



# Canadian Bioinformatics Workshops

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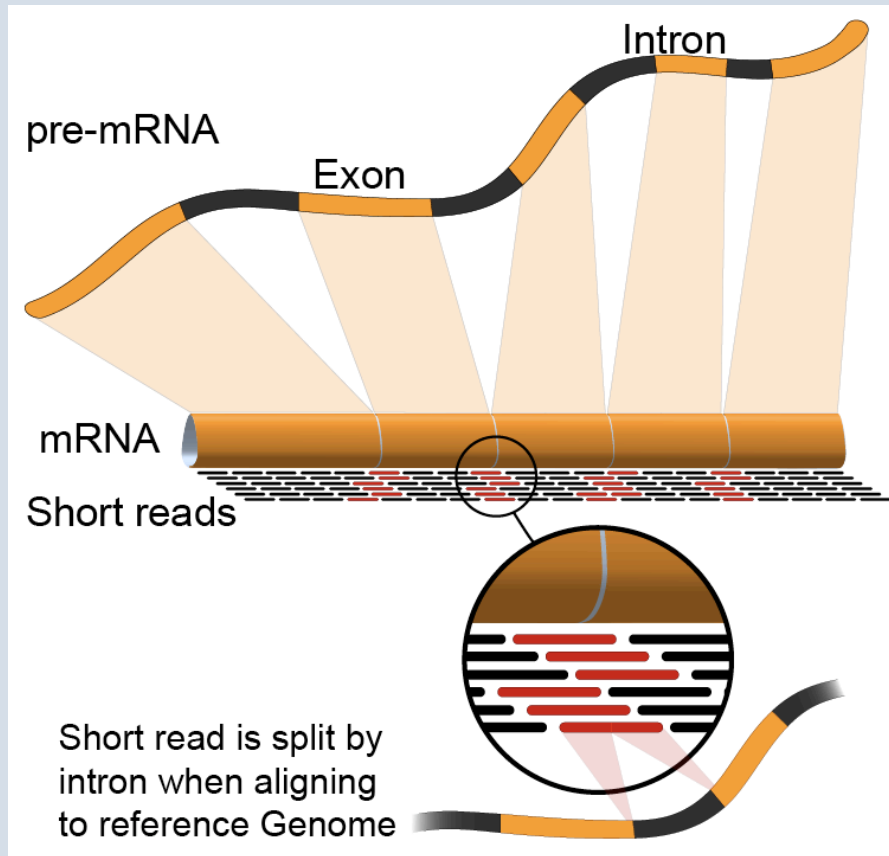
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# RNA-Seq Module 4

## Isoform Discovery and Alternative Expression (tutorial)

Malachi Griffith, Obi Griffith, Fouad Yousif  
Informatics for RNA-seq Analysis  
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# Learning Objectives of Tutorial

- Learn how to run Cufflinks in ‘reference only’, ‘reference guided’, and ‘de novo’ modes
- Learn how to use Cuffmerge to combine transcriptomes from multiple Cufflinks runs and compare assembled transcripts to known transcripts
- Learn how to perform differential splicing analysis with Cuffdiff
- Examine TopHat junctions counts and Cufflinks differential splicing files at the command line
- Visualize TopHat junction counts and Cufflinks assembled transcripts in IGV

# 5-i,ii. Running cufflinks in 'ref-guided' and 'de-novo' mode

- In Module 3 we ran cufflinks in 'ref-only' mode. This mode gives us an expression estimate for each known gene/transcript
- Now we want to be able to potentially identify novel genes, and novel isoforms of known genes
- To accomplish this we will re-run cufflinks in 'ref-guided' and 'de-novo' modes
  - In 'ref-guided' mode a known transcriptome will be used as a guide
  - In 'de-novo' mode no knowledge of the transcriptome will be used at all

# '-g', '-G' woe is me...

- tophat has a '-G' option
  - Used to supply a transcriptome GTF file
  - This will be used to **assist the alignment** step by allowing alignment to both transcriptome and genome sequences
  - Coordinates from alignments to transcriptomes will be converted back to genome coordinates
  - Even though we supply a transcriptome, tophat will not be limited in anyway to known transcripts
- tophat also has a '-g' option
  - Used to specify the maximum number of multiple mappings for a single read
- cufflinks has a '-G' option
  - Used to supply a transcriptome GTF file
  - If specified, cufflinks will quantitate against reference transcript annotations
  - We call this the 'ref-only' analysis mode
- cufflinks also has a '-g' option
  - Use to supply a transcriptome GTF file
  - Use reference transcript annotations to **guide assembly**
  - We call this 'reference-guided' analysis mode
- Running cufflinks with neither '-G' or '-g'
  - We call this 'de-novo' analysis mode
- cuffdiff requires a GTF file but it is not specified with a '-G' or '-g' option, but rather is simply supplied as a file path when you run cuffdiff

# The tophat 'junctions.bed' file

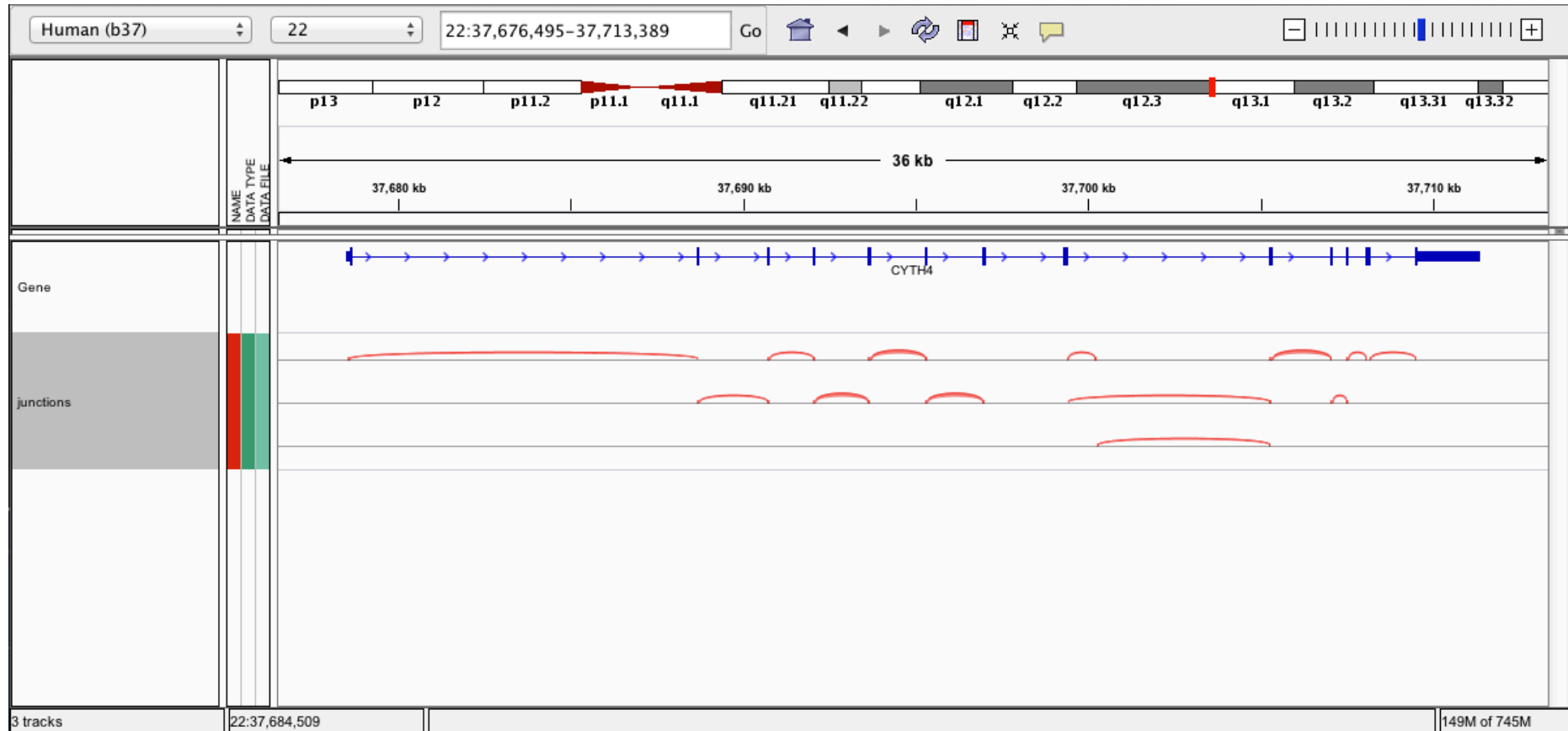
- After alignment, tophat creates a summary of all reads that support exon-exon junctions
  - e.g. exon1-exon2 has 5 reads
  - e.g. exon1-exon3 has 9 reads
- This file reports all of the unique exon-exon junctions observed and the read counts for each
  - In BED format

```
track name=junctions description="TopHat junctions"
22 17062079 17063415 JUNC00000001 3 - 17062079 17063415 255,0,0 2 98,19 0,1317
22 17092740 17095057 JUNC00000002 5 + 17092740 17095057 255,0,0 2 43,91 0,2226
22 17117940 17119543 JUNC00000003 6 + 17117940 17119543 255,0,0 2 40,75 0,1528
22 17152466 17156100 JUNC00000004 3 - 17152466 17156100 255,0,0 2 12,88 0,3546
22 17525819 17528242 JUNC00000005 1 + 17525819 17528242 255,0,0 2 71,29 0,2394
22 17528261 17538007 JUNC00000006 1 + 17528261 17538007 255,0,0 2 55,45 0,9701
22 17566071 17577976 JUNC00000007 10 + 17566071 17577976 255,0,0 2 48,25 0,11880
22 17577951 17578785 JUNC00000008 24 + 17577951 17578785 255,0,0 2 25,99 0,735
22 17578093 17578710 JUNC00000009 1 + 17578093 17578710 255,0,0 2 76,24 0,593
```



Junction read count

# Viewing the junctions.bed in IGV

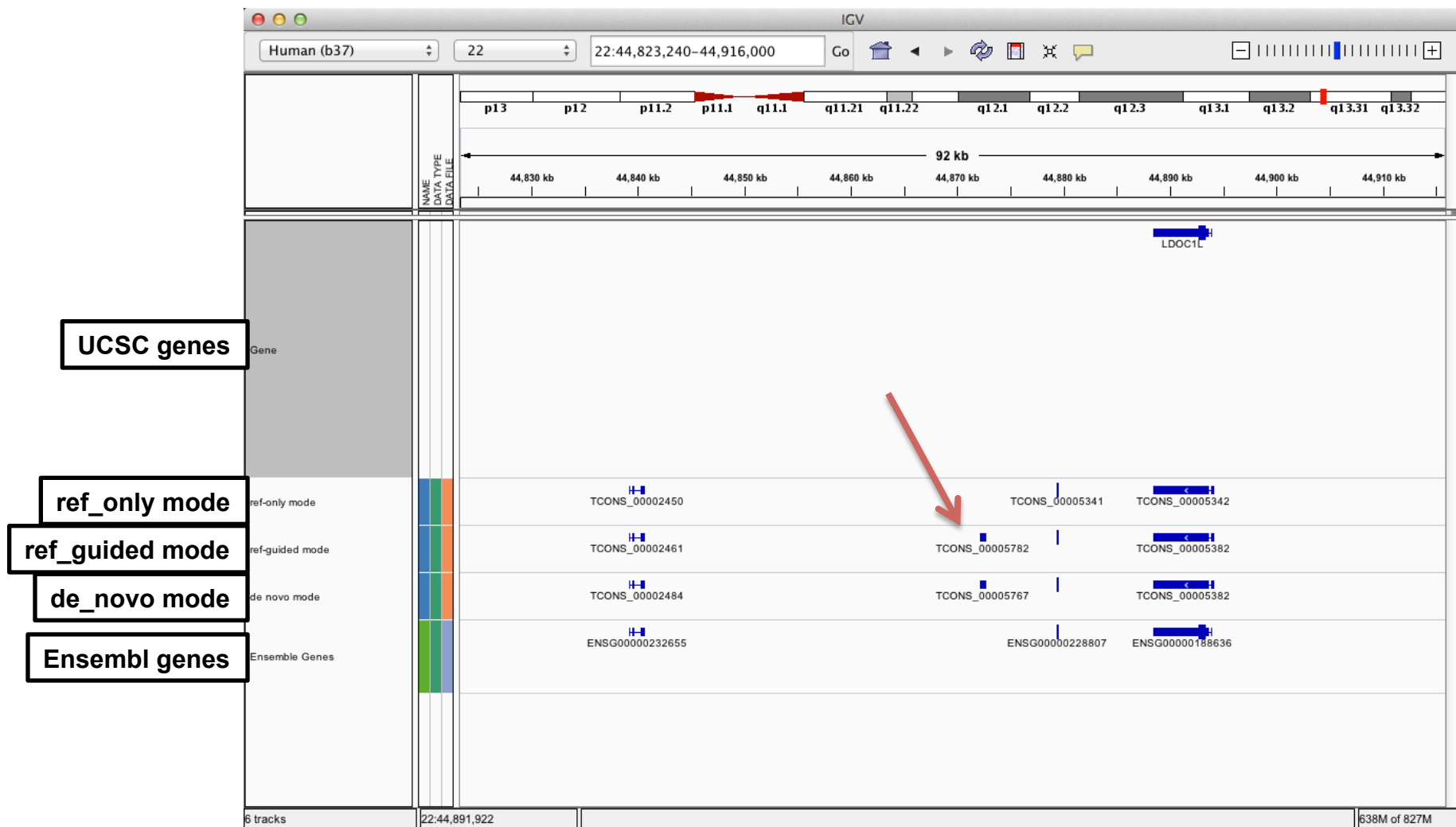




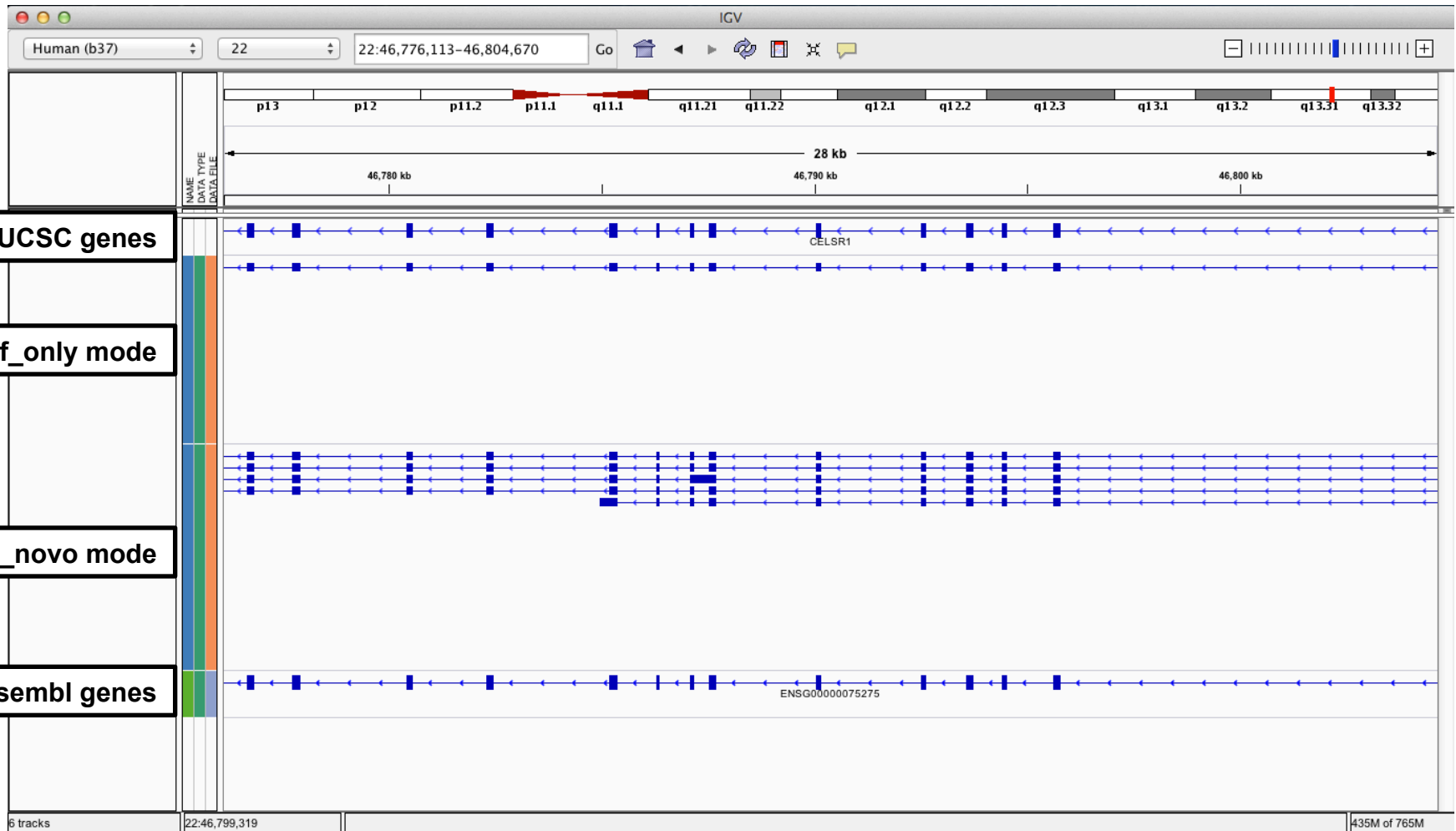
# 5-iii,iv. Cuffmerge

- <http://cufflinks.cbcb.umd.edu/manual.html#cuffmerge>
- Cuffmerge combines transcripts predicted from multiple RNA-seq data sets into one view of the transcriptome
  - Do this before running cuffdiff to compare between multiple conditions
- Cuffmerge can also simultaneously compare transcripts to the known transcripts GTF file from Ensembl, etc.
  - [http://cufflinks.cbcb.umd.edu/manual.html#class\\_codes](http://cufflinks.cbcb.umd.edu/manual.html#class_codes)

# 5-v. Comparison of merged GTFs from each cufflinks mode



# Comparison of merged GTFs from each cufflinks mode



# What if I return to my lab and can not get this to work on my own data?

- Refer to the materials provided with this course for clues
- Refer to the Nature Protocols tutorial (Trapnell et al. 2012)
  - In particular refer to the troubleshooting table (next slide)
- Search BioStars, SeqAnswers, and Google
  - <http://www.biostars.org/>
  - <http://www.seqanswers.com>
- If your question is not already answered on BioStars...
  - Ask it! Then follow up so that others that have the same problem in the future know whether this solution worked

# TopHat/Cufflinks/Cuffdiff troubleshooting table

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1	TopHat cannot find Bowtie or the SAM tools	Bowtie and/or SAM tools binary executables are not in a directory listed in the PATH shell environment variable	Add the directories containing these executables to the PATH environment variable. See the man page of your UNIX shell for more details
2	Cufflinks crashes with a 'bad_alloc' error Cufflinks takes excessively long to finish	Machine is running out of memory trying to assemble highly expressed genes	Pass the <code>-max-bundle-frags</code> option to Cufflinks with a value of <code>&lt;1,000,000</code> (the default). Try 500,000 at first, and lower values if the error is still thrown
5	Cuffdiff crashes with a 'bad_alloc' error Cuffdiff takes excessively long to finish	Machine is running out of memory trying to quantify highly expressed genes	Pass the <code>-max-bundle-frags</code> option to Cuffdiff with a value of <code>&lt;1,000,000</code> (the default). Try 500,000 at first, and lower values if the error is still thrown
	Cuffdiff reports FPKM = 0 for all genes and transcripts	Chromosome names in GTF file do not match the names in the BAM alignment files	Use a GTF file and alignments that has matching chromosome names (e.g., the GTF included with an iGenome index)

# We are on a Coffee Break & Networking Session

