/Desktop/TSS_vnz/downstream_antisense/18h_downstream.bed", sep = '\t', header = FALSE)
downstream_24h <- read.delim("~/Desktop/TSS_vnz/downstream_antisense/24h_downstream.bed", sep = '\t', header = FALSE)
```

Subset Primary/Antisense downstream TSSs

Now we want to clean these files up before making any meaningful comparisons. We will start by subsetting the “primary” TSS to be within 250bp of their given genes.

```
#column 16 is the distance from the TSS to its closest gene, forcing same strandedness
#and ignoring "upstream" of TSS position; i.e. the gene must be downstream of the TSS

primary_10h <- primary_10h[primary_10h[,16] <= 250,]
primary_14h <- primary_14h[primary_14h[,16] <= 250,]
primary_18h <- primary_18h[primary_18h[,16] <= 250,]
primary_24h <- primary_24h[primary_24h[,16] <= 250,]
```

Now we also want to subset the downstream to be within 40bp of their closest gene (which reads in the opposite direction of the TSS, but is downstream of it)

```
downstream_10h <- downstream_10h[downstream_10h[,16] <= 40,]
downstream_14h <- downstream_14h[downstream_14h[,16] <= 40,]
downstream_18h <- downstream_18h[downstream_18h[,16] <= 40,]
downstream_24h <- downstream_24h[downstream_24h[,16] <= 40,]
```

Annotate orphan TSSs

Now, let's subset all of the time-points for “orphan” TSS; i.e. TSS that did not fall into any of the above categories

```

#read in the raw output .bed file of each TSSAR run
all_TSS_10h <- read.delim("~/Desktop/TSS_vnz/TSS_output/TSS_10h_new.bed", sep = '\t', header = FALSE)
all_TSS_14h <- read.delim("~/Desktop/TSS_vnz/TSS_output/TSS_14h_new.bed", sep = '\t', header = FALSE)
all_TSS_18h <- read.delim("~/Desktop/TSS_vnz/TSS_output/TSS_18h_new.bed", sep = '\t', header = FALSE)
all_TSS_24h <- read.delim("~/Desktop/TSS_vnz/TSS_output/TSS_24h_new.bed", sep = '\t', header = FALSE)

#remove the first row (commented descriptive output)
all_TSS_10h = all_TSS_10h[-1,]
all_TSS_14h = all_TSS_14h[-1,]
all_TSS_18h = all_TSS_18h[-1,]
all_TSS_24h = all_TSS_24h[-1,]

#make lists of all of the TSS which have been annotated in any of our conditions.
#Each timepoint names the TSSs as "TSS0001, etc" in column 4.
annotated_10h <- c(primary_10h$V4, internal_10h$V4, internal_antisense_10h$V4, downstream_10h$V4)
annotated_14h <- c(primary_14h$V4, internal_14h$V4, internal_antisense_14h$V4, downstream_14h$V4)
annotated_18h <- c(primary_18h$V4, internal_18h$V4, internal_antisense_18h$V4, downstream_18h$V4)
annotated_24h <- c(primary_24h$V4, internal_24h$V4, internal_antisense_24h$V4, downstream_24h$V4)

#filter full list by whether the TSS exists in the list. The
orphan_10h <- all_TSS_10h[!(all_TSS_10h[,4] %in% annotated_10h),]
orphan_14h <- all_TSS_14h[!(all_TSS_14h[,4] %in% annotated_14h),]
orphan_18h <- all_TSS_18h[!(all_TSS_18h[,4] %in% annotated_18h),]
orphan_24h <- all_TSS_24h[!(all_TSS_24h[,4] %in% annotated_24h),]

```

Plot Venn diagrams

Now onto some Venn diagrams (generally re-creating what was presented in the original paper)

```

#load venn viagram program
#For the sake of being able to properly knit the markdown this function is commented out
#simply remove the comment and run the function

#install.packages("VennDiagram")
library(VennDiagram)

## Warning: package 'VennDiagram' was built under R version 4.0.2
## Loading required package: grid
## Loading required package: futile.logger

#make a pretty colour pallet for any number of comparisons
library(RColorBrewer)
myCol7 <- brewer.pal(7, "Pastel2")
myCol6 <- brewer.pal(6, "Pastel2")
myCol5 <- brewer.pal(5, "Pastel2")
myCol4 <- brewer.pal(4, "Pastel2")
myCol3 <- brewer.pal(3, "Pastel2")

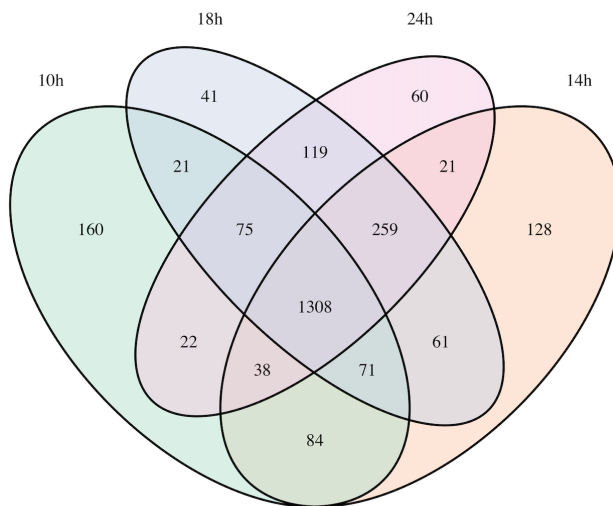
#the first comparisons we are making is how much the TSS's fluctuate between timepoints.
#This function calls the GENE ID that each TSS is associated with
 #(column 15; as the TSS names are obviously not comparable between timepoints).

```

```
#Primary TSSs
venn.diagram(
  x = list(primary_10h$V15, primary_14h$V15, primary_18h$V15, primary_24h$V15),
  category.names = c("10h", "14h", "18h", "24h"),
  filename = '~/Desktop/TSS_vnz/Images/primary_timpoints.jpg',
  fill= myCol4)
```

```
## [1] 1
```

```
knitr::include_graphics("Screenshots/primary.png")
```

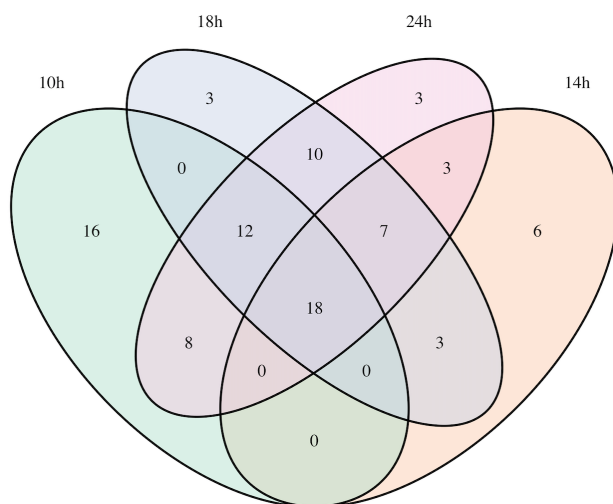


#Comparison of genes that are associated with primary TSSs between timepoints

```
#Antisense-downstream TSSs
venn.diagram(
  x = list(downstream_10h$V15, downstream_14h$V15, downstream_18h$V15, downstream_24h$V15),
  category.names = c("10h", "14h", "18h", "24h"),
  filename = '~/Desktop/TSS_vnz/Images/downstream_timepoints.jpg',
  fill= myCol4)
```

```
## [1] 1
```

```
knitr::include_graphics("Screenshots/downstream.png")
```



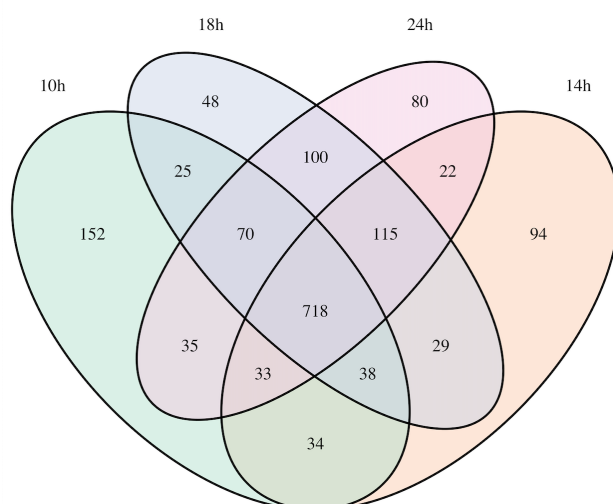
#Comparison of genes that are associated with downstream antisense TSSs between timepoints

#Internal TSSs

```
venn.diagram(
  x = list(internal_10h$V15, internal_14h$V15, internal_18h$V15, internal_24h$V15),
  category.names = c("10h", "14h", "18h", "24h"),
  filename = '~/Desktop/TSS_vnz/Images/internal_timepoints.jpg',
  fill= myCol14)
```

```
## [1] 1
```

```
knitr::include_graphics("Screenshots/internal.png")
```



#Comparison of genes that are associated with internal TSSs between timepoints

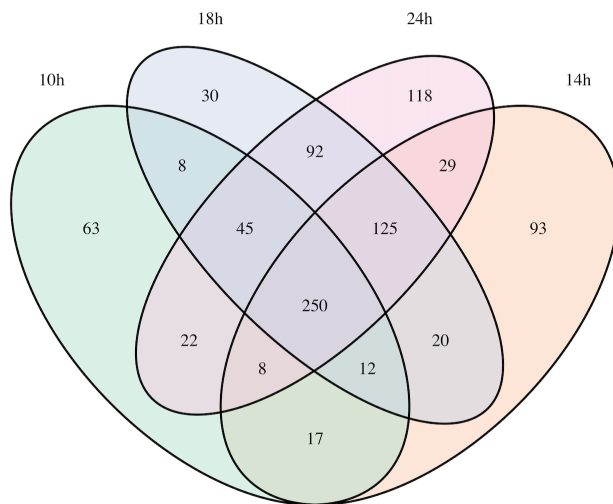
#Internal antisense TSSs

```
venn.diagram(
```

```
x = list(internal_antisense_10h$V15, internal_antisense_14h$V15, internal_antisense_18h$V15, internal_antisense_24h$V15),
category.names = c("10h", "14h", "18h", "24h"),
filename = '~/Desktop/TSS_vnz/Images/internal_antisense_timepoints.jpg',
fill= myCol4)
```

```
## [1] 1
```

```
knitr::include_graphics("Screenshots/internal_antisense.png")
```



#Comparison of genes that are associated with internal antisense TSSs between timepoints

Now since TSS can fall into many categories, lets see within each time-point how much overlap there is

#Combine antisense into one category - as done in (paper)

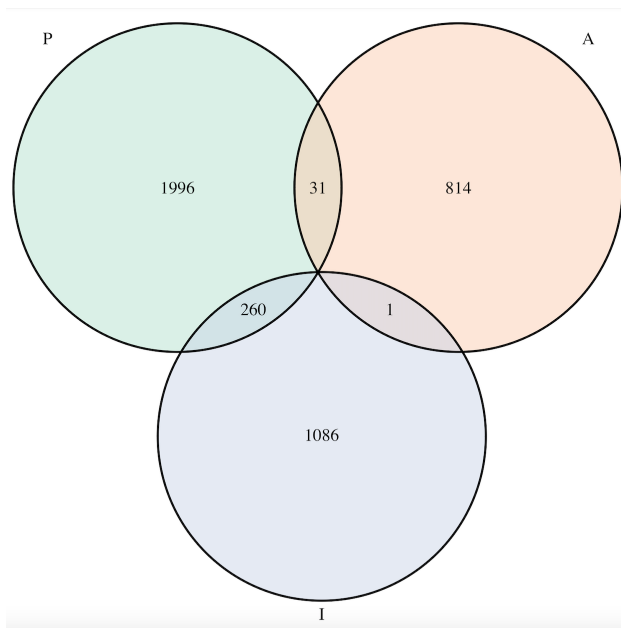
```
all_antisense_10h <- rbind(downstream_10h, internal_antisense_10h)
all_antisense_14h <- rbind(downstream_14h, internal_antisense_14h)
all_antisense_18h <- rbind(downstream_18h, internal_antisense_18h)
all_antisense_24h <- rbind(downstream_24h, internal_antisense_24h)
```

#10h categories, generating figures as made in post-processing by Amman et al. 2014

```
venn.diagram(
  x = list(primary_10h$V4, all_antisense_10h$V4, internal_10h$V4),
  category.names = c("P", "A", "I"),
  filename = '~/Desktop/TSS_vnz/Images/10h_TSS.jpg',
  fill= myCol3)
```

```
## [1] 1
```

```
knitr::include_graphics("Screenshots/10.png")
```

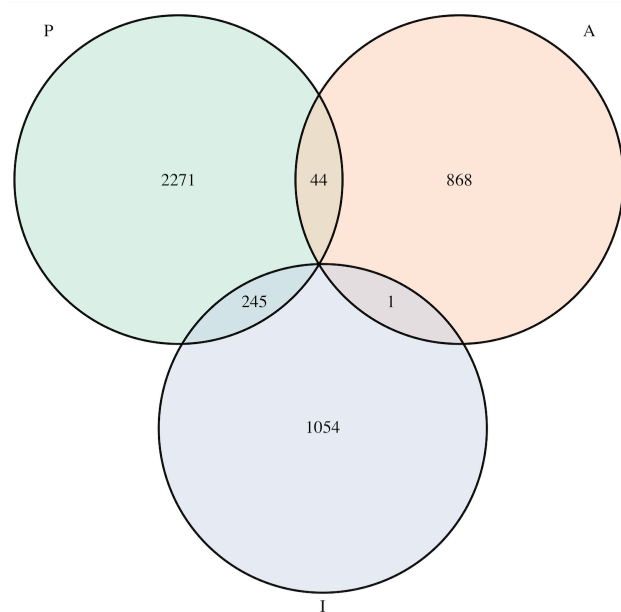


#Comparison of TSSs at 10h

```
#14h categories, generating figures as made in post-processing by Amman et al. 2014
venn.diagram(
  x = list(primary_14h$V4, all_antisense_14h$V4, internal_14h$V4),
  category.names = c("P", "A", "I"),
  filename = '~/Desktop/TSS_vnz/Images/14h_TSS.jpg',
  fill= myCol3)
```

```
## [1] 1
```

```
knitr::include_graphics("Screenshots/14.png")
```



#Comparison of TSSs at 14h


```
#18h categories, generating figures as made in post-processing by Amman et al. 2014
venn.diagram(
  x = list(primary_18h$V4, all_antisense_18h$V4, internal_18h$V4),
  category.names = c("P", "A", "I"),
  filename = '~/Desktop/TSS_vnz/Images/18h_TSS.jpg',
  fill= myCol3)
```

```
## [1] 1
```

```
knitr::include_graphics("Screenshots/18.png")
```

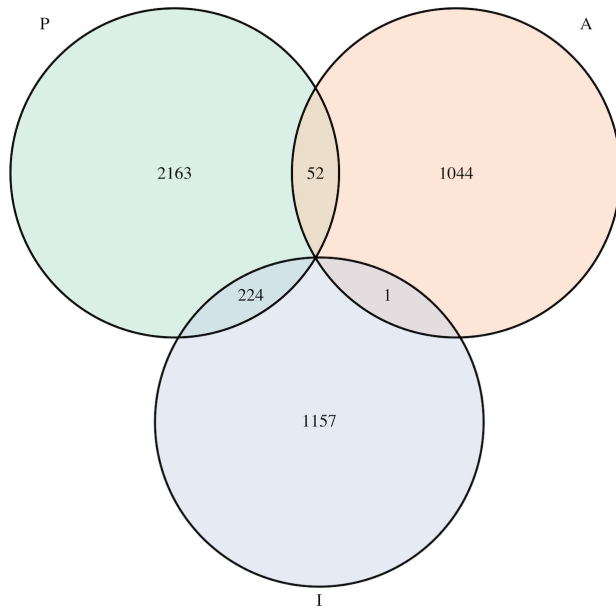


```
#Comparison of TSSs at 18h
```

```
#24h categories, generating figures as made in post-processing by Amman et al. 2014
venn.diagram(
  x = list(primary_24h$V4, all_antisense_24h$V4, internal_24h$V4),
  category.names = c("P", "A", "I"),
  filename = '~/Desktop/TSS_vnz/Images/24h_TSS.jpg',
  fill= myCol3)
```

```
## [1] 1
```

```
knitr::include_graphics("Screenshots/24.png")
```



#Comparison of TSSs at 24h

Create gff files for orphan TSSs

Lastly, as part of the TSS analysis we are interested in making genome feature files that describe the location of the orphan transcripts (which are potential ncRNAs of regulatory regions of long 5'UTRs. To do this, we will first extend the orphan TSS regions to 200bp (they are currently annotated as one nucleotide). This will facilitate the identification of differentially expressed potential ncRNAs by aligning RNA-sequencing data to the *S. venezuelae* genome, counting reads that align to our provided hypothetical 200bp transcripts, and identifying such transcripts that are differentially expressed.

To extend the genomic region of each TSS we will use the bedtools slop function (again as a program installed on your local computer accessed through the command line or on a remote server)

#first make the bed files

```
write.table(orphan_10h, "~/Desktop/TSS_vnz/orphan_TSS/orphan_10h.bed", col.names = F, row.names = F, qu
write.table(orphan_14h, "~/Desktop/TSS_vnz/orphan_TSS/orphan_14h.bed", col.names = F, row.names = F, qu
write.table(orphan_18h, "~/Desktop/TSS_vnz/orphan_TSS/orphan_18h.bed", col.names = F, row.names = F, qu
write.table(orphan_24h, "~/Desktop/TSS_vnz/orphan_TSS/orphan_24h.bed", col.names = F, row.names = F, qu
```

Bedtools slop and sort

#now start with the bedtools function

```
bedtools slop -i ~/Desktop/TSS_vnz/orphan_TSS/orphan_10h.bed -g ~/Desktop/TSS_vnz/ref/vnz_genome.fasta.
bedtools slop -i ~/Desktop/TSS_vnz/orphan_TSS/orphan_14h.bed -g ~/Desktop/TSS_vnz/ref/vnz_genome.fasta.
bedtools slop -i ~/Desktop/TSS_vnz/orphan_TSS/orphan_18h.bed -g ~/Desktop/TSS_vnz/ref/vnz_genome.fasta.
bedtools slop -i ~/Desktop/TSS_vnz/orphan_TSS/orphan_24h.bed -g ~/Desktop/TSS_vnz/ref/vnz_genome.fasta.
```

#looking at the files it seems they need to be sorted again (by genomic position, not +/-)

```
bedtools sort -i ~/Desktop/TSS_vnz/orphan_TSS/orphan_10h_extended.bed > ~/Desktop/TSS_vnz/orphan_TSS/orphan_10h_sort.bed
```

```
bedtools sort -i ~/Desktop/TSS_vnz/orphan_TSS/orphan_14h_extended.bed > ~/Desktop/TSS_vnz/orphan_TSS/orphan_14h_sort.bed
```

```
bedtools sort -i ~/Desktop/TSS_vnz/orphan_TSS/orphan_18h_extended.bed > ~/Desktop/TSS_vnz/orphan_TSS/orphan_18h_sort.bed
```

```
bedtools sort -i ~/Desktop/TSS_vnz/orphan_TSS/orphan_24h_extended.bed > ~/Desktop/TSS_vnz/orphan_TSS/orphan_24h_sort.bed
```

Now that we have our bed file which lists all **orphan TSSs as 200bp reads**, we want to convert the .bed file to a .gff file to use in our RNA-sequencing analysis.

Now this will require some manual conversion (there doesn't seem to be a program available)

GFF fields must have **9 tab-separated columns**. Also, all but the final field in each feature line must contain a value; "empty" columns should be denoted with a '.'

- 1) **seqname** - name of the chromosome or scaffold; chromosome names can be given with or without the 'chr' prefix.
- 2) **source** - name of the program that generated this feature, or the data source (database or project name)
- 3) **feature** - feature type name, e.g. Gene, Variation, Similarity
- 4) **start** - Start position* of the feature, with sequence numbering starting at 1.
- 5) **end** - End position* of the feature, with sequence numbering starting at 1.
- 6) **score** - A floating point value.
- 7) **strand** - defined as + (forward) or - (reverse).
- 8) **frame** - One of '0', '1' or '2'. '0' indicates that the first base of the feature is the first base of a codon, '1' that the second base is the first base of a codon, and so on..
- 9) **attribute** - A semicolon-separated list of tag-value pairs, providing additional information about each feature.

```
orphan_10h <- read.delim("~/Desktop/TSS_vnz/orphan_TSS/orphan_10h_sort.bed", header = F, sep = '\t')
```

```
orphan_14h <- read.delim("~/Desktop/TSS_vnz/orphan_TSS/orphan_14h_sort.bed", header = F, sep = '\t')
```

```
orphan_18h <- read.delim("~/Desktop/TSS_vnz/orphan_TSS/orphan_18h_sort.bed", header = F, sep = '\t')
```

```
orphan_24h <- read.delim("~/Desktop/TSS_vnz/orphan_TSS/orphan_24h_sort.bed", header = F, sep = '\t')
```

.Bed to .GFF conversion

Now let's consider what has to change to make it .gff file format: 1) **seqname**: the first column is the same! 2) **source** - not the same. Need to add a column that just says "TSSAR" the whole way down 3) **feature** - not the same. Need to add a column that just says "orphan_TSS" the whole way down 4) **start** - got it! TSS_vhV2(start)5) **end** - got it! TSS_vhV3(end) 6) **score** - got it! TSS_xhV5(score)7) **strand** - got it! TSS_xhV6(strand) 8) **frame** - One of '0', '1' or '2'. '0'. Bed files are base 1 so '1' the whole way. 9) **attribute** - I'll use TSS_xhV4 (the name of the TSS in that time-point for reference)

```
orphan_10h_gff <- data.frame(orphan_10h$V1,  
                             "TSSAR",  
                             "orphan_TSS",  
                             orphan_10h$V2,  
                             orphan_10h$V3,  
                             orphan_10h$V5,  
                             orphan_10h$V6,
```

```

1,
orphan_10h$V4)

orphan_14h_gff <- data.frame(orphan_14h$V1,
  "TSSAR",
  "orphan_TSS",
  orphan_14h$V2,
  orphan_14h$V3,
  orphan_14h$V5,
  orphan_14h$V6,
  1,
  orphan_14h$V4)

orphan_18h_gff <- data.frame(orphan_18h$V1,
  "TSSAR",
  "orphan_TSS",
  orphan_18h$V2,
  orphan_18h$V3,
  orphan_18h$V5,
  orphan_18h$V6,
  1,
  orphan_18h$V4)

orphan_24h_gff <- data.frame(orphan_24h$V1,
  "TSSAR",
  "orphan_TSS",
  orphan_24h$V2,
  orphan_24h$V3,
  orphan_24h$V5,
  orphan_24h$V6,
  1,
  orphan_24h$V4)

```

The last column needs not only to be the TSS number but has to be preceded by “ID=”

```

#Add "ID=" in front of the "TSS_XXXX" for all .gff files
library(dplyr)

```

```

## Warning: package 'dplyr' was built under R version 4.0.2
##
## Attaching package: 'dplyr'
## The following objects are masked from 'package:stats':
##
##   filter, lag
## The following objects are masked from 'package:base':
##
##   intersect, setdiff, setequal, union
orphan_10h_gff <- orphan_10h_gff %>%
  mutate_if(is.character,
    stringr::str_replace_all,
    pattern = 'TSS_',
    replacement = 'ID=TSS_')

```

```

orphan_14h_gff <- orphan_14h_gff %>%
  mutate_if(is.character,
    stringr::str_replace_all,
    pattern = 'TSS_',
    replacement = 'ID=TSS_')

orphan_18h_gff <- orphan_18h_gff %>%
  mutate_if(is.character,
    stringr::str_replace_all,
    pattern = 'TSS_',
    replacement = 'ID=TSS_')

orphan_24h_gff <- orphan_24h_gff %>%
  mutate_if(is.character,
    stringr::str_replace_all,
    pattern = 'TSS_',
    replacement = 'ID=TSS_')

#write .gff files to my local computer to later use for RNA-sequencing analysis!
write.table(orphan_10h_gff,
  "~/Desktop/TSS_vnz/orphan_TSS/10h_orphan.gff",
  quote = F,
  sep = '\\t',
  eol = '\\n',
  row.names = F,
  col.names = F)

write.table(orphan_14h_gff,
  "~/Desktop/TSS_vnz/orphan_TSS/14h_orphan.gff",
  quote = F,
  sep = '\\t',
  eol = '\\n',
  row.names = F,
  col.names = F)

write.table(orphan_18h_gff,
  "~/Desktop/TSS_vnz/orphan_TSS/18h_orphan.gff",
  quote = F,
  sep = '\\t',
  eol = '\\n',
  row.names = F,
  col.names = F)

write.table(orphan_24h_gff,
  "~/Desktop/TSS_vnz/orphan_TSS/24h_orphan.gff",
  quote = F,
  sep = '\\t',
  eol = '\\n',
  row.names = F,
  col.names = F)

```

Done! Next step will be the RNA-sequencing analysis.

This will be available in another markdown file!