ELB18F

Entry Level Bioinformatics

19-23 February 2018

(First 2018 run of this Course)

Basic Bioinformatics Sessions

Practical 6: Multiple Sequence Alignment

Tuesday 20 February 2018

Multiple Sequence Alignment

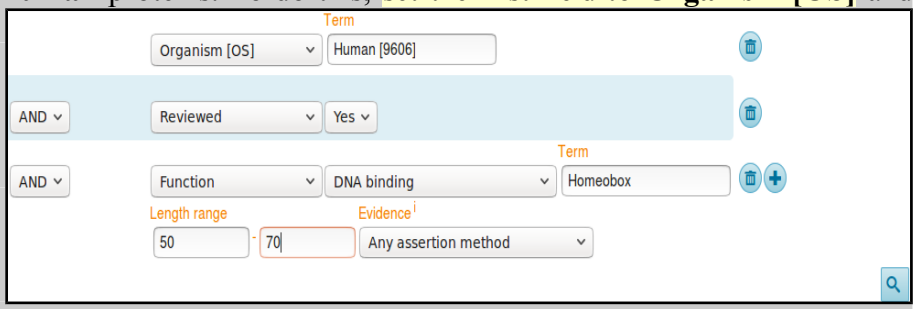
Here we will look at some software tools to align some protein sequences. Before we can do that, we need some sequences to align. I propose we try all the human **homeobox** domains from the well annotated section of **UniprotKB**. Getting the sequences is a trifle clumsy, so concentrate now! There used to be a much easier way, but that was made redundant by foolish people intent on making the future ever more tricky!!

So, begin by going to the home of **Uniprot**:

http://www.uniprot.org/

Choose the Advanced option of the Search button.

First specify that you are only interested in **Human** proteins. To do this, set the first field to **Organism [OS]** and **Term** to **Human [9606]**.



Set the second field selector to **Reviewed** and the corresponding **Term** to **Yes** (that is, choose to find only **SwissProt** entries).

Click on the button to request a further field selection option. Set the new field to **Function**. Set the type of **Function** to **DNA binding**. Set the **Term** selection to **Homeobox**.

From previous investigations, you should be aware that a **Homeobox** domain is **generally 60** amino acids in length. To avoid partial and/or really weird **Homeobox** proteins, set the **Length** range settings to recognise only **homeobox**s between **50** and **70** amino acids long.

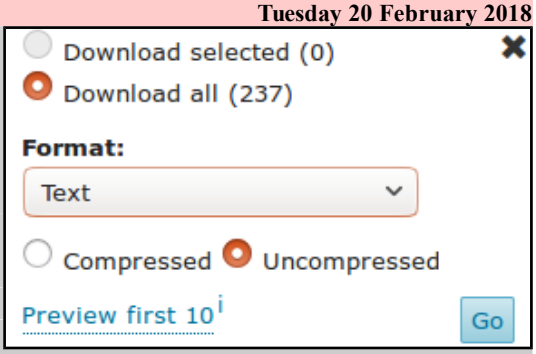
Leave the **Evidence** box as **Any assertion method**, one does not wish to be too fussy! Address the button with authority to get the search going.

BLAST | Align | Download | Add to basket | Columns | 1 to 25 of 237 | Show 25

Entry	Entry name	Protein names	Gene names	Organism	Length
P50222	MEOX2_HUMAN	**Homeobox protein MOX-2**	**MEOX2** GAX, MOX2	Homo sapiens (Human)	304
P52952	NKX25_HUMAN	**Homeobox protein Nkx-2.5**	**NKX2-5** CSX, NKX2.5, NKX2E	Homo sapiens (Human)	324
P26367	PAX6_HUMAN	**Paired box protein Pax-6**	**PAX6** AN2	Homo sapiens (Human)	422
P49639	HXA1_HUMAN	**Homeobox protein Hox-A1**	**HOXA1** HOX1F	Homo sapiens (Human)	335
Q99697	PITX2_HUMAN	**Pituitary homeobox 2**	**PITX2** ARP1, RGS, RIEG, RIEG1	Homo sapiens (Human)	317
Q99801	NKX31_HUMAN	**Homeobox protein Nkx-3.1**	**NKX3-1** NKX3.1, NKX3A	Homo sapiens (Human)	234
Q01860	PO5F1_HUMAN	**POU domain, class 5, transcription ...**	**POU5F1** OCT3, OCT4, OTF3	Homo sapiens (Human)	360
Q15475	SIX1_HUMAN	**Homeobox protein SIX1**	**SIX1**	Homo sapiens (Human)	284
Q01826	SATB1_HUMAN	**DNA-binding protein SATB1**	**SATB1**	Homo sapiens (Human)	763
P43699	NKX21_HUMAN	**Homeobox protein Nkx-2.1**	**NKX2-1** NKX2A, TITF1, TTF1	Homo sapiens (Human)	371

A fine miscellany of sequences will assemble upon you screen. Most seem to declare themselves in possession of a **Homeobox** or two (including **PAX6_HUMAN**), so I suggest a declaration of success.

Now save the entire list into a file using the Download button. Set the download to **uncompressed**. Make sure you have **all** sequences selected and that **Text** (i.e. **EMBL** or **SwissProt**) format selected. Press the **Go** button and do whatever it takes to ensure your results end up in a file residing on your **Desktop** called:



human_homeobox_proteins.emb

```
ID   MEOX2_HUMAN             Reviewed;         304 AA.
AC   P50222; A4D127; B2R8I7; O75263; Q9UPL6;
DT   01-OCT-1996, integrated into UniProtKB/Swiss-Prot.
DT   18-APR-2006, sequence version 2.
DT   25-OCT-2017, entry version 159.
DE   RecName: Full=Homeobox protein MOX-2;
DE   AltName: Full=Growth arrest-specific homeobox;
DE   AltName: Full=Mesenchyme homeobox 2;
GN   Name=MEOX2; Synonyms=GAX, MOX2;
OS   Homo sapiens (Human).
OC   Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
OC   Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
OC   Catarrhini; Hominidae; Homo.
OX   NCBI_TaxID=9606;
RN   [1]
RP   NUCLEOTIDE SEQUENCE [MRNA], AND VARIANT HIS-80 DEL.
RC   TISSUE=Embryo;
RX   PubMed=7607679; DOI=10.1016/0888-7543(95)80174-K;
RA   Grigoriou M., Kastrinaki M.-C., Modi W., Theodorakis K., Mankoo B.,
RA   Pachnis V., Karagogeos D.;
RT   "Isolation of the human MOX2 homeobox gene and localization to
RT   chromosome 7p22.1-p21.3.";
RL   Genomics 26:550-555(1995).
RN   [2]
RP   NUCLEOTIDE SEQUENCE [MRNA], AND VARIANT 79-HIS-HIS-80 DEL.
RC   TISSUE=Heart;
```

Take a swift look at the file you have just created. Your neat list of **Human Homeobox** sequences will have transformed into a flood of **many SwissProt** format **UniProtKB** entries. Ugly, but what is required.

Search (**Control F**) for the term **DNA_BIND**.

It should occur many times (at least once per sequence) in the **F**eature **T**ables and most often refer to a **Homeobox** region.

In the **DNA_BIND** **F**eature **T**able entries, the position of the **Homeobox**s are recorded and will be used by the next program to isolate the sequence of the **Homeobox**s.

```
FT   CHAIN         1    304       Homeobox protein MOX-2.
FT                                /FTId=PRO_0000049197.
FT   DNA_BIND    187    246       Homeobox. {ECO:0000255|PROSITE-
FT                                ProRule:PRU00108}.
FT   COMPBIAS     42     47       Poly-Ser.
FT   COMPBIAS     68     80       Poly-His.
FT   COMPBIAS     81     86       Poly-Gln.
FT   VARIANT      79     80       Missing. {ECO:0000269|PubMed:7713505}.
FT                                /FTId=VAR_026040.
FT   VARIANT      80     80       Missing. {ECO:0000269|PubMed:12690205,
FT                                ECO:0000269|PubMed:14702039,
FT                                ECO:0000269|PubMed:15489334,
FT                                ECO:0000269|PubMed:7607679}.
FT                                /FTId=VAR_026041.
FT   VARIANT     287    287       I -> L (in dbSNP:rs2237493).
FT                                /FTId=VAR_049585.
FT   MUTAGEN     236    236       Q->E: Abolishes DNA-binding. Does not
FT                                affect ability to activate expression of
FT                                CDKN2A. {ECO:0000269|PubMed:22206000}.
FT   CONFLICT     58     58       G -> D (in Ref. 2; AAA58497).
FT                                {ECO:0000305}.
SQ   SEQUENCE   304 AA;  33594 MW;  0C008479D6995389 CRC64;
     MEHPLFGCLR SPHATAQGLH PFSQSSLALH GRSDHMSYPE LSTSSSSCII AGYPNEEGMF
     ASQHHRGHHH HHHHHHHHHH QQQQHQALQT NWHLPQMSSP PSAARHSLCL QPDSGGPPEL
     GSSPPVLCSN SSSLGSSTPT GAACAPGDYG RQALSPAEAE KRSGGKRKSD SSDSQEGNYK
     SEVNSKPRKE RTAFTKEQIR ELEAEFAHHN YLTRLRRYEI AVNLDLTERQ VKVWFQNRRM
     KWKRVKGGQQ GAAAREKELV NVKKGTLLPS ELSGIGAATL QQTGDSIANE DSHDSDHSSE
     HAHL
//
```

Now to extract from the whole protein sequences you have saved in a file, the sequences of just the **Homeobox** domains. One way of doing this (possibly not the best), is to use an **EMBOSS** package program called **extractfeat**. This can be found in many places, including the Bioinformatics server at **Wageningen** in the Netherlands. Go to:

http://emboss.bioinformatics.nl/

Find the program **extractfeat** (in the **EDIT** section), and set it going.

Use the **Choose File** button to **upload** the **SwissProt** format sequences from **UniProtKB** that you saved in **the file**:

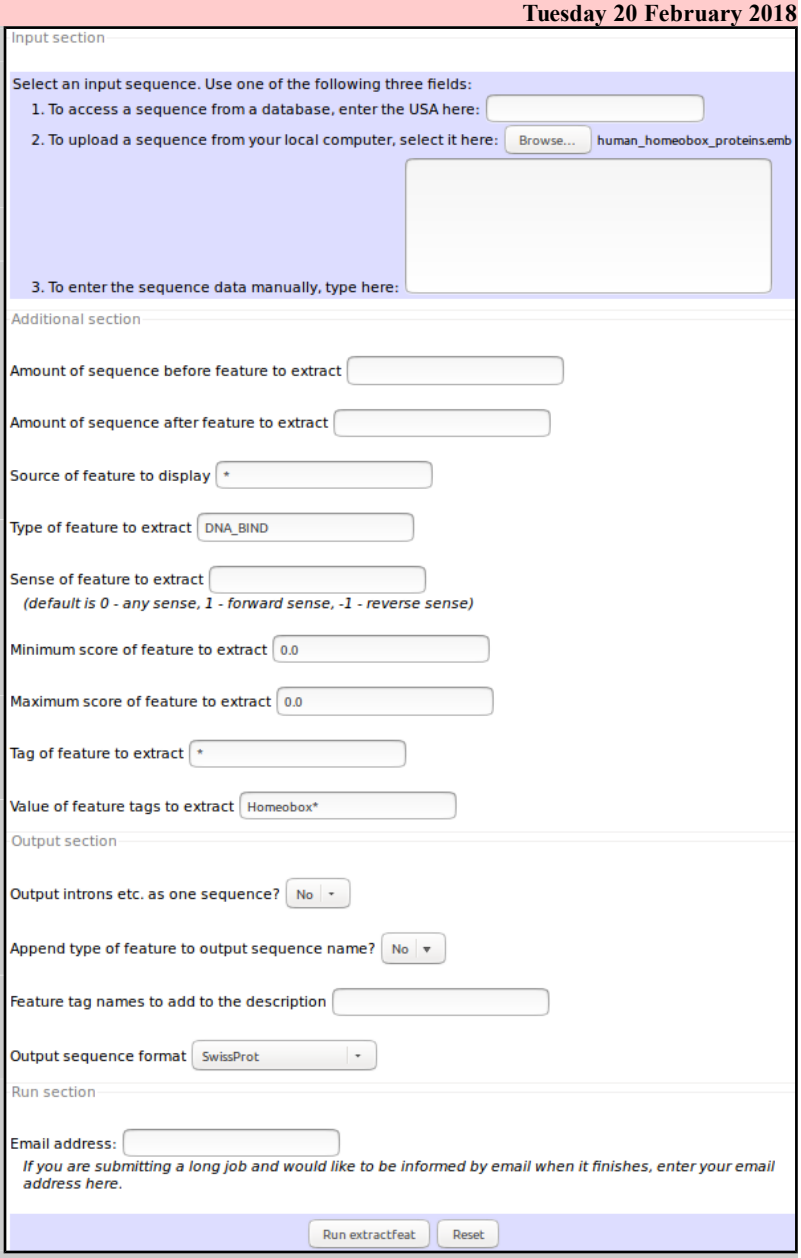
`human_homeobox_proteins.emb`.

Set **Type of feature to extract field** to **DNA_BIND** (Make sure you remove the "*").

Set **Value of feature tags to extract** to **Homeobox*** (Make sure you append the "*" to ensure hits with, for example "**Homeoboxes**").

Set the **Output sequence format** to **SwissProt** (**Fasta** would do, but **SwissProt** retains more annotation).

Click on the Run extractfeat button to start **extractfeat** going. Many sequences of **60** amino acids (or so) in length will leap into view.



Right click the outseq button and select **Save Link as...** . Do whatever it takes to save all your **Homeobox** domains into a file residing on your **Desktop** called:

`homeobox_human.emb`

Finally, we have some sequences with which to investigate the multiple sequence alignment programs.

Take a look at the file you have created. You should have many human **homeobox** domains in **SwissProt** format, looking rather as they did in your browser window. Happily **ClustalX**, the first multiple alignment program to be investigated, accepts multiple sequence **SwissProt** format files as input.

OUTPUT FILE outseq

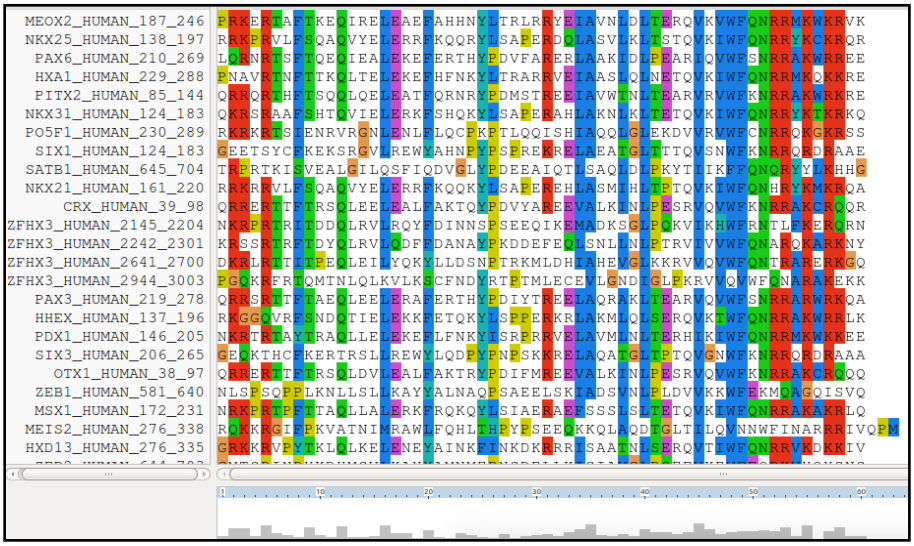
```
ID   MEOX2_HUMAN_187_246     Reviewed;          60 AA.
DE   [DNA_contact] Homeobox protein MOX-2 (Growth arrest-specific homeobox) (Mesenchyme homeobox 2)
SQ   SEQUENCE   60 AA;  7615 MW;  7AA1CEC5BBC0265F CRC64;
     PRKERTAFTK EQIRELEAEF AHHNYLTRLR RYEIAVNLDL TERQVKVWFQ NRRMKWKRVK
//
ID   NKX25_HUMAN_138_197     Reviewed;          60 AA.
DE   [DNA_contact] Homeobox protein Nkx-2.5 (Cardiac-specific homeobox) (Homeobox protein CSX) (Homeobox protein NK-2 homolog E)
SQ   SEQUENCE   60 AA;  7514 MW;  16EE564D071E5E8A CRC64;
     RRKPRVLFSQ AQVYELERRF KQQRYLSAPE RDQLASVLKL TSTQVKIWFQ NRRYKCKRQR
//
ID   PAX6_HUMAN_210_269      Reviewed;          60 AA.
DE   [DNA_contact] Paired box protein Pax-6 (Aniridia type II protein) (Oculorhombin)
SQ   SEQUENCE   60 AA;  7447 MW;  075C194DB9F33ED9 CRC64;
     LQRNRTSFTQ EQIEALEKEF ERTHYPDVFA RERLAAKIDL PEARIQVWFS NRRAKWRREE
//
ID   HXA1_HUMAN_229_288      Reviewed;          60 AA.
DE   [DNA_contact] Homeobox protein Hox-A1 (Homeobox protein Hox-1F)
SQ   SEQUENCE   60 AA;  7365 MW;  53E2BC59B06F544E CRC64;
     PNAVRTNFTT KQLTELEKEF HFNKYLTRAR RVEIAASLQL NETQVKIWFQ NRRMKQKKRE
//
```

ClustalX is a part of the mostly widely known family of **M**ultiple **S**equence **A**lignments (**MSA**) programs, originating in the **1980**s. Until relatively recently, it was the only real option. **ClustalX** still has merit, although it lacks some of the sophistication of more recent programs. **ClustalX** runs on effectively all workstations and has a nice **G**raphical **U**ser **I**nterface (**GUI**). A good place for us to start. It is, hopefully, installed on your workstations.

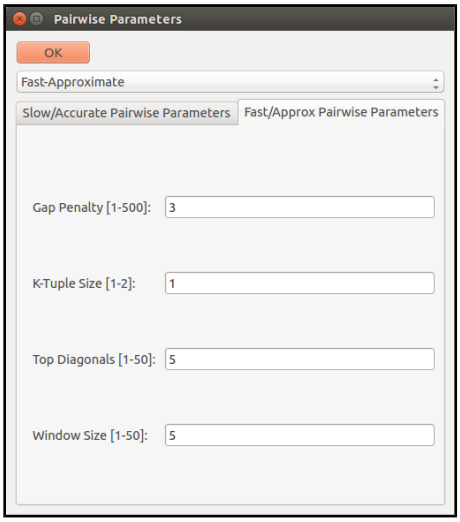
Start up the program **ClustalX**[1]. The **ClustalX** **G**raphical **U**ser **I**nterface (**GUI**) will regally mount your screen.

Select **Load Sequences** from the **File** pull down menu and load your file of **homeobox** domains (`homeobox_human.emb`**).**

The sequences will arrange themselves colourfully. Many of the **homeoboxes** are similar enough to look convincing even before alignment. Note the "Manhattan skyline" under the sequences indicating the varying degrees of conservation.



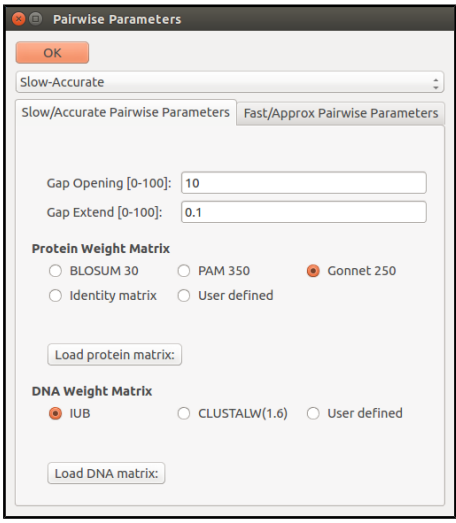
You might like to increase the **Font** size from the minute default setting designed for Hawks and Eagles, to something more comfortable. **24** works tolerably well for me.



From the **Alignment** pull down menu, go to the **Alignment parameters** menu and select **Pairwise Alignment Parameters**. Just for a moment, change the setting from **Slow-Accurate** to **Fast-Approximate**. Bring the corresponding parameters into view by clicking on **Fast/Approx Pairwise Parameters** tab[2].

Hopefully, we will have discussed the way **ClustalX** (and similar multiple alignment tools) work. Intuitively, it should not make a lot of difference how the initial pairwise comparison stage is conducted. However, it very often does.

Specifically for this set of proteins, as well as generally, **ClustalX** will give a noticeably better alignment if the initial pairwise alignment stage is done carefully. Accordingly, reverse your whimsical setting change by moving back from **Fast-Approximate** to **Slow-Accurate**.



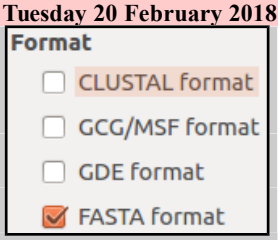
Click on the **Slow/Accurate Pairwise Parameters** tab for a final look at the default parameters to be used. The **Slow-Accurate** option is essentially a version of **Global Alignment** algorithm we will have discussed previously. Hopefully, all the parameter options will therefore be familiar to you.

I will assume both sets of parameters at least ring a bell? If not please ask. The default **Slow/Accurate Pairwise Parameters** you now have in view are fine. Click the **OK** button to dismiss the **Pairwise Parameters** window.

1 Of course, you could run **Clustal** from websites all over the world if you wished. Specifically, it is available at the Bioinformatics server at **Wageningen**. Try it if you have time. You get the same results but will, sadly, lose the pretty interface.
http://www.bioinformatics.nl/tools/clustalw.html
The **EBI** no longer offer basic **Clustal**.

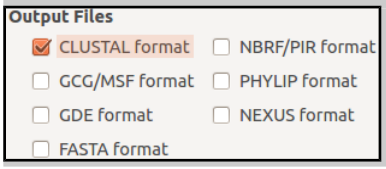
2 The **Fast-Approximate** algorithm is essential that which the database searching program **fasta** employs. Assuming we have discussed how **fasta** (or **blast**) works, little further explanation should be required here.

Before proceeding, save the **homeobox** sequences in **FASTA** format, which will better suit the other **MSA** programs we will try. Do this by selecting **Save sequences as...** from the **File** pull down menu. Deselect **CLUSTAL format**, select **FASTA format**.



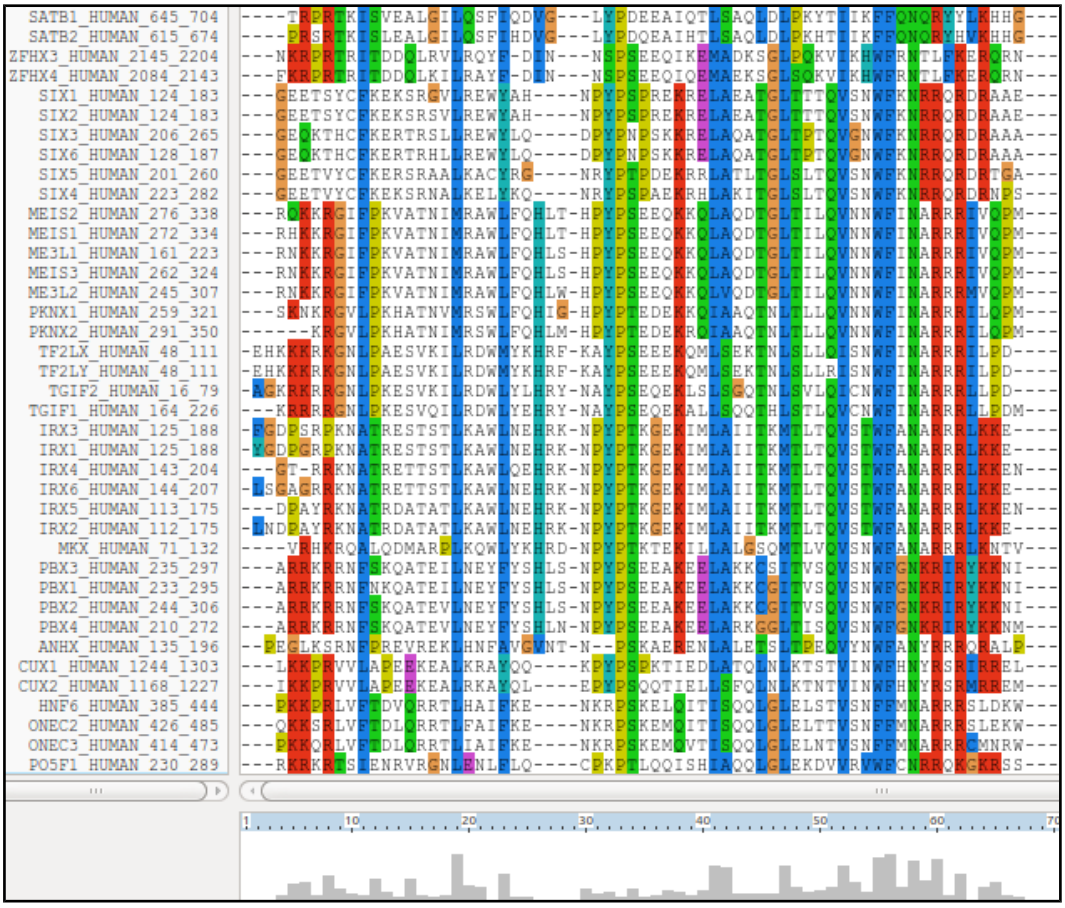
Change the default file output file name to `homeobox_human_full`

Click **OK**. A file called `homeobox_human_full.fasta` will be created. Take a look to check it is as you would expect.



Strangely, saving your sequences in **FASTA** format convinces **clustalx** that it should now output its alignments in **FASTA** format. To prevent this, select **Output Format Options** from the **Alignments** pull down menu. Deselect **FASTA format** and select **CLUSTAL format**. Click **OK**.

From the **Alignment** pull down menu, select **Do Complete Alignment**. Accept the default names for output files and click on the **OK** button. **ClustalX** will start to think deeply and eventually come up with it view of how the **homeobox** domains should be aligned.



Note the display at the bottom of the **ClustalX** window in which the preliminary pairwise comparisons of all sequences is monitored. The scores from these comparisons are used to compute the **Guide Tree**.

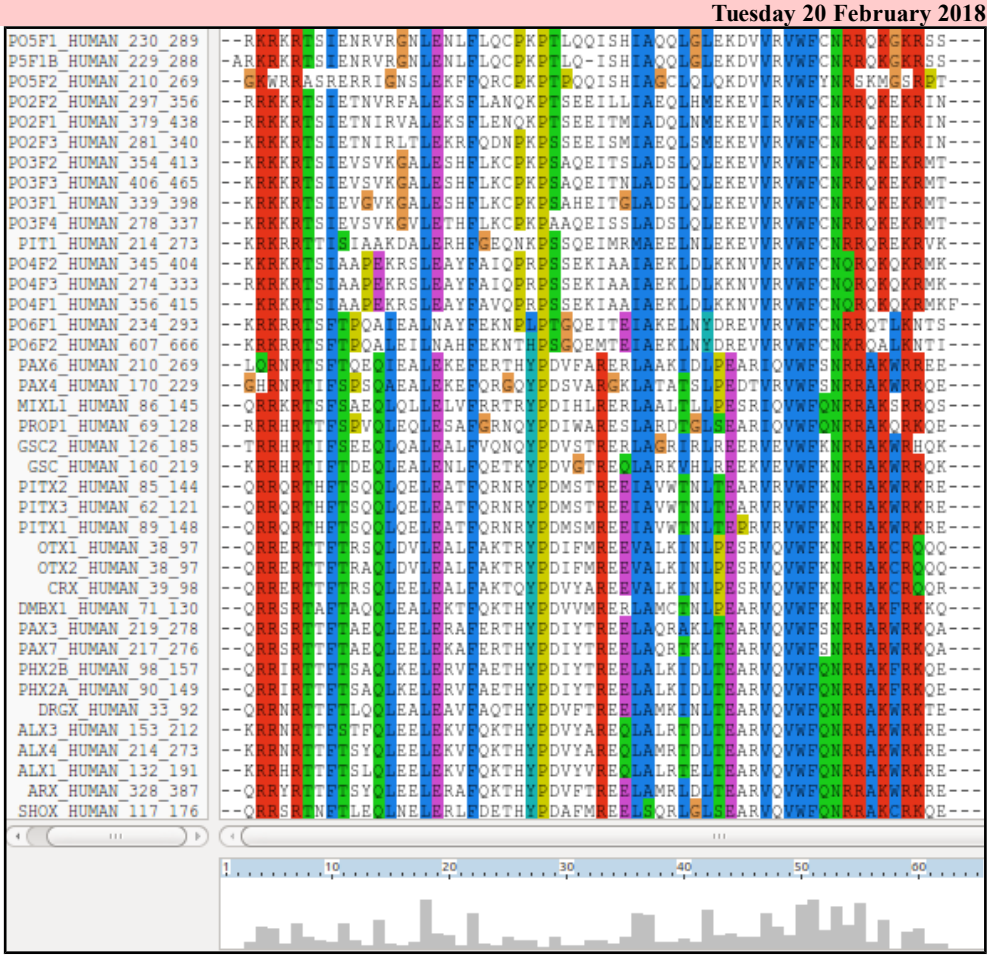
Not a bad first try. From an entirely non scientific, cosmetic, viewpoint, the ragged ends offend a trifle, as does the gap just before position **30**!

In reality, these features might be interesting, but here I go for pretty!

Just to investigate the possible, select all the **homeobox** sequences that are causing the gap around position **30** by clicking on their names (quite a lot of them I fear). Hold the **Ctrl** key down to allow multiple selection.

All selected, go to the **Edit** pull down menu and select **Cut Sequences**. Then select **Remove Gap-Only columns** from the **Edit** pull down menu. Nasty gap gone … along with all scientific credibility, but … never mind.

You could recompute the same alignment from scratch for the reduced sequence set. To justify this assertion, select **Select All Sequences** from the **Edit** menu. Then select **Remove All gaps** from the **Edit** menu and confirm your intentions. You are now back where you started, but without the sequences that mess up the alignment.



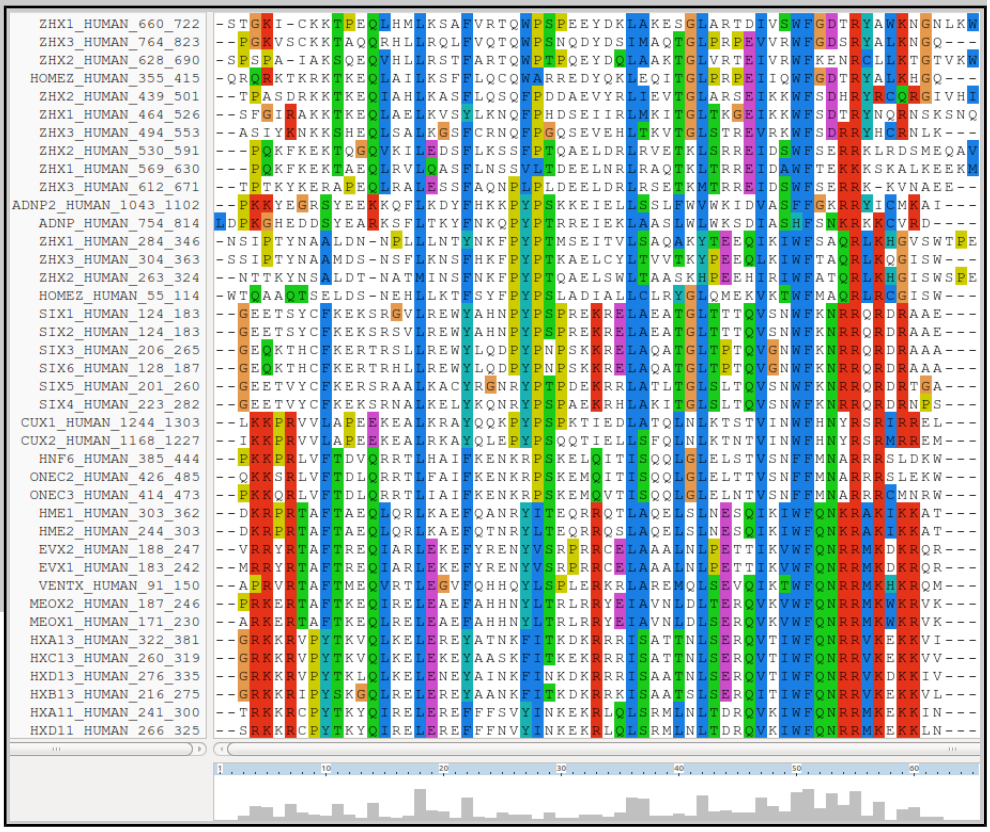
Save your filtered set of sequences. From the **File** menu select **Save Sequences as...** . Choose **FASTA** format only. This time, create a file with the default name:

`homeobox_human.fasta`

The full original set of sequences was saved in a differently named file, as a precaution. I am convinced the sequences eliminated would not align convincingly with any of the tools we have at hand. Let us lose them! Press the **OK** button.

From the **Alignment** menu, select **Output Format Options** and then select **CLUSTAL format** only.

From the **Alignment** menu, select **Do Complete Alignment**. Accept the default names for the output files. This will overwrite your previous efforts, but no matter. Well, I got back to where I was, no gaps around position **30** but still the ragged ends!

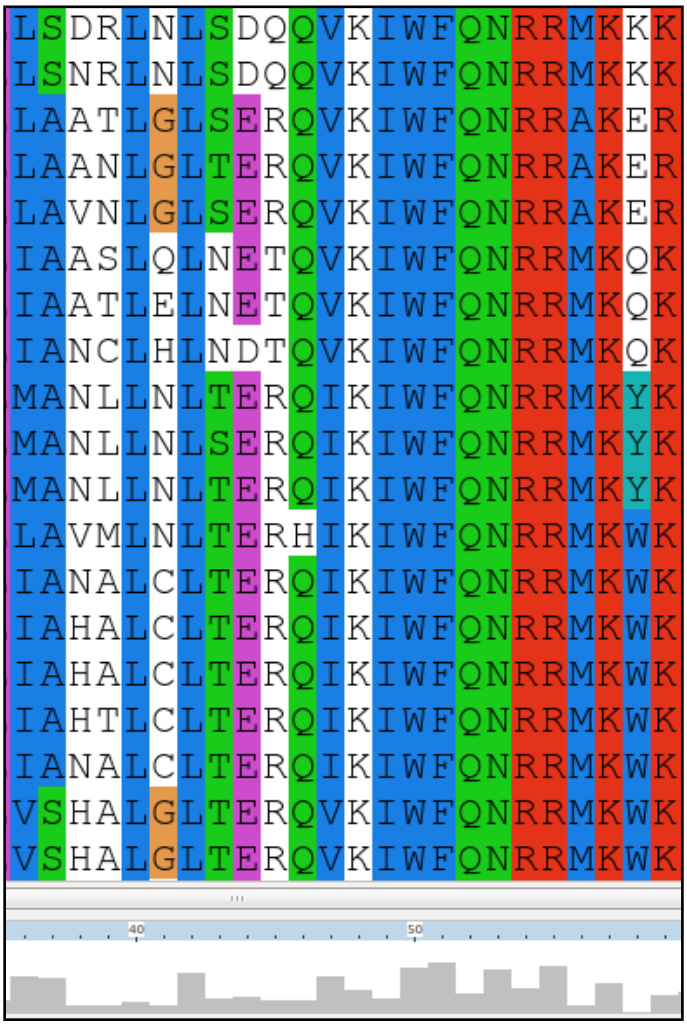


It is difficult to prove you have exactly the same alignment as previously as the order of the **MSA** will be different. This order being determined by the pairwise comparison stage of the **ClustalX MSA** computation.

The **Prosite** motif database uses Patterns to represents protein features (in addition to **HMM**s). The pattern for a **homeobox** is the ever memorable:

[LIVMFYG]-[ASLVR]-x(2)-[LIVMSTACN]-x-[LIVM]-{Y}-x(2)-{L}-[LIV]-[RKNQESTAIY]-[LIVFSTNKH]-W-[FYVC]-x-[NDQTAH]-x(5)-[RKNAIMW]

Any speculations as to how this might be interpreted? Quick Hint?

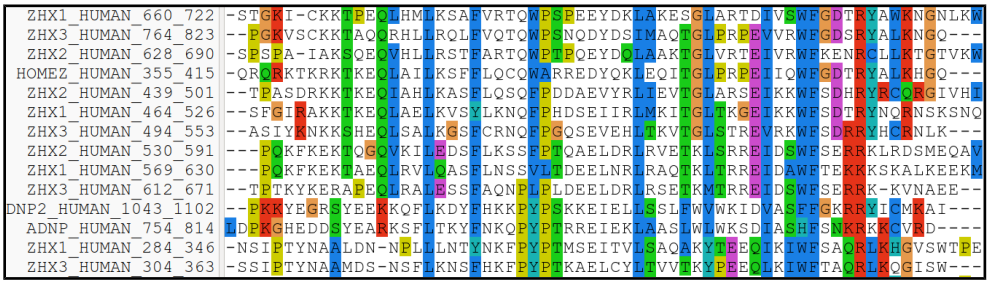


This pattern corresponds to positions **36** to **59** in my alignment. See that the "Manhattan Skyline" is encouraging in the parts of this region that matter.

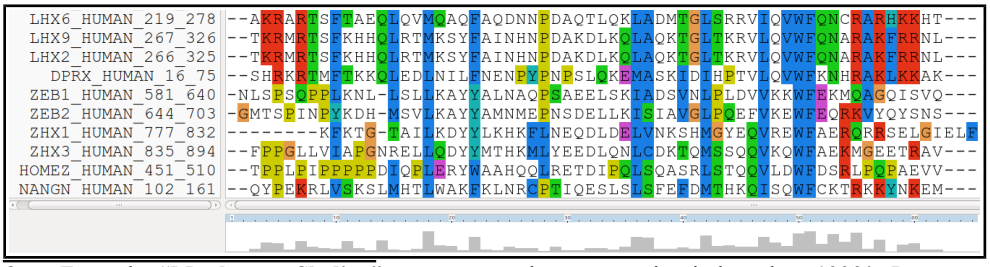
Note that the profile **Tryptophan**, in position **50**, is **very** consistent, but not quite **100%** as suggested by the **Prosite** pattern[3]. The **W** was even conserved in the sequences that were cosmetically removed.

Position **52** is not conserved ("**-x-**") according to the **Prosite** pattern. In the alignment segment offered here, it looks like a pretty consistent **Q**. However, the "**Manhattan skyline**" at this position is quite low, suggesting that the sequences in view might not be typical of the whole alignment set. Which, upon checking …. they are not!

Looking through this alignment, I get the feeling I could design a better, stricter pattern for the region between **36** and **59**. Possibly true, but remember the pattern in **Prosite** aims to represent the conservation of **Homeobox** domains in **ALL** organisms. Here we have only sequences from **Human**.



Of course, things are not quite so convincing throughout. If you look at the top and bottom few sequences, you will see that **ClustalX** had its moments of uncertainty.



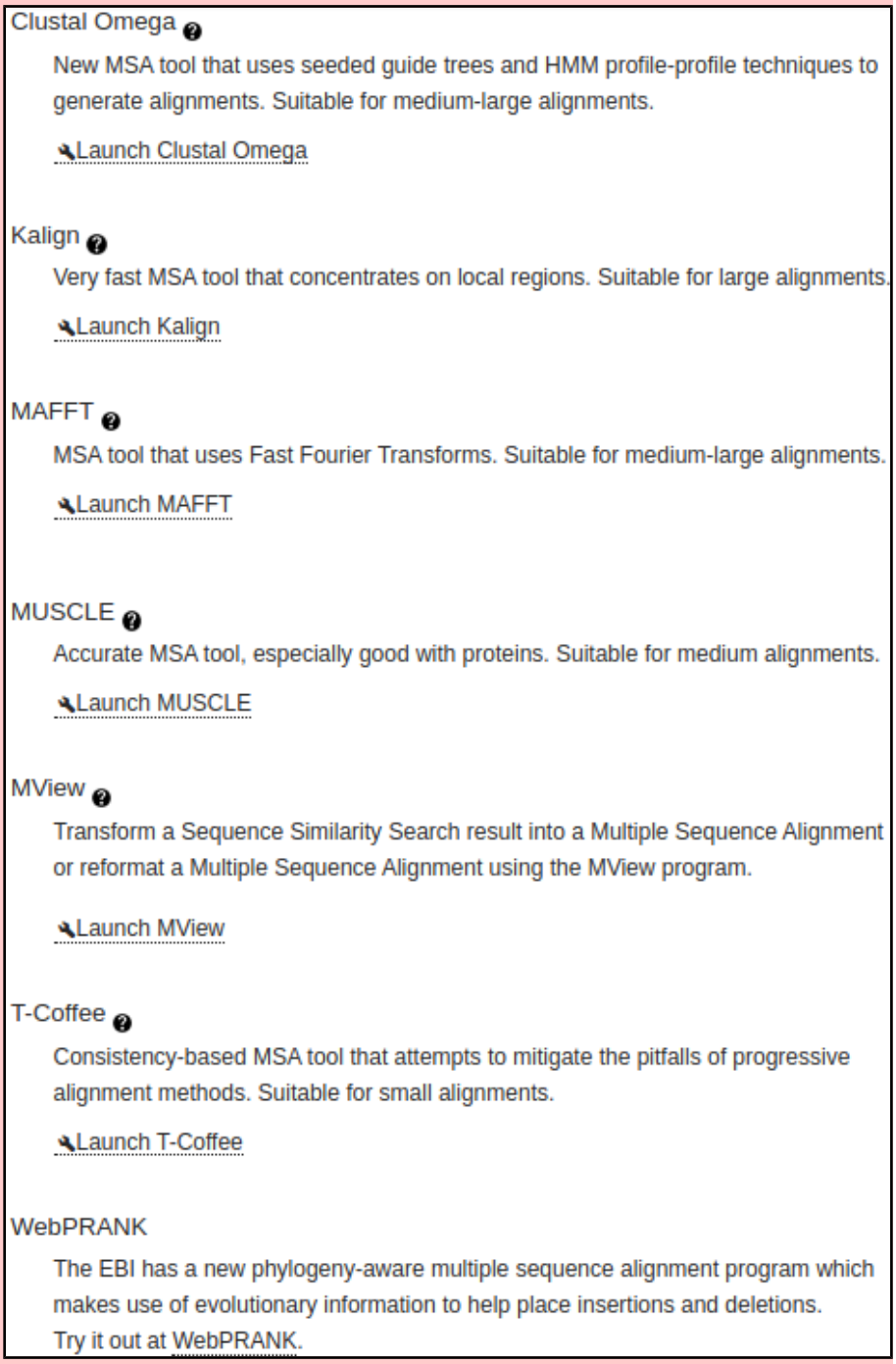
Note, however, the consistent **W** in position **50** despite the surrounding crumble.

3 From the "**Manhattan Skyline**", you can see the conservation is less than **100%**. Less conserved than the **F** that immediately follows in fact? Look at your alignment, the "**Manhattan Skyline**" does not seem to reflect reality? The **W** is **very** well conserved, although the scoring matrices would regard any deviation from **W** as serious? I need to find out more about how the **Skyline** is computed.

Now to show existence of some **msa** program options available on the web. There are many. They are available from a number of server sites. An obvious place to start has to be the **EBI** page dedicated to **MSA**. Go to:

`http://www.ebi.ac.uk/Tools/msa/`

Offered here is a selection of popular, current generation **MSA** tools. Each is accompanied by advice to guide the choice of tool to best fit the circumstances. Each tool is provided with a link to its **Launch** interface. All the **Launch** interfaces are very consistent. Once you have run one of the **MSA** options, you should have no trouble running any of the others.

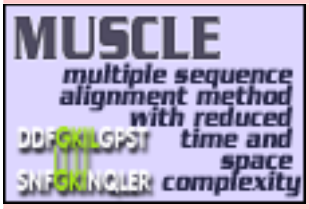


Here I intend to align again the human **homeboxes** with just one of the tools on offer. Then take a quick look at how the machine generated multiple alignment can be manually edited using **Jalview**, a program that is probably installed on your workstation and definitely available as a web service. You might have already used **Jalview** as an alignment viewer when investigating **Pfam** and/or **Jpred**.

Then I will invite you to try a few of the other options for yourself and see that they do not all produce the same alignment! Differences reflect not only the parameters selected, which we will have discussed, but also the particular objectives of the program selected. For example, a multiple protein sequence alignment optimal for investigating conservation of protein structure might well not be identical to one best representing protein evolution.

Used to align the **Homeobox** sequences used in this exercise, I do not expect you will see much difference between the outputs of any of these options. They will all work sufficiently on such a simple data set.

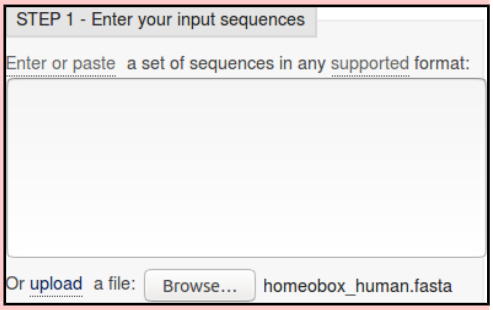
The program whose use I choose to describe carefully, leading on to a short **Jalview** exercise is **MUSCLE**. I choose thus as **MUSCLE** is now the first choice of most of the people with whom I work. Also popular are **Clustal Omega**, **MAFFT** and, for **phylogeny**, **WebPRANK**.

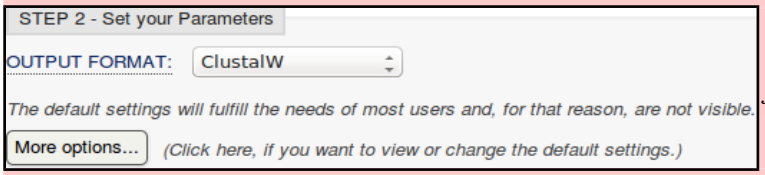


So the plan now is to use **MUSCLE**[4] to align again the **homeobox** sequences previously aligned with **ClustalX**. **MUSCLE** works in a way similar to **clustalX** but it takes rather more care in the generation of the **Guide Tree** used to control the order of pairwise construction of the final multiple alignment[5]. Particularly for more difficult alignments, **MUSCLE** should do a better job than **ClustalX**. The alignment you will generate here will certainly be different. I leave you to judge for yourselves whether it is better.

Start by requesting to Launch MUSCLE.

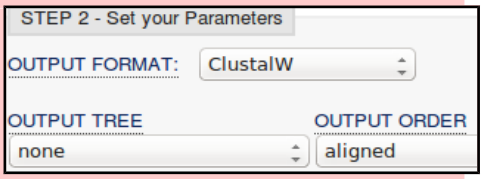
Use the Browse... button to upload the file containing the **FASTA** format **homeobox** sequences, **homeobox_human.fasta**. This file should not included the sequences with a mess around position **30**.

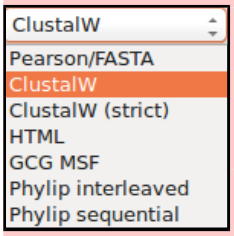




Take a look at the **Set your Parameters** section of the page. I find the claim that "*The default settings will fulfill the needs of most users and, for that reason, are not visible*" a little strange? What about the users who are not in the category "*most*"? I want control over all the programs that their creators deemed sensible to make available[6]?

The default settings behind the More options... button are not those that affect the computation of the **MSA**. I confess myself confused at the lack of any meaningful options to consider? I was expecting at least the **gap open** and **gap extension penalty** options (available elsewhere, including **Wageningen**), plus a way to change the **scoring matrix**. I have inquired why things are as they are (most recently **2016.04.17**). No practical issue here, as I intended to suggest the defaults whatever they were. Look at the range of settings for the **OUTPUT TREE** parameter. **none** is indeed the thinking persons choice, but … one or the other (but not both?) of the **Guide Trees** that **MUSCLE** will compute can be saved if you wish[7]. You may also set the **OUTPUT ORDER** to **aligned** or … **aligned**?





There are a number of **OUTPUT FORMATS** offered. For a quick glance at your results, both **ClustalW** or **HTML** are fine. Here I suggest it would be nice to generate an output that can be downloaded and viewed in **Jalview**[8]. The default **ClustalW** or **Pearson/FASTA** serve for this purpose. As **ClustalW** looks more like an alignment in the web page, I choose **ClustalW**[9].

How do the options for the **OUTPUT TREE** relate to the output files of **ClustalX** and the difference between the way that **ClustalX** and **muscle** work?

Comment on how one might choose between the range of options offered for the aligned parameter?

4 More available from a variety of websites in addition to the **EBI**, including the Bioinformatics server at **Wageningen**:
`http://www.bioinformatics.nl/tools/muscle.html`

5 As discussed, superficially at least, previously. I hope.

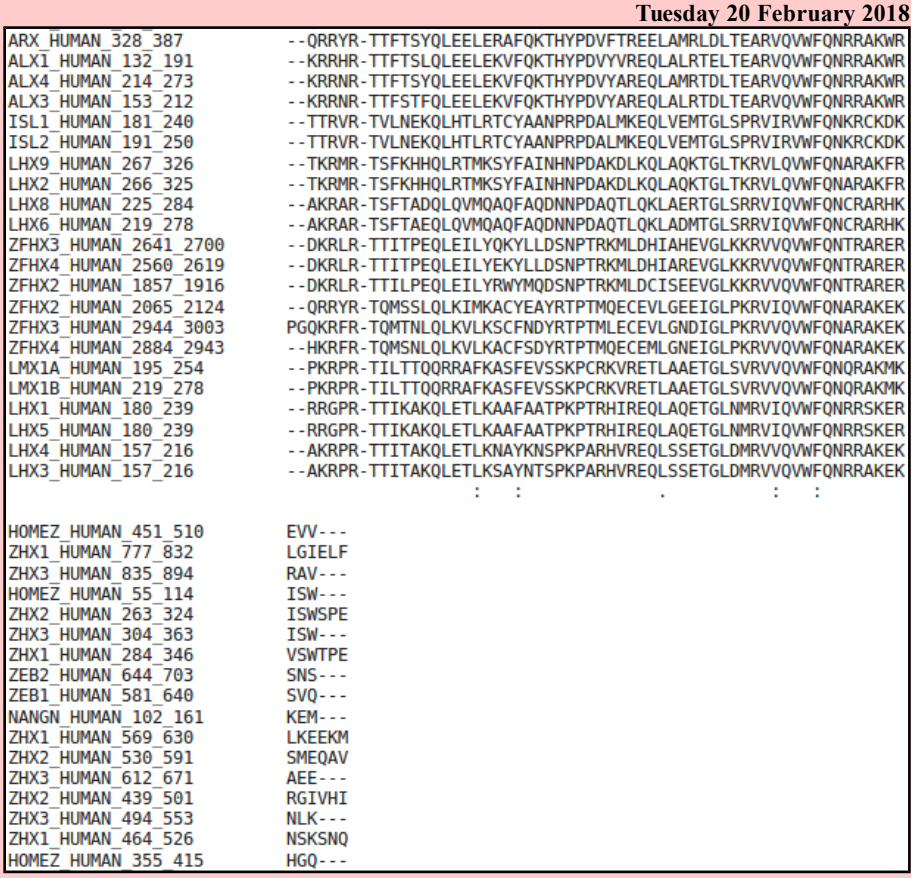
6 I have asked the **EBI** about their policy (the same for all the locally provided **MSA** options). Discussion is ongoing (**2016.04.20**).

7 A useful option if you thought it possible you might want to rerun **MUSCLE** with different parameter setting for the stages after the **Guide Tree(s)** are generated. The same possibilities exist for **ClustalX**. Of course, utterly pointless if it is impossible to control the relevant parameters …. so I really cannot see the point of any of the **More options** section? I am open to elucidation from all/any sources.

8 A widely used **java** alignment editor and viewer.

9 But feel free to try the others. **HTML** is the default at **Wageningen**. The **Phylip** formats are the best if you are going to analyse your output further with the phylogeny programs of the **PHYLIP** package.

After considering these enigmas, or before if you prefer, Click on the Submit button and sit back to admire **muscle** in action.



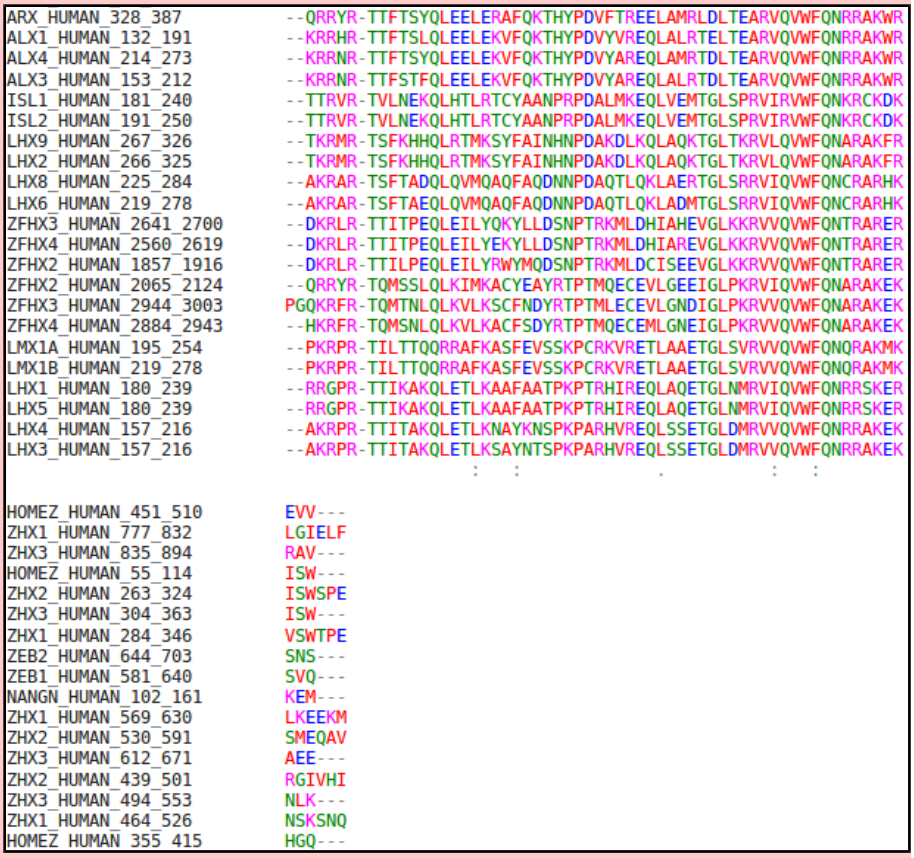
The alignment that is computed is, superficially at least, similar to that offered by **ClustalX**.

The alignment is irritatingly split into two sections. A nice extra parameter might have been "How wide would you like your alignment to be"? A problem with the format rather than the program, to be fair.

At the very bottom of the page, **muscle** whines:

PLEASE NOTE: Showing colors on large alignments is slow.

So click the **Show Colors** button at the top of the page and try to live with the pain of such gross Trans-Atlantic inept spelling in a European site!!! Good Grief! They get everywhere!!



Well, an improvement I suppose? Colours are very useful (even slow ones) in the interpretation of alignments. Various colour schemes are used to clarify the message of alignments. Colouring can indicate shared amino acid properties not immediately evident when the letter representations differ.

But any decoration available here is far short of what can be achieved with **Jalview**, so click on the **Download Alignment File** button to save you alignment in a file on your **Desktop** called:

```
homeobox_human_muscle.aln
```

Jalview can be easily installed under all commonly used operating systems and run locally. For these exercises, I attempt to use services available freely from the **INTERNET** wherever possible, so let us run **Jalview** from the web here by first going to:

http://www.jalview.org/

and selecting the Launch Jalview Desktop link at the top of the page. And agree with all the many questions you will be asked.

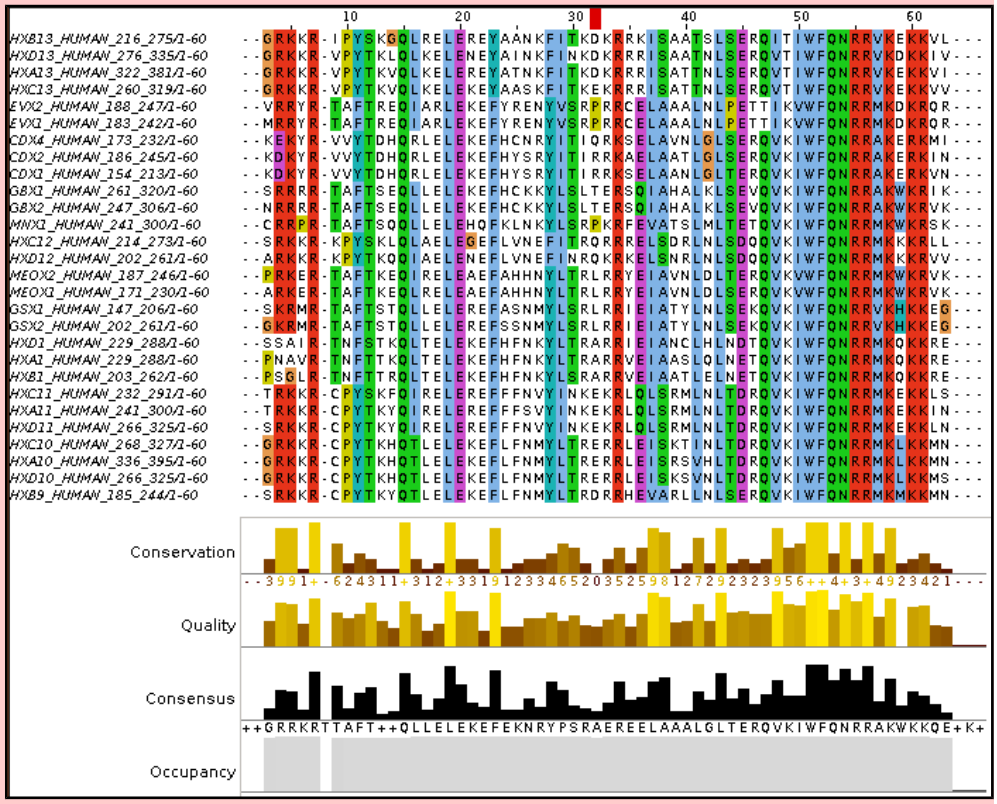
Close down all the example outputs **Jalview** sees fit to show you on start up. From the **File** pull down menu choose **from File** from the **Input Alignment** option. Locate and load the file:

`homeobox_human_muscle.aln`

You might need to adjust the file name filter to included **.aln** files.

The default view is a trifle bland. Try a few of the options from the **Colour** pull down menu.

You could try the default colour scheme used by **ClustalX**, for example.



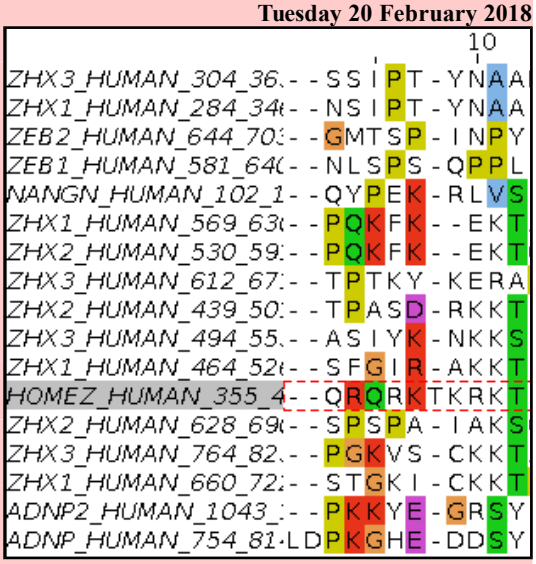
The **MUSCLE** and massaged **ClustalX** alignments now look very similar! In the nicely aligned regions at least.

There are many **Jalview** features that merit investigation. Have a look around if you have time. In particular, **Jalview** will compute simple phylogenetic trees for you employing a number of methods (**Calculate Tree** from the **Calculate** pull down menu). Try it, but be aware this is only sensible if you were very sure of your alignment (and have more meaningfully selected sequences maybe?).

Jalview is made by the same group as produce **Jpred** (an extremely effective **Secondary Structure Prediction** system). You could send your alignment for **Secondary Structure Prediction** via the **Web Service** pull down menu, if you wished.

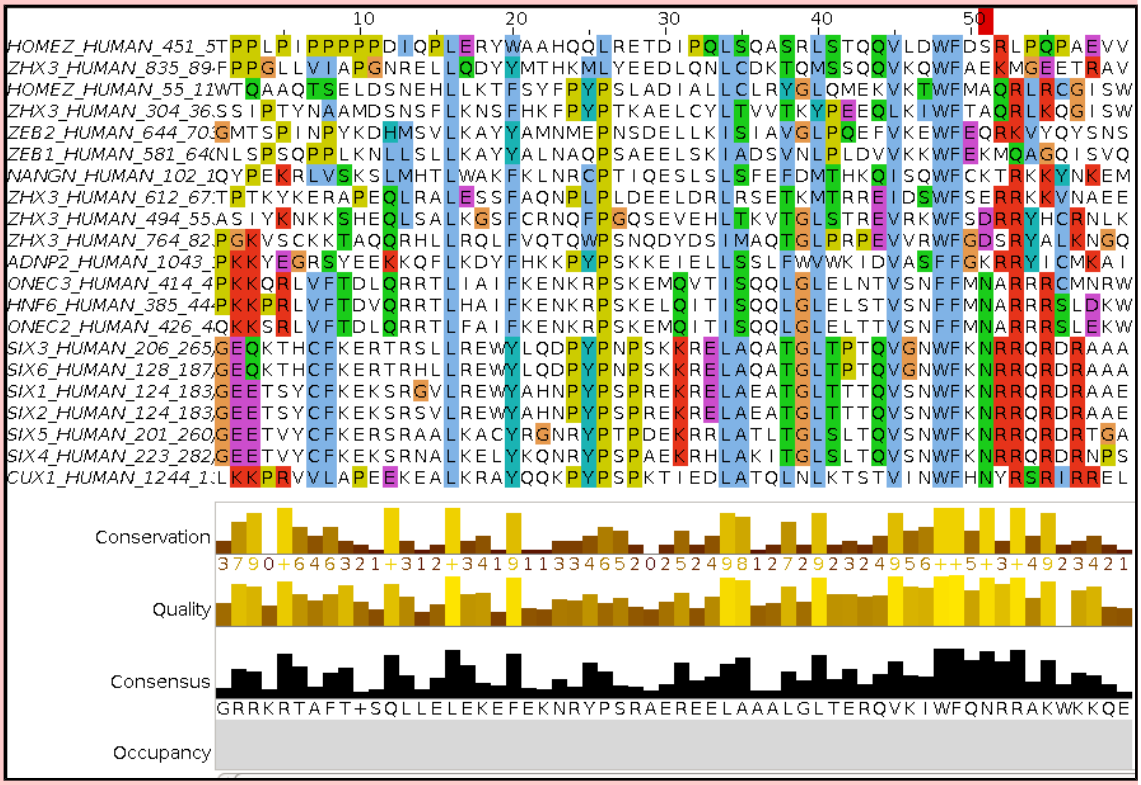
A central purpose of **Jalview** is to allow users to edit alignments as well as just to view them. For example, hold down the **Shift** key, click and hold on any amino acid at the edge of a gap, slide left and right and see that you can introduce and/or alter the position of gaps. It is very important to be able to edit alignments generated by even the best of programs. As I hope has been made clear, the alignment algorithms are crude. If you know something about the sequences you are aligning it is very reasonable to suppose you can improve upon the computer's alignments. **Jalview** tries to make this possibility easy. Look through some of the other **Edit** pull down menu options, maybe to increase the font size in particular!, it does not matter how much you mangle your alignment, you can always make another one.

Finally, take a look at the **Jalview** "**Manhattan Skyline**" for the highly conserved **W** at position **51**. This seems better quality than **clustalX** managed? I am not sure how one can make further comment without knowing what parameters were used. Is there really an improvement? If so, is it due to the improved algorithm or more appropriate choice of parameters? Impossible to discuss further as the parameters used for **MUSCLE** are not revealed.



In my alignment, the **W** at position **51** was at position **50**, according to **clustalx**. This slippage to the right is due to **MUSCLE** introducing an extra gap, inspired by just one sequence at position **8**. Is this sensible? No idea … exactly when it might be good idea to investigate the effect of lighter/heavier gap penalties?

You can also **Select** and **Cut** sequences in a way similar to that you employed with **clustalx**. I could not resist it! I removed all the ugly sequences that caused the gaps at the start and finish of the alignment, ***and*** the sequence that messed up column **8** (just select their names and then select **Cut** or **Delete** from the **Edit** menu). I achieved the gap-free beautiful alignment illustrated.



Of course, **Jalview** does not compute alignments, so once I had removed all the unfortunate proteins, I had to use an **Edit** option to tidy up my meddling. I used **Remove Empty Columns** to get rid of the gap columns at the start of the alignment. The gaps at the end just melted away once the sequences that supported their presence were removed.

Science is easy! Once you remove the need for honesty that is.

If it could be done slightly more meaningfully, I would suggest you might try some of the other **MSA** tools offered by the **EBI**, to investigate the differences in the alignments computed. Any differences might be due to different parameter selection or differences in the algorithms of the tool you select.

For full control, you really need to download the various tools and run them locally. The **EBI** is not the only site that hides significant parameters from their users. To be fair, one could argue that the web site should only set out to provide draft answers? Maybe the, relatively few users that need/desire full control should epect to download the software, read the manual and do things the hard way?

I am not sure I am sufficiently convinced, particularly when faced with pull down menues with one option and the chance to create data files I cannot use. Make your own mind up.

DPJ – 2017.12.23

Model Answers to Questions in the Instructions Text.

Notes:

For the most part, these "**Model Answers**" just provide the reactions/solutions I hoped you would work out for yourselves. However, sometime I have tried to offer a bit moer back ground and material for thought? Occasionally, I have rambled off into some rather self indulgent investigations that even I would not want to try and justify as pertenent to the objective of these exercises. I like to keep these meanders, as they help and entertain me, but I wish to warn you to only take regard of them if you are feeling particularly strong and have time to burn. Certainly not a good idea to indulge here during a time constrained course event!

Where things have got extreme, I am going to make two versions of the answer. One starting:

Summary:

Which has the answer with only a reasonably digestible volume of deep thought. Read this one.

The other will start:

Full Answer:

Beware of entering here! I do not hold back. Nothing complicated, but it will be long and full of pedantry.

This makes the Model answers section very big. **BUT**, it is not intended for printing or for reading serially, so I submit, being long and wordy does not matter. Feel free to disagree.

From your investigations of **Multiple Sequence Alignment**

How do the options for the **OUTPUT TREE** relate to the output files of **ClustalX** and the difference between the way that **ClustalX** and **muscle** work?

I leave this question here in the hope that one day I will be able to offer a full and sensible answer. First draft answer below.

Essentially, both **ClustalX** and **MUSCLE** work in two stages. First they create **Guide Tree(s)**. Then they create a multiple alignment by pairwise steps ordered by most refined the **Guide Tree**.

ClustalX just computes one based exclusively on the pairwise comparison of its input sequence set.

MUSCLE will create a **Guide Tree** that is the rough equivalent of that computed by **ClustalX**. Then it will offer to refine this **Guide Tree** from computed draft **MSA**s until a user selected maximum number of iterations is met or no further improvement is possible.

ClustalX saves the **Guide Tree** it computes by default. **MUSCLE** offers to save its **Guide Tree** from its first or second refinement iteration.

The purpose of saving the **Guide Tree(s)** to a file is to enable a rerun of the second phase with new parameter settings without having to first recalculate the **Guide Tree**. Of course, as mentioned previously, utterly pointless if there is no way to change the parameters to allow a guide tree to be used as input? but that is the theory.

More investigation by me and expansion of this answer required. Discussion with EBI current (2016.04.20).

Comment on how one might choose between the range of options offered for the aligned parameter?

I cannot … beyond suggesting it simply does not make sense? Going by what is offered at **Wageningen**, the choice should be between **aligned** and **input order**. i.e. the order of the original set of sequences to be aligned or the order after they have all been compared with each other and arranged into a **Guide Tree** … or two.

Currently, the only way of which I am aware to run **muscle** with full flexibility, is to download it. It is available for **Windows**, **Linux** or **Mac** operating systems but has no pretty **GUI** front end. You have to read the manual carefully and run from the command line.

To attempt (with pain) to be fair, one might suggest that web services are for creating draft results primarily. If one wanted to get serious and have full control over the software and record properly all the settings one has chosen, it would make sense to download the software and run in locally.

That still does not excuse offering selections that only have one option and/or save files that cannot serve any function. I think I give up trying to persuade the **EBI** guys of this and just live with "what is". So much more restful (**2017.05.01**).

DPJ – 2017.12.23

Discussion Points and Casual Questions arising from the Instructions Text.

Notes:

Work in progress I fear.

The intention is to provide a full consideration of some issues skimmed over in the exercise proper.

If you are attending a "supervised" presentation of the exercise, I would hope to have conducted a live discussion of all these issues to an extent that reflects:

- the depth that seems appropriate
- the time available
- the degree to which the issues seem to match the interests of the class
- how many of you are awake

Here, I hope to write out very full answers were such a response exists. Accordingly, I suggest you will not need to read much of many of these discussions. There will be much detail of interest to rather few of you. Possibly a bit self indulgent, but I wish to make a note of all the background I have discovered while writing these exercises.

In a nutshell, the exercises are trying to make very general points avoiding too much detail. Nevertheless, I record the detail outside the main exercise text, just in case it might be if interest. Some of the answers to the "**Casual Questions**" are exceedingly trivial. Some of the "**Discussion Points**" are exceedingly long and rambling. You have been warned.

Discussion of the way **ClustalX** (and similar multiple alignment tools) work.

…

Explanation of **clustalX FAST/APPROXIMATE** parameters.

…

Explanation of **clustalX Global Alignment** parameters.

…

The interpretation of the **Homeobox Prosite Pattern**?

```
[LIVMFYG]-[ASLVR]-x(2)-[LIVMSTACN]-x-[LIVM]-{Y}-x(2)-{L}-[LIV]-
[RKNQESTAIY]-[LIVFSTNKH]-W-[FYVC]-x-[NDQTAH]-x(5)-[RKNAIMW]
```

After reference to the Quick Hint mentioned in the text, the boring answer (taking each element in turn, after removing the optional "-" signs) is:

Pattern Position	*Pattern Element*	*Interpretation*
1	[LIVMFYG]	Any of the bracketed amino acid codes are acceptable
2	[ASLVR]	Any of the bracketed amino acid codes are acceptable
3	x(2)	Any amino acid is acceptable in the next 2 position
4	[LIVMSTACN]	Any of the bracketed amino acid codes are acceptable
5	x	Any amino acid is acceptable in this position
6	[LIVM]	Any of the bracketed amino acid codes are acceptable
7	{Y}	Any amino acid *EXCEPT* Y (Tyrosine) is acceptable in this position
8	x(2)	Any amino acid is acceptable in the next 2 position
9	{L}	Any amino acid *EXCEPT* L (Leucine) is acceptable in this position
10	[LIV]	Any of the bracketed amino acid codes are acceptable
11	[RKNQESTAIY]	Any of the bracketed amino acid codes are acceptable
12	[LIVFSTNKH]	Any of the bracketed amino acid codes are acceptable
13	W	The *ONLY* acceptable amino acid code in this position is a W (Tryptophan)
14	[FYVC]	Any of the bracketed amino acid codes are acceptable
15	x	Any amino acid is acceptable in this position
16	[NDQTAH]	Any of the bracketed amino acid codes are acceptable
17	x(5)	Any amino acid is acceptable in the next 5 position
18	[RKNAIMW]	Any of the bracketed amino acid codes are acceptable

Note the lack of flexibility of these patterns. An amino acid code is either allowed or not. No reflection of relative frequency of residues in the region of **MSA** from which they are designed (typically by hand).

Note that this particular pattern, though long, is too weak for **Interpro** take take very seriously. As discussed earlier, **Interpro** records a "**Conserved site**" when a match is discovered with this pattern. It is not considered strong enough, by itself, to indicate a **Homeobox** domain.

To examine a few more features of **Prosite**, particularly the very wide degree of relevance to be associated with matches with the patterns, I include a quick exercise to compare all of **Prosite** with the **Human PAX6** protein. In this exercise protein sequence motifs and protein domains will be sought using just **Prosite** and its associated searching software.

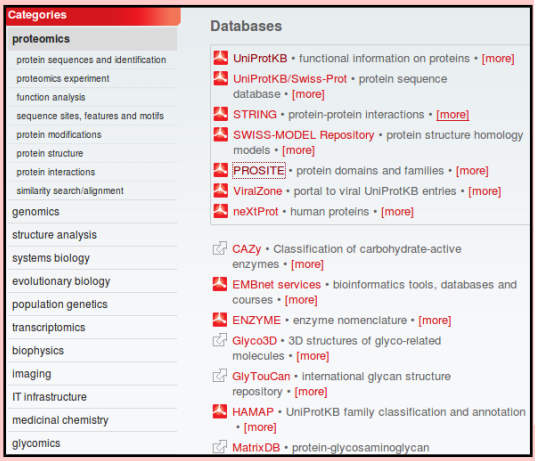
Please do not use class time to go through this. I would hope to discuss the issues briefly anyway. The full instructions are really for people who are going through the exercises by themselves.

Searching

A major database for both motifs and domains is PROSITE. Sequence motifs include examples that are extremely simple, and short. These represent such common phenomena possible sites for post-translational modifications (e.g. **glycosylation** or **phosphorylation**). Motifs are generally represented by "Patterns" of characters adhering to some very trivial rules.

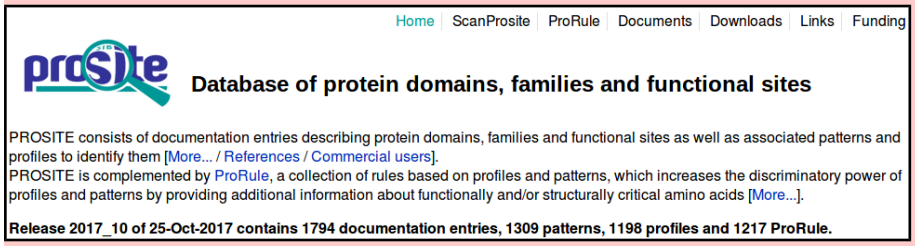
For a swift experience of using **Prosite**, try the following. Go to the ExPASy[10] site at:

http://www.expasy.org



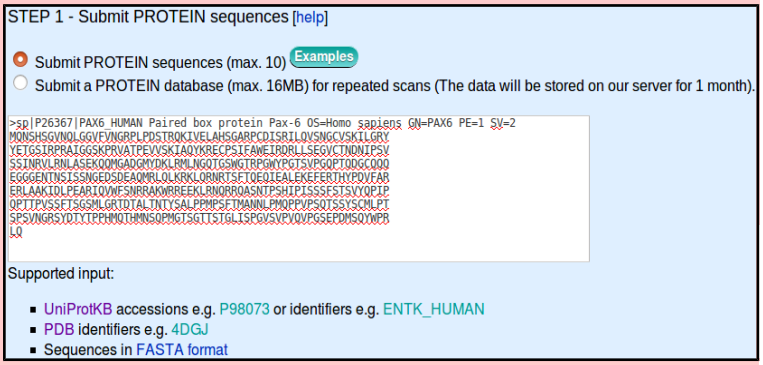
Select **proteomics** from the list of **Categories**.

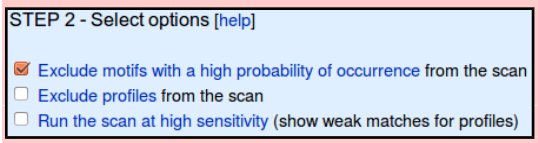
Select **PROSITE** from the **Databases** section.



Click on the **ScanProsite** link at the top of your page.

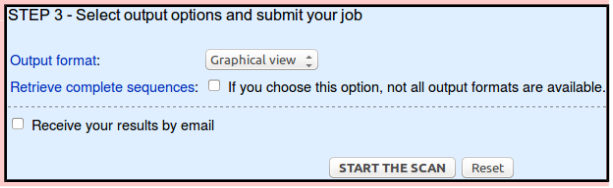
Enter **pax6_human** in the **STEP 1 - Submit PROTEIN sequences** section.





In the **STEP 2 - Select options** section, ensure that the **Exclude motifs with a high probability of occurrence** box is ticked.

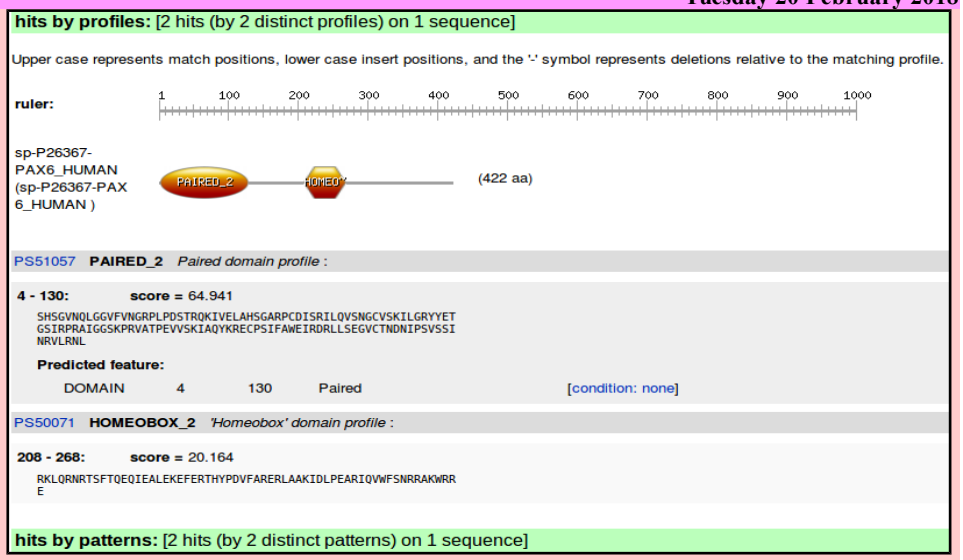
The defaults offered in the **STEP 3 - Select output options and submit your job** section are fine so just click on the **START THE SCAN** button. In but a few moments, your results will burst forth.



10 **Expasy** is a major site for protein based research in Switzerland. As the all knowing Wikipedia puts it:

"**ExPASy** is a **bioinformatics** resource portal operated by the **S**wiss **I**nstitute of **B**ioinformatics (**SIB**) and in particular the **SIB Web Team**. It is an extensible and integrative portal accessing many scientific resources, databases and software tools in different areas of life sciences. Scientists can access a wide range of resources in many different domains, such as **proteomics**, **genomics**, **phylogeny/evolution**, **systems biology**, **population genetics**, and **transcriptomics**."

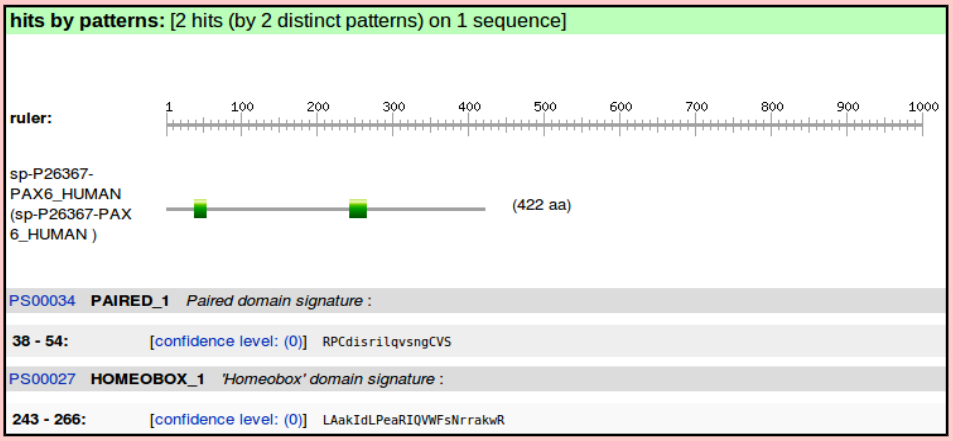
Two hits with **PROSITE** profiles suggesting the familiar domains in their familiar places.



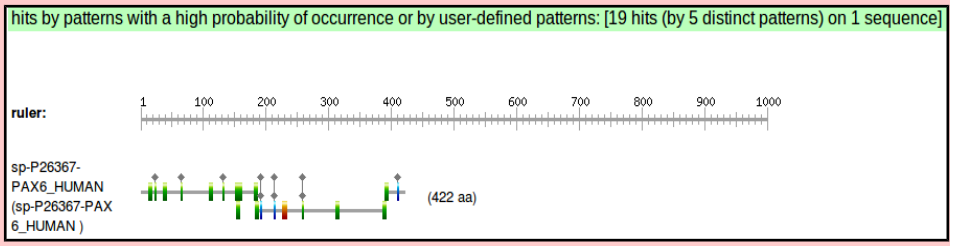
Two hits with **PROSITE** patterns confirm the same domains by matching highly conserved subregions.

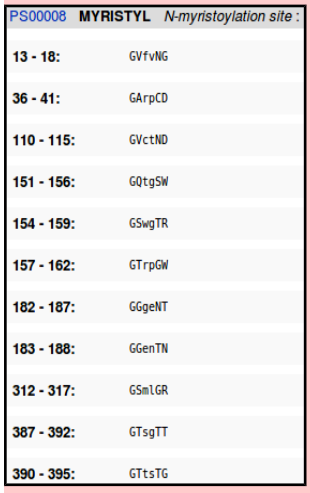
This confirms what has already been discovered more than once, by reading database annotations, by running **Interpro** and by running other individual database search program(s) manually.

Note that **Prosite** is happy to accept the **HOMEOBOX** pattern hit as sufficient to predict the presence of a **Homeobox domain**. **Interpro** regards exactly the same evidence to register only a "**Homeobox conserved site**". I suspect the caution of **Interpro** is justified.



Move back to the search submission. In **Step 2**, deselect **Exclude patterns with a high probability of occurrence**. **START THE SCAN**.





This time you will see many more hits with very short patterns.

Follow the link to the documentation for an **N-myristoylation site** (**PS00008**).

See that the pattern is just **6** positions wide. **2** of those positions can be any amino acid. Only one position is fully specified. Not too demanding on the whole. I would expect this to match most proteins of any size and not always because there was an **N-myristoylation** site.

The pattern is explained in the database thus.

- The N-terminal residue must be glycine.
- In position 2, uncharged residues are allowed. Charged residues, proline and large hydrophobic residues are not allowed.
- In positions 3 and 4, most, if not all, residues are allowed.
- In position 5, small uncharged residues are allowed (Ala, Ser, Thr, Cys, Asn and Gly). Serine is favored.
- In position 6, proline is not allowed.

The description is not entirely an honest reflection of the information to which the scanning software will respond. The software is given to understand that **ANY** amino acid can occur in positions **3** and **4**. The software has no way to know that "**Serine** is favoured" in position **5**! Maybe you think that my pointing out these transparent truths makes me an intolerable pedant? Well … so is the computer!

PROSITE predicts **11 N-myristoylation** sites in the **Human PAX6** protein. A site every **40** amino acids or so. Without considerable further effort, it is not really possible to suggest how many of these predictions might be "real". The evidence of this exercise alone is most certainly insufficient. Intuitively, I would expect a large number of false positives from as weakly specified motif as this one. It has been suggested (**May** of **2011**) of this **PROSITE** pattern, by researchers looking at more sophisticated detection methods, that:

> "**PS00008** of **PROSITE** constructed from a small dataset … produces a great number of not only false positive but false negative predictions."

This is good enough to believe the majority of these predictions to be unreliable. It is not good enough for me to hazard a meaningful guess as to how many real sites would be expected in this particular protein.

Consider for a few moments the **Prosite Paired Box** pattern, **R-P-C-x(11)-C-V-S**, specifically its location within the **Paired Box** domain.

At the top of your **ScanProsite Results** page, you will find the **canonical** version of **PAX6 Human** displayed. If you hover over the graphic indicating the position of the **Profile** match for the **Paired Box**, the position of the whole **Paired Box** domain will be highlighted. If you hover over the graphic for the **Pattern** match for **Paired box**, the position of the pattern will be illustrated.

My illustration is of these two views superimposed on each other and prettied up a trifle.

The pattern **RPC**xxxxxxxxxx**CVS** within the entire domain is clear.

```
MQNSHSGVNQLGGVFVNGRPLPDSTRQKIVELAHSGARPCDISRILQVSNGCVSKILGRYYETGSI
RPRAIGGSKPRVATPEVVSKIAQYKRECPSIFAWEIRDRLLSEGVCTNDNIPSVSSINRVLRNLAS
EKQQMGADGMYDKLRMLNGQTGSWGTRPGWYPGTSVPGQPTQDGCQQQEGGGENTNSISSNGEDSD
EAQMRLQLKRKLQRNRTSFTQEQIEALEKEFERTHYPDVFARERLAAKIDLPEARIQVWFSNRRAK
WRREEKLRNQRRQASNTPSHIPISSSFSTSVYQPIPQPTTPVSSFTSGSMLGRTDTALTNTYSALP
PMPSFTMANNLPMQPPVPSQTSSYSCMLPTSPSVNGRSYDTYTPPHMQTHMNSQPMGTSGTTSTGL
ISPGVSVPVQVPGSEPDMSQYWPRLQ
```

If you were to repeat this whole exercise with the **isoform 5a** version of **Human PAX6** (*please do not!*), the equivalent picture would be as illustrated.

```
MQNSHSGVNQLGGVFVNGRPLPDSTRQKIVELAHSGARPCDISRILQTHADAKVQVLDNQNVSNGC
VSKILGRYYETGSIRPRAIGGSKPRVATPEVVSKIAQYKRECPSIFAWEIRDRLLSEGVCTNDNIP
SVSSINRVLRNLASEKQQMGADGMYDKLRMLNGQTGSWGTRPGWYPGTSVPGQPTQDGCQQQEGGG
ENTNSISSNGEDSDEAQMRLQLKRKLQRNRTSFTQEQIEALEKEFERTHYPDVFARERLAAKIDLP
EARIQVWFSNRRAKWRREEKLRNQRRQASNTPSHIPISSSFSTSVYQPIPQPTTPVSSFTSGSMLG
RTDTALTNTYSALPPMPSFTMANNLPMQPPVPSQTSSYSCMLPTSPSVNGRSYDTYTPPHMQTHMN
SQPMGTSGTTSTGLISPGVSVPVQVPGSEPDMSQYWPRLQ
```

The **14** amino acid insertion of **isoform 5a** (**THADAKVQVLDNQN**, corresponding to the entire **3rd** coding exon being spliced into the **mRNA**) has landed right in the middle of the pattern! It surely cannot match as intended when used with an **isoform 5a PAX** domain.

From your **ScanProsite Results** page, follow the link to the documentation for this pattern (PS00034). Find and read the description of the pattern where it is claimed that the pattern matches all **58** true **Paired Boxes** in **SwissProt**[11].

PAIRED_1, PS00034; Paired domain signature (PATTERN)

- Consensus pattern:
  R-P-C-x(11)-C-V-S
- Sequences in UniProtKB/Swiss-Prot known to belong to this class: 58
  - detected by PS00034: 58 (true positives)
  - undetected by PS00034: 0 (false negative or 'partial')
- Other sequence(s) in UniProtKB/Swiss-Prot detected by PS00034:
  7 false positives.
- Retrieve an alignment of UniProtKB/Swiss-Prot true positive hits:

This is bold claim can only be true if ***none*** the **PAX** domains in **Swissprot** are **isoform 5a** domains. Unsurprisingly, this is the case. All **PAX** proteins are recorded in **Swissprot** in their "**canonical form**". **Isoform 5a** variants are always only acknowledged in the annotation as "**Features**". **ScanProsite** is not clever enough to assemble and search all variations of a **Swissprot** entry. It just searches the main ***canonical*** sequence. Yes, it finds all **58** ***canonical*** **SwissProt PAX** proteins, but it would not find any **isoform 5a PAX** proteins if they were stored as separate entries in **SwissProt** (or input to **ScanProsite** as an independent protein sequence). The **PAX Prosite Pattern** is not as effective as its documentation claims.

11 This claim is, of course, only possible if all the annotation of **SwissProt** is assumed to be **100%** accurate.

In order to detect just the **PAX isoform 5a**, the pattern would have to be:

R-P-C-x(25)-C-V-S

To detect both isoforms, using just one pattern:

R-P-C-x(11,25)-C-V-S

would work, but would be insufficiently specific and would generate far too many false positives. These sort of patterns are useful, but only with caution. They are valuable because of their simplicity, but they are very fragile.

In the **Prosite Paired domain documentation page**, just below the **Pattern** description, is the **Profile** description.

- Sequences in UniProtKB/Swiss-Prot known to belong to this class: 58
  - detected by PS51057: 58 (true positives)
  - undetected by PS51057: 0 (false negative or 'partial')
- Other sequence(s) in UniProtKB/Swiss-Prot detected by PS51057: NONE.

The claim here is also to find all the **58 PAX** domains in **SwissProt**. This time, with **0** false positives (the **Pattern** had to admit to **7**). A clear but small improvement, but, the real superiority of the **Profile** over the **Pattern** is that its allows enough flexibility to find **Paired** boxes that have the relatively large **14** amino acid **isoform 5a** insertion. The documentation cannot boast that this is true as there are no instances in SwissProt to allow the case to be proven, However, it is true … because I say so!

This flexibility of the probabilistic approach employed by **pHMM**s was also illustrated when we glanced at **PFAM**. The **PFAM pHMM** for **PAX** was computed from a **5** sequence alignment including no representation of any **isoform 5a** sequence, yet it too will match **isoform 5a PAX domains**.

Comments on **Jalview** as an alignment viewer/editor in various contexts (e.g. **Pfam** and **Jpred**).

Jalview has appeared in the exercises twice already. Not however, in particularly high profile sections, so you might have yet to be introduced formally. Here I attempt brief correction of any inappropriate informality.

Alignment algorithms are crude.

DPJ – 2017.12.23

References for further extension:

https://en.wikipedia.org/wiki/Multiple_sequence_alignment