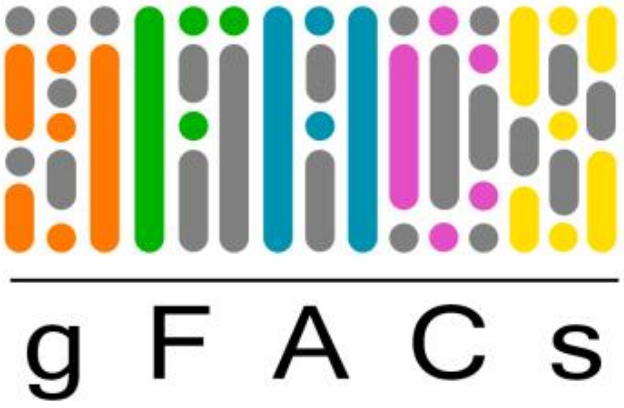


Gene filtering, analysis, and conversion.

User guide

Madison Caballero
University of Connecticut
https://gitlab.com/PlantGenomicsLab/gFACs.git

Version date: 08/23/2018

What is new?

Version 08/23/18

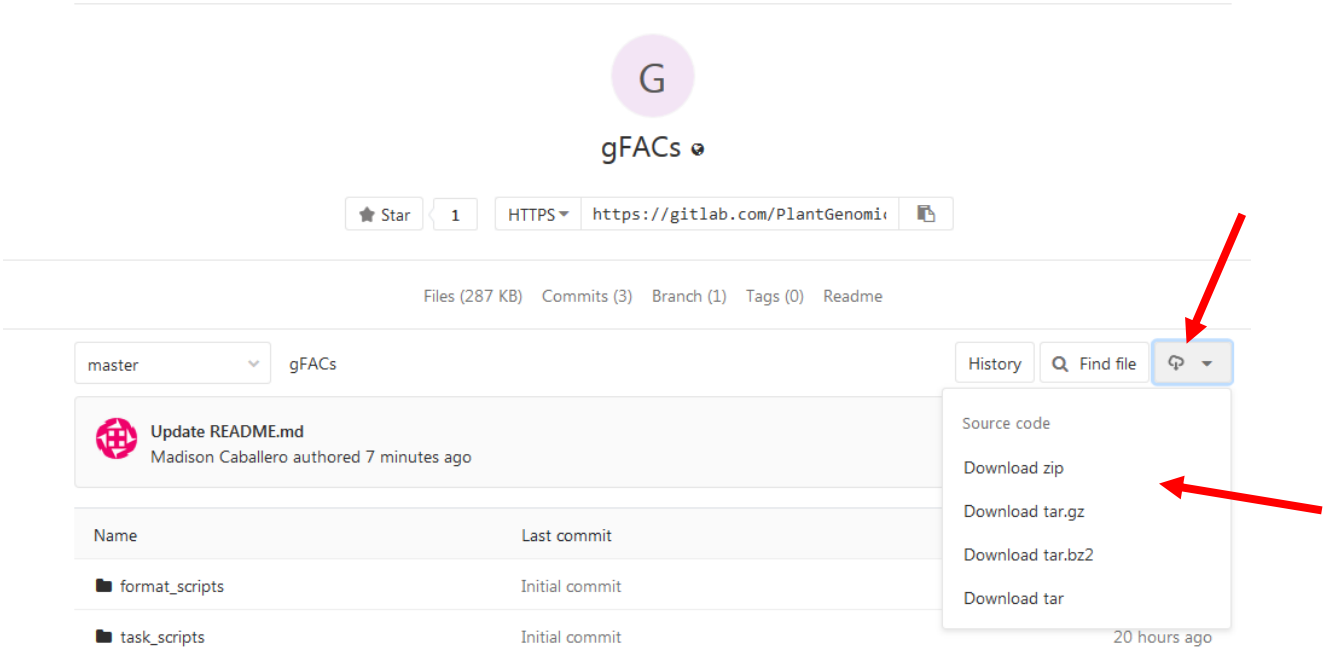
- Pseudo_gFACs.pl has been added as a way to run gFACs but without the filters being additive.
- The option to have distributions on exon and intron positions has been added.
- A few changes make fasta-dependent commands run faster.
- Error message for not having a fasta will print to the log.
- Raw data for exon and intron position is available.
- Slight modifications to script locations

Contents

DOWNLOAD

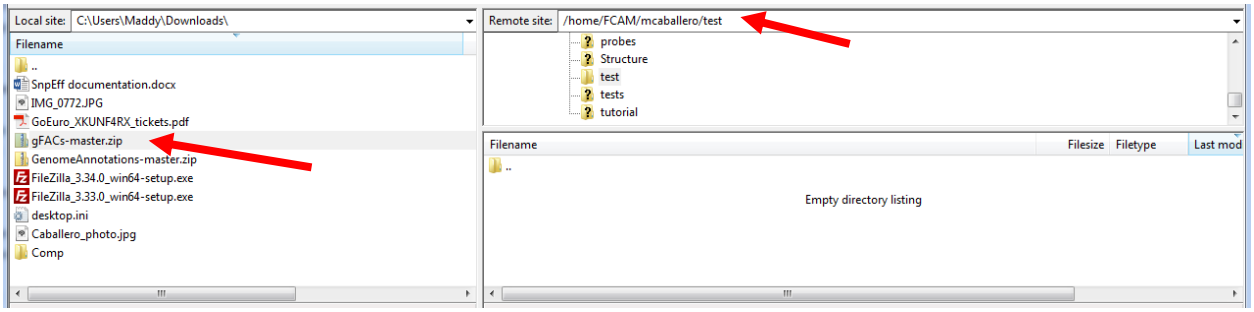
gFACs is currently publicly available here:
https://gitlab.com/PlantGenomicsLab/gFACs.git

1. **Download the directory from git lab.**



2. **Place the file onto the cluster for your use.**

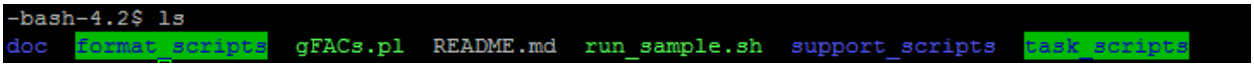
I downloaded this as a zip file and extracted the files on my personal computer. Then you can use filezilla to transfer it onto the cluster (or whatever you use to transfer files).



You should now have a folder called gFACs-master.zip:

```
-bash-4.2$ ls
gFACs-master.zip
```

You can unzip it and look inside. Inside that folder, the layout looks like this:



3. **Test to see it if works.**

Make sure to module load perl first.

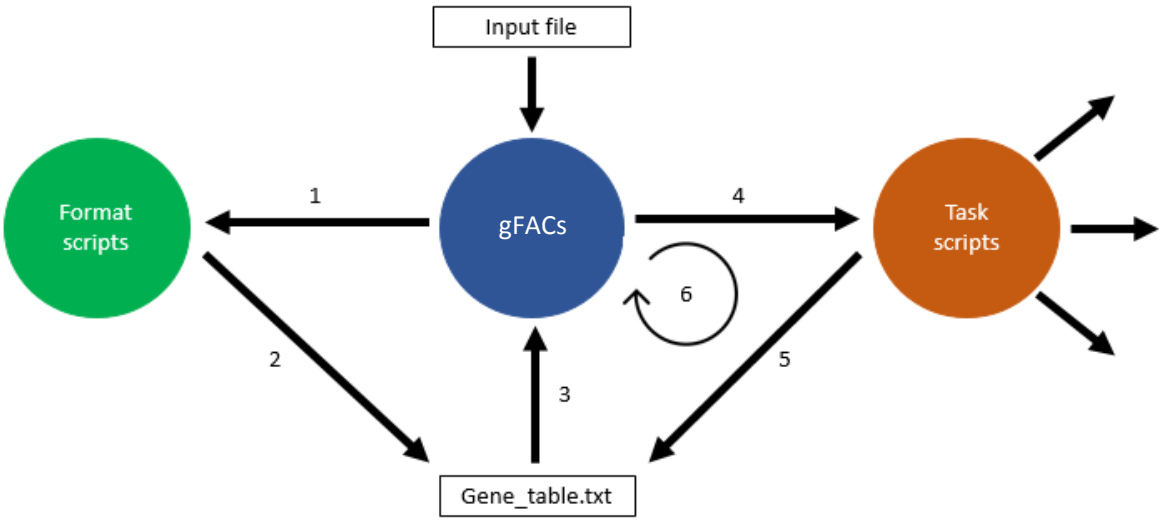
To get the command line manual, type this command:

```
$ perl gFACs.pl
```

A manual that is a lighter version of the attached pdf you are reading now should appear.

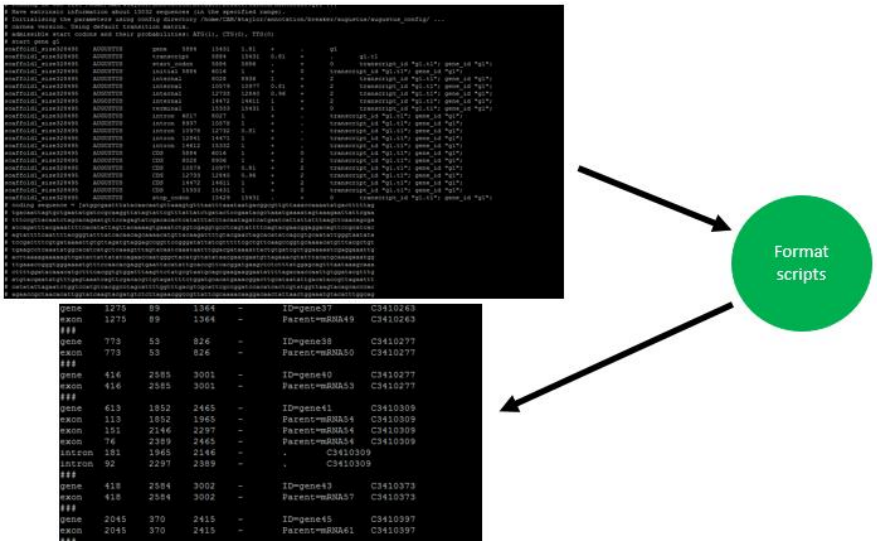
If you have issues, send me an email: Madison.caballero@uconn.edu

GENERAL FLOW



The flow of gFACs.pl is controlled by the master script gFACs.pl. Flags and input files are processed by the master script, and then a series of task-specific scripts are called upon to edit and filter gene or alignment models. It is designed this way to allow for modules and formats to be built in while minimizing the potential complexity of one do-all script. Fixing bugs is also more manageable as each task-specific script or format script can be run independently.

The inputted file is a gene-file, either as gene transfer (gtf) or general feature (gff/gff3). Since these file types have no regulation, formats designed to fit a particular software's output must be created. Specific scripts in the folder format_scripts/ are used to convert the input into a median file type called gene_table.txt.



gene_table.txt (referred to also as the gene table) is not an official format and is created to hold the minimum amount of information for the tasks this program can do. Some file types, like gff, give more information than the typical gff3 file type. However, given that the goal of this program is to work across many file types, information from the lowest common denominator limits the median file type.

The gene table is the most important file for this program as it is used and edited in every step. Each flag or task in evaluation needs the format of the gene table to work successfully. The gene table will always have the gene models or alignments that are retained.

Here is an example of gene table format:

```
###
gene    1206    144168  145373  -       ID=g28960.t1;geneID=g28960      scaffold124501
exon    250     144168  144417  -       Parent=g28960.t1        scaffold124501
exon    373     144565  144937  -       Parent=g28960.t1        scaffold124501
exon    286     145088  145373  -       Parent=g28960.t1        scaffold124501
intron  147     144418  144564  -       .       scaffold124501
intron  150     144938  145087  -       .       scaffold124501
###
gene    375     76279   76653   +       ID=g28961.t1;geneID=g28961      scaffold124508
exon    375     76279   76653   +       Parent=g28961.t1        scaffold124508
###
```

The columns go:

1. Gene part
2. Length
3. Start
4. Stop
5. Strand
6. ID (8th column from input file)
7. Scaffold/chromosome (needed for fasta commands)

Further scripts expect the gene table to be in the output directory called gene_table.txt. If you are using a prefix, it will look for the file with the prefix. Multiple task-scripts create their own out file, notably if things are being separated, such as potential splice transcripts. **However, the retained genes will always be renamed or concatenated onto the gene table.**

How are introns predicted?

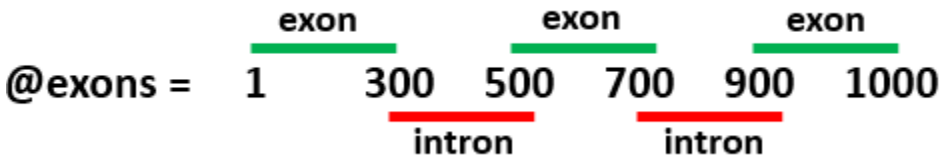
The creation of the gene table has intron information that is not *always* found in the input file. Even if the input format does provide introns, they will always be recalculated based on the positions of predicted exons.

In the format script, a temporary file is created that will eventually be deleted. The purpose of this step is to add a divider between gene families. The ### line allows for a clear break between different genes. The script then revisits the temp file, calculates introns and makes final formatting shifts.

Here is an example of the temporary file:

```
###
scaffold124501  AUGUSTUS        gene    5717    6235    .       -       .       ID=g28956.t2;geneID=g28956
scaffold124501  AUGUSTUS        mRNA    5717    6235    .       -       .       ID=g28956.t2;geneID=g28956
scaffold124501  AUGUSTUS        exon    5717    6235    0.61    -       .       Parent=g28956.t2
scaffold124501  AUGUSTUS        CDS     5717    6235    .       -       0       Parent=g28956.t2
###
scaffold124501  AUGUSTUS        gene    6831    8759    .       +       .       ID=g28957.t1;geneID=g28957
scaffold124501  AUGUSTUS        mRNA    6831    8759    .       +       .       ID=g28957.t1;geneID=g28957
scaffold124501  AUGUSTUS        exon    6831    7127    0.97    +       .       Parent=g28957.t1
scaffold124501  AUGUSTUS        exon    7301    7392    1.00    +       .       Parent=g28957.t1
scaffold124501  AUGUSTUS        exon    8400    8435    0.56    +       .       Parent=g28957.t1
scaffold124501  AUGUSTUS        exon    8579    8759    0.32    +       .       Parent=g28957.t1
scaffold124501  AUGUSTUS        CDS     6831    7127    .       +       0       Parent=g28957.t1
scaffold124501  AUGUSTUS        CDS     7301    7392    .       +       0       Parent=g28957.t1
scaffold124501  AUGUSTUS        CDS     8400    8435    .       +       1       Parent=g28957.t1
scaffold124501  AUGUSTUS        CDS     8579    8759    .       +       1       Parent=g28957.t1
###
```

The question of determining introns begins with *where* introns are. By this script's definition, they are the sequence between exons. Lengths and start and stop coordinates then needs to be calculated based on exon information. To accomplish this, exon lengths are pushed into an array and called by position. This method is more universally reliable but prone to errors involving overlapping exons. This can be resolved with a flag --splice-rescue.

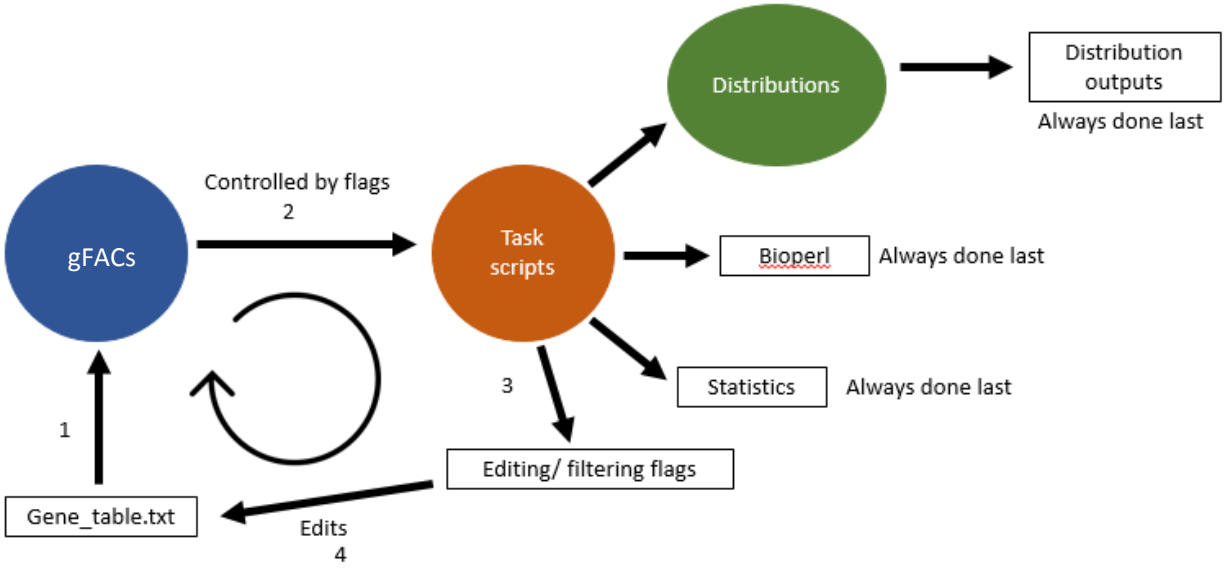


Introns start on the base immediately following the exon and end on the base before the next exon. In the example above, the first intron would span 301-499 and have a length of 199 bases.

How do task scripts work?

Once the format script has completed, and the gene table has been created, filtering as designated by flags is done cyclically on the gene table until the final set of genes is produced. The order of filtering flags is pre-determined although does not change the final result. For example, whether or not monoexonic genes are removed first or last will not change how many monoexonics appear in the final iteration of the gene table (spoiler alert: none).

Once all filtering is done, other commands are activated that involve processing or analyzing the final sets. This includes statistics, bioperl commands such as analyzing splice types, or distributions.



In addition to the gene table, the **gFACs_log.txt** file is created every time the script is run, no exceptions. If a prefix is included, the log will have this prefix. The log file is reported by the master script and is appended with information regarding filtering at each step. It will also report what flags are being activated and the corresponding system commands.

The log may be helpful for the user to see what is happening and the results of a particular filter. It is also helpful for noticing bugs and verifying script efficacy.

HOW TO RUN

To get the manual

```
perl gFACs.pl
```

→ The manual will also pop up if the word “help” is found *anywhere* in the input. So --help and -help will work (but not -h). Also, don’t have help in your file name or make it your prefix.

To run this script

```
perl gFACs.pl \
-f <format> \
[options] \                                  # All flags go here
-O <output directory> \                      # Must be second to last
<input_file>                                 # Must be last
```

Mandatory inputs

-f <format>	I need to know the format of your input. All gff/gtf/gff3 files are different so I need to call the right script.
-O <output directory>	I need to know where to put the output files since the script runs in a shared space. Please write the output ending with a / as the SECOND TO LAST argument
<input_file>	There needs to be an input file as the LAST argument.

Output files always created

gFACs_log.txt : What's happening
gene_table.txt : A very readable table with information from your input

Supported Formats

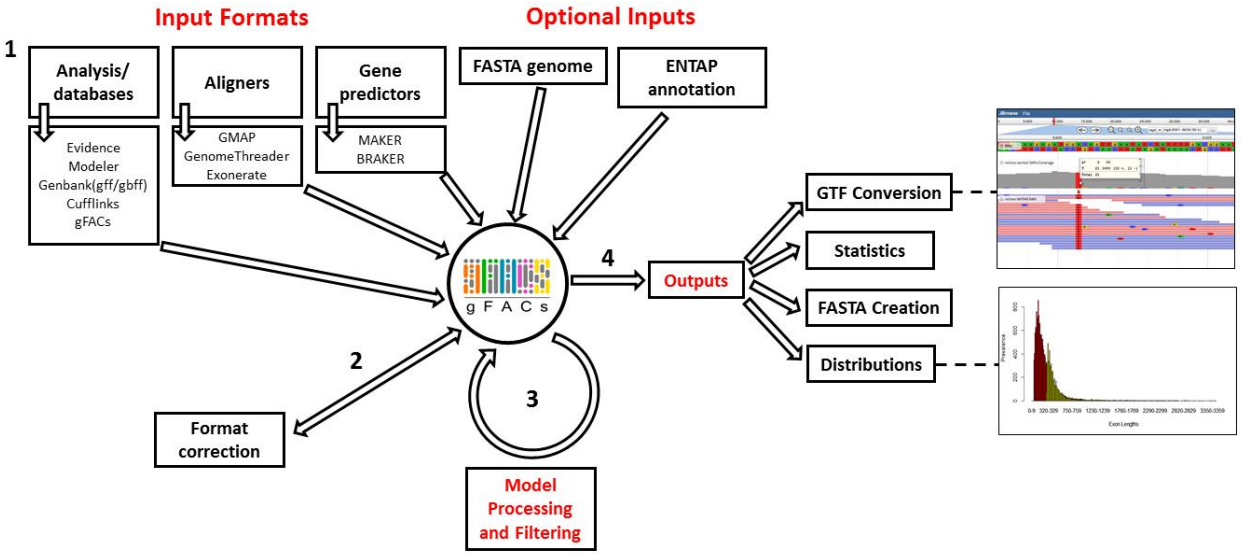
-f [format] Specifying a format: A mandatory step to call upon the right script.

Available formats (code to be entered in green column):

Source	Type	Version	Format	-f [code]	Notes
GMAP	Aligner	03/17/2017	.gff3	gmap_2017_03_17_gff3	
Braker	Gene predictor	2.05	.gtf	braker_2.05_gtf	
		2.05	.gff	braker_2.05_gff	
		2.05	.gff3	braker_2.0_gff3	
		2.0	.gtf	braker_2.0_gff	
		2.0	.gff	braker_2.0_gtf	
		2.0	.gff3	braker_2.0_gff3	

Maker	Gene predictor	2.31.9	.gff	maker_2.31.9_gff	Maker may provide other information such as blastx and protein2genome information. Currently, only maker models of genes and exons will be considered.
Genome threader	Aligner	1.6.6	.gff3	genomethreader_1.6.6_gff3	
Gffread (cufflinks)	Analyzer	0.9.12	.gff3	gffread_0.9.12_gff3	
Exonerate	Aligner	2.4.0	.gff	exonerate_2.4.0_gff	
Evidence Modeler	Analyzer	1.1.1	.gff3	EVM_1.1.1_gff3	
gFACs	Analyzer	all	gene_table.txt	gFACs_gene_table	You can input a gene table from gFACs, any version. However, the prefix on the input will NOT be retained.
	Analyzer	all	out.gtf	gFACs_gtf	
NCBI refseq	Database	all	.gff (.gff3)	refseq_gff	CDS only taken.
NCSBI genbank	Database	all	.gbff	genbank_gbff	UTR is removed. (CDS sequences only considered)

<u>** If your file does not have a clear format or does not work, see format_diagnosis.pl at the end.</u>



Basic run

```
#!/bin/bash
#SBATCH --job-name=gFACs
#SBATCH --partition=general
#SBATCH --mail-user=email@email.com
#SBATCH -o gFACs_%j.o
#SBATCH -e gFACs_%j.e

module load perl

perl gFACs.pl \
-f gffread_0.9.12_gff3 \
--splice-rescue \
-O /path/to/your/output/directory/ \
/path/to/the/gffread/gff3/file.gff3
```

→ Always run as a bash script
→ For more advanced run options, see flags. However, I always recommend using –splice-rescue. Without it, the fasta files and statistics may be off. It is not mandatory, however, as it is possible your gene files may already be filtered for non-overlapping models.

Tips

- *Always always* use --splice-rescue.
- For large files that are alignments or if your genome is large, specify more memory. Nothing is too computationally advanced but parsing and storing the information may cause the script to die.
- **Check the log!** Information will be printed there such as numbers removed by filtering. Always check to see if they make sense.
- If your format is edited or modified, you can *try* different format types to see if one will work. However, be wary of using non-matching formats and manually check some results. Just because it returns numbers does NOT mean they will be correct.
- To sequester monoexonics from multiexonis, run the script twice. One with –rem-monoexonics and one with –rem-multiexonics. Use a different prefix for each run. Presto.

FLAGS OVERVIEW

You can include as many flags as you want in *any* order. However, the order in which the flags are run is **predetermined** by the gFACs.pl script. This section is designed to tell you what the flags do conceptually. This is not the true order. See the log file for the order.

-p [prefix]

All files created will have your designated prefix. For example, if you provide the prefix “test”, your gene table will be called test_gene_table.txt. Every file (even temporary files) will have this prefix. However, it is not a mandatory argument.

FILTER FLAGS THAT DO NOT NEED A FASTA

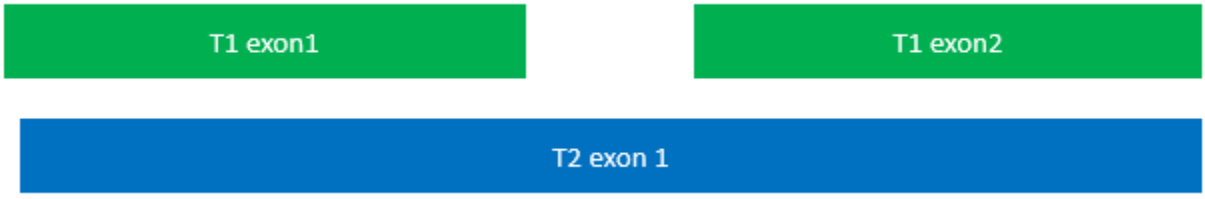
--splice-rescue

I always recommend using this flag. This parameter involves two scripts: task_scripts/overlapping_exons.pl and task_scripts/splice_variants.pl. The first analyzes the gene table for overlapping exon space and then the second analyzes genes with overlapping exon space for evidence that these are separate transcripts or models.

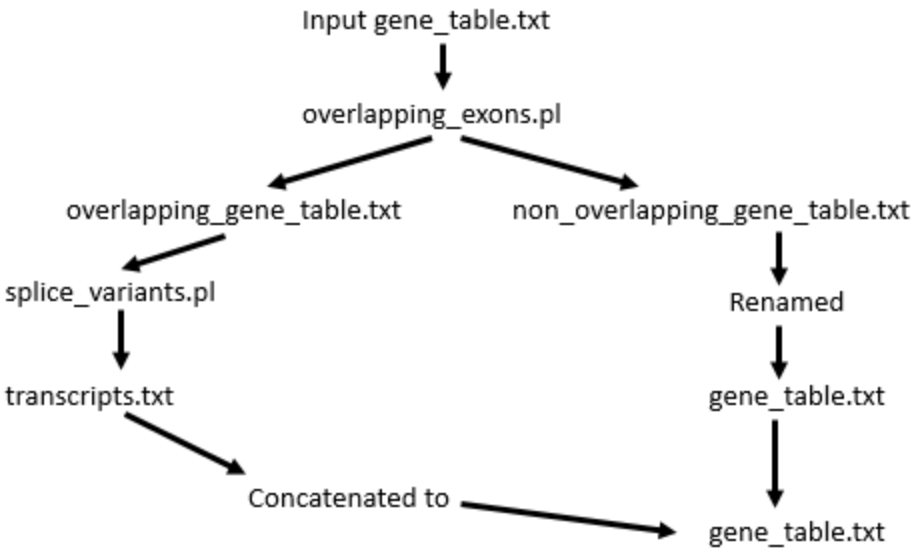
Take this particular gene for example:

scaffold124501	AUGUSTUS	mRNA	5314	6235	.	-	.	ID=g28956.t1;geneID=g28956
scaffold124501	AUGUSTUS	exon	5314	5707	0.70	-	.	Parent=g28956.t1
scaffold124501	AUGUSTUS	exon	5799	6235	0.73	-	.	Parent=g28956.t1
scaffold124501	AUGUSTUS	CDS	5314	5707	.	-	1	Parent=g28956.t1
scaffold124501	AUGUSTUS	CDS	5799	6235	.	-	0	Parent=g28956.t1
scaffold124501	AUGUSTUS	mRNA	5717	6235	.	-	.	ID=g28956.t2;geneID=g28956
scaffold124501	AUGUSTUS	exon	5717	6235	0.61	-	.	Parent=g28956.t2
scaffold124501	AUGUSTUS	CDS	5717	6235	.	-	0	Parent=g28956.t2

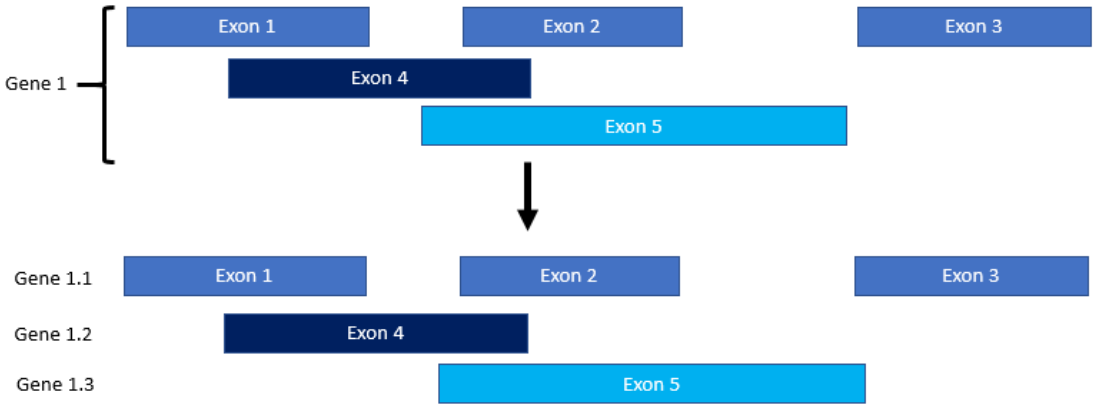
Although three exons are called, they overlap as the third is supported by a different parent transcript. The first two claim an intron while the third spans over that particular intron like this:



Intron prediction, when not taking into account that these are *separate* models of the gene space, would be wrong. To solve this, genes that show exon overlap are separated out into their own file. Then they are evaluated.



The way splice variants are confirmed is due to the labeling on the 6th column in the gene table. If an exon has a different transcript ID (often labeled t1, t2, t3…) then the exon is separated into its own "batch". What was originally "gene22" with three separate transcripts then becomes gene22.1, gene22.2, and gene22.3.



Introns are then recalculated for each of the separate isoforms. If some of the models are incomplete, they can be filtered out with other flags since they are now treated as separate "genes". (This is because they are separated by a ### partition).

Passing genes will remain in the gene table. Results of this filter are printed in the log.

To resolve transcripts back to unique genes (selecting largest, if available, or first transcript) can be done with the –unique-genes-only flag.

Following this step, another script called task_scripts/gene_table_fixer.pl is implemented. This is done to check that exons and introns are in positive strand ascending order. Without it, printing the fastas will be problematic. It is done regardless of whether or not you use splice rescue.

--rem-start-introns

This option is controlled by the script task_scripts/remove_starting_introns.pl. It is designed to pull out genes that start with “intronic” space. This is almost always because of missing evidence due to missing scaffold sequence.

Take this for example:

```
###
gene    22546   1       22546   +       g193    super417
exon    73      86      158     +       transcript_id "g193.t1"; gene_id "g193";        super417
exon    325     925     1249    +       transcript_id "g193.t1"; gene_id "g193";        super417
```

The very first exon begins 86 nucleotides into the proposed gene space. You can also see that the gene “begins” at position 1 in super417. This particular model came from BRAKER 2.0.

This script finds genes where gene start and first exon start do not agree. Passing genes will remain in the gene table. Results of this filter are printed in the log.

NOTE: This script takes into account **directionality**. Meaning a positive strand gene without a start intron would occur at the start of the gene (all gene coordinates are positive stranded). In a negative strand gene, missing the start intron would occur at the end of the gene.

--rem-end-introns

This option is controlled by the script task_scripts/remove_ending_introns.pl. It is nearly identical to removing start and also takes into account strandedness and directionality. For another example in the exact same BRAKER 2.0 gene table:

```
gene    3088    1231    4318    +       g59947  jcf7180001216318
exon    117     1231    1347    +       transcript_id "g59947.t1"; gene_id "g59947";    jcf7180001216318
exon    105     1547    1651    +       transcript_id "g59947.t1"; gene_id "g59947";    jcf7180001216318
exon    78      1833    1910    +       transcript_id "g59947.t1"; gene_id "g59947";    jcf7180001216318
exon    117     1231    1347    +       transcript_id "g59947.t2"; gene_id "g59947";    jcf7180001216318
exon    105     1547    1651    +       transcript_id "g59947.t2"; gene_id "g59947";    jcf7180001216318
exon    41      1847    1887    +       transcript_id "g59947.t2"; gene_id "g59947";    jcf7180001216318
intron  115     1232    1346    +       .       jcf7180001216318
intron  199     1348    1546    +       .       jcf7180001216318
intron  103     1548    1650    +       .       jcf7180001216318
intron  181     1652    1832    +       .       jcf7180001216318
intron  39      1848    1886    +       .       jcf7180001216318
###
```

Here you can see that the last exon ends at *1887 but the gene claims to end at 4318*.

Sometimes this occurs because of the scaffold ending as before, but further fasta involvement can find incomplete genes due to codon evidence.

Passing genes will remain in the gene table. Results of this filter are printed in the log.

--rem-extra-introns

Performs the tasks of --rem-start-introns and --rem-end-introns. See above for what each task does individually. Passing genes will remain in the gene table. Results and commands of this filter are printed in the log as if each command was run separately.

--rem-monoexonics

Removes monoexonics based off the presence of **introns**. All multiexonic genes then remain in the gene table. The script that does this command is task_scripts/remove_monoexonics.pl.

--rem-multiexonics

Remove multiexonics based off the presence of **introns**. All monoexonics genes then remain in the gene table. The script that does this command is task_scripts/remove_multiexonics.pl.

--min-exon-size [number]

Default: 20

Creates a filter to remove genes with exons below a certain size. The script that performs this command is task_scripts/minimum_exon.pl. If you do not provide a following number, 20 is used as a benchmark for an exon that is suspiciously too small.

Passing genes will remain in the gene table. Results of this filter are printed in the log.

--min-intron-size [number]

Default: 20

Creates a filter to remove genes with introns below a certain size. The script that performs this command is task_scripts/minimum_intron.pl. If you do not provide a following number, 20 is used as a benchmark for an intron that is suspiciously too small. However, it might be *technically* possible to have introns that are less than 20 nucleotides.

Passing genes will remain in the gene table. Results of this filter are printed in the log.

--min-CDS-size [number]

Default: 74

Creates a filter to remove genes with a coding sequence (CDS) below a certain nucleotide length. Introns do not count, only exon sequence size. The default is based off the smallest known gene and will be used if no input is provided.

Passing genes will remain in the gene table. Results of this filter are printed in the log.

--unique-genes-only

This option will collapse directly overlapping genes and resolve transcripts created using –splice-rescue.

When using --splice-rescue, multiple transcripts are created that represent the *same gene*. They may be isoforms of one gene or the exact same gene model repeated due to multiple pieces of

evidence. Since separation treats each transcript as if it was its own gene for statistics and file-creation steps, this step will return only unique genes.

This is done by the script task_scripts/unique_genes.pl. It separates out transcripts denoted by their .1, .2, etc... modification. For those representing the same gene, the **largest** transcript is selected **if available**. Otherwise it will just take the first one.

When not dealing with transcripts, if two separate genes with different IDs share the exact same space, the **first one** numerically will be chosen. This only affects genes with 100% overlap where each gene is the same size and starts and ends at the same coordinates.

This step is done before any outputs are created such as statistics, fastas, output tables, or gtf files. Unique genes will remain in the gene table. Results of this filter including genes in, transcripts present, unique transcripts, non-transcript duplicates, lost, and final returned genes are printed in the log.

FILTER FLAGS THAT REQUIRE AN ENTAP ANNOTATION

--entap-annotation /path/to/your/final_annotation.tsv

Provide the path to the output of the protein annotation. The first column should be the name of a gene in the "g###.t1" format. As of this version, **only braker 2.05 outputs** annotated with ENTAP are recognized due to building resources. It will refuse to run with other formats.

The trailing t1 is disregarded and there *should* be no recognition issues if everything is run with the latest version of gFACs. Additionally, all ENTAP steps are done before fasta steps so all outputs will reflect filters that require the annotation.

The annotation should look something like this:

```
g60348.t1

g60343.t1

g60341.t1       sp|F4JLP5|PLPD2_ARATH   80.6    572     92      5       1       568     1       557     2.8e-252        92.400000       sp|F4JLP5|PLPD2_ARATH Dihydrolip
oyl dehydrogenase 2, chloroplastic OS=Arabidopsis thaliana GN=LPD2 PE=2 SV=2    Arabidopsis thaliana    /UCHC/LABS/Wegrzyn/WalnutGenomes/Regia/outfiles/similarity_searc
h/blastp_RegiaCompleteMultiExonics_final_uniprot_sprot.out              No      Yes     29760.VIT_05s0077g01210.t01     2.8e-277        959.5           Viridiplantae
1BH5S@strNOG,1DUCK@virNOG,COG1249@NOG,KOG1335@euNOG     dihydrolipoyl dehydrogenase     Biosynthesis of secondary metabolites (01110), Pyruvate metabolism (00620), Meta
bolic pathways (01100), Citrate cycle (TCA cycle) (00020), Glycine, serine and threonine metabolism (00260), Glycolysis / Gluconeogenesis (00010), Microbial metabolism
in diverse environments (01120), Valine, leucine and isoleucine degradation (00280)     PFAM (Pyr_redox_2, Pyr_redox_dim, Pyr_redox, GIDA, FAD_binding_2)       GO:00090
58-biosynthetic process(L=2),GO:0044237-cellular metabolic process(L=2),GO:0044710-single-organism metabolic process(L=2),GO:0071704-organic substance metabolic process
(L=2),  GO:0043227-membrane-bounded organelle(L=2),GO:0043228-non-membrane-bounded organelle(L=2),GO:0043233-organelle lumen(L=2),GO:0044422-organelle part(L=2),GO:0044
464-cell part(L=2),     GO:0016491-oxidoreductase activity(L=2),
g60340.t1
```

--annotated-all-genes-only

Only genes that have an associated similarity search OR EggNOG annotation are kept. Done by the script task_scripts/annotated_all_genes_only.pl. Passing genes will remain in the gene table. Results of this filter are printed in the log.

--annotated-ss-genes-only

Only genes that have an associated similarity search annotation are kept. Done by the script task_scripts/annotated_ss_genes_only.pl. Passing genes will remain in the gene table. Results of this filter are printed in the log.

FILTER FLAGS THAT REQUIRE A FASTA

These scripts require that there is a fasta, because sequence is being evaluated. The gFACs.pl script will index your fasta, and then task scripts that require sequence will find and use that index. If there is already an index, the indexing step will be skipped.

To specify a fasta:

--fasta /path/to/your/nucleotide/fasta.fasta

Bioperl will create an index with the ending ".fasta.idx". It is a fairly fast process. The file may end with .fa or .fasta, but no other naming formats can be recognized.

NOTE: This fasta **MUST MUST MUST** be the same fasta used when making your particular gff3/gtf/gff. Bioperl needs to recognize the name on the fasta info line to the sixth column in the gene table.

--canonical-only

Analyzes introns for a canonical splice sites (GT-AG on the positive strand). The script that performs this task is task_scripts/canonical_only.pl.

To pass, **all** introns in a gene must have canonical splice sites. Monoexonics will also pass this filter because they do not have the evidence to be pulled out. Of course, monoexonic genes can be removed by –rem-monoexonics filter.

Genes that pass this filter are kept in the gene table and results are printed in the log.

NOTE: Splice sites take into account directionality and reverse compliment.

--rem-genes-without-start-codon

The first three nucleotides of the sequence are analyzed to match ATG. Currently, no alternate start codons are taken into account. This task is performed by task_scripts/rem_genes_without_start.pl. Again, gene directionality is considered.

Genes that pass this filter are kept in the gene table and results are printed in the log.

--rem-genes-without-stop-codon

The last three nucleotides of the sequence are analyzed to match TAA, TAG, and TGA. Currently, all end codons are assumed to be **within** the reported gene. This task is performed by task_scripts/rem_genes_without_stop.pl. Again, gene directionality is considered.

Following this step, a script called task_scripts/frame_detection.pl is run. It is designed to pick out any genes that technically have a stop codon as the last three nucleotides, but it is not real because the codon is actually out of frame. These are **rare** occurrences, often happening on negative strand genes that run into the beginning of a scaffold where the first three nucleotides of the scaffold are a reverse complement stop codon. To solve this, any gene whose CDS is *not* divisible by 3, is removed.

Genes that pass this filter are kept in the gene table and results are printed in the log.

--allowed-inframe-stop-codons [number]

Default: 0

Creates a filter that removes genes based on the presence of a stop codon that is **not** the last codon in the gene. For example, setting this parameter as 1 will allow one other stop codon between the methionine and the terminating stop codon.

If you are not filtering for start and stop codons, this will still work so long as there are stop codons within the amino acid sequence but not necessarily at the end.

Genes that pass this filter are kept in the gene table and results are printed in the log.

--splice-table

To understand splice usage, a splice-site table is printed to the log that tells the frequency of every type of splice site used. This command is performed by task_scripts/splice_table.pl.

The splice table will look something like this:

```
        gt_ag   113699  99.286%
        at_ac   39      0.034%
        gc_ag   779     0.680%
```

This splice table will show you *everything* present in the file adjusted to lower case letters including N-bases. If you specify canonical genes only, the table will only show you gt_ag counts.

--nt-content

The CDS (all exon sequences) is analyzes for GC, AT, and N content by percent composition. This information is printed to the log. Here is an example of the output:

```
GC content:     46.708%
AT content:     53.271%
N content:      0.021%
```

OUTPUT FLAGS THAT DO NOT NEED A FASTA

--statistics

Statistics will be run on the gene table and printed to statistics.txt. This command is performed by task_scripts/classic_stats.pl. If a prefix is used, the statistics file will be named accordingly.

These are all the potential statistics in the reported format:

Number of genes:
Number of monoexonic genes:
Number of multiexonic genes:

Number of positive strand genes:
Monoexonic:
Multiexonic:

Number of negative strand genes:
Monoexonic:
Multiexonic:

Average overall gene size:
Median overall gene size:
Average overall CDS size:
Median overall CDS size:
Average overall exon size:
Median overall exon size:

Average size of monoexonic genes:
Median size of monoexonic genes:
Largest monoexonic gene:
Smallest monoexonic gene:

Average size of multiexonic genes:
Median size of multiexonic genes:
Largest multiexonic gene:
Smallest multiexonic gene:

Average size of multiexonic CDS:
Median size of multiexonic CDS:
Largest multiexonic CDS:
Smallest multiexonic CDS:

Average size of multiexonic exons:
Median size of multiexonic exons:
Average size of multiexonic introns:
Median size of multiexonic introns:

Average number of exons per multiexonic gene:
Median number of exons per multiexonic gene:
Largest multiexonic exon:
Smallest multiexonic exon:
Most exons in one gene:

Average number of introns per multiexonic gene:
Median number of introns per multiexonic gene:
Largest intron:
Smallest intron:

If your set is only monoexonics, a smaller version of the statistics will be printed that only contain the categories where monoexonic genes are evaluated.

--statistics-at-every-step

A statistical analysis of the gene table is run following every *filtering* step. This information is in the same format as regular --statistics but prints to the log following the information line for each flag. To ensure statistics.txt is created at the end, make sure to include –statistics in your command.

OUTPUT FLAGS THAT REQUIRE A FASTA

--get-fasta-with-introns

The nucleotide fasta sequence is printed to genes_with_introns.fasta. The genes are always printed on the positive strand. This fasta will contain the **intron sequences** so number of genes printed will be the **same** for both with and without introns. The header for each sequence is the fifth column of the gene line in the gene table.

This command is performed by task_scripts/get_fasta_with_introns.pl. If a prefix is specified, the output fasta will be named accordingly.

--get-fasta-without-introns

The nucleotide fasta sequence is printed to genes_without_introns.fasta. The genes are always printed on the positive strand. This fasta will **not** contain the intron sequences. The header for each sequence is the fifth column of the gene line in the gene table.

This command is performed by task_scripts/get_fasta_with_introns.pl. If a prefix is specified, the output fasta will be named accordingly.

--get-protein-fasta

A protein fasta of the genes is created called genes_without_introns.fasta.faa. Genes never include the introns (because of course not). All genes are printed in the N-terminus to C-terminus orientation (so M would be first) but reverse complementation of the negative strand is considered to choose the correct amino acids. Stop codons are depicted as *. The header for each sequence is the fifth column of the gene line in the gene table.

This command is performed by task_scripts/get_protein_fasta.pl. If a prefix is specified, the output fasta will be named accordingly.

--create-gtf

A gtf file called out.gtf is created. If a prefix is specified, the gtf file will have it. This step is done with two scripts, task_scripts/add_start_stop_to_gene_table.pl and task_scripts/gtf_creator.pl.

Since GTF files (as a general rule) require start and stop codon information, the locations of the start and stop codon (if found) are added to the gene table and the final gtf. CDS scores that correspond to an exon are retrieved from the original input file if found and the “exon” attribute is returned to “CDS”. Introns currently remain.

Here is an example of what the gtf looks like:

```
scaffold124501  GSTATS  gene        5314    6235    .       -       .       ID=g28956.t1;geneID=g28956
scaffold124501  GSTATS  start_codon     6233    6235    .       -       .       .
scaffold124501  GSTATS  stop_codon      5314    5316    .       -       .       .
scaffold124501  GSTATS  CDS         5314    5707    0.61    -       .       Parent=g28956.t1
scaffold124501  GSTATS  CDS         5799    6235    0.73    -       .       Parent=g28956.t1
scaffold124501  GSTATS  intron      5708    5798    .       -       .       .
scaffold124501  GSTATS  gene        16664   17628   .       +       .       ID=g28959.t1;geneID=g28959
scaffold124501  GSTATS  start_codon     16664   16666   .       +       .       .
scaffold124501  GSTATS  stop_codon      17626   17628   .       +       .       .
scaffold124501  GSTATS  CDS         16664   17047   0.94    +       .       Parent=g28959.t1
scaffold124501  GSTATS  CDS         17200   17628   0.59    +       .       Parent=g28959.t1
scaffold124501  GSTATS  intron      17048   17199   .       +       .       .
```

The source line does say gFACs. Not to steal the credit, it just might be helpful to know where the information is coming from particularly after filtering and rearranging.

DISTRIBUTIONS

--distributions [option] [option] …

Activates the ability to create distributions. This task is always done last on the final version of the gene table. If a prefix is specified, all output files will reflect that.

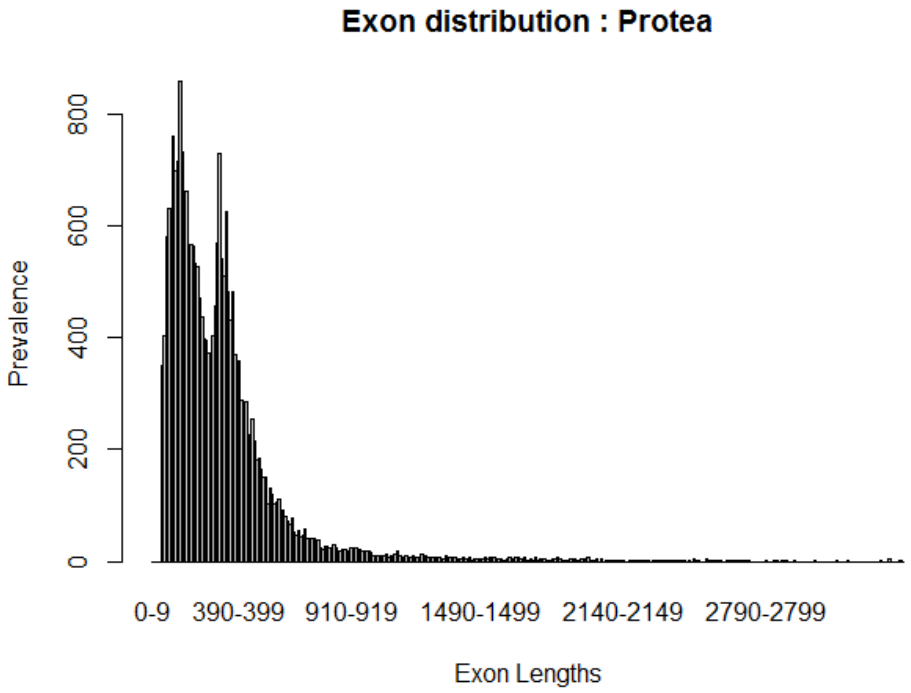
All outputs are printed in a .tsv file that can be opened for viewing on excel or R. The options available for distributions are as follow:

exon_lengths

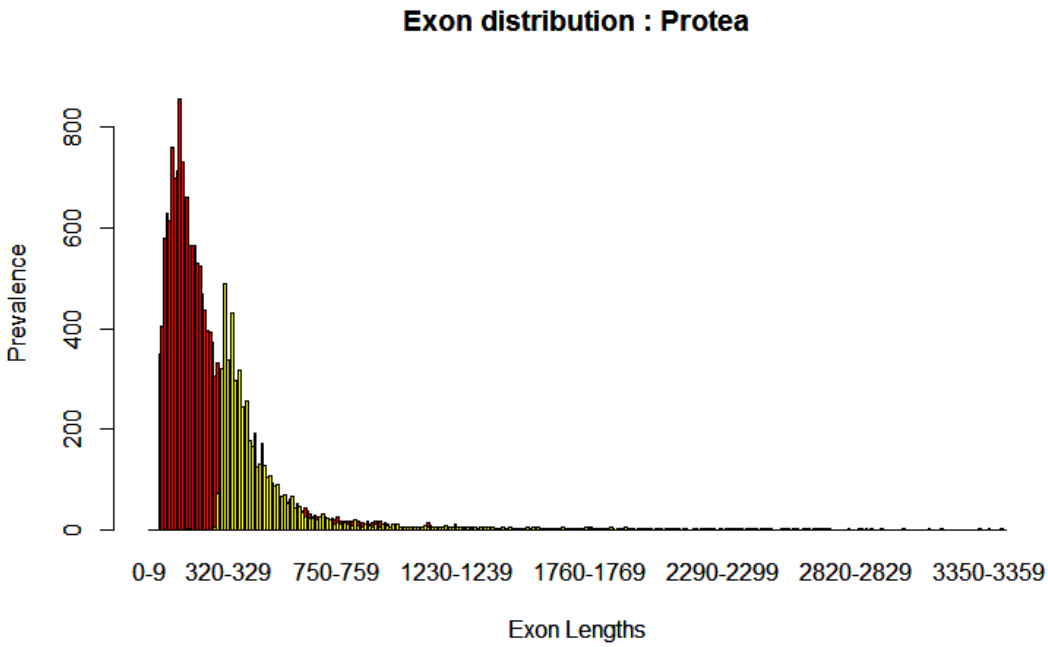
Creates the file exon_lengths_distributions.tsv. In it, a range of exon lengths and the corresponding representation is printed. In this example, --min-exon size was set to 40, which is reflected in the numbers:

exon_lengths	N
0-9	0
10-19	0
20-29	0
30-39	0
40-49	350
50-59	404
60-69	580
70-79	630
80-89	614
90-99	760

The above data, when rendered into a histogram using R, looks like this:



Notice that the curve is bimodal, which is indicative of the mono and multiexonic genes. Utilizing two runs one with --rem-monoexonics (red) and one with --rem-multiexonics (yellow) you can see the curves are indeed the difference in gene type where smaller exon lengths are in multiexonic genes:



Advanced: Zoom of exon lengths can be controlled with a trailing number. This changes the size of the step. In the example above, the range of values as the cluster for the distribution is 10, but it can be controlled like this:

exon_lengths 5

This would change the above table to:

exon_lengths	N
0-4	0
5-9	0
10-14	0
15-19	0
20-24	0
25-29	0
30-34	0
35-39	0
40-44	185
45-49	165
50-54	217
55-59	187
60-64	286
65-69	294
70-74	302
75-79	328
80-84	303
85-89	311
90-94	374

The default, if no number is chosen, is decided by the maximum exon length of the provided data. For a maximum length that is less than 100 nucleotides, the step is 1. For a maximum value of exon length that is more than 100 but less than 1,000, the step is 10 and so on.

Changing this step number should not *drastically* change the time it takes to run. However, the file will be larger and have more lines when a smaller number is used!

intron_lengths

Creates the file intron_lengths_distributions.tsv. In it, a range of intron lengths and the corresponding representation is printed. The outputs and applications are identical to exon_lengths.

Advanced: Zoom of intron lengths can be controlled with a trailing number. This changes the size of the step. It can be controlled like this:

intron_lengths 20

The default, if no number is chosen, is decided by the maximum intron length of the provided data. For a maximum length that is less than 100 nucleotides, the step is 1. For a maximum value of intron length that is more than 100 but less than 1,000, the step is 10 and so on.

Changing this step number should not *drastically* change the time it takes to run. However, the file will be larger and have more lines when a smaller number is used!

CDS_lengths

Creates the file CDS_lengths_distributions.tsv. In it, a range of CDS lengths and the corresponding representation is printed. The outputs and applications are identical to exon_lengths.

Advanced: Zoom of CDS lengths can be controlled with a trailing number. This changes the size of the step. It can be controlled like this:

CDS_lengths 25

The default, if no number is chosen, is decided by the maximum CDS length of the provided data. For a maximum length that is less than 100 nucleotides, the step is 1. For a maximum value of CDS length that is more than 100 but less than 1,000, the step is 10 and so on.

Changing this step number should not *drastically* change the time it takes to run. However, the file will be larger and have more lines when a smaller number is used!

gene_lengths

Creates the file gene_lengths_distributions.tsv. In it, a range of gene lengths and the corresponding representation is printed. These sequence lengths do include all introns. The outputs and applications are identical to exon_lengths.

Advanced: Zoom of gene lengths can be controlled with a trailing number. This changes the size of the step. It can be controlled like this:

gene_lengths 1000

The default, if no number is chosen, is decided by the maximum gene length of the provided data. For a maximum length that is less than 100 nucleotides, the step is 1. For a maximum value of gene length that is more than 100 but less than 1,000, the step is 10 and so on.

Changing this step number should not *drastically* change the time it takes to run. However, the file will be larger and have more lines when a smaller number is used!

exon_position

Analyzes and creates an output that evaluates exon position in a gene to its size. Position meaning which exon comes first. In positive strand genes, these are in the order they appear in the gene table. For reverse strand genes, the first exon is the last one to appear in the gene table.

Creates the output file exon_position_distributions.tsv. The output looks like this:

exon_position	n(Individual exons)	n(Max exons in gene)	average_size	median_size	Min	Max
1	70923	28762	335.161 294	20	4955	
2	42161	19901	229.762 170	20	4975	
3	22260	8596	212.085 146	20	3210	
4	13664	4376	195.893 131	20	8028	
5	9288	2557	177.604 119	20	4056	
6	6731	1656	172.398 114	20	6544	

Exon position goes from 1 to whatever the maximum number of exons in one gene is. It will match what a statistics output would say. The second column is how many exons are representative of that position. The first exon support (70,923 above) will always be equal to the overall number of genes because even monoexonics have a first exon. (You can remove those, of course). You can also say there are 70,923 *first* exons, 42,161 *second* exons, etc…

The third column is how many genes have the first column number as their *maximum* number of exons. So, in the last row shown, there are 1,656 genes that have 6 *total* exons. There are 28,762 monoexonics then as well by this same logic.

The third and fourth columns are average and median size of an exon at that positon. The last two are minimum and maximum. If you use a minimum exon parameter (as I did above) it will be reflected!

exon_position_data

Provides the raw data in data_intron_position_distributions.tsv on exon positions alongside exon_position_distributions.tsv produced from the command above. This set of data can be used to make boxplots.

The data appears like this:

1	4379	694	117	806	993	605	492	102	106	93	5699	4578	593	1578	378	886	922	944	83	709
2	664	115	82	229	2882	444	115	240	4030	745	80	197	211	771	98	4333	8060	9910	189	531
3	383	2926	351	6307	96	202	1133	138	456	102	1067	140	276	743	191	2031	1309	1299	131	1567
4	98	455	78	1150	1413	1071	104	93	526	176	199	197	5313	2038	1641	179	1525	3193	126	4120
5	570	137	99	115	93	125	123	108	115	312	1904	622	104	613	198	83	490	6888	483	1712
6	430	97	120	104	1760	81	157	96	83	695	76	606	409	83	126	1329	96	190	1807	148
7	228	125	547	325	553	2870	87	167	2959	1358	90	146	167	332	1133	89	84	6996	149	524
8	136	533	356	3615	454	15203	923	148	211	88	115	6295	701	83	94	380	2746	133	4643	440
9	369	707	801	93	112	119	178	160	566	87	136	8994	189	167	1147	5560	82	78	2175	124
10	79	328	1341	2514	87	313	412	84	222	162	121	81	934	98	86	779	314	217	135	218
11	152	127	423	111	196	7459	242	383	3965	214	95	482	120	1338	1624	158	1657	108	184	88
12	96	252	923	122	476	89	101	117	530	103	889	1796	701	879	2862	288	135	3676	95	1508
13	1883	205	15194	881	782	6060	2702	94	269	82	140	113	3335	90	672	358	160	2220	116	2187
14	76	88	1756	94	275	153	59	91	92	417	82	413	4907	80	2328	104	93	829	103	395
15	678	653	100	107	101	740	86	1058	103	101	112	596	257	200	119	393	278	198	11154	1714
16	1420	116	96	106	416	407	172	4972	79	82	113	1229	1103	128	88	93	136	1325	1007	153
17	71	105	767	234	986	102	89	153	425	93	287	116	470	913	920	91	103	89	105	83
18	93	135	235	129	670	85	136	114	141	517	162	239	428	70	235	624	893	91	191	431
19	928	165	553	344	185	84	1152	100	768	369	94	88	322	75	4456	915	1771	310	1654	1027
20	106	5654	90	90	126	436	7128	620	1961	839	275	775	1039	198	105	145	996	90	100	90
21	4446	2071	94	1636	775	2233	2396	43	4235	132	162	149	433	116	765	85	2750	102	586	79
22	192	80	134	399	100	127	122	91	127	138	1718	646	3001	176	99	1744	134	73	5776	128
23	286	172	654	997	84	91	86	89	664	117	70	114	105	80	559	1526	79	154	102	3086
24	97	298	117	416	1032	77	271	1423	437	432	1681	553	108	3784	298	149	97	96	84	120
25	998	210	1651	130	142	85	1279	81	410	110	90	2690	3853	106	84	188	87	108		
26	888	788	253	95	1946	121	921	2258	115	86	89	1032	178	1806	569	299				
27	181	80	3183	603	1046	784	227	115	79	173	133	1440	5005							
28	154	439	125	1410	131	92	78	240	288	110	9226									
29	236	152	86	100	98	104	103	80	6718											
30	87	91	93	1894	93	485	110	137	3452											
31	689	122	84	90	1908	96	4998													
32	104	94	107	16344	2053															

The first column is the exon position and the following values in the row are the sizes of exons (non-sorted). The row will have as many columns as exon position data points. Notice how 32 (the last visible row) has only 5 numbers, showing there are only 5 genes that have a 32nd exon where the values are the sizes.

intron_position

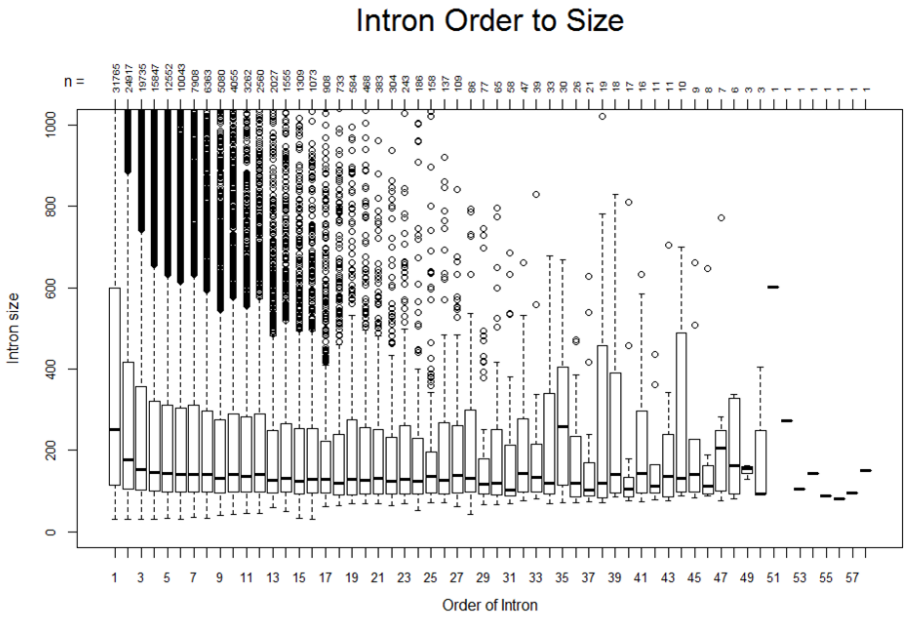
Intron positioning works identically to exon positions. However, this will only include multiexonic genes! All header names have the same meaning as exon position.

Creates the output file intron_position_distributions.tsv.

intron_position_data

Intron position raw data works identically to exon positions and will also only include multiexonic genes. Creates the output file data_intron_position_distributions.tsv and the default intron_position_distributions.tsv.

A sample of a boxplot that can be created:



*For how I created the boxplot, feel free to contact me!

Full run example:

```
#!/bin/bash
#SBATCH --job-name=gFACs
#SBATCH --qos=general
#SBATCH --mail-type=ALL
#SBATCH --mail-user=email@uconn.edu
#SBATCH -o gFACs_%j.o
#SBATCH -e gFACs_%j.e


module load perl

perl gFACs.pl \
-f gffread_0.9.12_gff3 \
--statistics \
-p gff3_test \
--splice-rescue \
--rem-start-introns \
--rem-monoexonics \
--rem-end-introns \
--canonical-only \
--splice-table \
--min-exon-size 40 \
--min-intron-size 70 \
--min-CDS-size 100 \
--unique-genes-only \
--rem-genes-without-start-codon \
--rem-genes-without-stop-codon \
--get-fasta-without-introns \
--get-fasta-with-introns \
--get-protein-fasta \
--create-gtf \
--distributions exon_lengths 10 intron_lengths 100 intron_position_data \
--entap-annotation /path/to/my/ENTAp/file.tsv \
--annotated-ss-genes-only \
--fasta /path/to/my/fasta.masked.3k.fasta \
-O /path/to/where/I/want/the/output/ \
/path/to/my/gffread_0.9.12.gff3
```

SUPPORT SCRIPTS

Found within the folder support_scripts/

FORMAT DETERMINATION: format_diagnosis.pl

To determine what format you have, if it is ambiguous, can be done with format_diagnosis.pl. The script will output information that you can compare with the table below to see if another format may work for you.

To use the script:

$ perl format_diagnosis.pl [input_file]

The output will look something like this:

```
-bash-4.2$ perl format_diagnosis.pl /UCHC/LABS/Wegrzyn/gstats/format_dropoff/trimmed_i_trans_gstats.gff3
Format  partition       gene    mRNA    exon    CDS     intron  start_codon     stop_codon      Other third column
Your input      no[0]   yes[278]        yes[278]        yes[1583]       yes[1609]       no[0]   no[0]   no[0]   ()
```

The data tells you what information is present followed by the observed quality of the feature. In the above output, the line of the “gene” feature, comes up 278 times and matches that with mRNA. This is not always the case. It also has exon lines and CDS lines but NOT at the same frequency. So CDS will be the more important feature.

Given the comparison, you could choose several formats that might work. braker_2.05_gff3, braker_2.05_gtf, gFACs_gtf, and several more.

FORMATS properties:

Format	partition	gene	mRNA	exon	CDS	intron	start_ codon	stop_ codon	Other third column
gmap_2017_03_17_ gff3	Yes [6026]	Yes [6026]	Yes [6026]	Yes [31116]	Yes [30948]	No [0]	No [0]	No [0]	()
braker_2.05_gff3	No [0]	Yes [37757]	Yes [39297]	Yes [137280]	Yes [137280]	Yes [104809]	Yes [32571]	Yes [32936]	(initial single internal terminal)
braker_2.05_gff	No [0]	Yes [37757]	No [0]	No [0]	Yes [137280]	Yes [104809]	Yes [32571]	Yes [32936]	(terminal internal initial transcript single)
braker_2.05_gtf	No [0]	Yes [37757]	No [0]	Yes [137280]	Yes [137280]	Yes [104809]	Yes [32571]	Yes [32936]	(initial single transcript terminal internal)
braker_2.0_gff3	No [0]	Yes [60358]	Yes [63356]	Yes [270267]	Yes [270267]	Yes [209203]	Yes [61583]	Yes [61894]	()
braker_2.0_gff	No [0]	Yes [60358]	No [0]	No [0]	Yes [270267]	Yes [209203]	Yes [61583]	Yes [61894]	(transcript)

Format	partition	gene	mRNA	exon	CDS	intron	start_ codon	stop_ codon	Other third column
braker_2.0_gtf	No [0]	Yes [60358]	No [0]	Yes [270267]	Yes [270267]	Yes [209203]	Yes [61583]	Yes [61894]	(transcript)
maker_2.31.9_gff	Yes [118474]	Yes [34322]	Yes [34322]	Yes [64118]	Yes [64118]	No [0]	No [0]	No [0]	(translated_nucleotide_match contig three_prime_UTR five_prime_UTR expressed_sequence_match match_part protein_match)
genomethreader_1.6 .6_gff3	Yes [598452]	Yes [598452]	Yes [816371]	Yes [1182140]	Yes [511162]	No [0]	No [0]	No [0]	(three_prime_cis_splice_site five_prime_cis_splice_site)
gffread_0.9.12_gff3	No [0]	No [0]	Yes [44128]	Yes [90451]	Yes [90451]	No [0]	No [0]	No [0]	()
exonerate_2.4.0_gff	No [0]	Yes [2126]	No [0]	Yes [3843]	Yes [3843]	Yes [1717]	No [0]	No [0]	(similarity splice5 splice3)
EVM_1.1.1_gff3	No [0]	Yes [20678]	Yes [20678]	Yes [66408]	Yes [66408]	No [0]	No [0]	No [0]	()
gFACs_gtf	No [0]	Yes [42798]	No [0]	No [0]	Yes [110386]	Yes [67588]	Yes [42798]	Yes [42798]	()
refseq_gff	Yes [1]	Yes [54981]	Yes [60516]	Yes [413261]	Yes [318259]	No [0]	No [0]	No [0]	(match region sequence_feature cDNA_match lnc_RNA pseudogene primary_transcript rRNA transcript tRNA miRNA)

NOTE: In the above table, know that each format example is NOT the same file. The information inside the [brackets] should be seen as a ratio within a format. **Your file will not fit the actual numbers in the brackets above**, but the **ratio** between a format's exon to CDS counts may be the same. These were derived from my own collection of sample files across different species and projects.

OVERVIEW FILTERING: pseudo_gFACs.pl

Before committing to a gFACs run, it may be important to understand what gFACs is planning on removing from the original set of models. When gFACs filters, it removes sequentially from a shrinking pool of models. However, it may be important to know how many gene models *would* be removed from the **original** set. How many overall do not have a stop codon? How many overall are of a certain CDS length?

Pseudo gFACs runs identically to gFACs but only implements the filtering flags and always resets to the original set of genes in the gene table. Therefore, the log will print what is removed and retained but never follows through with filtering. Resulting files will be the gene table completely unedited and the typical log but with resetting numbers.

It is run identical to gFACs.pl and can also print the manual by running the script with no arguments.

NOTE: pseudo_gFACs.pl does **NOT** keep the results of splice rescue nor unique genes only. This is problematic with **overlap** when isoforms or multiple RNA evidence models are within the same called gene. Sequence pull filters like in-frame stop codon, fasta creation, or analysis can then be

wrong. For example, when setting in-frame stops to 0, all genes with isoforms will print one after another in a long sequence string that will have as many stop codons as models presented. This may create the appearance of increased removal of models. To work around this problem, run classic gFACs on your set using splice rescue and unique genes only. Then run pseudo_gFACs on that parsed gene table output. Feel free to contact me if you have problems!