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Advanced Sequencing Technologies & Applications

http://meetings.cshl.edu/courses.html

Cold Spring Harbor Laboratory

Module 4
Isoform discovery and alternative expression (tutorial)

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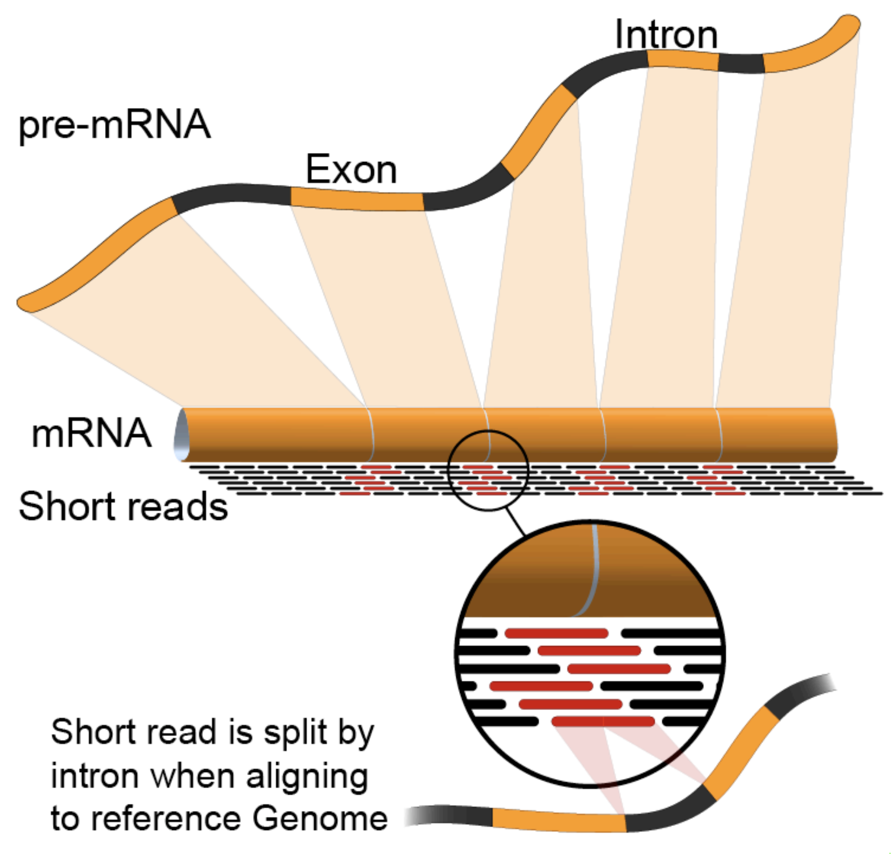
Advanced Sequencing Technologies & Applications
November 11-23, 2014


Intron
pre-mRNA
Exon
mRNA
Short reads
Short read is split by intron when aligning to reference Genome

THE GENOME INSTITUTE at Washington University

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

Running cuffinks in 'ref-guided' and 'de-novo' mode

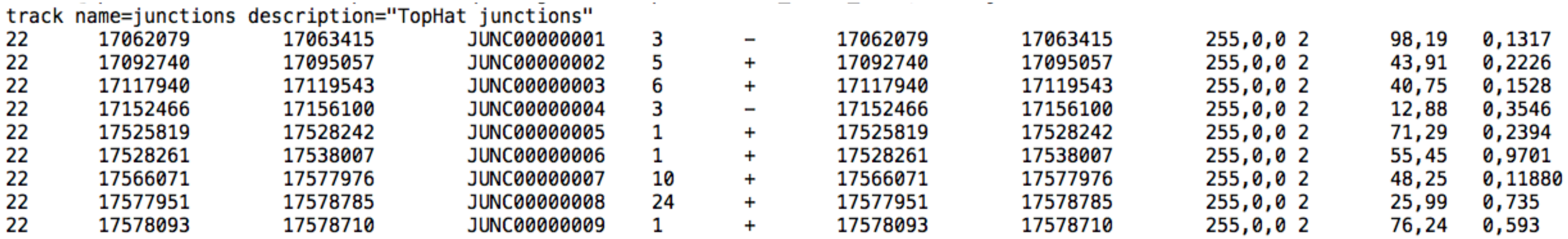
- In Module 3 we ran cufflinks in 'ref-only' mode. This mode gives us an expression estimate for each known gene/transcript
- Now we want to be able to potentially identify novel genes, and novel isoforms of known genes
- To accomplish this we will re-run cufflinks in 'ref-guided' and 'de-novo' modes
  - 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

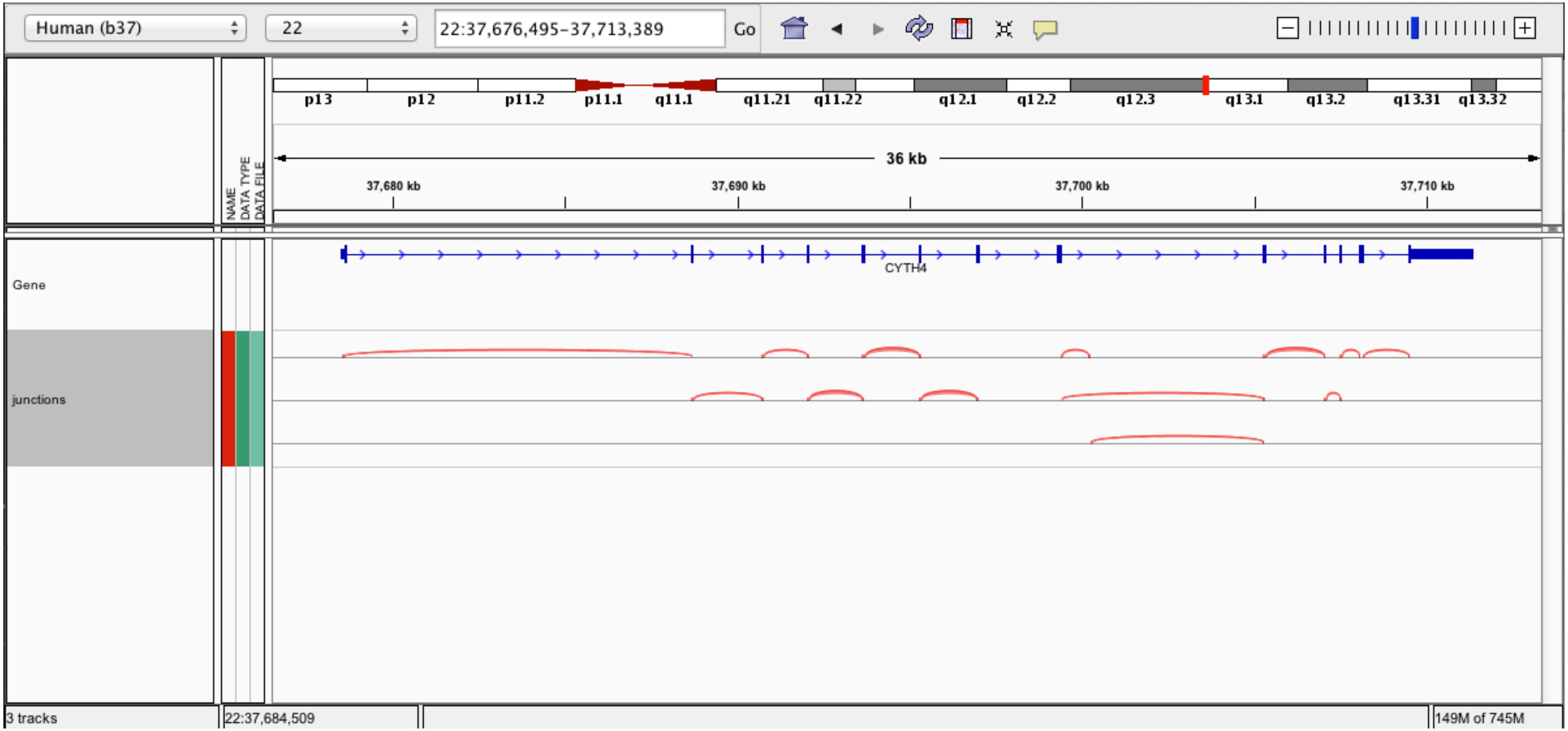
- After alignment, tophat creates a summary of all reads that support exon-exon junctions
  - e.g. exon1-exon2 has 5 reads
  - e.g. exon1-exon3 has 9 reads
- This file reports all of the unique exon-exon junctions observed and the read counts for each
  - In BED format



```
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

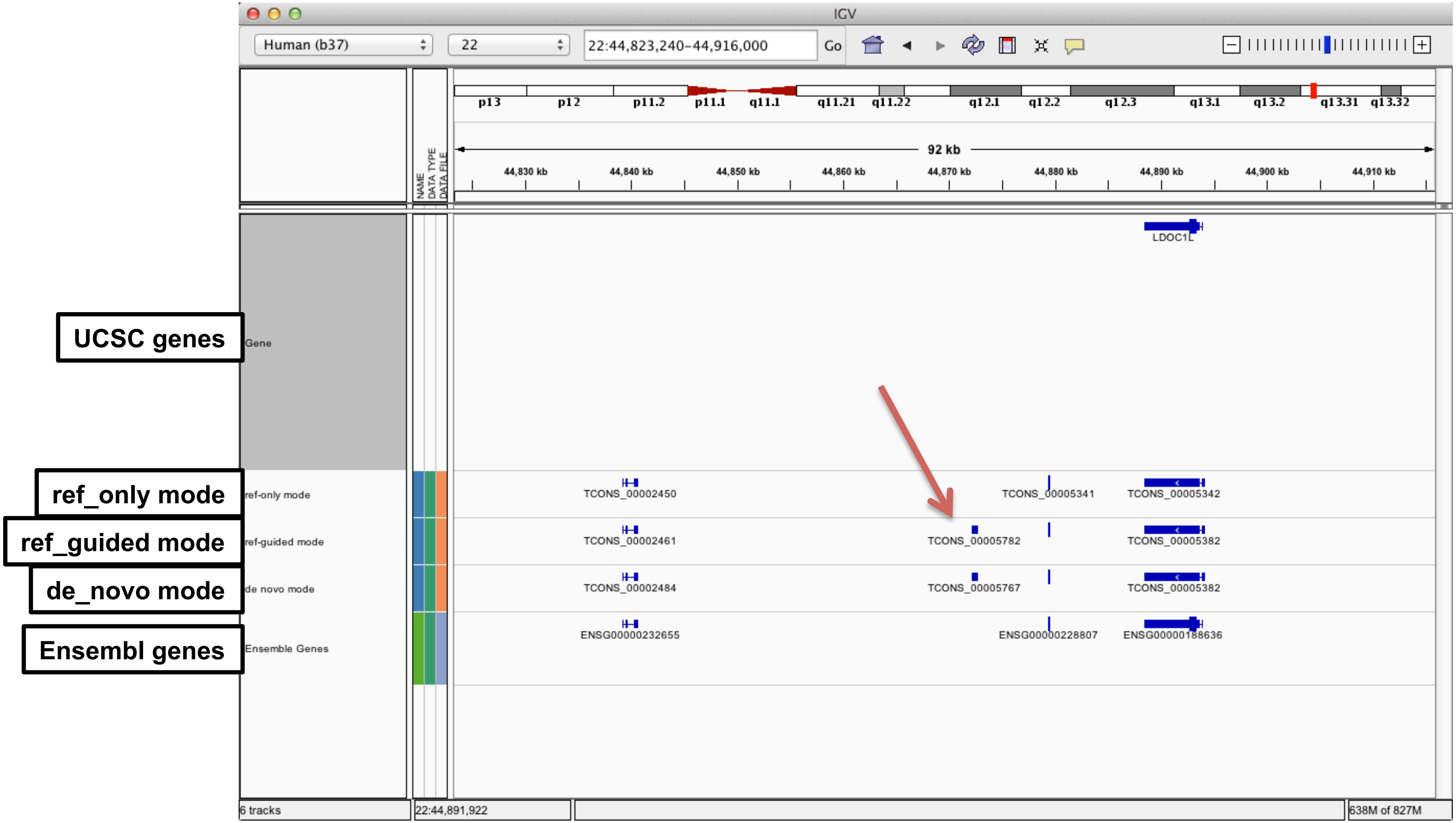


Cuffmerge

- 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

Comparison of merged GTFs from each cufflinks mode



UCSC genes

ref_only mode

ref_guided mode

de_novo mode

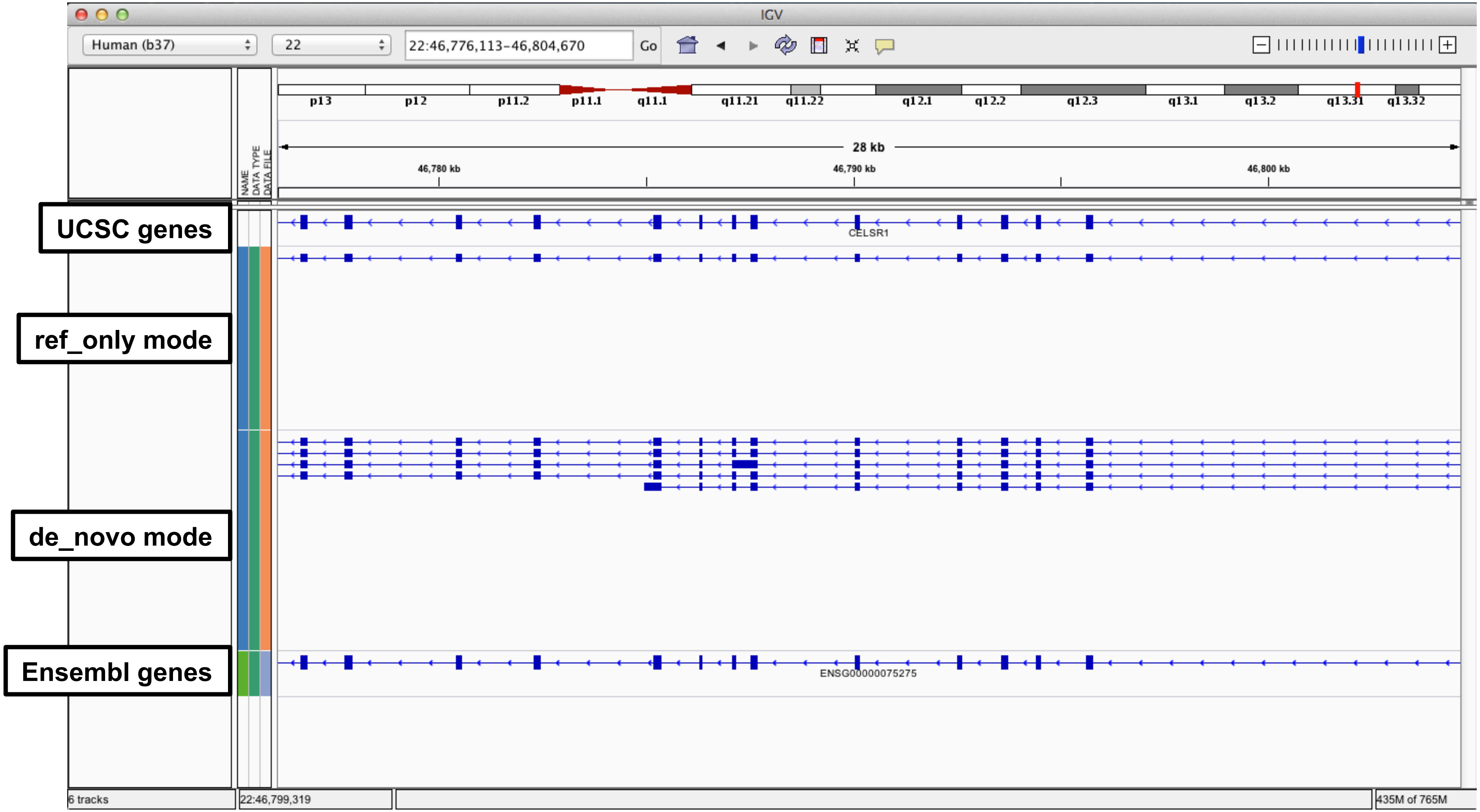
Ensembl genes

Human (b37)

22

22:44,823,240-44,916,000

Comparison of merged GTFs from each cufflinks mode



UCSC genes

ref_only mode

de_novo mode

Ensembl genes

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

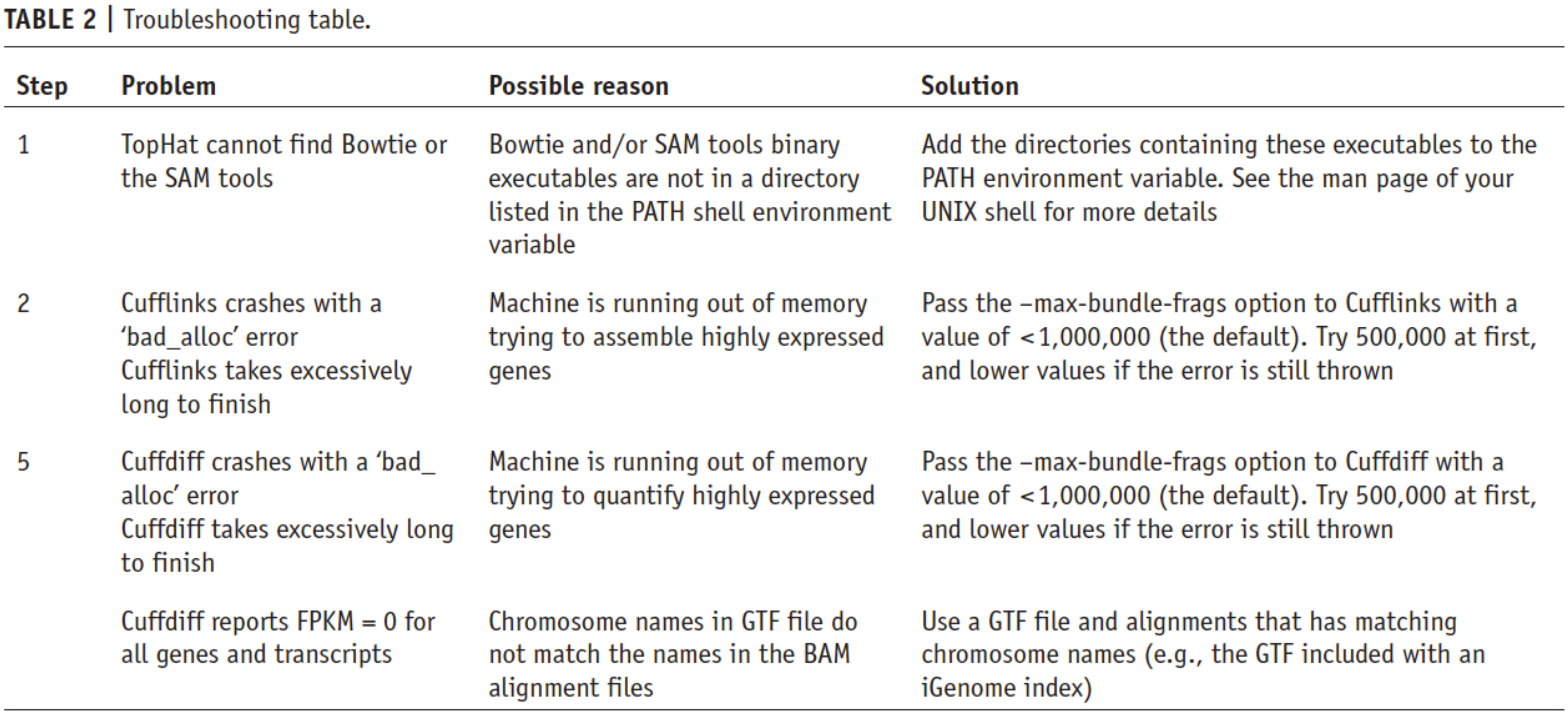


TABLE 2 | Troubleshooting table.

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 –max-bundle-frags option to Cufflinks with a value of <1,000,000 (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 –max-bundle-frags option to Cuffdiff with a value of <1,000,000 (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)

Break