Annotation of Conserved Motifs in *Drosophila*

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Prerequisites

- Annotation of Transcription Start Sites in *Drosophila*
- Introduction to Motifs and Motif Finding (Lecture)

Resources

Website	Web Address
FlyBase	https://flybase.org/
FlyFactorSurvey	https://mccb.umassmed.edu/ffs/
Patser	http://stormo.wustl.edu/consensus/

Files for this walkthrough

The package containing the files for this walkthrough are available for download through the "Annotation of Conserved Motifs in *Drosophila*" page on the GEP website.

Introduction

The *D. melanogaster* Muller F element is unusual because it appears to be packaged as heterochromatin, but the genes that reside in this domain exhibit expression patterns that are similar to euchromatic genes (RIDDLE *et al.* 2012). This dichotomy suggests that Muller F element genes either have some unique characteristics, or they have a more robust version of the gene activation machinery that enables them to function in a heterochromatic environment. For example, Muller F element genes might have distinct transcription factor binding sites (TFBS) near the transcription start sites (TSS) or they could simply have more enhancers around the TSS that facilitate the activation of a gene.

To begin to explore these hypotheses, the GEP Muller F element motif project will tabulate the types and the locations of known TFBSs within 2kb of the TSS of Muller F and Muller D element genes in multiple *Drosophila* species. Comparison of the motifs found in these two regions might identify motifs that are either enriched or depleted in Muller F element genes.

In this walkthrough, we will illustrate the protocol for annotating conserved motifs by searching for TFBSs near the promoter of *onecut* in the *D. biarmipes* project **contig35** [Aug. 2013 (GEP/Dot) assembly]. Because of the lack of experimental data for *D. biarmipes*, we can only infer the locations of TFBSs based on sequence conservation with *D. melanogaster*.

Identify the putative transcription start site of *onecut*

Before we can search for TFBSs, we need to annotate the coding exons and identify the putative TSS of the *onecut* gene. The TSS annotation protocol is described in the "Annotation of Transcription Start Sites in *Drosophila*" walkthrough. In the current walkthrough, we assume that the annotator has already produced the gene annotations for *onecut* on the *D. biarmipes* Muller F element project contig35. The *onecut* gene is on the minus strand on contig35 and the first transcribed exon (onecut:3) of the A isoform has been placed at 18924-21599. The TSS for both isoforms of *onecut* is placed at **21,599**.

Identify the transcription factor binding sites in *D. melanogaster*

Similar to the annotation of the coding and untranslated regions, the first step of our analysis is to determine the list of TFBSs that are present in the *D. melanogaster* gene. The modENCODE project has produced a map of TFBSs for 38 site-specific transcription factors in *D. melanogaster* early embryos. To summarize the TFBS results, the modENCODE project has also defined a list of High Occupancy Target (HOT) regions based on the number and the proximity of TFBSs in a given genomic region (NÈGRE *et al.* 2011).

To view the list of HOT regions for the *onecut* gene, open a new web browser window and navigate to FlyBase (https://flybase.org/). Enter "***onecut***" into the "Jump to Gene" (J2G) search box in the top navigation bar and then click "Go". Click on the "***GBrowse***" button under the "Genomic Location" section of the FlyBase Gene Report for *onecut* (Figure 1).

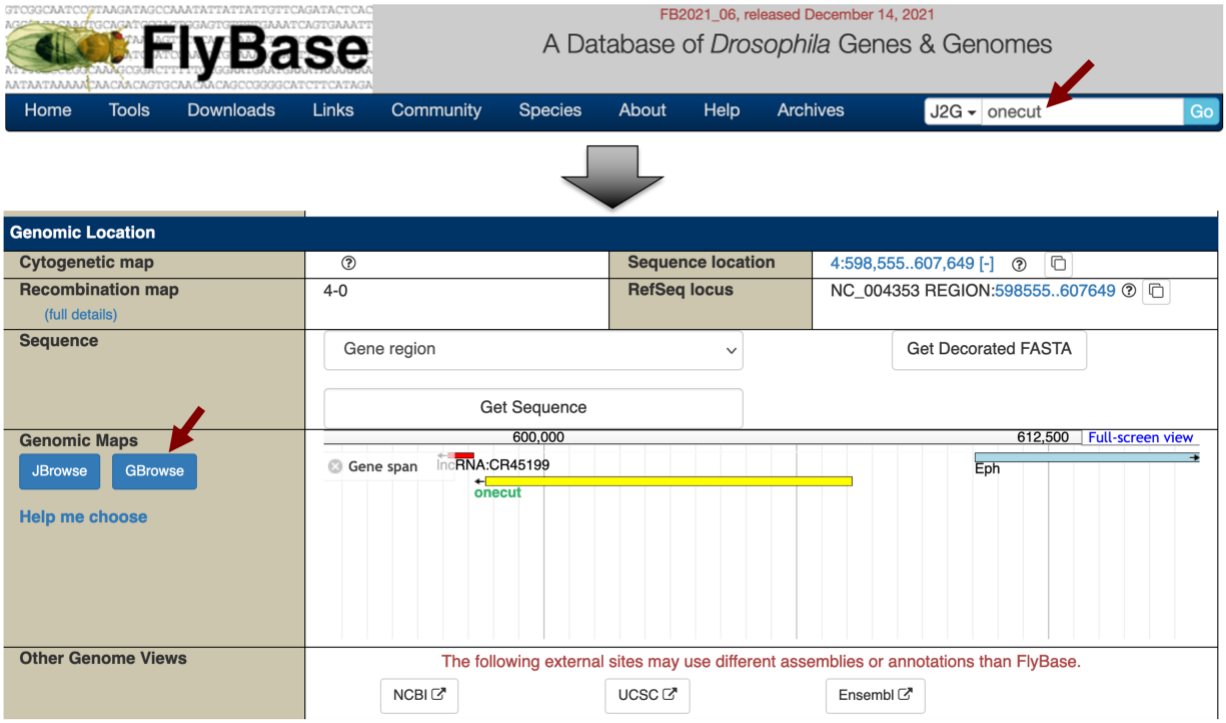


Figure 1 Search for the *onecut* gene on FlyBase and then click on the "*GBrowse*" button to view the genomic region surrounding the *onecut* gene in *D. melanogaster*.

Click on the "**Select Tracks**" tab (next to the "Browser" tab). Scroll down and select the "ChIP (whole embryo) - TF HOT spot analysis" track under the "Noncoding Features" section (Figure 2). Click on the "**Back to Browser**" link at the top of the page (Figure 2) or the "**Back to Browser**" button at the bottom of the page to return to the graphical view of *GBrowse*.

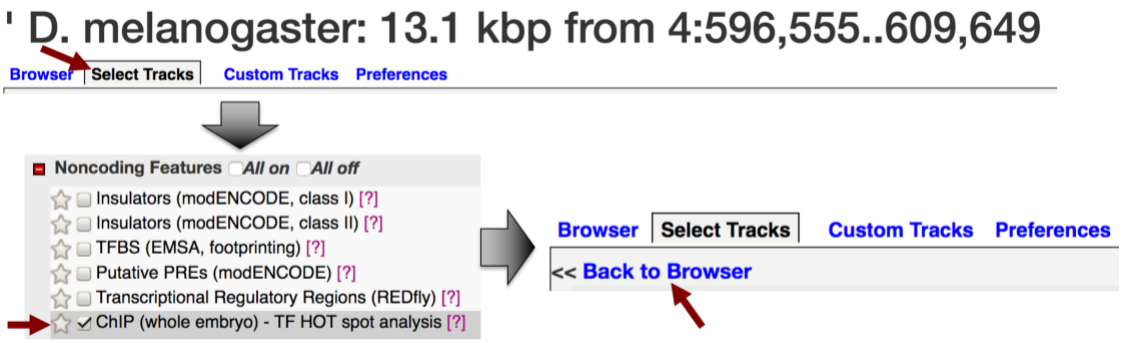


Figure 2 Enable the "ChIP (whole embryo) - TF HOT spot analysis" track on FlyBase *GBrowse*.

The "ChIP (whole embryo) - TF HOT spot analysis" track shows multiple HOT spots that overlap with the transcribed exons of the *D. melanogaster onecut* gene. There are also HOT spots in the regions immediately upstream and downstream of this gene. You can click on each of these HOT spots to view the complexity score and the transcription factors that are associated with each HOT spot (listed under Experimental Data ➔ Binding data). The complexity score of a HOT spot is correlated with the distribution and the number of transcription factor binding sites (TFBS). A HOT spot with more TFBSs typically has a higher complexity score than a HOT spot with fewer TFBSs [see (NÈGRE *et al.* 2011)].

When we hover the mouse over the HOT spot that overlaps with the TSS of *onecut* (at ~607kb), a tooltip appears which indicates that this HOT spot is a binding site for the transcription factors *GATAe*, *twist* (*twi*), and *dorsal* (*dl*) (Figure 3). By contrast, the HOT spot at ~609kb is a binding site for only the transcription factor *dl*. We can click on the links within the tooltip to learn more about each transcription factor from the FlyBase Gene Report. In this walkthrough, we will only explore one of these transcription factors (*dorsal*) in more detail. In your own projects, you should analyze all the transcription factors that are associated with each HOT spot.

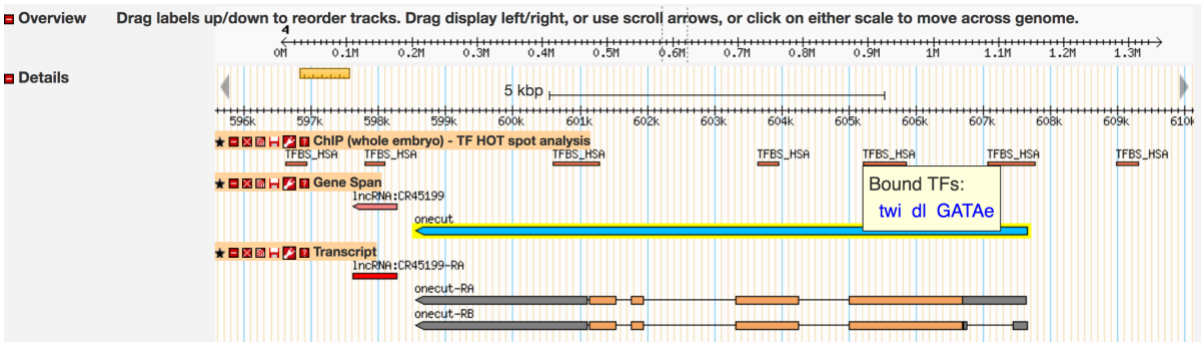


Figure 3 Transcription factor binding site HOT spots for the *D. melanogaster onecut* gene. One of the HOT spots (at ~607kb) overlaps with the 5' untranslated region of *onecut*, and is a binding site for *twist* (*twi*), *dorsal* (*dl*), and *GATAe*.

Using FlyFactorSurvey to determine the motif for the TFBS

Examination of the two HOT spots surrounding the TSS of *onecut* [one that overlaps with the 5' untranslated region (UTR) of *onecut* and one upstream of the TSS (at ~609kb)] shows that they both contain binding sites for *dl* (*dorsal*). To test whether these binding sites also exist in the *D. biarmipes* ortholog, we need to first determine the binding site motif for *dl* in *D. melanogaster*.

The FlyFactorSurvey database contains the binding site motifs for many transcription factors in *D. melanogaste*r. These motifs have been experimentally determined using the bacterial one-hybrid method (ZHU *et al.* 2011). To obtain the binding site motif for *dl*, navigate to the FlyFactorSurvey website at https://mccb.umassmed.edu/ffs/. Under the "Search For Transcription Factors" section, change the "Search Term" field to "**Gene Symbol**" and the "Search Value" to "**dl**". Then click on the "Search" button. Click on the magnifying glass icon under the "View" column to view the record (Figure 4).

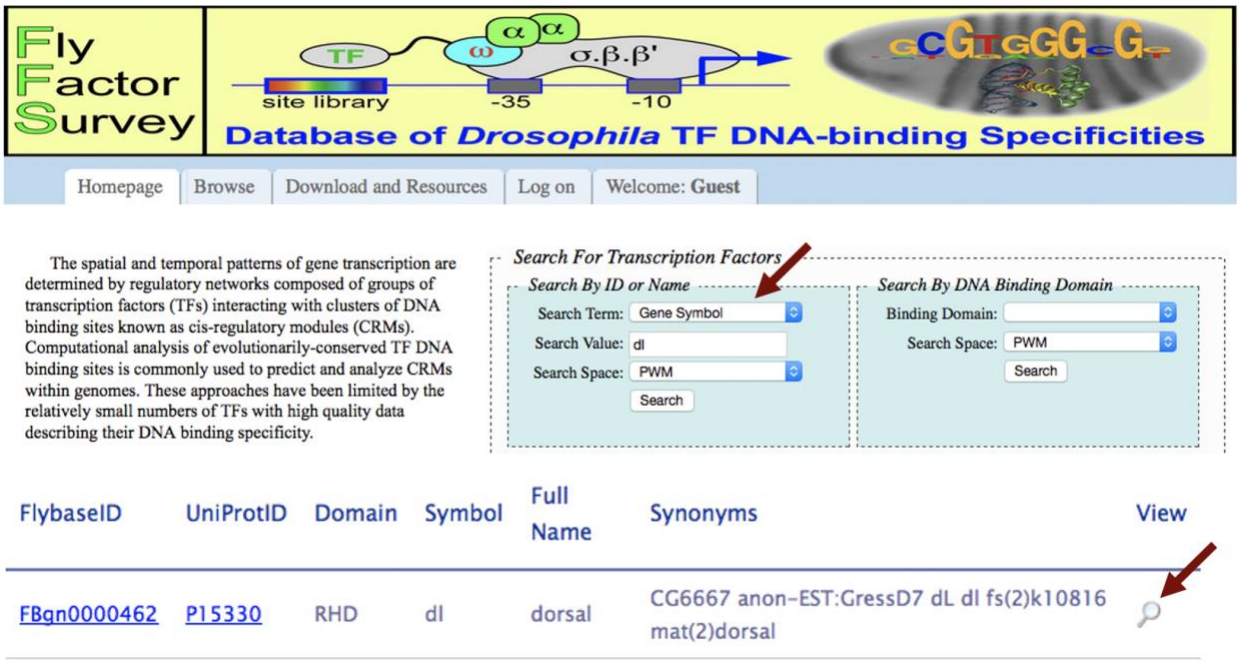


Figure 4 Use FlyFactorSurvey to determine the binding site for the transcription factor *dl* in *D. melanogaster*.

The Detail Viewer page shows two motifs for the TFBS of *dl* that were defined by two different studies. The "Source" field in the "Other Information" panel shows that the first motif is determined by the bacterial one-hybrid method (B1H) while the second motif is determined by DNase I footprinting. Each motif is shown as a sequence logo under the "Motif" column (SCHNEIDER and STEPHENS 1990). The sequence logo is constructed by aligning a collection of sequences (identified by B1H or DNase I, respectively) that contain a *dl* binding site. The *x*-axis corresponds to the aligned position of the nucleotides in the motif and the height of the letters correlate with the frequency of the nucleotide at each aligned position. For example, the sequence logo for the first *dl* motif (identified by B1H, Figure 5, top) shows that the first and second positions of the motif are always a G while the fourth and fifth positions have almost the same probability of being either an A or a T.

The total height of each column of the sequence logo corresponds to the information content (in bits) for that position. Since there are four nucleotides, the maximum amount of information for a position within a motif is two bits [i.e., $\log_2(4)$]. A motif position has two bits of information when all the motif instances used to construct the sequence logo have the same nucleotide at that position of the motif. The total information content of a motif corresponds to the sum of the information content for all the columns of a motif.

For the first *dl* motif, the sequence logo shows that the first position contributes ~1.8 bits of information (Figure 5, top). For the second *dl* motif, the sequence logo shows that the first position has lower information content than the first *dl* motif, as it contributes ~0.4 bits of information (Figure 5, bottom). When a motif is compared against a genome to identify potential binding sites, the alignment score for each position is weighted according to the information content of each column. For example, while the G nucleotide appears most frequently in the first column of both the first and second *dl* motifs, an alignment which contains a mismatch to the first column will incur a higher penalty for the first *dl* motif than then second *dl* motif.

Based on the total heights of the two sequence logos for *dl*, the motif defined by B1H (Figure 5, top) contains more information than the motif defined by DNase I (Figure 5, bottom). Since a motif with higher total information content produces fewer spurious matches than a motif with lower information content (i.e., has higher specificity), we will use the *dl* motif defined by B1H in this exercise.

We can retrieve the count matrix that was used to construct the sequence logo of the first *dl* motif by clicking on the "**Horizontal Count**" button (Figure 5). Save this file on your Desktop. The count matrix shows the frequency of each nucleotide at each position of the motif. Depending on your web browser settings, the file might be saved automatically in your **Downloads** folder. (For teaching purposes, we have included the matrix file **UmassPGFE_PWMfreq_dl_NBT_Horizontal.txt** in the exercise package.)

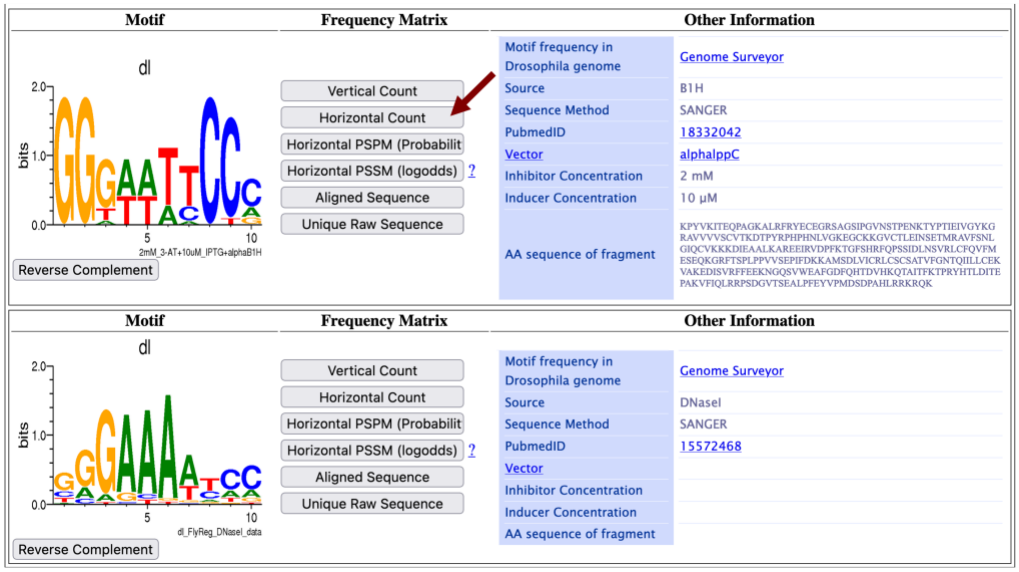


Figure 5 FlyFactorSurvey shows two different motifs for the TFBS of *dl*.

Using *Patser* to search for TFBS in *D. biarmipes*

Because the sequence logo shows that the *dl* TFBS consists of a short and degenerate sequence, we cannot use *blastn* to find the putative binding sites for *dl* in our *D. biarmipes* project. In addition, we will find many spurious matches in our search results when searching for TFBSs because short sequences are more likely to occur by chance. Hence we need to use a tool that can identify the subset of matches that are statistically significant.

We will use *Patser* (http://stormo.wustl.edu/consensus/) to search for potential binding sites of *dl* in the region surrounding the TSS of the *onecut* ortholog in *D. biarmipes*. Because two of the *dl* binding sites overlap with the initial exon (i.e., onecut:3) of the A isoform of *onecut* in *D. melanogaster*, we will search for potential binding sites of *dl* in the region that encompasses the entire first transcribed exon to 2kb upstream of the putative TSS in our *D. biarmipes* ortholog (i.e., contig35:18924-23599). (Note that the search region is defined by the distribution of the TFBS HOT spots in the *D. melanogaster* ortholog so the search region will differ from gene to gene.)

Open a new tab on your web browser and then navigate to the Consensus server (http://stormo.wustl.edu/consensus/). Click on the "**Enter**" link and then click on the "**Advanced**" link under the "*Patser*" section on the left navigation bar (Figure 6).

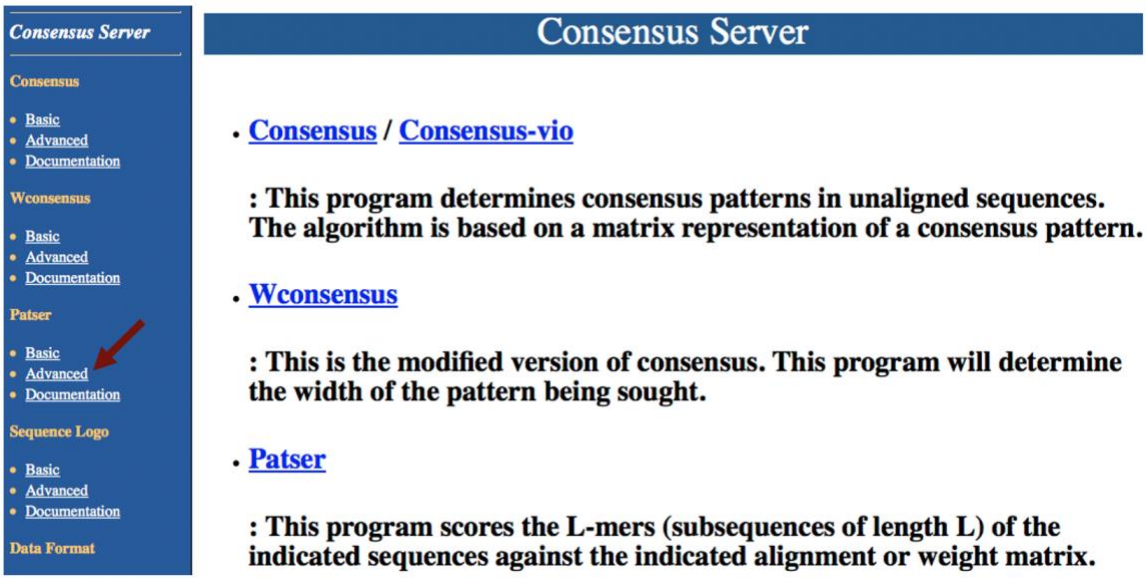


Figure 6 Access the "Advanced" version of the *Patser* web service using the Consensus Server.

In order to perform the motif search with *Patser*, we need to specify the genomic sequence to search and the TFBS count matrix. We will use the GEP's Mirror of the *UCSC Genome Browser* to retrieve the genomic sequence surrounding the *onecut* gene in *D. biarmipes*. Open a new tab and then navigate to GEP's Mirror of the *UCSC Genome Browser* at https://gander.wustl.edu. Click on the "Genome Browser" link. Enter "***D. biarmipes***" into the "Enter species or common name" field. Select "**Aug. 2013 (GEP/Dot)**" under the "*D. biarmipes* Assembly" field, and enter "**contig35**" into the "Position/Search Term" field (Figure 7). Click on the "Go" button.

Figure 7 Navigate to the *D. biarmipes* contig35 project on GEP's Mirror of the *UCSC Genome Browser*.

Click on the "DNA" button (under "View") in the main navigation bar. Enter "**contig35:18924-23599**" into the "Position" field and then click on the "**get DNA**" button (Figure 8). "Select all" the sequence and copy the sequence onto the clipboard. (For teaching purposes, we have included the sequence file **contig35_onecut_upstream.fasta** in the exercise package.)

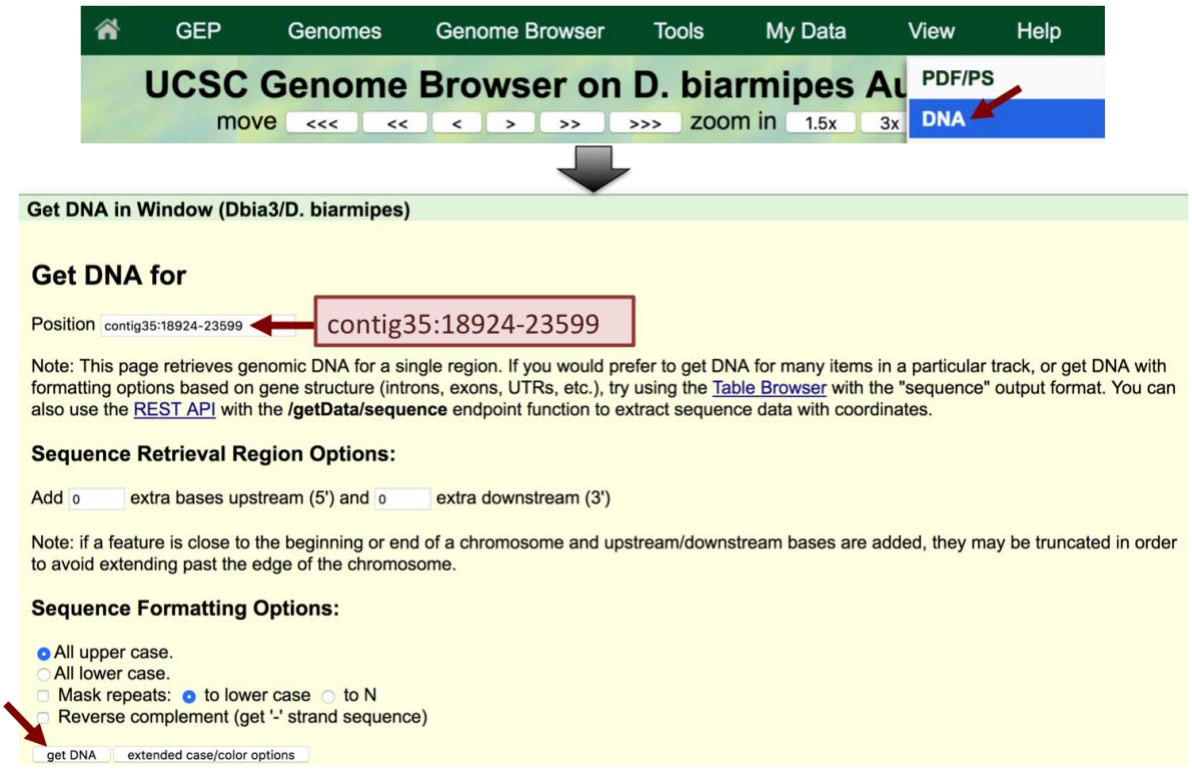


Figure 8 Retrieve the genomic region that spans from 2kb upstream of exon onecut:3 to the end of the exon in *D. biarmipes* contig35.

Go back to the *Patser* web browser window. Paste the *D. biarmipes* sequence into the "Enter the name of sequence file" text box. Change the definition line (i.e., the line that begins with '>' at the beginning of the sequence) to "**>contig35:18924-23599**". Open the matrix file (i.e., UmassPGFE_PWMfreq_dl_NBT_Horizontal.txt) we have saved earlier in a text editor (*e.g.*, WordPad on MS Windows, TextEdit on macOS). Copy only the four lines that begin with a nucleotide (i.e., skip the definition line which begins with '>') and then paste it into the "Enter your matrix from Consensus" text box (Figure 9).

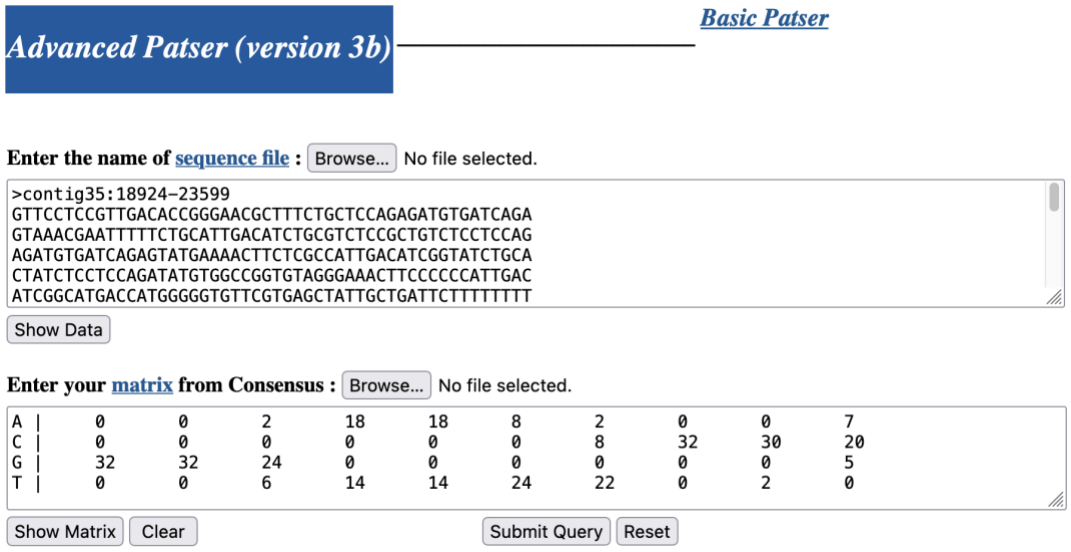


Figure 9 Enter the sequence extracted from contig35 and the matrix for the *dl* transcription factor into the *Patser* configuration form.

> The *Patser* web interface has very stringent requirements for the format of the sequence and the matrix. The sequence header (which begins with ">") must contain only alphanumeric characters, colons, and dashes. In addition, the first line of the matrix must begin with a nucleotide. If you encounter an error when you run *Patser*, please verify that the sequence and matrix are in the correct format.

Scroll down to the "Alphabet Options" section. Because the statistical significance of a match depends on the overall sequence composition, we need to specify the background base frequencies when we run *Patser*. The base composition for the *D. biarmipes* genome assembly is 58.2% A+T and 41.8% G + C. As an approximation, we will enter "**a:t 3 c:g 2**" in the "Seq. Alphabet and Normalization Information" field (Figure 10). (These values correspond to 60.0% A+T and 40.0% G+C.)

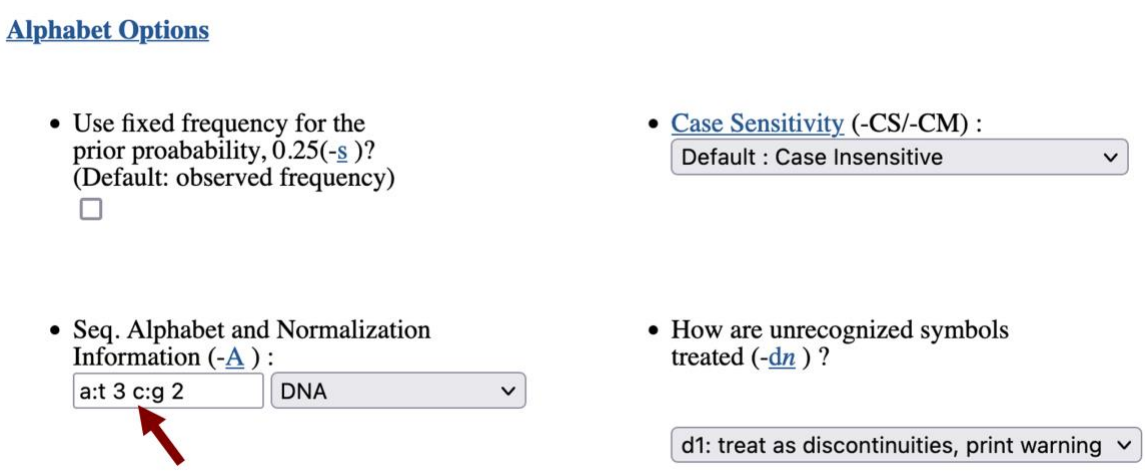


Figure 10 Enter the background base frequencies for the *D. biarmipes* genome (a:t 3 c:g 2).

Scroll down to the "Output Options" section. By default, *Patser* will report the match score for every nucleotide in the query sequence. To show only the subset of matches that have positive scores, we will change the "Lowest score to print (-l)" to **1**. Because transcription factors could bind in either orientation relative to the TSS, we will also select the option to "Score complementary sequence" (Figure 11).

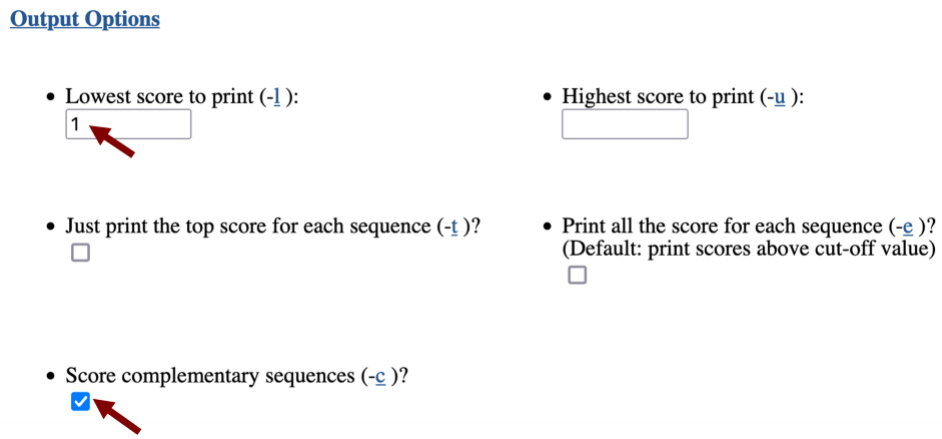


Figure 11 Change the *Patser* output options to search the complementary sequence and limit the output to motif matches that have a score greater than or equal to 1.

Click on the "Submit Query" button at the bottom of the page to run the search. (For teaching purposes, the output from *Patser* is also available in the file **Patser_onecut_dl_Dbiarmipes.txt** in the exercise package.)

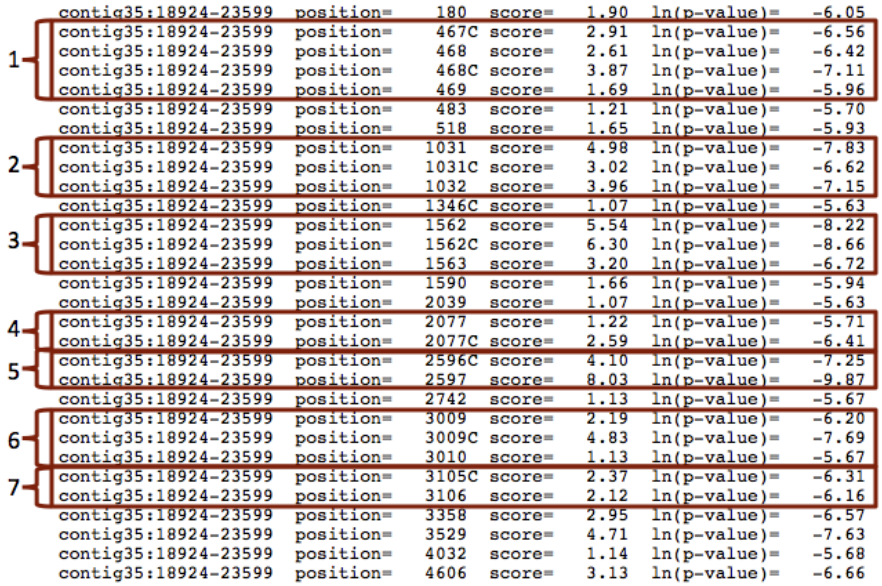
Interpreting the *Patser* results

Part of the *Patser* output includes a suggested cutoff score for statistically significant motif matches. In this case, the suggested ln(cutoff p-value) after adjusting for the expected information content from an arbitrary alignment of random sequences (i.e. sample size) is -9.312 (Figure 12), which means that only matches with a ln(p-value) smaller (i.e., more negative) than -9.312 are significant. Among the 30 motif matches reported by *Patser*, only one match (at position 2597) has a ln(p-value) that is less than -9.312.

```
Matrix Pattern: GGGAATTCCC
Information content (base e):   9.795
Sample size adjusted information content
     (information content minus the average information
     expected from an arbitrary alignment of random sequences):   9.312
Information content after adding pseudo-counts:   8.883

                                  maximum score:   11.632
                                  minimum score:  -34.965
            range of scores:   11.632 -  -34.965 =   46.597

          minimum score for calculating p-values:    0.000
       maximum ln(numerically calculated p-value):   -5.151
       minimum ln(numerically calculated p-value):  -14.473

ln(cutoff p-value) based on sample size adjusted information content:   -9.312
              numerically calculated cutoff score:    7.204
        ln(numerically calculated cutoff p-value):   -9.326
average score above numerically calculated cutoff:    8.430
```

Figure 12 *Patser* suggests that matches with a ln(p-value) less than -9.312 are statistically significant.

The suggested cutoff values calculated by *Patser* provide a good starting point for investigating potential candidate TFBS. However, the significance of a match depends on the level of motif conservation across different species, the degree of degeneracy of the binding site, and the size of the search region. Hence motif matches that are not statistically significant might nonetheless be biologically interesting.




```
contig35:18924-23599  position=    180  score=   1.90  ln(p-value)=   -6.05
contig35:18924-23599  position=    467C score=   2.91  ln(p-value)=   -6.56
contig35:18924-23599  position=    468  score=   2.61  ln(p-value)=   -6.42
contig35:18924-23599  position=    468C score=   3.87  ln(p-value)=   -7.11
contig35:18924-23599  position=    469  score=   1.69  ln(p-value)=   -5.96
contig35:18924-23599  position=    483  score=   1.21  ln(p-value)=   -5.70
contig35:18924-23599  position=    518  score=   1.65  ln(p-value)=   -5.93
contig35:18924-23599  position=   1031  score=   4.98  ln(p-value)=   -7.83
contig35:18924-23599  position=   1031C score=   3.02  ln(p-value)=   -6.62
contig35:18924-23599  position=   1032  score=   3.96  ln(p-value)=   -7.15
contig35:18924-23599  position=   1346C score=   1.07  ln(p-value)=   -5.63
contig35:18924-23599  position=   1562  score=   5.54  ln(p-value)=   -8.22
contig35:18924-23599  position=   1562C score=   6.30  ln(p-value)=   -8.66
contig35:18924-23599  position=   1563  score=   3.20  ln(p-value)=   -6.72
contig35:18924-23599  position=   1590  score=   1.66  ln(p-value)=   -5.94
contig35:18924-23599  position=   2039  score=   1.07  ln(p-value)=   -5.63
contig35:18924-23599  position=   2077  score=   1.22  ln(p-value)=   -5.71
contig35:18924-23599  position=   2077C score=   2.59  ln(p-value)=   -6.41
contig35:18924-23599  position=   2596C score=   4.10  ln(p-value)=   -7.25
contig35:18924-23599  position=   2597  score=   8.03  ln(p-value)=   -9.87
contig35:18924-23599  position=   2742  score=   1.13  ln(p-value)=   -5.67
contig35:18924-23599  position=   3009  score=   2.19  ln(p-value)=   -6.20
contig35:18924-23599  position=   3009C score=   4.83  ln(p-value)=   -7.69
contig35:18924-23599  position=   3010  score=   1.13  ln(p-value)=   -5.67
contig35:18924-23599  position=   3105C score=   2.37  ln(p-value)=   -6.31
contig35:18924-23599  position=   3106  score=   2.12  ln(p-value)=   -6.16
contig35:18924-23599  position=   3358  score=   2.95  ln(p-value)=   -6.57
contig35:18924-23599  position=   3529  score=   4.71  ln(p-value)=   -7.63
contig35:18924-23599  position=   4032  score=   1.14  ln(p-value)=   -5.68
contig35:18924-23599  position=   4606  score=   3.13  ln(p-value)=   -6.66
```

Figure 13 *Patser* identifies 7 regions where the TFBS for *dl* matches on both the positive and negative strand.

A closer examination of the *Patser* results shows that there are some positions within the search region that matched the *dl* TFBS motif on both the positive and negative strands. The "C" after the position denotes a match on the minus strand, *i.e.*, the complement. We also find that some of the motif matches are located immediately adjacent to each other (Figure 13).

Many transcription factors bind to the DNA as either a homodimer (i.e., two of the same transcription factor) or heterodimer (i.e., two different transcription factors); previous studies have shown that *dl* binds to DNA as a homodimer (GOVIND *et al.* 1992). Consequently, while the seven regions identified by *Patser* are not statistically significant, they still merit further investigation (e.g. via wet lab experiments) to determine if they correspond to functional binding sites of *dl*.

The positions reported by *Patser* are with respect to the extracted region and the motif of *dl* consists of 10 nucleotides. We can transform the coordinates reported by *Patser* so that they are with respect to the start of the contig35 sequence by adding 18,923 to all of the matched positions. For example, converting the position of the match at 2597 to the contig35 coordinate system (i.e., 2597+18924-1) means that the putative *dl* binding site begins at 21,520 in contig35.

However, because the motif match can be in either orientation, you must take the orientation of the motif match into account when you calculate the span of each motif

match. (The Excel workbook "Patser_onecut_dl_Dbiarmipes.xlsx" in the exercise package contains additional details on how to transform the coordinates reported by *Patser* to the coordinates with respect to the entire contig35 sequence.) The list of putative binding sites for *dl* is summarized in the table below:

Motif Candidate	Region
1	19381-19401
2	19945-19964
3	20476-20495
4	20991-21009
5	21510-21529
6	21923-21942
7	22019-22038

Conclusions

This walkthrough illustrates how we can identify conserved TFBS in the putative ortholog of the *onecut* gene on the *D. biarmipes* Muller F element. Using FlyBase *GBrowse*, we examined the modENCODE HOT spot annotations near the TSS of the *onecut* gene in *D. melanogaster*. For each of the TFBS associated with the HOT spot, we can retrieve the corresponding horizontal count matrix from the FlyFactorSurvey database. We can then use this count matrix with *Patser* to determine if the TFBS is also present in the region surrounding the TSS of the *D. biarmipes onecut* ortholog.

This walkthrough illustrates how we can annotate the putative TFBS for *dl* in the region surrounding the TSS of the *D. biarmipes onecut* ortholog. We can apply this search strategy to the other TFBSs (i.e., *twi*, *GATAe*, and *sens*) that are found within the HOT spots that overlap with exon onecut:3 in *D. melanogaster* in order to determine if these binding sites are also conserved in *D. biarmipes*.

The comparative analysis so far suggests that the *dl* transcription factor likely plays an important role in regulating the transcription of the *onecut* gene in both *D. melanogaster* and *D. biarmipes.* However, we do not have any experimental data examining the evolution of the *dl* consensus binding site, which could differ in *D. biarmipes*, so this remains a hypothesis until further testing.

Bibliography

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