Identification of Conserved Regions in CRISPR Protein Family

02-712 Final Project

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Abstract

In the past few years, CRISPR/*Cas* system has enjoyed exponential growth in terms of studies and applications due to its sequence-level recognition. However, because this is a relatively new topic, while there are many publications demonstrating successful application of *Cas9*, limited studies are currently available on the protein family as a whole. While *Cas9* currently is the CRISPR protein *du jour*, other *Cas* proteins offer much more divergence in terms of PAM sequences, as well as possible reduction in terms of components required and size of the protein complex for *Cas* applications, and could potentially address some of the challenges that have come up in applications of *Cas9*. Therefore, it is imperative that much more studies are done on the *Cas* family as a whole rather than just applications. In this project, we explored the capability of the following three approaches, pairwise sequence alignment, Gibbs Sampling, and HMM profiling, in our attempt to identify conserved regions, or motifs, in the *Cas* family. However, due to limited data and and high divergence of *Cas* proteins, it was proven to be challenging to discover consistent motifs, especially in more complex models but nevertheless offer us insight into CRISPR/*Cas* protein family.

1 Introduction

This report explores the evolution and relationship of various *Cas* (CRISPR-associated) proteins. CRISPR is a prokaryotic adaptive immune system that works by base-pair recognition of foreign genetic material and subsequent nuclease activity on the non-self genome. This has been adopted for various applications including genome engineering, and the fact that CRISPR's recognition mechanism based on base-pairing (as opposed to protein-DNA recognition of ZNF or TALENs) result in improved accuracy and reduced costs (no protein engineering involved). CRISPR is as diverse as the species that carry CRISPR in its genome, but ultimately have the same function of adaptive immunity against foreign agents. While *Cas9* (isolated from *Streptococcus pyogenes*) is currently the Cas protein of choice for such applications due to smallest number of involved components, it would be beneficial to study the other *Cas* proteins as well, since *s.pyogenes Cas9* is limited in terms of PAM (Protospacer Adjacent Motif), of `-NGG`), large size of *Cas9* provides limits in some applications, as well as expanding available options for CRISPR engineering.

Cas9 currently is predominantly used for genome engineering because it was the first CRISPR protein that was successfully adapted for genome engineering, chosen for its relative simplicity (single gene for all required protein components). In other CRISPR system, the CRISPR functionality is split into multiple *Cas* proteins rather than a single protein, such as in *Cas9*. Now that we know more about the CRISPR system and somewhat better understanding of what each domain does, it may be beneficial to explore using some of the other CRISPR proteins to achieve same goal : use *Cas* protein and its base-pair recognition capacity for various applications ranging from scientific research to genetic therapy.

Cas9's big size (1368AA residues) has been a source of concern for therapeutic applications. Prominent delivery tool for *in vivo* and *in vitro* for gene therapy has been adeno-associated virus. While there have been attempts to utilize such delivery method for *Cas9* such as [12] or [13], its prohibitively large size has limited number of studies reporting successful gene therapy using both *Cas9* and AAV. Other families of *Cas* proteins are predicted to have different functions split up into multiple genes. As some function of CRISPR system is not necessarily for the purpose of genetic engineering (such as initial cleavage and insertion of foreign genetic material into CRISPR complex), it would be greatly beneficial to identify and isolate specific regions of interest, as well as individual *Cas* proteins that represent those regions, that are directly applicable for genome studies. By identifying conserved motifs between different CRISPR proteins, we can hopefully identify regions of corresponding activity in those CRISPR proteins, and compare to *Cas9* which has been studied in greater detail in terms of structure [10] or function [6] compared to other *Cas* proteins. Using other *Cas* proteins would not only allow for greater choice for PAM motifs, but also possibly smaller *Cas* proteins that can accomplish same goals, with less hindrance from the size of protein.

In this report, we employ 3 different approaches to identify motifs, or conserved regions of significance in terms of *Cas* function for better understanding of the *Cas* protein family and mechanism of each component.

1.1 CRISPR/Cas

CRISPR(Clustered regularly interspaced short palindromic repeats) is a microbial adaptive immune system. While bacteria and archaea utilize CRISPR system to store foreign genetic material to distinguish self vs non-self, this system has been adopted and exploited by scientists since 2012 genome engineering tool, as discussed in [7] and [8]. CRISPR initially began as next-generation tool to replace ZNFs and TALENs, it has since then been modified for non-genome engineering purposes such as CRISPRi [9]. There also have been attempts to reduce off-target effects by modifying the nuclease domain [11] .

Naturally in bacteria or archaea, CRISPR proteins have distinct roles in the three phases of CRISPR system as follows :

1. Acquisition : foreign genetic material enters microbe, which is cut by CRISPR protein and inserted into CRISPR array. This fragment is now a *spacer* separated by *repeats*, hence the name
2. Expression : CRISPR array, which include multiple spacers separated by repeats, is expressed as a single RNA. This is then cleaved into individual units known as *crRNA*, which contain single spacer. *crRNA* forms complex with one or more CRISPR proteins (depending on the CRISPR system)

3. Interference : upon recognition of specific foreign genetic material via base-pairing with the spacer in crRNA, CRISPR protein in complex with the spacer cleaves the foreign genetic material.

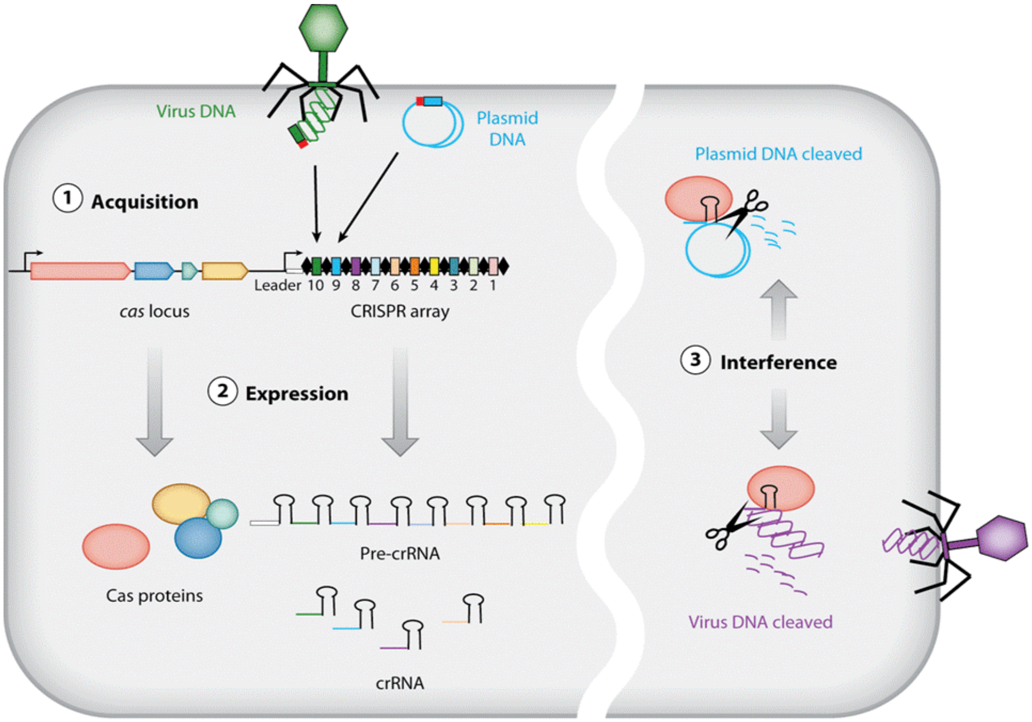

Figure 1: Overview of CRISPR proteins and their function as described in 1.1 [5]

1.2 Past Approaches

Functionally related regions can be clustered by evidences in experimental data. As summarized in Figure 2. Previous work has found conserved regions on the sequence level using sequence alignment and structural information [4] inside each sub-type of the *Cas* system but not across the whole *Cas* family.

1.3 Goal of Project

In this paper, we would like to answer the question that whether or not the proteins in *Cas* family share some sequence level similarity. And more specifically, as Cas9 is a multi-domain protein with multiple functions and each function can be achieved by other single-function protein in *Cas* family, we would like to explore if we can map such functional domain similarity on the basis of primary sequence similarity between Cas9 and other cas proteins. Informally, we tend to solve the following problem:

- **Input**: Two sets of sequences $C_1 = \{p_1, ..., p_m\}$ and $C_2 = \{q_1, ..., q_n\}$

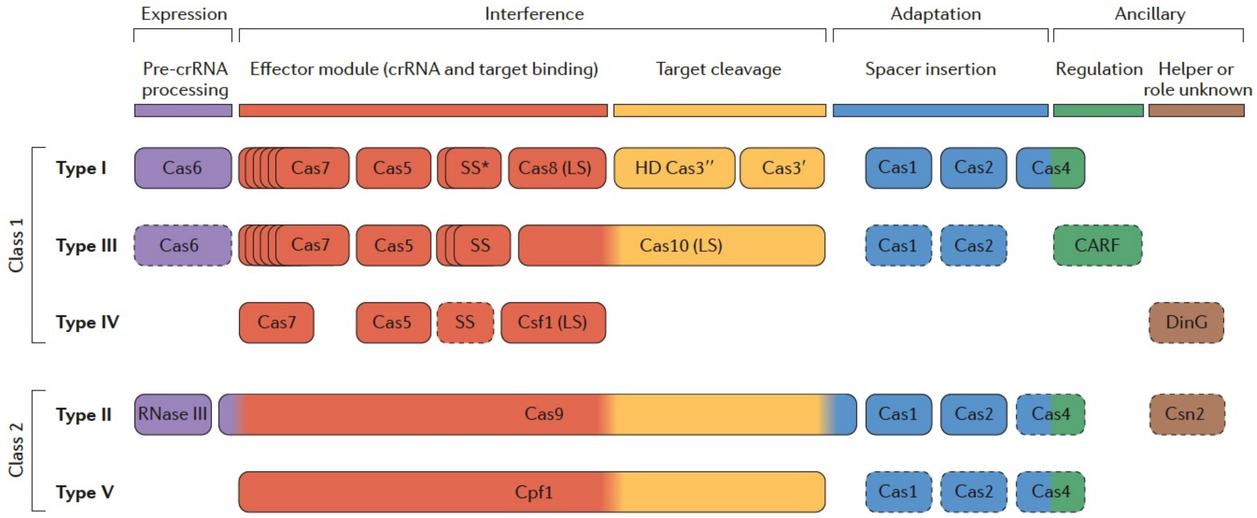

Figure 2: Conserved building blocks of *Cas* family proteins [1]

- **Output**: A set of regions $R = \{r_1, ..., r_k\}$ such that r_i *occurs* and is *representative* in both C_1 and C_2

Here *occurs* and *representative* can be explained in different ways under different strategies. In the following section we will discuss the two strategies we proposed to solve this problem.

1.4 Approaches in This Project

In this paper, we propose the following two strategies: i) alignment; ii) motif finding. First of all, our problem is naturally a multiple sequence alignment problem. Notice that other *Cas* proteins is like a substring of Cas9, then to recognize such local similarity, both semi-global alignment and local alignment is suitable in this case. And here *occurs* and *representative* mean that r_i is optimal in alignment.

Besides profile-based local alignment or semi-global alignment techniques, an alternative approach is to make use of motif information. Motif finding problem is defined as to find representative pattern in a collection of sequences. Following this idea, we can first find motif in C_1 and perform pattern recognition in C_2. The motif found in C_1 carries the *representative* signature of C_1 and the recognition in C_2 tests whether it meets the requirement to be *occur* in both C_1 and C_2. Furthermore, for motif analysis, we propose two widely used methods: i) Gibbs sampling; ii) Hidden Markov Models.

2 Methods

All code and output files are available on `https://github.com/cookie223/CAS_project`.

Any reference to files in this report indicate filepath based on root of the repository.

In this paper, we use 3 different approaches, each with different strengths and limits as for discovering patterns and information from multiple related sequences. Each method has its own section which discusses overview of algorithm/model, pros and cons of given model, detailed protocol and parameters, and analysis performed.

Protein sequences were used (as opposed to DNA), to uncover preservation of *Cas* protein's functional motifs. Protein sequence analysis much more appropriate for such purpose than DNA sequence especially for distantly related sequences.

2.1 Data Retrieval

Gene sequences for *Cas* family including *Cas1* through *Cas10* were searched and downloaded from NCBI. For each gene, a variety of species were selected to have all the sequences of the proteins in the *Cas* system of one species and also maintain a certain level of variety. The selection is also subject to the availability in NCBI. For domain-specific profile HMM, domain markers were fectched from EMBL-EBI (http://www.ebi.ac.uk).

2.2 Sequence Alignment using Dynamic Programming

Semiglobal alignment using Needleman-Wunsch [2] and local alignment using Smith-Waterman Algorithm [3] were implemented for pairwise sequence comparison. This is the most intuitive and straight-forward approach for identifying regions of similarity or conserved patterns. However, as simple as the method is, it does have some limitations as discussed in detail below. Because amino acid sequences were used for alignment, it does allow for discrimination between varying degree of conservation or divergence of residues or patterns. While *Cas1, Cas2* and *Cas4* are not part of interference domain and typically not considered in studies for applications of CRISPR/Cas, I included these proteins in pairwise sequence alignment against *s.pyogenes Cas9* to explore whether there is any sequence similarity within the CRISPR proteins that are currently predicted to be separate genes, and possibly repeated functionality.

2.2.1 Model & Algorithm Overview

Semiglobal alignment and local alignment were performed on various *Cas* sequences. Because certain families of *Cas* proteins are composed of multiple genes, it is impossible to do a global alignment, or multiple sequence alignment with all *Cas* sequences. Instead, *s.pyogenes Cas9* was used as a reference sequence, to which all other *Cas* sequence was aligned to in pairwise sequence alignment.

Semiglobal alignment aimed to identify which region of the *Cas* protein as a whole fit to *Cas9*, or the reference sequence (which part of *Cas9* it is most similar to as a whole). As *Cas9* has been studied extensively relative to other *Cas* proteins, this approach aimed to possibly map the function of individual *Cas* protein to a specific region of *Cas9* protein with regions with identified functions.

Local alignment aimed to identify specific regions that may or may not be smaller than the *Cas* protein itself, compared to *Cas9*. This alignment should be fairly similar in case of highly conserved sequences. However, it may result in *Cas* protein mapping to different regions in *Cas9* depending on the size or the degree of divergence.

2.2.2 Pros and Cons

Because alignment were done against *s.pyogenes Cas9* rather than a progressive alignment, for *Cas* sequences highly divergent from *s.pyogenes Cas9* may be aligned to an inaccurate site. Each alignment is guaranteed to return the global maximum or the most optimal alignment with given parameters, which may or may not be the actual corresponding motif. Also, this method assumes site independence, and does not discriminate conserved regions (which motifs would likely be part of) as opposed to fast-evolving regions.

2.2.3 Protocol

Following parameters were used for sequence alignment :

- Scoring Matrix = BLOSUM62 (BLASTP default)
- Affine Gap Penalty = -10 (BLASTP default is -11)
- Gap Extension Penalty = -1 (BLASTP default)
- End Gap Penalty (for Semi-Global only) = -3

Gap opening / affine gap penalty was slightly lowered to relax requirement for opening gap, as this experiment is for identifying local regions rather than strict sequence search.

2.2.4 Analysis

After each *Cas* sequence was aligned to *s.pyogenes Cas9*, it was then analyzed for the following values :

- Start position (row, col) of traceback
- End position (row, col) of traceback
- Alignment score (based on parameters discussed in section 2.2
- Average Score per base : alignment score / number of bases in alignment
- % Sequence Aligned : length of alignment / length of query (non-reference) sequence

This was done for both semi-global and local alignment outputs.

2.3 Gibbs Sampling

2.3.1 Model & Algorithm Overview

Gibbs sampling approach is based on position-specific scoring matrix [15], or PSSM, is one of the ways to model a motif. Suppose we are working on sequence set with Σ as alphabet and the length of the motif is w. Then PSSM is a $|\Sigma|$-by-w matrix with entry:

$$S_c(A) = \log \frac{\Pr(A \text{ is at } c\text{th column}|\text{motif})}{\Pr(A \text{ is at } c\text{th column}|\text{background})}$$

, where $S_c(A), A \in \Sigma$ is the score of alphabet A appearing at cth position in the motif and the score is a log odds ratio.

Under PSSM setup, the motif is ungapped with a fixed length and each position is scored independently to each other. PSSM provides a way to parameterize motif and, furthermore, the motif finding problem can be cast as an optimization problem as follow:

$$\max_{S,o} \sum_{i=1}^{n} \sum_{c=1}^{w} S_c(q_i[o_i + c]) \tag{1}$$

, where $q_i[x]$ is the xth character of ith sequence in the collection and o_i indicates the starting site of the motif for ith sequence. With various size of the sequence set, this problem is NP-hard and [16] has proposed a Gibbs sampling approach to solve it.

Note that with known o, solving for S is reduced to a maximum likelihood estimation, which is trivial to solve, so the core of this problem is to find the optimal $o^\star$. Let S^o denote the scoring matrix induced by o and $f_S(o)$ denote the objective in Equation (1) (with S as scoring matrix), then we can encode the probability distribution of o according to $f(o)$:

$$\Pr(o) \propto e^{f_{S^o}(o)}$$

Gibbs sampler can be used to sample from this distribution with transition probability as follow:

$$\begin{aligned}
&\log q(o_1, ..., o'_i, ..., o_k | o_1, ..., o_i, ..., o_k) \\
=&\log \frac{1}{k} \Pr(o_1, ..., o'_i, ..., o_k | o_1, ..., o_i, ..., o_k) \qquad (2) \\
=&S^{(o_1,...,o'_i,...,o_k)}(o'_i) \sum_{j \neq i}^{k} S^{(o_1,...,o'_i,...,o_k)}(o_j) + const \\
\approx&S^{o_{-i}}(o'_i) \sum_{j \neq i}^{k} S^{o_{-i}}(o_j) + const \\
=&S^{o_{-i}}(o'_i) + const \qquad (3)
\end{aligned}$$

, where $S^{o_{-i}}$ is the scoring matrix derived from o without o_i and $S(o_i)$ is the score of o_i based on S. With this setup, [16] proposed Algorithm 1.

Algorithm 1 Gibbs sampler for motif finding

```
Input: sequence set $q$, motif width $w$
Output: set of starting points $o$
initialize $o$
$o^\star \leftarrow o$
while forever do
    pick $i$ from $1, ..., |q|$ with uniform distribution
    update $S \leftarrow S^{o_{-i}}$
    compute transition probability $p(o'_i) \leftarrow \exp\{S(o'_i)\}$
    update $o_i \sim \text{Multinomial}(p, 1)$
    update $o^\star$ if there is any improvement
end while
```

2.3.2 Pros and Cons

Gibbs sampling approach is easy to implement and it converges to optimal solution as the running time goes to infinity. Besides, PSSM representation of a motif is easy to understand and visualize. While, the downside of this approach is that it cannot bear gap. If the sequence collection shares a gapped motif, PSSM based Gibbs sampler will fail to recognize it. Furthermore, the length of the motif and the number of steps are two hyper-parameters which should be specified by the user. In practice, it takes extra computing time to search for a suitable width and to stop early or later is instance specific, and has no general strategy to follow.

2.3.3 Protocol

Following steps were applied to the analysis:

1. Implement a Gibbs sampler described in Algorithm 1
2. Train a set of motifs with various widths using *Cas5* and *Cas7*
3. Score Cas9 sequences based on learned motifs

The widths used started from 10 to up to 190 and the size of interval was 10. To retrieve not only the most representative motif but other sub-optimal ones, for every width we repeated the Gibbs sampling procedure five times and recorded the best output for every run. For every Markov chain, we ran 600 steps because it was sufficient to reach a suboptimal with 600 steps for all instances in analysis.

2.3.4 Analysis

To show our Gibbs sampler converge, we track the objective value along the Markov chain. And to show our motif finding based strategy works, we performed the proposed procedure on simulated sequences and Globin sequences given in Hmmer [14] where two sets of sequences share similar primary sequence patterns and checked whether our approach could recover such similarity.

2.4 Domain-specific profile HMM

2.4.1 Model & Algorithm Overview

To find out whether a sequence of amino acid belongs some domain, we can build a model of the domain and try to match the sequence of the model. Profile Hidden Markov Model is one of the models we can build to figure out whether a sequence contains the domain. The model of profile HMM is shown in Figure 3.

2.4.2 Pros and Cons

- Pros
  1. We can leverage the abundant prior knowledge of Cas9 domain markers by building profile HMM using the alignment of the domains rather than the full sequence.

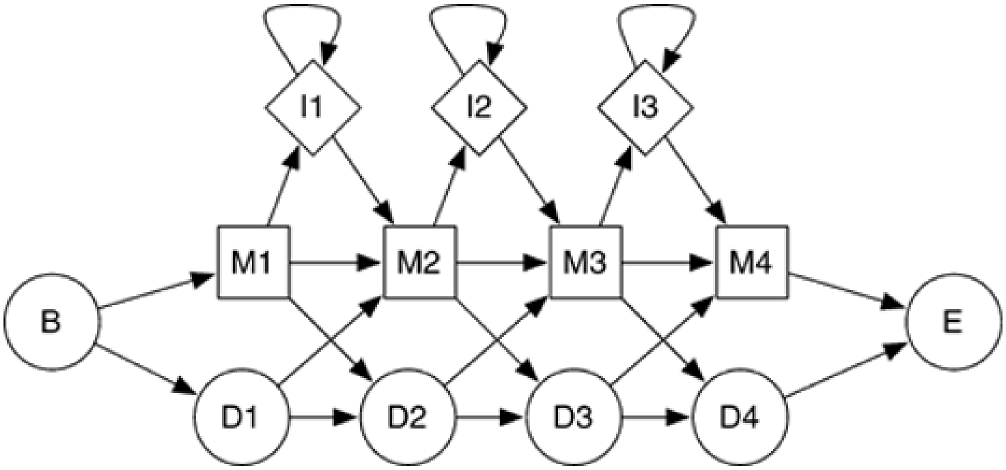

Figure 3: Profile Hidden Markov Model [14]

2. Compared with pair-wise sequence alignment, HMM can find more cases of distantly related sequences.
3. As shown in the figure, HMM can model insertions and deletions.

- Cons
  1. To leverage the prior knowledge of domain markers, those markers need to be fetched separately from other data source rather than learned by the algorithm.
  2. The number of parameters is very large and they need to be optimized.

2.4.3 Protocol

a. A set of *Cas9* or *Cas5* sequences and their domain markers are fetched from EMBL-EBI (http://www.ebi.ac.uk).

b. Sequences of each of the shared domains are subtracted from the full *Cas9* or *Cas5* sequences.

c. For each domain, multiple sequences from different *Cas9* or *Cas5* are aligned by Clustal Omega (http://www.clustal.org/).

d. A profile HMM is built on the multiple sequence alignment for each domain by Hmmer (http://hmmer.org/) [14].

e. Search for matches using the profile HMM in the sequences of all previously downloaded *Cas* family proteins.

2.4.4 Analysis

For method testing, a set of Globin sequences given in Hmmer [14] is used. Since there are *Cas9* and *Cas5* in the previously downloaded *Cas* family proteins sequences, they also act as positive control since the method should be able to find the domains in these proteins.

For output analysis, HMM is able to give the probability of given sequence emitted from the underlying domain profile HMM.

3 Results

Each of the three approaches for identifying motifs of *Cas* proteins and the resulting data are presented below.

3.1 Sequence Alignment using Dynamic Programming

Table 1: Semi-Global Alignment Output

Gene Name	Alignment Score	Average Score / base	% Sequence aligned	Traceback Start	Traceback End
Cas10_Mtuberculosis	234	0.212148685	1	[1365][810]	[330][1]
Cas10_Phorikoshii	335	0.249813572	1	[1368][762]	[41][1]
Cas10_Ssolfataricus	364	0.27063197	1	[1322][1046]	[62][1]
Cas10_Tvolcanium	343	0.305976806	1	[1203][776]	[146][1]
Cas1_DvsH_plasmid	160	0.282186949	1	[1297][344]	[734][1]
Cas1_Gvaginalis	163	0.332653061	1	[1367][321]	[886][1]
Cas1_K-12	105	0.243055556	0.911764706	[428][306]	[1][28]
Cas1_Tdenticola	176	0.387665198	1	[519][291]	[79][1]
Cas2_K-12	65	0.5	1	[528][95]	[400][1]
Cas2_Lsalivarius	64	0.444444444	1	[617][102]	[476][1]
Cas2_StB20-like	68	0.586206897	1	[217][88]	[102][1]
Cas3_DvsH_plasmid	152	0.145315488	1	[1059][703]	[32][1]
Cas3_Gvaginalis	260	0.229681979	0.858199753	[1119][811]	[1][116]
Cas3_K-12	233	0.185805423	1	[1254][889]	[29][1]
Cas4_K-12	171	0.341317365	1	[1323][364]	[833][1]
Cas4_Ssolfataricus	87	0.294915254	1	[679][203]	[387][1]
Cas4_TtenaxKra	57	0.230769231	1	[573][191]	[330][1]
Cas5_Gvaginalis	140	0.309050773	1	[1185][292]	[733][1]
Cas5_K-12	79	0.302681992	0.8	[261][225]	[1][46]
Cas6_Cbotulinum	158	0.478787879	1	[740][230]	[414][1]
Cas6_Hvolcanii_plasmid	99	0.25848564	1	[1309][273]	[929][1]
Cas6_Ssolfataricus	118	0.280952381	1	[1117][288]	[701][1]
Cas7_Hvolcanii_plasmid	164	0.316602317	1	[899][341]	[390][1]
Cas7_K-12	171	0.341317365	1	[1323][364]	[833][1]
Cas7_Ssolfataricus	147	0.267272727	1	[950][312]	[404][1]
Cas8_LsV	136	0.265625	1	[1289][314]	[783][1]
Cas8_Pdistasois	237	0.295511222	1	[1342][573]	[559][1]
Cas8_Pgingivalis	212	0.280794702	1	[1340][500]	[609][1]
Cas9_Bthermosphacta	1726	1.249818972	1	[1365][1301]	[47][1]
Cas9_Cindologenes	476	0.282157676	1	[1366][1444]	[3][1]
Cas9_Cochracea	375	0.276344878	0.650315347	[1322][1427]	[1][500]
Cas9_Hpullorum	180	0.27820711	1	[643][345]	[4][1]
Cas9_Hpullorum_2	330	0.391459075	1	[1347][703]	[549][1]
Cas9_Kkingae	520	0.370634355	0.997172479	[1341][1061]	[1][4]
Cas9_Movipneumoniae	519	0.340998686	0.988216811	[1349][1273]	[1][16]
Cas9_Nlactamica	505	0.353889278	0.994459834	[1368][1083]	[1][7]
Cas9_Pacidlactici	1786	1.209207854	0.999267399	[1366][1365]	[1][2]
Cas9_Pnultocida	510	0.365068003	0.997161779	[1348][1057]	[1][4]
Cas9_Ranatipestifer	391	0.239143731	1	[1366][1406]	[3][1]
Cas9_Sgallolyticus	4529	3.274765004	0.999271137	[1368][1372]	[1][2]
Cas9_Smoniliformis	514	0.327597196	1	[1368][1260]	[3][1]
Cas9_Spaucimobilis	415	0.290616246	1	[1362][1091]	[3][1]

Method as discussed in section 2.2. Visualization of data is available in Figure 4

Table 2: Local Alignment Output

Gene Name	Alignment Score	Average Score / base	% Sequence aligned	Traceback Start	Traceback End
Cas10_Mtuberculosis	317	0.239064857	0.988888889	[1345][808]	[46][8]
Cas10_Phorikoshii	313	0.246650906	0.874015748	[1368][705]	[105][40]
Cas10_Ssolfataricus	432	0.316020483	0.864244742	[1365][929]	[16][26]
Cas10_Tvolcanium	362	0.312878133	0.923969072	[1312][749]	[165][33]
Cas1_DvsH_plasmid	152	0.290630975	0.959302326	[1237][342]	[728][13]
Cas1_Gvaginalis	120	0.270880361	0.99376947	[1332][320]	[904][2]
Cas1_K-12	107	0.29558011	0.666666667	[1340][263]	[983][60]
Cas1_Tdenticola	124	0.294536817	0.965635739	[1096][287]	[687][7]
Cas2_K-12	43	0.651515152	0.663157895	[1119][86]	[1055][24]
Cas2_Lsalivarius	77	0.611111111	0.794117647	[621][101]	[496][21]
Cas2_StB20-like	72	1.028571429	0.568181818	[738][87]	[669][38]
Cas3_DvsH_plasmid	210	0.195712954	0.944523471	[1099][683]	[48][20]
Cas3_Gvaginalis	261	0.244382022	0.937114673	[1317][790]	[272][31]
Cas3_K-12	248	0.245787909	0.750281215	[1232][873]	[265][207]
Cas4_K-12	177	0.353293413	0.848901099	[1355][325]	[856][17]
Cas4_Ssolfataricus	100	0.304878049	0.827586207	[1308][180]	[981][13]
Cas4_TtenaxKra	60	0.810810811	0.277486911	[883][175]	[811][123]
Cas5_Gvaginalis	159	0.42513369	0.863013699	[797][274]	[424][23]
Cas5_K-12	77	0.292775665	0.68	[493][184]	[233][32]
Cas6_Cbotulinum	132	0.371830986	0.82173913	[453][215]	[99][27]
Cas6_Hvolcanii_plasmid	93	0.322916667	0.648351648	[755][225]	[468][49]
Cas6_Ssolfataricus	121	0.292978208	0.954861111	[1208][286]	[799][12]
Cas7_Hvolcanii_plasmid	177	0.5	0.692082111	[416][297]	[63][62]
Cas7_K-12	177	0.353293413	0.848901099	[1355][325]	[856][17]
Cas7_Ssolfataricus	139	0.445512821	0.769230769	[1144][311]	[837][72]
Cas8_LsV	168	0.305454545	0.984076433	[592][313]	[43][5]
Cas8_Pdistasois	222	0.308333333	0.848167539	[772][572]	[65][87]
Cas8_Pgingivalis	192	0.309677419	0.666	[690][498]	[75][166]
Cas9_Bthermosphacta	1736	1.260711692	0.996156802	[1361][1296]	[47][1]
Cas9_Cindologenes	658	0.443396226	0.810249307	[1366][1170]	[3][1]
Cas9_Cochracea	493	0.361172161	0.62789068	[1352][896]	[3][1]
Cas9_Hpullorum	174	0.280645161	0.985507246	[616][342]	[6][3]
Cas9_Hpullorum_2	351	0.445997459	0.928876245	[1345][701]	[614][49]
Cas9_Kkingae	528	0.372881356	0.97737983	[1368][1042]	[3][6]
Cas9_Movipneumoniae	620	0.417508418	0.865671642	[1357][1118]	[2][17]
Cas9_Nlactamica	580	0.420594634	0.874422899	[1359][955]	[3][9]
Cas9_Pacidlactici	1793	1.214769648	0.998534799	[1365][1364]	[1][2]
Cas9_Pnultocida	577	0.416907514	0.904446547	[1361][961]	[3][6]
Cas9_Ranatipestifer	529	0.354795439	0.826458037	[1368][1162]	[3][1]
Cas9_Sgallolyticus	4540	3.289855072	0.997084548	[1366][1369]	[1][2]
Cas9_Smoniliformis	543	0.378133705	0.843650794	[1368][1063]	[3][1]
Cas9_Spaucimobilis	404	0.289191124	0.973418882	[1332][1063]	[4][2]

Method as discussed in section 2.2. Visualization of data is available in Figure 5

3.2 Gibbs Sampling

3.2.1 Proof of concept

The simulated sequences contain a motif with length 16 at various locations in the set of sequences and the sequences in the set contain either this motif or this motif and another motif shared by half some of the sequences (see Figure 6). We trained motif with lengths 5, 10, 15, 16 (the correct length), 20, and 30. The results are shown in Figure 7. It shows that our strategy can successfully recognize and recover the position of the conserved region. And furthermore, as the motif length matches exactly the underlying truth, the number of outliers is minimized and if the length is close enough or slightly longer than the truth, the method is still robust. From the accuracy perspective, also, the accuracy is minimized if we use the right width but if the width is chosen within an appropriate range, then the true position is roughly within 10 amino acids to the predicted ones, which is still acceptable for our goal.

Besides, simulated data, the similar analysis was done using Globin data as well. We performed the analysis using width 10, 30, 50, ..., 130. Figure 8 shows the results of pattern recognition using learned motif with width 10, 50, 110, where we learned 10 motifs with width 10 and 5 for others. Here the score in each cell is defined as the maximum score among all the scores obtained by any possible window such position evolving with any motifs learned. It turns out that our method can find the conserved region at test time even though the motif is short, which implies that our method has enough sensitivity for our task.

3.2.2 Motif finding in *Cas5* and *Cas7*

The optimization curves of motif finding in *Cas5* and *Cas7* with width 10, 50, 110 are shown in Figure 9 and Figure 10. From the curves we can see the Markov chain almost converges in each run.

3.2.3 Pattern recognition in Cas9

The results of pattern recognitions are shown in Figure 11 and Figure 12. It turns out that for motif with width 10, we some noises and for motif with width 50 and 110, we get no single at all.

3.3 Domain-specific profile HMM

3.3.1 Protocol testing

For method quality control purpose, some protocol was applied to Globins4.fasta (a file of 4 sequences of Globins generated from a tutorial file of HMMer). We built the profile Hmm and save it in golbins4.hmm. Using this profile HMM, we searched Globins45.fa (a tutorial file of HMMer) and the result is saved into golbins.search. The summary of found domains is in the header part of the file as shown in Figure. 13. An example alignment of the found domain is shown in Figure. 14. We can see that a domain is found in HBB_MANSP. The profile HMM model can also be read from the alignment. Full result is available in golbins.search file.

3.3.2 Finding domains of *Cas9*

After subtracting and aligning all the shared domains of *Cas9* marked in EMBL-EBI. We built profile HMMs from these multi-sequence alignments and save them into data/domains_Cas9/IPR032239.hmm, etc. Then we use these models to find the domains of *Cas9* in all *Cas* family protein sequences, hoping to find similar domains in other proteins in different subtypes. The summary of one of the search results can be seen in Figure. 15. The profile HMM can find similar domains in other *Cas9* proteins, which is as expected. But all the models failed to find any similar domains in other *Cas* family proteins other than *Cas9*.

3.4 Finding domains of *Cas5*

We also tried the other direction. That is to use domain markers of *Cas5*, which is a component of Type I system, and to find similar domains in *Cas9*. The protocol is the same and one of the summaries of the results can be found in Figure 16. The result is similar, the model is able to find some similar domains in other *Cas5* proteins but not other *Cas* family proteins.

4 Conclusion

In sequence alignment model, successful mapping of various *Cas9* protein to *Cas9* was observed to depend on the length of the query sequence. *Cas10*, the biggest protein in CRISPR Class 1, mapped to *Cas9* most accurately in both local and semi-global alignment. What was interesting is how much of *Cas1, Cas2* and *Cas4* mapped to *Cas9*, despite the fact these three proteins are part of adaption functionality rather than interference functionality [1]. Another example of both local alignment and semiglobal alignment successfully mapping is *Cas9* sequence of *Helicobacter pullorum*, where the *Cas9* that has been split into two parts, mapped neatly to the *s.pyogenes Cas9*. In this alignment, difference in the semiglobal(green in figure5) and local(purple in figure5) alignment show the actual conserved regions in purple, and the regions of semiglobal alignment that does not have corresponding local alignment would indicate some extraneous sequence at either ends of the protein.

However, in some cases, local and global alignment disagreed as for where to map to in *s.pyogenes Cas9*. One example would be *Cas8*, where all three sequences of *Cas8* aligned disagreed on semiglobal vs local alignment. Given that both local alignment and semiglobal alignment were performed using the same substitution matrix (`BLOSUM62`) and same gap penalties with the only difference being except for end gap penalty and traceback startpoint, we would be more inclined to trust the local alignment in terms of motif-finding, because average score per position for such conflicting alignments is much higher for local alignment, and because it does not penalize overall sequence alignment for certain highly divergent regions within the gene. Default BLAST parameters, such as `BLOSUM62`, were used due to limited predictions currently available for accurate phylogenetic model of CRISPR family. These parameters have been shown to have the best performance overall when there is limited information about evolutionary distance and divergence. By modifying parameters to fit the actual evolutionary distance when such data is available (including, but not limited to substitution matrix, gap costs, etc.), much more accurate alignment and motif detection through sequence alignment could be obtained.

The Gibbs sampling model was verified to successfully identify conserved regions in simulated sequences and Globin data, which indicates that the motif finding approach with PSSMs is capable of finding underlying conserved regions in sequences. However, the same motif finding approach fails in the analysis of *Cas* protein family. The main reason is that the conserved region shared by *Cas* family might be evolutionarily far to divergent compared to the Globin family and the signal is hard to be captured by the default PSSM without utilizing any molecular evolution information. Also, the size of our training data is small and it may prevent discovery of motifs in sequences that are divergent too divergent. This pitfall leads to the possibility that the discovered motifs might not be representative of the entire *CRISPR* protein family, and might be too stringent and overfitted to particular input data.

Profile-HMM was also verified to successfully identify conserved domains in the Globin-family. In addition, it successfully identified the domains in the same subtype as the model source. For example, the model built from *Cas9* can find most domains in *Cas9* in other species. But it failed to find similar domains across different subtypes in the *Cas* family. One possible reason for the failure is that the limited number of training sequences makes the model too rigid to be able to fit remotely related sequences. Another possible reason is that even though different proteins in different subtypes have similar functionalities, they evolved separately and have low level sequence similarity.

Pairwise sequence alignment model implicitly incorporates biophysical properties while making alignment and motif predictions, but assumed sequence independence. Gibbs Sampling or HMM profiling do not take biophysical properties into consideration, but both consider multiple sequence for each position. Gibbs sampling is easy to implement and much more intuitive to understand and require relatively less data compared to HMM, but also assumed positional independence in evolution. HMM profiling addresses many of the features lacking in pairwise sequence alignment or Gibbs sampling, but generally requires much more data. However, there is finite number of *Cas* sequences and even less in terms of actually available sequences.

In case of Gibbs and HMM models, both were shown to accurately predict conserved regions with the control data : Globin family and simulated sequences. However, Globin proteins belong to eukaryotes whose evolutionary history is much shorter than that of the prokaryotes - bacteria and archaea - which possess the CRISPR system. It may be that these models as-is are too stringent for identifying evolutionary relationships and motifs in much more distant protein family than a highly conserved protein like Globin, in a relatively short evolutionary timestep. Given that highly complex multicellular eukaryotes have evolved 0.5 billion years ago, whereas some of the prokaryotic CRISPR systems represent billions of years of evolutionary time distance, coupled with much faster rate of reproduction, it is not surprising that CRISPR system would be much more divergent than the Globin family of eukaryotes [17]. Performance of all current methods for identifying similarities or motif finding decrease as evolutionary diversity and divergence of the protein family being studied increases. Much more work can and should be done in development of models specifically for studying and identifying the relationship and conservation of motifs among distantly related organisms.

Our study has many potential directions it could take for improvement and expansions. One approach may be to increase our dataset to include even greater number of *Cas* protein sequences. Also, all three models would greatly benefit from a more updated prediction of evolutionary timeline and relationship between the *Cas* proteins for additional parameter tuning for more accurate motif discovery. Recall that the motif finding approach fails in the task with the potential lack of power in motif representation and the lack of data. One of the ways to overcome this pitfall is to reduce the size our alphabet. For example, instead of using the whole alphabet (20 amino acids), we can use 5-character alphabet where 5 characters indicate the biochemical properties of the residues. This can partly solve the problem of limited data by reducing the complexity of our model, and furthermore, it also automatically makes use of biochemical property and evolutionary

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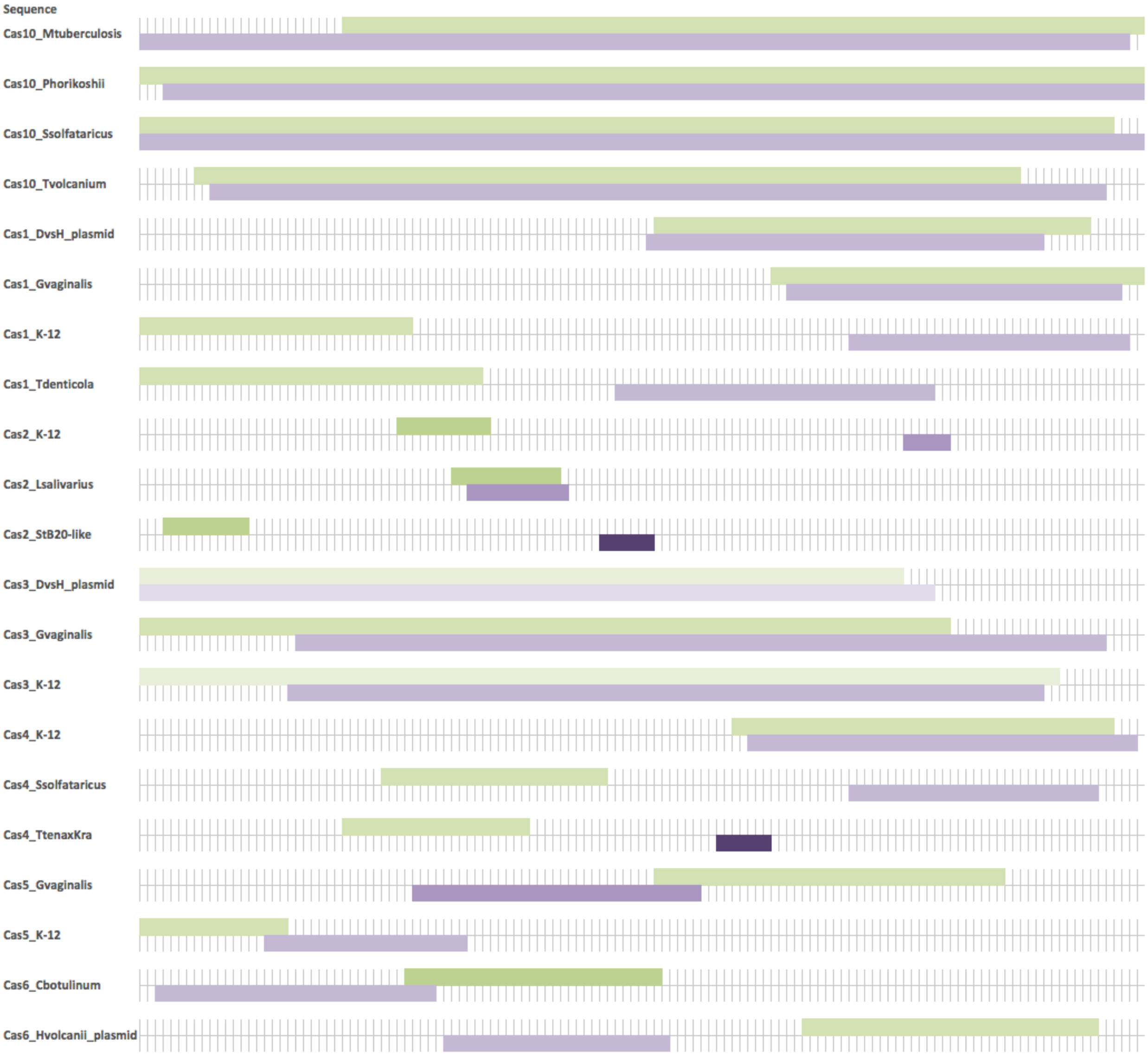


Figure 4: Pairwise sequence alignment of various *Cas* protein sequences against *s. pyogenes Cas9* protein sequence. Green bars show coverage of semi-global alignment of individual sequence against *s. pyogenes Cas9*. Purple bars show coverage of local alignment of individual sequence against *s. pyogenes Cas9*. Darker color indicates higher average score per base, and therefore higher sequence similarity. Each grey marker represents 10 amino acid residues

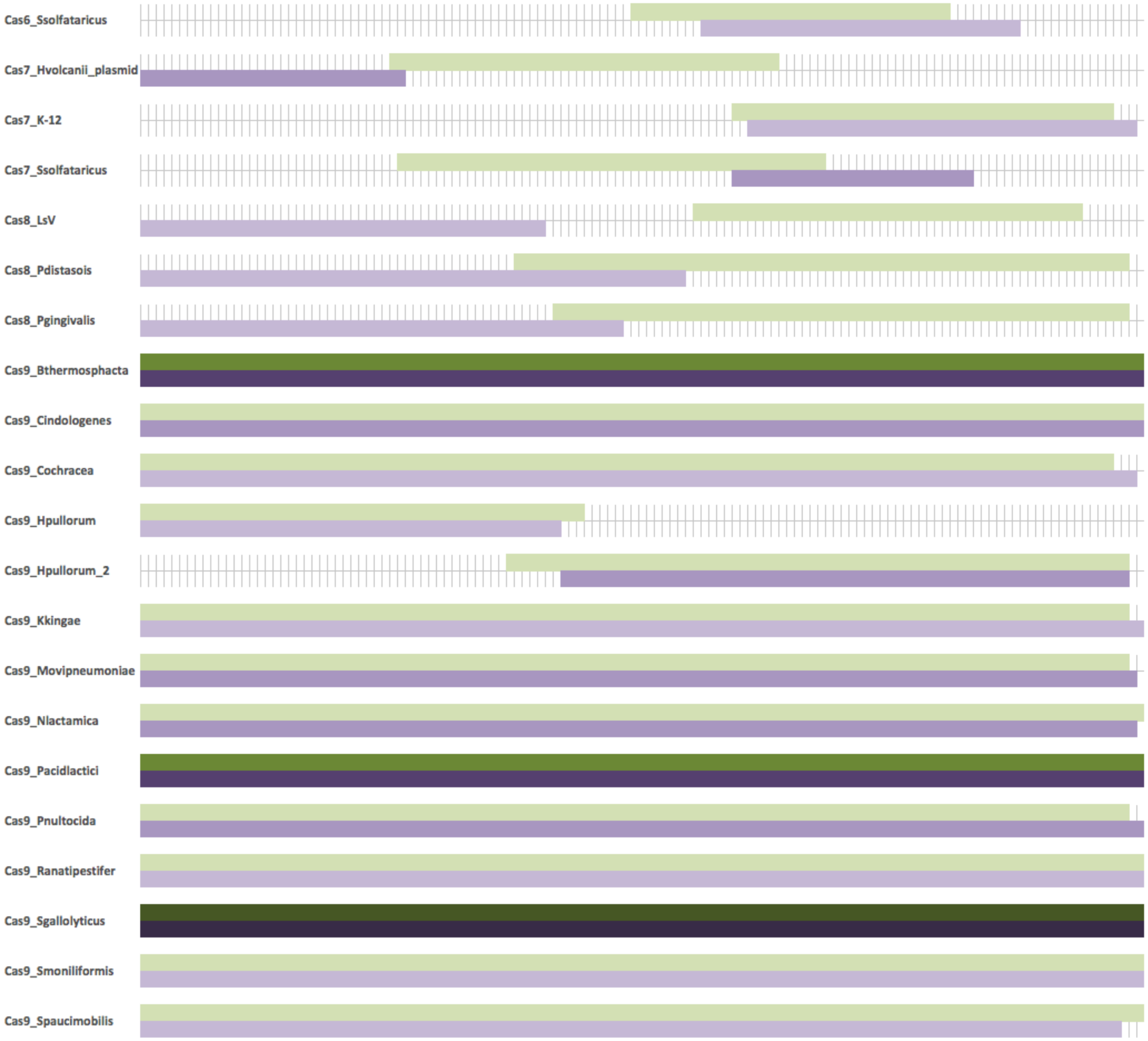
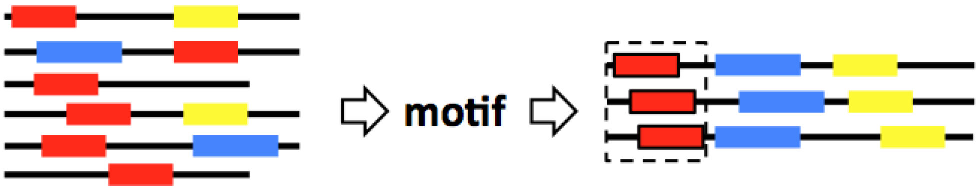


Figure 5: Pairwise sequence alignment of various *Cas* protein sequences against *s. pyogenes Cas9* protein sequence. Green bars show coverage of semi-global alignment of individual sequence against *s. pyogenes Cas9*. Purple bars show coverage of local alignment of individual sequence against *s. pyogenes Cas9*. Darker color indicates higher average score per base, and therefore higher sequence similarity. Each grey marker represents 10 amino acid residues



Simulated data has three motifs and every sequence at training time share the same motif (red) but may or may not contain other motifs (yellow/blue). At test time, the sequences share similar pattern and we chech if the trained motif can successfully recover the shared pattern (red).

Figure 6: Gibbs sampling approach - The overview of the analysis design for simulated data

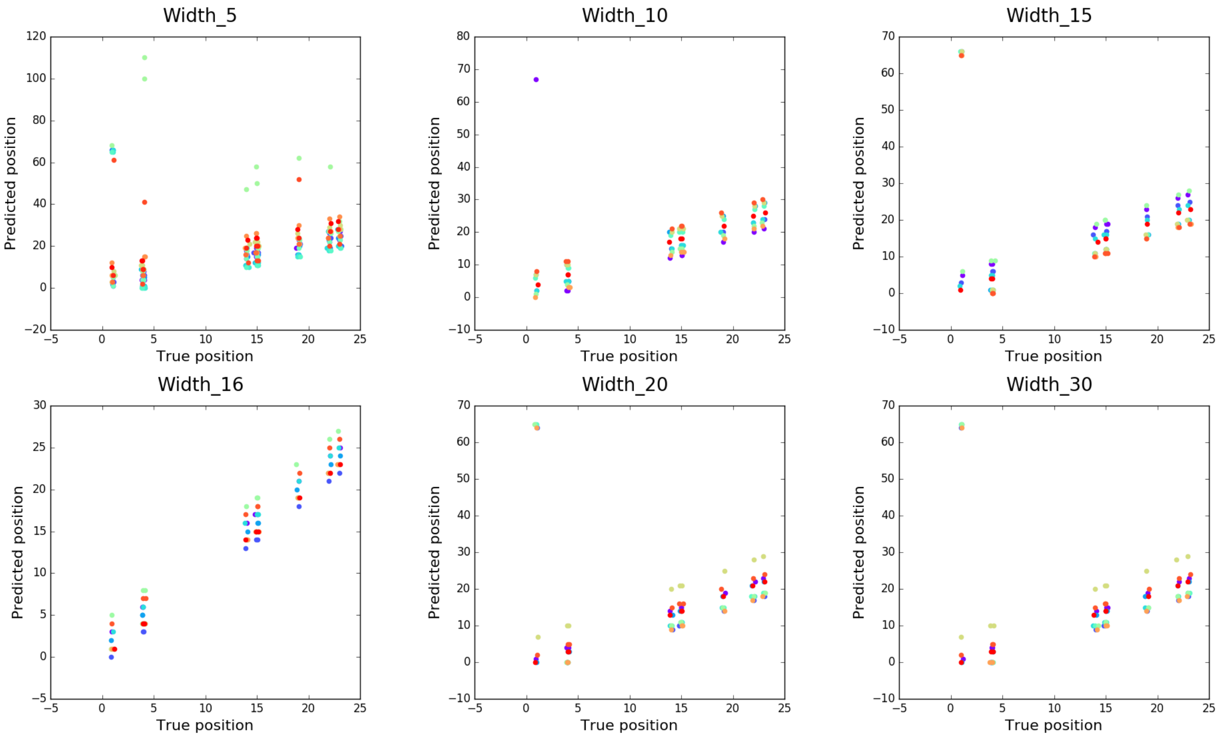


Figure 7: Gibbs sampling approach - The results of conserved region finding in simulated sequences

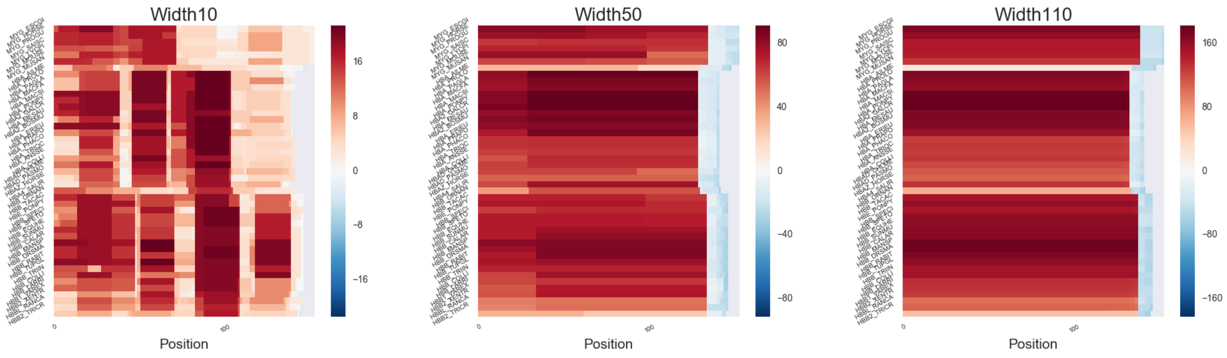
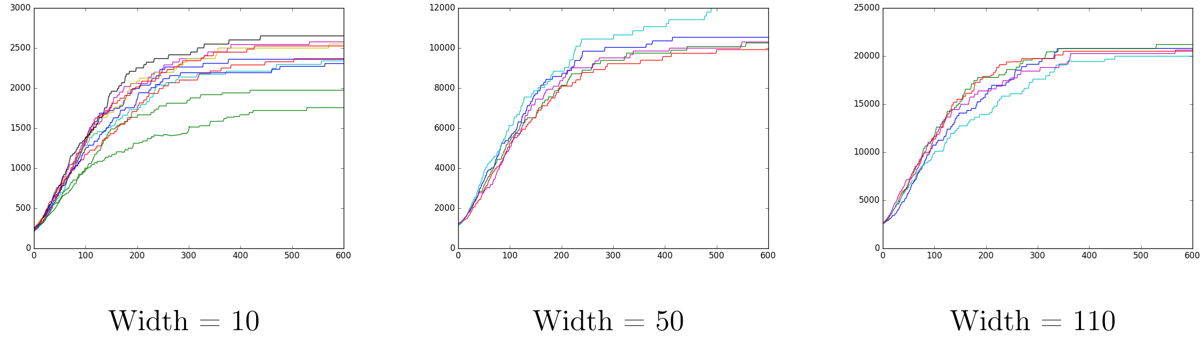
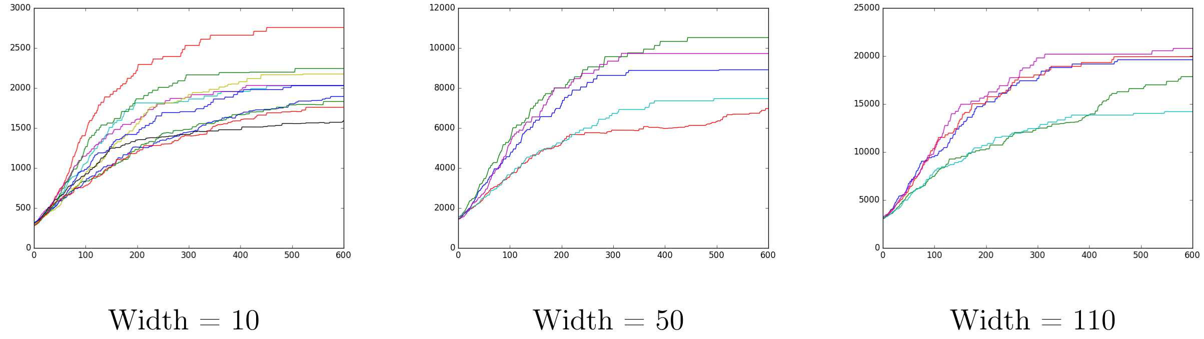


Figure 8: Gibbs sampling approach - The pattern recognition scores of Globins



Width = 10 Width = 50 Width = 110

Figure 9: Gibbs sampling approach - The optimization curves for motif finding in *Cas5*



Width = 10 Width = 50 Width = 110

Figure 10: Gibbs sampling approach - The optimization curves for motif finding in *Cas7*

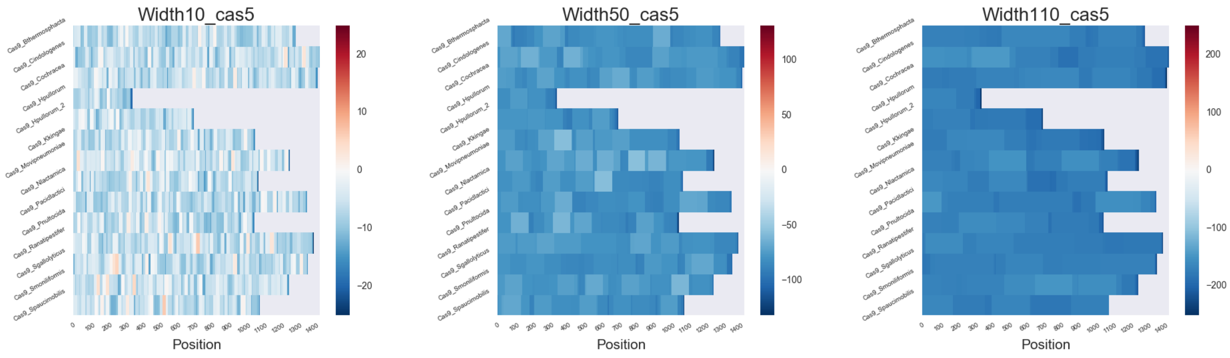


Figure 11: Gibbs sampling approach - The pattern recognition scores of Cas9 with motifs found in *Cas5*

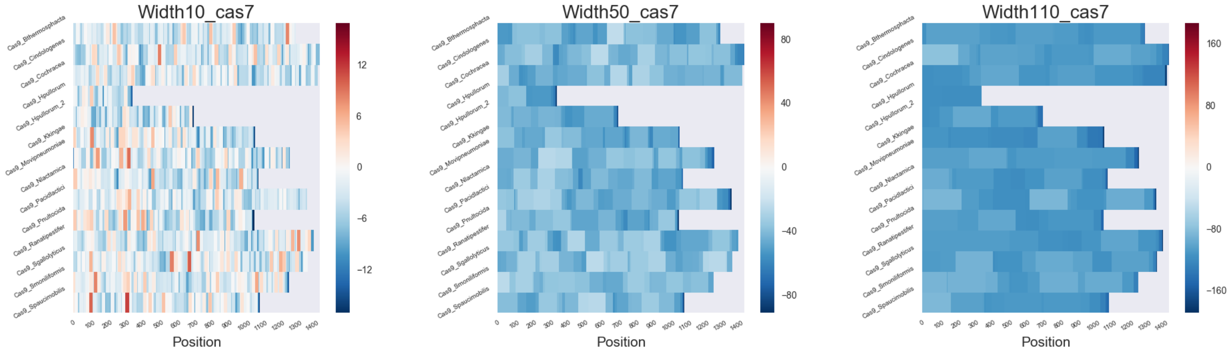
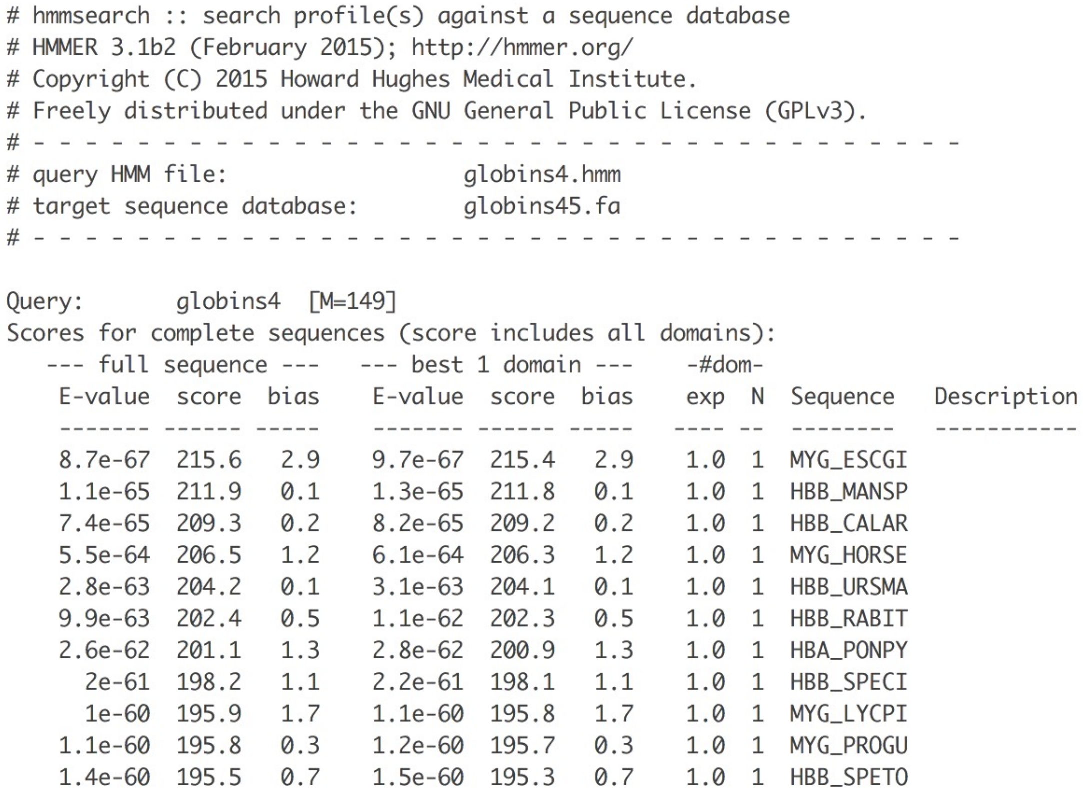


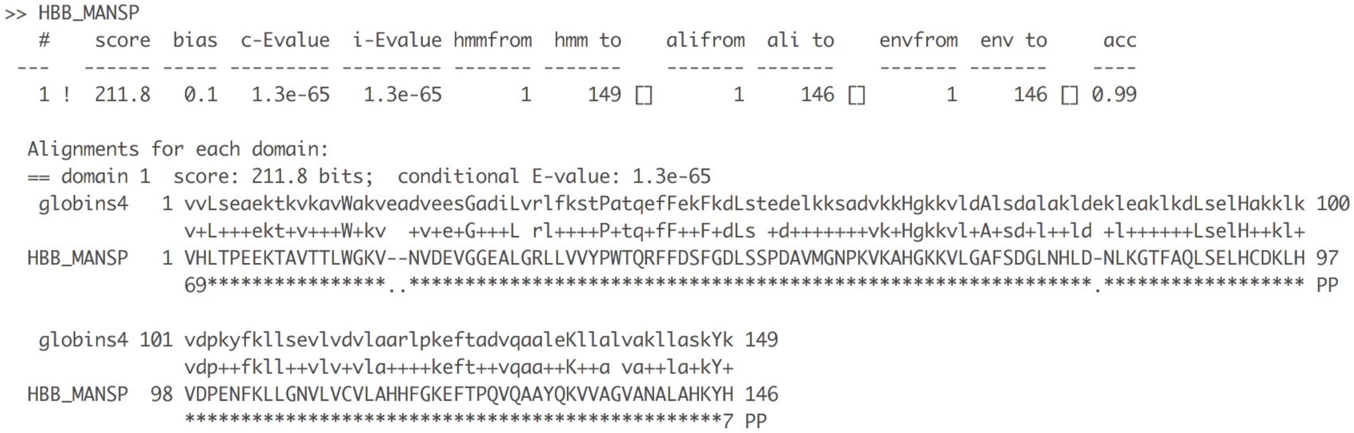
Figure 12: Gibbs sampling approach - The pattern recognition scores of Cas9 with motifs found in *Cas7*



```
# hmmsearch :: search profile(s) against a sequence database
# HMMER 3.1b2 (February 2015); http://hmmer.org/
# Copyright (C) 2015 Howard Hughes Medical Institute.
# Freely distributed under the GNU General Public License (GPLv3).
# - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
# query HMM file:                  globins4.hmm
# target sequence database:        globins45.fa
# - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

Query:       globins4  [M=149]
Scores for complete sequences (score includes all domains):
   --- full sequence ---   --- best 1 domain ---    -#dom-
    E-value  score  bias    E-value  score  bias    exp  N  Sequence   Description
    ------- ------ -----    ------- ------ -----   ---- --  --------   -----------
    8.7e-67  215.6   2.9    9.7e-67  215.4   2.9    1.0  1  MYG_ESCGI
    1.1e-65  211.9   0.1    1.3e-65  211.8   0.1    1.0  1  HBB_MANSP
    7.4e-65  209.3   0.2    8.2e-65  209.2   0.2    1.0  1  HBB_CALAR
    5.5e-64  206.5   1.2    6.1e-64  206.3   1.2    1.0  1  MYG_HORSE
    2.8e-63  204.2   0.1    3.1e-63  204.1   0.1    1.0  1  HBB_URSMA
    9.9e-63  202.4   0.5    1.1e-62  202.3   0.5    1.0  1  HBB_RABIT
    2.6e-62  201.1   1.3    2.8e-62  200.9   1.3    1.0  1  HBA_PONPY
      2e-61  198.2   1.1    2.2e-61  198.1   1.1    1.0  1  HBB_SPECI
      1e-60  195.9   1.7    1.1e-60  195.8   1.7    1.0  1  MYG_LYCPI
    1.1e-60  195.8   0.3    1.2e-60  195.7   0.3    1.0  1  MYG_PROGU
    1.4e-60  195.5   0.7    1.5e-60  195.3   0.7    1.0  1  HBB_SPETO
```

Figure 13: profile HMM search results summary in Globins.search

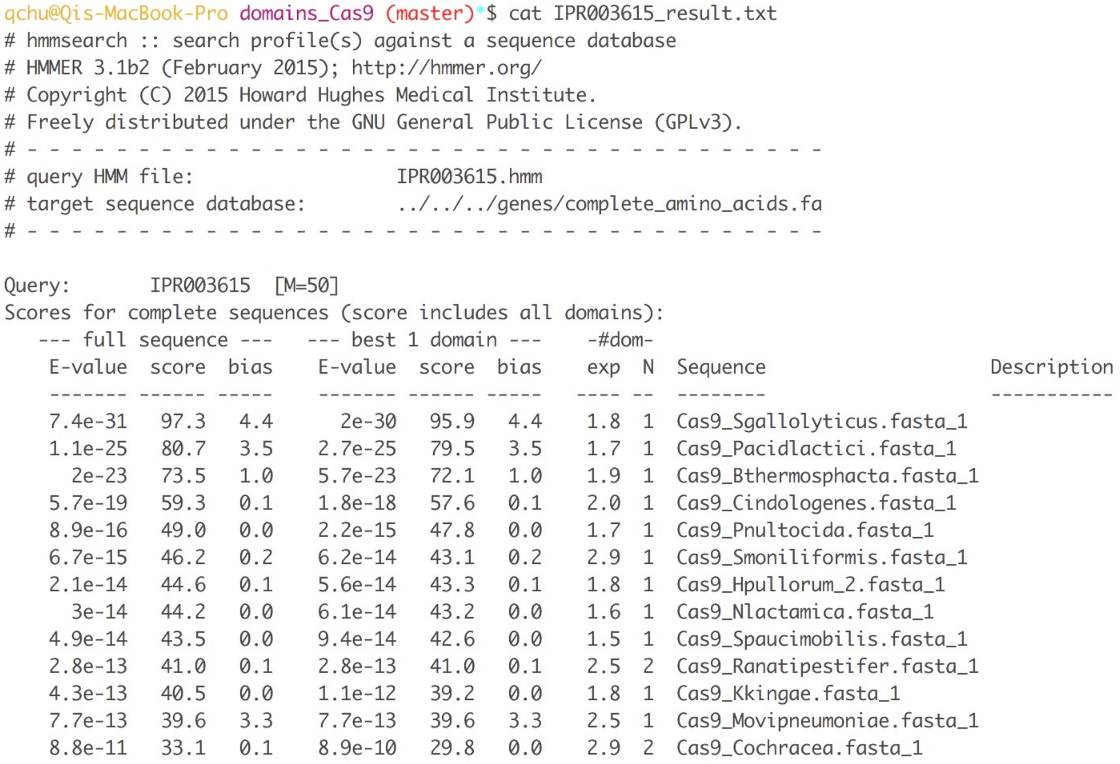


```
>> HBB_MANSP
   #    score  bias  c-Evalue  i-Evalue hmmfrom  hmm to    alifrom  ali to    envfrom  env to     acc
 ---   ------ ----- --------- --------- ------- -------    ------- -------    ------- -------    ----
   1 !  211.8   0.1   1.3e-65   1.3e-65       1     149 []       1     146 []       1     146 [] 0.99

  Alignments for each domain:
  == domain 1  score: 211.8 bits;  conditional E-value: 1.3e-65
   globins4   1 vvLseaektkvkavWakveadveesGadiLvrlfkstPatqefFekFkdLstedelkksadvkkHgkkvldAlsdalakldekleaklkdLselHakklk 100
                v+L+++ekt+v+++W+kv  +v+e+G+++L rl++++P+tq+fF++F+dLs +d+++++++vk+Hgkkvl+A+sd+l++ld +l++++++LselH++kl+
  HBB_MANSP   1 VHLTPEEKTAVTTLWGKV--NVDEVGGEALGRLLVVYPWTQRFFDSFGDLSSPDAVMGNPKVKAHGKKVLGAFSDGLNHLD-NLKGTFAQLSELHCDKLH 97
                69****************..*************************************************************.****************** PP

   globins4 101 vdpkyfkllsevlvdvlaarlpkeftadvqaaleKllalvakllaskYk 149
                vdp++fkll++vlv+vla++++keft++vqaa++K++a va++la+kY+
  HBB_MANSP  98 VDPENFKLLGNVLVCVLAHHFGKEFTPQVQAAYQKVVAGVANALAHKYH 146
                ************************************************7 PP
```

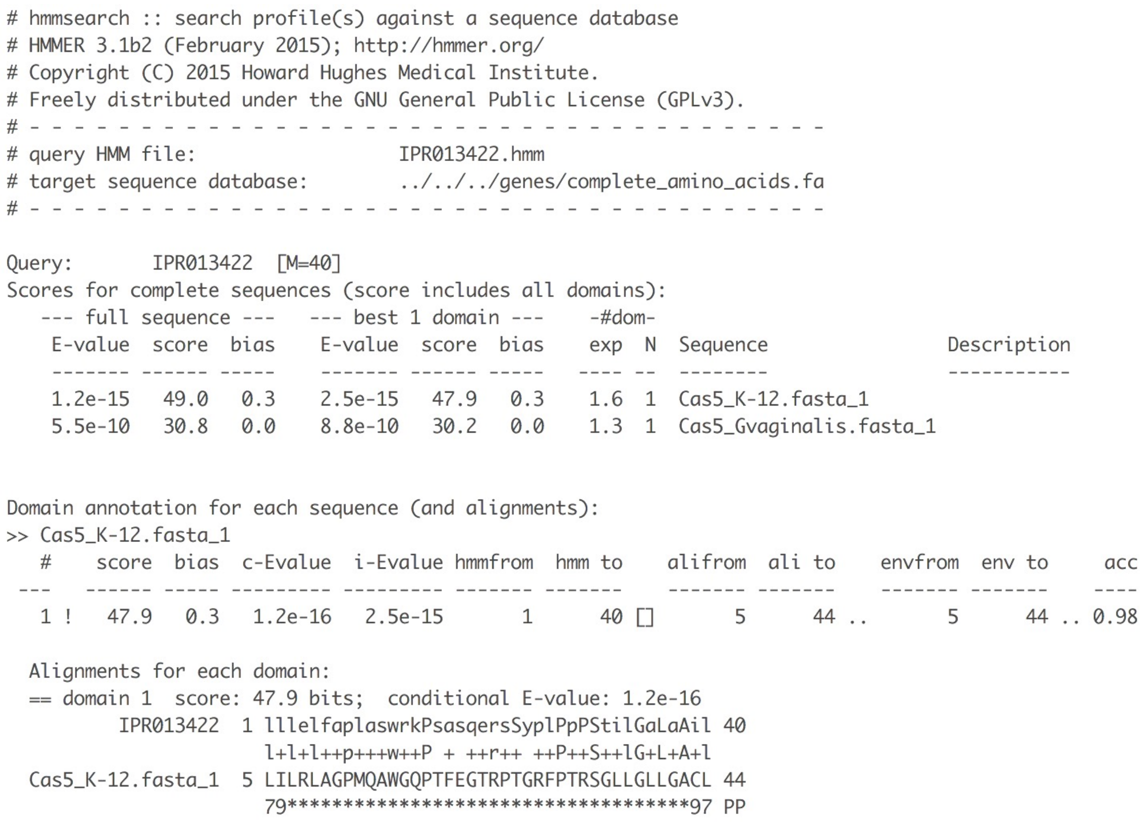
Figure 14: profile HMM approach - Domain alignment in Globins.search



```
qchu@Qis-MacBook-Pro domains_Cas9 (master)*$ cat IPR003615_result.txt
# hmmsearch :: search profile(s) against a sequence database
# HMMER 3.1b2 (February 2015); http://hmmer.org/
# Copyright (C) 2015 Howard Hughes Medical Institute.
# Freely distributed under the GNU General Public License (GPLv3).
# - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
# query HMM file:                  IPR003615.hmm
# target sequence database:        ../../../genes/complete_amino_acids.fa
# - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

Query:       IPR003615  [M=50]
Scores for complete sequences (score includes all domains):
   --- full sequence ---   --- best 1 domain ---    -#dom-
    E-value  score  bias    E-value  score  bias    exp  N  Sequence                    Description
    ------- ------ -----    ------- ------ -----   ---- --  --------                    -----------
    7.4e-31   97.3   4.4      2e-30   95.9   4.4    1.8  1  Cas9_Sgallolyticus.fasta_1
    1.1e-25   80.7   3.5    2.7e-25   79.5   3.5    1.7  1  Cas9_Pacidlactici.fasta_1
      2e-23   73.5   1.0    5.7e-23   72.1   1.0    1.9  1  Cas9_Bthermosphacta.fasta_1
    5.7e-19   59.3   0.1    1.8e-18   57.6   0.1    2.0  1  Cas9_Cindologenes.fasta_1
    8.9e-16   49.0   0.0    2.2e-15   47.8   0.0    1.7  1  Cas9_Pnultocida.fasta_1
    6.7e-15   46.2   0.2    6.2e-14   43.1   0.2    2.9  1  Cas9_Smoniliformis.fasta_1
    2.1e-14   44.6   0.1    5.6e-14   43.3   0.1    1.8  1  Cas9_Hpullorum_2.fasta_1
      3e-14   44.2   0.0    6.1e-14   43.2   0.0    1.6  1  Cas9_Nlactamica.fasta_1
    4.9e-14   43.5   0.0    9.4e-14   42.6   0.0    1.5  1  Cas9_Spaucimobilis.fasta_1
    2.8e-13   41.0   0.1    2.8e-13   41.0   0.1    2.5  2  Cas9_Ranatipestifer.fasta_1
    4.3e-13   40.5   0.0    1.1e-12   39.2   0.0    1.8  1  Cas9_Kkingae.fasta_1
    7.7e-13   39.6   3.3    7.7e-13   39.6   3.3    2.5  1  Cas9_Movipneumoniae.fasta_1
    8.8e-11   33.1   0.1    8.9e-10   29.8   0.0    2.9  2  Cas9_Cochracea.fasta_1
```

Figure 15: profile HMM approach - One example result for searching for *Cas9* in all *Cas* family proteins



```
# hmmsearch :: search profile(s) against a sequence database
# HMMER 3.1b2 (February 2015); http://hmmer.org/
# Copyright (C) 2015 Howard Hughes Medical Institute.
# Freely distributed under the GNU General Public License (GPLv3).
# - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
# query HMM file:                  IPR013422.hmm
# target sequence database:        ../../../genes/complete_amino_acids.fa
# - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

Query:       IPR013422  [M=40]
Scores for complete sequences (score includes all domains):
   --- full sequence ---   --- best 1 domain ---    -#dom-
    E-value  score  bias    E-value  score  bias    exp  N  Sequence                Description
    ------- ------ -----    ------- ------ -----   ---- --  --------                -----------
    1.2e-15   49.0   0.3    2.5e-15   47.9   0.3    1.6  1  Cas5_K-12.fasta_1
    5.5e-10   30.8   0.0    8.8e-10   30.2   0.0    1.3  1  Cas5_Gvaginalis.fasta_1


Domain annotation for each sequence (and alignments):
>> Cas5_K-12.fasta_1
   #    score  bias  c-Evalue  i-Evalue hmmfrom  hmm to    alifrom  ali to    envfrom  env to     acc
 ---   ------ ----- --------- --------- ------- -------    ------- -------    ------- -------    ----
   1 !   47.9   0.3   1.2e-16   2.5e-15       1      40 []       5      44 ..       5      44 .. 0.98

  Alignments for each domain:
  == domain 1  score: 47.9 bits;  conditional E-value: 1.2e-16
          IPR013422  1 lllelfaplaswrkPsasqersSyplPpPStilGaLaAil 40
                       l+l+l++p+++w++P + ++r++ ++P++S++lG+L+A+l
  Cas5_K-12.fasta_1  5 LILRLAGPMQAWGQPTFEGTRPTGRFPTRSGLLGLLGACL 44
                       79************************************97 PP
```

Figure 16: profile HMM approach - One example result for searching for *Cas5* in all *Cas* family proteins