Manual to Online FISH Probe Optimization Tool

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This manual describes the details of the FISH probe optimization algorithm starting with the processing of the user supplied data and finishing with the output report generation. It will help unfamiliar users understand the principle of the algorithm and help them understand its functions and ways to modify them by changing the default configuration file.

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1 Introduction

The website http://www.nanoimaging.uni-jena.de/fish/ is the user interface to the FISH probe search algorithm. It gives the user the option to select a target species and input a DNA sequence to be analyzed for the presence of potential FISH targets.

The sequence together with other optional parameters are analyzed and a report is generated. It includes a comprehensive graphical presentation of the analyzed DNA sequence with suitable FISH probes highlighted alongside the relevant genes on a background map of unique and similar sequences. Primer pairs necessary for the production of the FISH probes and their exact sequences are also delivered.

The user-supplied DNA sequence referred to as the "query" is aligned to the genome of the selected species. Query sequences found in only a single position in the entire genome are considered "*unique*". These "*unique regions*" of sufficient length are selected for the design of FISH probes. Any parts of the query which are found in the genome in multiple similar copies are referred to as "*similar*". If several *similar* sequences overlap or are separated by a gap of a maximum permitted length, they are grouped into a single *"similar region*". These *similar regions* are analyzed for the presence of a subclass of *"unique similar"* sequences. Such sequences are found in multiple similar copies within the genome, but all of them localize to the same *similar region* and make suitable repetitive FISH probe targets. Both *unique* and *unique similar regions* are partitioned into segments of user defined length range for production of for single-copy *unique* and repetitive *unique similar* FISH probes. PCR primers with user-determined characteristics are designed to enable either PCR-mediated cloning of the FISH probe templates or direct synthesis of the FISH probes.

2 Interaction with the User

A successful query submission through the web page results in a project directory being produced on the web server. In this directory the query sequence for analysis is saved along with the selected target species, the user's email address, a default or user-modified configuration file, and an optional file with the reference sequences such as genes. This project directory content is imported and the analysis of the input files is initiated while a new web page is send to the user's web browser to display regularly updated information about the progress of the data processing. Once the analysis is successfully finished, a report is posted on the website and the user receives a notification email. If any processing error occurs, the user is also informed.

3 Sequence for Analysis

The user must provide at least one query sequence to be aligned with the genome for identification of the specific FISH probe targets. Additionally, a reference sequence can be provided. This may consist of multiple shorter sequences of genes, regulatory sequences or any other interesting parts of the query. They will be aligned to the query sequence and their positions will be displayed in the graphical output for easier interpretation of the identified FISH probes positions. Both the query sequence and the optional reference must conform with the standard DNA FASTA format[1]. In simplified words, the FASTA format consists of a string of bases (ACGT) preceded by a line initiated by the greater-than character “>” followed by the name of the sequence. Multiple sequences with their names can be concatenated into one file.

4 Configuration File Import

The user has the option to provide a custom configuration file or rely on the default one. The configuration file allows modification of large range of parameters of the analysis. It is based on the XML format in which XML tags are surrounded with explanatory text. Each XML tag consists of a parameter and a value organized in the following format:

```
<parameter>value</parameter>
```

The user may vary any of the values if desired and the algorithm will adopt to this change. The parameters in the configuration file are grouped into those defining Mega BLAST alignment parameters, search parameters for the *unique* and *unique similar* FISH probes, graphical output parameters, and primer optimization parameters. The default configuration file with annotations describing in detail each entry is included in Abstract **??**.

If any XML tags are missing from the configuration file, they are corrupted or the user-specified values are out-of-range or otherwise unacceptable, they are replaced

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[1] http://www.ncbi.nlm.nih.gov/blast/fasta.shtml, December 2010

by hardwired default values of which the user is notified. These modified tags are appended to the end of the configuration file which is returned to the user at the end of the analysis.

Anything distinguished by a `fixed-width typeface` throughout the rest of this chapter refers to a parameter used in the configuration file.

5 Sequence Alignment

The supplied sequences are aligned with the genome of the specified organism by Mega BLAST algorithm **?**. It is initiated with default parameters except for the disabled low complexity sequence filtering and the expectation value lowered from 10 to 0.01. The filtering undesirably removes some repetitive sequences which potentially could represent suitable FISH probe targets. Lowering the expectation value cutoff saves processing time by increasing the stringency of search for similar sequence; in other words more dissimilar sequences are omitted from the alignment result.

Further details of the sequence alignment are discussed later in this chapter in Sections 16 and 17.

6 *Similar* and *Unique* Sequences

Each alignment contains information about the bit score, the starting and ending positions in the query, and the starting and ending positions of the similar hit in the genome. Alignments obtained from all sequence databases (e.g. chromosomes) are pooled together and sorted according to their bit scores (details in Section 17). The alignment with the highest score specifies the position of the query sequence within the genome and is not considered in the further analysis. The remaining alignments are mapped onto the query by marking any sequence between each alignment start and end positions as a *similar* sequence. The *similar* sequences are those which are found in the query and one or more similar copies identified by Mega BLAST elsewhere in the genome (Section 5). This way the target region becomes divided into stretches of *unique* and *similar* sequences as illustrated in Figure 7.1**a**) which displays the *unique* sequences in white while the *similar* ones in light gray.

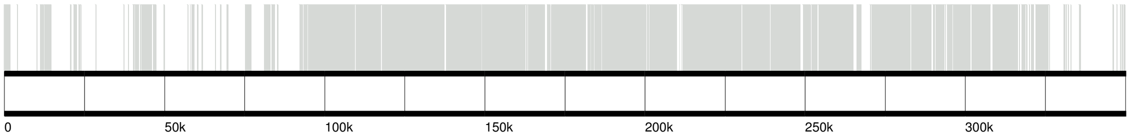
7 Merging Adjacent Similar Sequence Stretches

As introduced in the Section **??**, the algorithm searches for *unique similar* sequences which are found within only a single *similar region* of the query sequence. These *unique similar* sequences are suitable targets for repetitive yet unique FISH probes which bind to a number of places within the *similar region* but not elsewhere in the entire genome. The boundaries of such *similar region* must be selected carefully in order for the algorithm to correctly identify the *unique similar* sequences. A set of *unique similar* sequences might be localized into a single limited region of a chromosome, but if separated by even a short stretch of unique sequence, they would be interpreted as belonging to different *similar region*s and not considered being *unique similar*. Therefore a tolerance for short unique sequence gap within a single *similar region* is introduced. This allows merging of several *similar* sequences separated by short gaps into a single *similar region*.

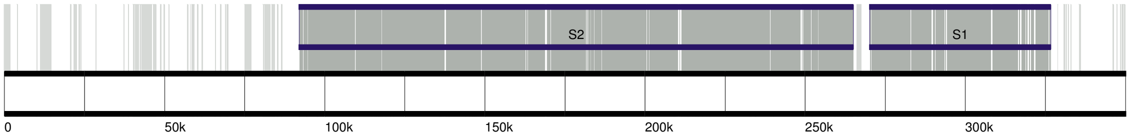
The length of this permitted gap is specified by `max_sim_gap` parameter in the configuration file. It set to 1000 by default which suited the needs of the project introduced in Chapter .... In general, this parameter is dependent on the distribution of the *unique* and *similar* sequences in each query and can only be determined empirically by the user. Selecting `max_sim_gap` as large as the length of the entire query might be desirable to detect the presence of any *unique similar* sequences in case none were identified using the default value. The pay-off for the large `max_sim_gap` is the loss of any *unique* sequences found in the gaps between the *similar regions* which could otherwise be potentially available as suitable FISH probe targets.

8 Region Length Filtering

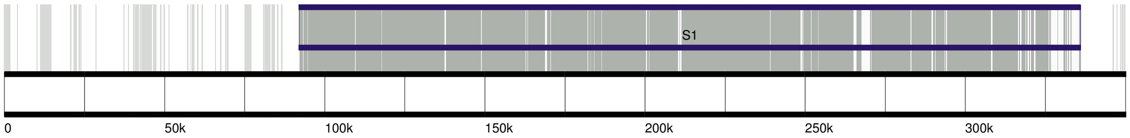
Each region, whether *unique* or *similar*, might contain sequences potentially suitable as FISH targets. The maximum permissible length of these target is determined by the



(a) `max_sim_gap` 0



(b) `max_sim_gap` 1000



(c) `max_sim_gap` 3000

Figure 7.1: **Non-contiguous *similar region* grouping**. *Similar* sequences separated by gaps consisting of *unique* sequences are merged into a single *similar region* if the gap length is less than the `max_sim_gap` value. **(a)** With `max_sim_gap` set to 0, the *similar* sequences shown in light gray are fragmented without presence of any *similar unique* sequences. **(b)** The default value of 1000 for `max_sim_gap` yields two *similar regions* S_1 and S_2 **(c)** `max_sim_gap` of 3000 results in merging most of the *similar* sequences into a single *similar region* S_1.

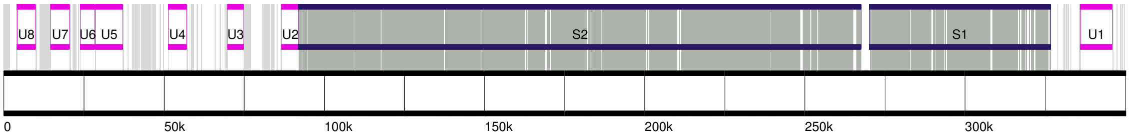


Figure 8.1: ***Unique* and *similar regions*** of satisfactory lengths are highlighted by pink and blue rectangles, respectively. FISH probes are sought within these regions.

length of each region. The fluorescence intensity obtainable from each FISH probe is proportional to the length of its target and therefore only sufficiently long targets can justify the labor intensive and expensive FISH probe production. A length threshold for the regions is therefore introduced to only use the sufficiently long ones. It is determined by the `min_uniq_length` and `min_sim_length` parameters by default set to 4000 and 30000 for the *unique* and *similar* regions, respectively. The selection of the regions of fulfilling length is illustrated in Figure 8.1.

9 Histogram of *Unique Similar* Sequence Repeats

Each *similar region* is divided into *unique similar* sequences which occur in one or more similar repeats found exclusively in the *similar region* and those which are similar to sequences localized to other parts of the genome. To identify them, a histogram is generated which displays the number of *similar* sequence repeats within each *similar region* of the query. It has a bin for each base of the query sequence. The value in each bin corresponds to the number of similar repeats of its surrounding sequence found the same *similar region*. The histogram bin value is **zero** if the surrounding sequence is similar to any genomic sequence not contained in the *similar region*. The non-zero histogram bins define the *unique similar* sequence positions in the query. An example of such histogram is presented in Figure 9.1 with the *unique similar* sequence highlighted in the cyan color.

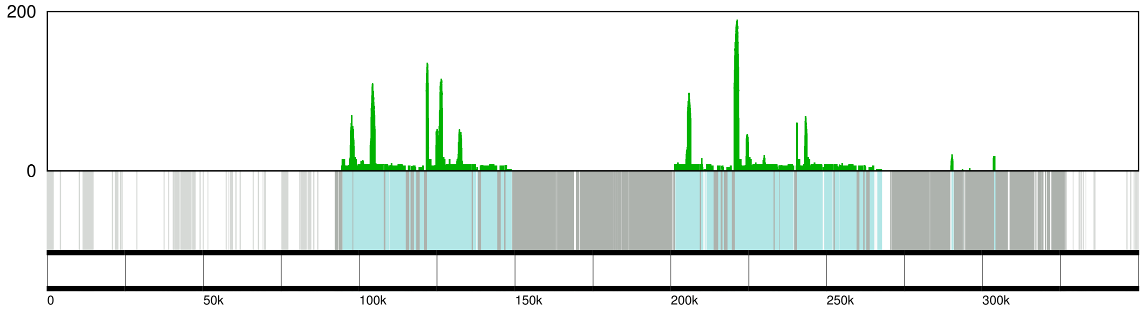


Figure 9.1: ***Similar unique* sequence histogram** value represents the number of similar repeats of given sequence stretch within each *similar region* of the query sequence. The histogram value is zero for sequences which are similar to any part of the genome outside the scrutinized *similar region*. The *unique similar* sequences with non-zero histogram bins are highlighted by cyan color in the sequence map.

10 PCR Product Search

The algorithm serves the main purpose of designing PCR primers to clone suitable sequences for specific FISH probe production. The obtained PCR products are tiled across to the selected *unique* and *unique similar* sequences (Sections 8 and 9). The permissible PCR product length and the overlap between neighboring PCR products can be defined in the configuration file.

10.1 PCR Templates Selection

Multiple parameters decide which sequences should be used for PCR cloning and subsequent FISH probe production. The templates for the PCR primer design are selected from the *unique* and *unique similar regions*. `Min_uniq_pcr` and `min_sim_pcr` parameters define the minimum permissible length of the PCR template within these regions. If the given region is not sufficiently long, the PCR primers will not be designed. Similarly, `max_uniq_pcr` and `max_sim_pcr` limit the maximum allowed length of the PCR template. If the scrutinized region is longer than this value, it is first divided into the lowest possible number of templates of length within the allowed range. The region division depends on the value of the `pcr_overlap` parameter which defines the requirement for overlap between the neighboring PCR products and its extent.

10.2 Neighboring Template Overlap

The primers are sought only in the terminal tails of the selected PCR templates. Initially, the length of these tails is limited to 100 bases. If no primers could be identified within the tails, they are incrementally extended by another 100 bases and the search is repeated until a suitable primer pair is found or the absolute value of the `pcr_overlap` parameter is reached. If no primers could be found within the extended tails, the sequence search window is shifted by the absolute value of the `pcr_overlap` parameter. The search for primers, again starting with only 100 bases long tails, is repeated until successful PCR primer pair is identified or the end of the region is reached. The length of the terminal tails into which the primers localize is purposely minimized to lessen the gaps between individual PCR products. The search window and the terminal tails are always chosen such that both PCR primers stay within the studied region to ensure specificity of the resulting FISH probes.

If the `pcr_overlap` value is negative, the PCR primer search of the second and successive PCR products starts beyond the end of the previous PCR product such that neighboring PCR products never overlap. This is particularly beneficial for the design of the CyDNA FISH probes which should not compete for the same overlapping binding sites. On the contrary, positive `pcr_overlap` gives rise to overlapping neighboring PCR products which are desirable for FISH probes generated by nick translation or random priming. Such FISH probes are generated randomly and therefore are inherently overlapping and competing for their binding sites anyway. The overlap of the PCR products increases the span of the FISH probe binding sites by eliminating any gaps between the individual PCR products.

10.3 Primer Search Parameters

The primers search referred to in the previous paragraphs is conducted by the Primer3 program **?**. The user can vary the primer search parameters such as the permissible length and annealing temperatures of the primers by modifying the configuration file (summarized in Table 1). The detailed information about each of each of these

Configuration parameter	Description	Default
`primer_opt_size`	Primer optimum length	20
`primer_min_size`	Primer minimum length	15
`primer_max_size`	Primer maximum length	30
`primer_opt_temp`	Primer optimum temperature	66
`primer_min_temp`	Primer minimum temperature	58
`primer_max_temp`	Primer maximum temperature	68
`primer_salt_corrections`	Primer salt corrections (0) Breslauer, (1) SantaLucia, (2) Owczarzy	2
`primer_gc_clamp`	Primer GC clamp	2
`primer_min_gc`	Primer minimum GC content	30
`primer_max_gc`	Primer maximum GC content	70
`primer_max_tm_diff`	Maximum difference between primer melting temperatures	3

Table 1: **Primer parameters in the configuration file.** The details of each parameter can be found in the Primer3 release 2.2.2 README file.

parameter is available in the Primer3 release 2.2.2 README file[2].

10.4 Restriction Endonuclease Recognition Site Search

Each PCR product is scrutinized for the presence of selected restriction endonuclease recognition sites. Their absence determines the optimal restriction sites for incorporation into the cloning PCR primers. The user may select a comma separated list of names from the Restriction Enzyme Database **?**[3] and include it through the `enzyme` parameter for analysis.

11 PCR Products for *Unique* Regions

Designing FISH probes for *unique* sequences is straightforward. For *unique regions* with length between `min_pcr_size` and `max_uniq_pcr` a primer pair is identified near

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[2] http://sourceforge.net/projects/primer3/files/primer3/2.2.2-beta/, January 2011

[3] http://rebase.neb.com/, January 2011

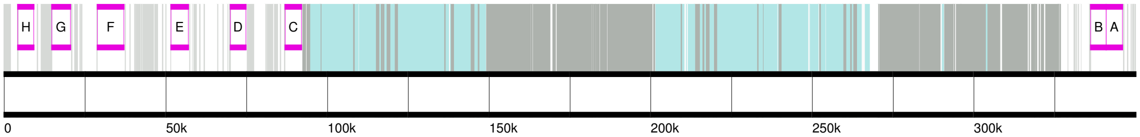


Figure 11.1: **Unique FISH Probes.** The optimized unique FISH probes are made of sequences highlighted by the empty pink rectangles. They bind unique sequences which are present only in a single copy in the entire genome.

its ends to allow PCR-mediated cloning of the intermediate sequence. *Unique regions* longer than the `max_uniq_pcr` are divided into multiple templates and a PCR primer pair is designed to clone each of them (Section 10).

The `min_uniq_pcr` and `max_uniq_pcr` values should be selected according to the type of experiment planned. If FISH probes are generated by nick translation of random priming the overall length of the FISH probe target should be in the order of tens of kilobases for reliable fluorescence detection. A PCR product less than a few kilobases in length would contribute rather insignificantly to the desired overall length of the FISH probe. Hence, the `min_uniq_pcr` is by default set to 4000. If the unique regions, on the other hand, are very fragmented, and no contiguous regions of sufficient length are identified, this default value might need to be decreased. The `max_uniq_pcr` value depends mainly on the feasibility of the PCR-mediated cloning. To save time and cost, lower number of longer PCR products to cover a given region is desirable. However, the length of the PCR amplicons is limited by the efficiency of the PCR and the subsequent cloning. The upper limit is set by default to 8500.

For CyDNA based FISH probes in which individual FISH probe molecules are directly synthesized in a PCR reaction with E10 polymerase, the `min_pcr_size` and `max_pcr_size` values limit the length of the produced FISH probes. In the experiments described in Chapter .... the goal was to obtain FISH probes around 500 bases long which was achieved by setting `min_uniq_pcr` to 500 and `max_uniq_pcr` to 700.

12 PCR Products for *Unique Similar* Regions

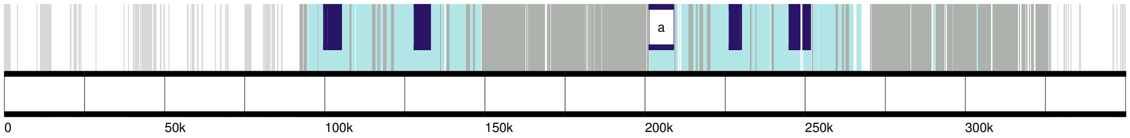
More complicated is the design of FISH probes for *unique similar* sequences. These FISH probes bind multiple similar targets that are all located within a *single similar* region. The *unique similar* sequences are defined by the non-zero regions in the histogram of the unique repeats (Section 9).

The PCR product length is limited by the `min_sim_pcr` and `max_sim_pcr` parameters. These values are selected as described in the previous Section 11 with the difference in mind, that these FISH probes bind repetitive targets and thus bind sequences of overall length exceeding their own. *Unique similar* FISH probes prepared from shorter PCR products can be tolerated because they give rise to stronger fluorescence signal compared to the *unique* probes. The default `min_sim_pcr` value is 2000.

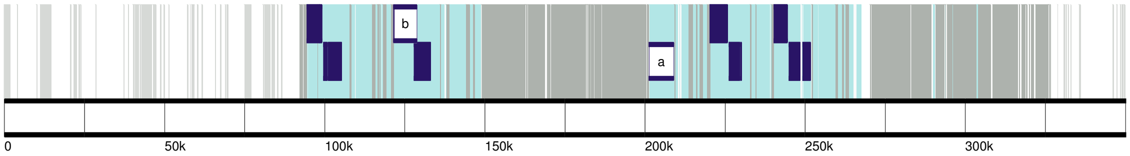
Providing that any *unique similar* sequences are present within the query, a large number of suitable PCR products is likely to be identified. Each of these PCR products or its portions are similar to multiple sites within the same *similar region.* The FISH probes obtained from these PCR products would also bind sequences exceeding their own length. The PCR product which is similar to the longest overall pool of sequences is selected as the best PCR product for *unique similar* FISH probe production (Figure 12.1**a**). Additionally, combinations of 2 to 5 best PCR products are selected from the pool (Figure 12.1**b**: only two best FISH probes are displayed). This offers the user the alternative to decide on the number of FISH probes that must be produced to a label sufficient amount of DNA for adequate fluorescence signal.

13 Restricted Sequences

The user is given the option to select a range of positions within the query which should be excluded from the analysis. It becomes desirable if the DNA template for the PCR reaction (BAC or plasmid) is available for only part of the query and the cloning primers must be selected only form this template. The range of sequences restricted from the search is specified by the `restrict_sequence` parameter obeying the following format. The start and end positions of the restricted sequence within the query are separated



(a) Single unique repetitive FISH probe binds five similar sites.



(b) Two best unique repetitive FISH probes extend the labeled length of the five similar sites.

Figure 12.1: **Unique Similar FISH Probes.** The optimized unique similar FISH probes are made of sequences highlighted by the empty blue rectangles. Their binding sites also span all sequences similar to each probe which are emphasized by the filled blue rectangles displayed in the same row as the respective FISH probe.

by a comma (,). Semicolon (;) separates several restricted sequence in each query and the pipe symbol (|) separates restricted sequences if multiple queries are submitted simultaneously.

Restricted sequences are automatically defined for positions of the query sequence which contain other values than the standard bases ACGT. An example of this would be the human T cell receptor β locus which is not fully sequenced and it contains two regions with sequences entirely consisting of undefined bases N (Figure **?? b**).

14 Graphical Output

While the PCR cloning primers for FISH probe templates production are the most important results of the analysis, a graphical output is crucial for understanding and interpreting the results. It provides a highly-customizable graphical representation of the query sequence with the distribution of *unique*, *similar* (Section 6), and *unique similar* (Section 9) sequences. The the binding sites for the optimized FISH probes (Sections 11 and 12) are emphasized along with the optional reference sequences such as genes (Section 14.4). The configuration file parameters allow the user to choose which features should be displayed, enable their annotation, and modify their colors. By default all the options are enabled and a standard colors are provided. The detailed description of each parameter is described in the available configuration file. Only the most important ones are introduced here.

14.1 Similar Sequence Distribution

The background of the graphical output is composed of a map splitting the query into *unique* sequences, *similar* sequences, and *unique similar* sequences. They are further distinguished by a darker tint if they are part of a similar region (Figure 9.1). Each sequence type can be assigned a different color by the parameters `uniq_color`, `sim_color`, `tint_uniq_color` `tint_sim_color`, and `uniq_sim_color`. The colors are specified by comma separated vector of its RGB components ranging from 0 to 255. The background map can be enabled and disabled by the `plot_area` parameter.

14.2 Sequence Grouping into Regions

The contiguous *unique regions* of the minimum required length (see Section 8) can be highlighted by rectangles spanning each region by setting the `plot_uniq_reg` parameter. They are label Uxx where xx is an integer distinguishing one from another. The color of the rectangle is specified by `uniq_pcr_color` parameter. Similarly, the contiguous *similar regions*-marking rectangles can be enabled by the `plot_sim_reg` parameter. They are labeled Sxx where xx are the distinguishing integers. Their color is determined by the `sim_pcr_color` parameter. Distribution of regions in the default sequence is displayed in Figure 8.1.

14.3 PCR Products

The positions of the PCR products for *unique* FISH probe production (Section 11) are displayed if `plot_uniq_pcr` is set (Figure 11.1). Their positions in the query are highlighted by rectangles of color is defined by `uniq_pcr_color`. The naming strategy for the PCR products changes if the *unique regions* are displayed or not (see previous

Section). When disabled (`plot_uniq_reg` is 0), the PCR products are labeled with the letters of alphabet applied in an increasing order (i.e. A-Za-z). Otherwise, they are given the name of the respective region and a letter if more than one PCR product is contained in a single region.

Similar labeling convention applies to the *unique similar* PCR products (Section 12) enabled by the `plot_sim_pcr` parameter (Figure 12.1). Their color is determined by the `sim_pcr_color` parameter. Unlike with the *unique* PCR products which are plotted side-by-side, each *unique similar* PCR product is plotted on a separate line of the graphical output (Figure 12.1**b**). This provides space for marking the positions of sequences similar to each PCR product. These similar sequences are highlighted by filled rectangles with the color defined by `mistarget_color` if `plot_mistarget` is set.

14.4 Reference Sequences

Apart from the analyzed query sequence, the user may provide a second FASTA file containing one or more reference sequences such as genes, their exons or regulatory sequences. These reference sequences are plotted in their respective positions as empty rectangles with their color specified by the `genes_color` parameter. Each sequence in the user-supplied FASTA file[4] is preceded by its name which is displayed inside the rectangle (Figure 14.2). The sequence names can take advantage of the advanced formatting options offered by LaTeX which for example allows to display Greek letters by typing a backslash followed by their English name, i.e. "\mu" or "\sigma". Underscore "_", i.e. "_{your-text}" would define subscripts, while the power symbol "^" superscript. The line defining the sequence name for the IGHG3 gene would then look the following: ">\gamma_{3}" and it would display γ_3 in the graphical output.

14.5 Horizontal Axis

The readability and arrangement of the query features are enhanced by the horizontal axis divided into multiple intervals. It can be enabled and disabled by the `plot_axis`

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[4] http://www.ncbi.nlm.nih.gov/blast/fasta.shtml, December 2010

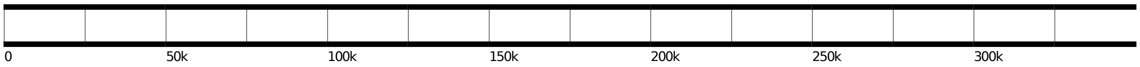


Figure 14.1: **Horizontal Axis** simplifies visual alignment of features in the resulting graphical output.

parameter. Its text annotation position below (1) or above (0) the axis is determined by `axis_topbottom`. The axis normally starts with 0 but can be offset by any value by `axis_offset` if desired.

14.6 Annotation of the Output

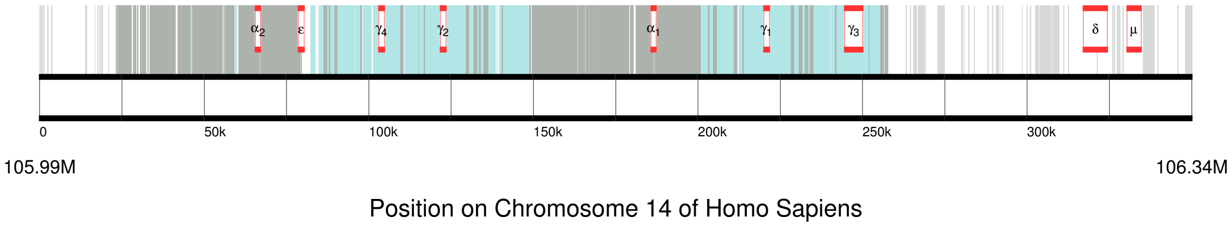
Depending on the `plot_labels` value, the graphical output can be annotated with a title bearing the user-provided name of the query, the chromosome number and the studied species and the X-axis and histogram would be labeled with their limiting values. The letter sizes used for the annotation and their type-face can also be defined through the configuration file.

14.7 Output Format

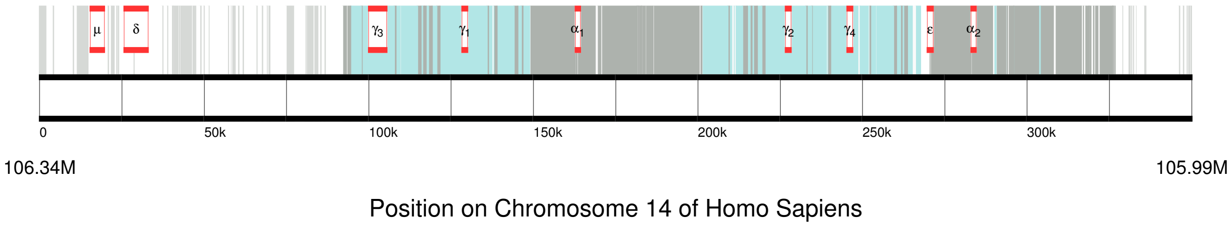
The graphical output is produced as an image in EPS vector format which allows scaling without resolution limits and even manual editing of the content. Optionally, it can be converted into a bitmap formatted image using the `graph_format` parameter and selecting one of the following options: -dpng, -djpeg, -dtiff or the other formats listed in the configuration file. `dpi_res` determines the resolution of the bitmap file in DPI.

14.8 Output Mirroring

Sometimes the genes contained in the query sequence are organized on the chromosome in a reversed order compared to the intuitive expectation. For instance, the human IgM immunoglobulin heavy chain constant region gene is functionally upstream of the other constant region genes but it is localized closer to the end of the chromosome. To overcome this confusing arrangement, the displayed sequence can be mirrored by setting the `rev_xaxis` to 1 instead of the default 0.



(a) `rev_xaxis` 0



(b) `rev_xaxis` 1

Figure 14.2: **Output mirroring** enabled by `rev_xaxis` parameter mirrors the content of the graphical output. **a**) Human immunoglobulin heavy chain constant region genes as they are ordered and oriented on human chromosome 14. Their order is contra-intuitive because first to the left is the IgA$_2$ (α_2) gene while the last one is IgM (μ). **b**) The same query sequence with genes was horizontally mirrored to obtain the expected order.

File ending	Content
.primer.txt	Summary of all primer sequences.
.unique_segs.txt	Primer sequences for *unique* FISH probes, complete PCR product sequences, restriction endonuclease site presence.
.nonunique_segs.txt	Primer sequences for all *unique similar* FISH probes, complete PCR product sequences, restriction endonuclease site presence.
.nonunique_segs-N.txt	Selection of primer sequences for the N best *unique similar* FISH probes, complete PCR product sequences, restriction endonuclease site presence.
XXX.primers.txt	Detailed analysis for each primer pair including alternative primer pairs.
.genconf	Configuration file generated in the analysis.
.log	Log file of the analysis.

Table 2: **Textual reports.** The analysis report is provided in a number of files whose names and contents are summarized in this table.

15 Textual report

Text reports are generated alongside the graphical output. These files contain all information about the cloning PCR primers designed for the production of specific FISH probe templates. Their contents are summarized in Table 2. They provide details on the primer sequences, their positions in the query sequence, the complete PCR products sequences which are necessary for verification of any plasmids produced using these primers, and the presence of restriction endonucleases recognition sites within the PCR products.

16 Mega BLAST Alignment Parameters

The query sequence is aligned to the genome using the Mega BLAST algorithm. It was introduced in the Section 5 without providing any details which would disrupt the flow of the Chapter and they will be introduced in the following Sections with the assumption that the reader is already familiar with the algorithm and its requirements.

The genome sequence used for the alignment is obtained from the Ensembl project[5]. The quality of the genome assembly varies among the species. Some of the less investigated genomes are assembled into a single contiguous database not accounting for the individual chromosomes. In others, it is assembled into sequences of the individual chromosomes, mitochondrial, and nonchromosomal DNA. The nonchromosomal DNA databases contain assembled sequences that could not have been ordered or oriented onto one of the chromosomes so far. The most studied genomes also include "patch" and "haplotype" sequences. The patch sequences contain newly sequenced parts of the genomes and amended sequences obtained by improved techniques which might in the future be incorporated into the main chromosomal assemblies. However, certain parts of the genome are highly susceptible to changes between different organisms of the same species. This prevents the determination of the invariable genome sequence for that species. Typically, this occurs with polymorphic genes such as the natural killer cell immunoglobulin-like receptor (Section **??**), the major histocompatibility complex (MHC), or the color pattern of butterflies. The "haplotype" databases contain such alternative sequences of the variable loci acquired from different organisms (**?** and **?**).

The type and quality of the genome assembly influences the function and performance of the algorithm and is discussed in the next two paragraphs.

16.1 Genomes in Single Sequence

The genome sequences assembled into a single file not accounting for the individual chromosome are divided number of sequence database files to speed up the alignment to such genomes. The genome fractioning is done at random positions which would impede alignment if the query sequence would overlap with one of these breaks. Due to the size of each genome, and the very small number of such artificial breaks, it is highly unlikely to ever occur.

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[5] ftp://ftp.ensembl.org/pub/current_fasta/

16.2 Genomes with Patches and Haplotypes

The patches and haplotypes for the chromosomal sequences are typically not used for the alignment to save computation time and to avoid undesired introduction of duplicated or similar sequences. The user has the option to enable them by setting `include_patch` and `include_haplo` to 1.

17 Mega BLAST Alignment Import

This Section discusses an important `bit_score` parameter which determines the threshold between *similar* and *unique* sequences. It is linked to the alignment introduced above (Section 16).

The distribution of the *similar* and *unique* sequences detected in the query will depend on the setting of this threshold. It also influences the specificity of the produced FISH probes which is partially determined by the presence of similar sequences throughout the genome. There is no direct characterized link between the sequence similarity obtained by Mega BLAST and the FISH probe's tolerance for mismatches and therefore it must be determined empirically. To obtain it, it was assumed that the similar sequence stretch should be at least the length of the FISH probes and exceed similarity of 85 %.

The Mega BLAST delivers positions of the similar sequences sorted by their bit score[6] value which reflects the length and the number of mismatches and gaps between the two compared sequences. The minimum required bit score for an alignment to be processed is defined by the `bit_score` value. By default, it is set to 200. This sets the

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[6] Bit score is calculated from the raw score being the sum of scores of all mismatches, gaps, and extensions of the compared sequences. The penalties for a mismatch, gap occurrence and gap extension depend on a lookup table called the substitution matrix whose values are proportional to the natural likelihood of occurrence of these mutations, insertions or deletions. The raw score is dependent on the parameters of the substitution matrix and therefore raw scores obtained from alignments using different substitution matrices are not comparable. The bit score unlike the raw score is normalized in respect to the substitution matrix values and therefore even bit scores obtained using different substitution matrices can be directly compared. Both scores are closely related to the expectation value introduced in the Section 5. This is equivalent to the number of alignments with raw scores better than the current one which would occur in the query and database sequences by random chance.

lower limit for the similar sequences in the human genome to 260 ± 40 bases in length with the percentage similarity of 85.2 ± 1.5. The length matches the FISH probe size which is preferably between 100 and 300 bases.

Lower `bit_score` value might be desirable for designing *unique* FISH probes (Figure 17.1**a**). The lower bit score threshold decreases the stringency of the search for the similar sequences, making the resulting FISH probes more specific for their targets and less likely to bind other parts of the genome.

Higher `bit_score`, on the other hand, might increase the quality of the designed *unique similar* FISH probes as long as it does not mask presence of similar sequences found elsewhere in the genome which would lead to production of nonspecific *unique similar* FISH probes. Therefore, the `bit_score` can be increased only if it does not result in longer overall stretch of *unique similar* FISH (Figure 17.1**c**).

The implications of changing the `bit_score` values should be understood and taken into account when designing the FISH probes. Repeating the algorithm with several `bit_score` values might be desirable before deciding which is the optimal set of FISH probes to be used. All FISH probes used in the experiments presented in this thesis were designed with the `bit_score` value of 200.

18 WWW Interface

The user interface of the FISH probe optimization algorithm is achieved through an Internet page[7]. The page has four main sections. The Introduction explains the usage and applications of the algorithm while Protocols provide technical advice on cloning and FISH probe production. The Standard Designer allows the user to upload a query sequence, select a target species, provide an email address, and upload optional reference sequence file. The Advanced Designer in addition requires upload of an user-supplied configuration file (Figure 18.1). Once analysis is successfully completed, a new page with summary of the results is provided (Figure 18.2) and a notification email is sent to the user-specified address. The data is kept on the server for one week for the user

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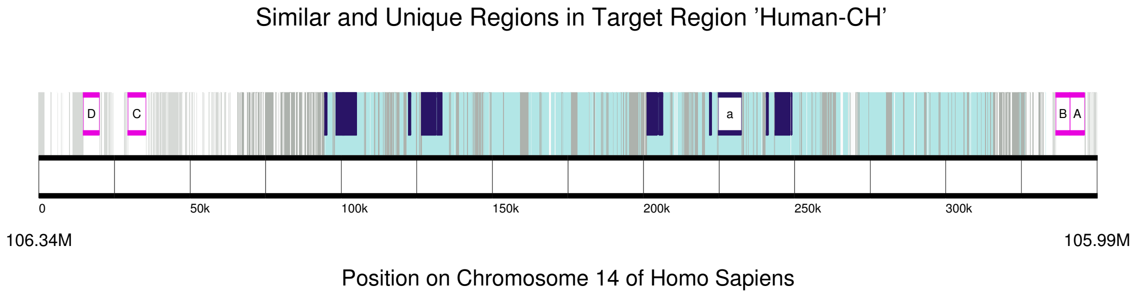
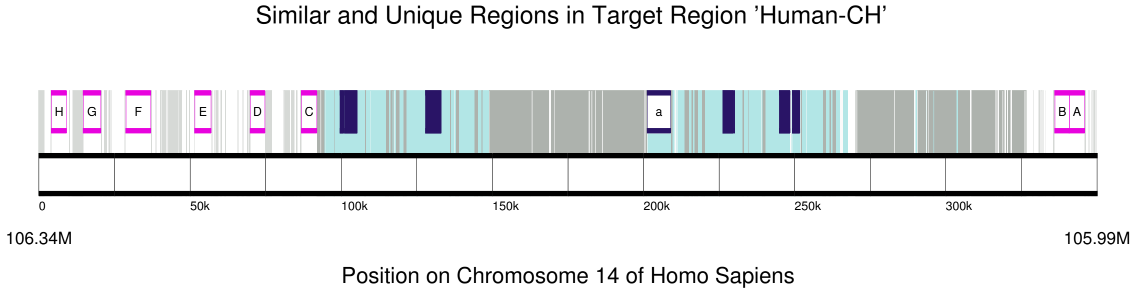
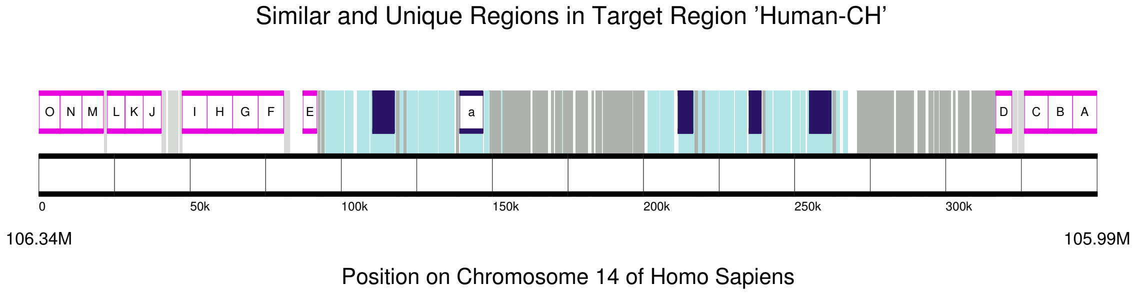
[7] http://www.nanoimaging.uni-jena.de/fish/



(a) `bit_score` 0: High stringency for unique FISH probes



(b) `bit_score` 200: Compromise value



(c) `bit_score` 1000: High stringency for unique similar FISH probes

Figure 17.1: **FISH Probe dependence on `bit_score`.** Highly specific unique FISH probes are obtained with low `bit_score` values **(a)**. Highly similar unique FISH probes are obtained with higher `bit_score` values **(c)**. `Bit_score` of 200 offers a compromise between both extremes **(b)**. It reflects the average minimum cut-off value of 260 bases for similar sequences which is similar to the size of the FISH probe molecules.

for viewing and download.

18.1 Data Safety

The design of the web site offers a compromise between data safety on one side and the usability with simplicity on the other. The user stays anonymous without the need to provide any login details or further information and the results are accessible exclusively to the user for one week following their completion. None of the data submitted to the web server is publicly available, yet it could be potentially viewed by a third-party exerting a concentrated hacking effort.

Privacy Protection Means

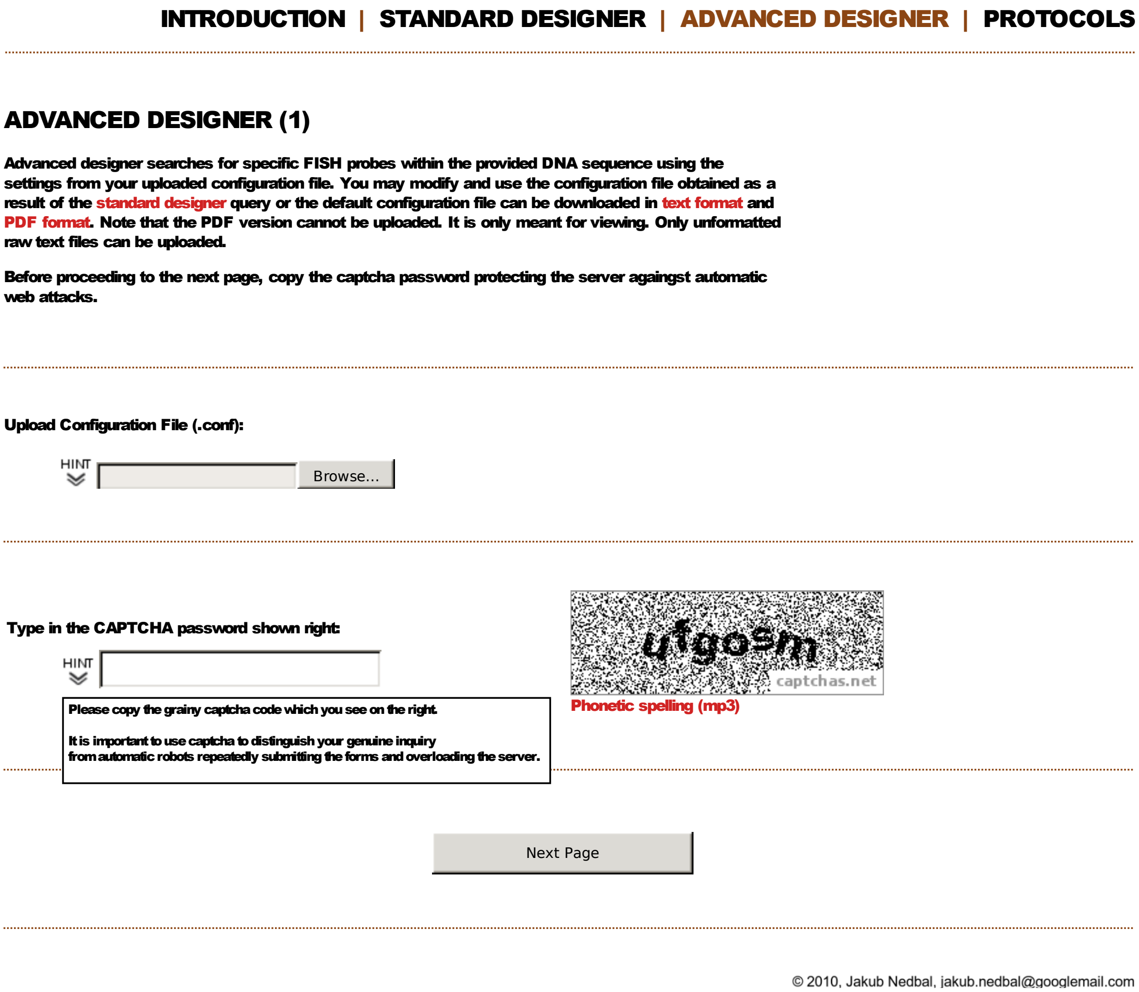
Each user submission to the web server is given a unique 11 to 16 digit tag without which the data cannot be accessed by anybody else. It consists of an ever increasing 10 digit number distinguishing the queries by the time of their submission. This is followed a randomly generated 1 to 6 digit number separated by a dash.

The user submitted query including the DNA sequences, configuration files and the email address are kept on the server only for a short time before it is safely stored for analysis. The email address is only kept during the analysis and destroyed after sending the notification email. The results are irrecoverably destroyed a week after finishing the analysis.

The web server communicates with the Internet browser of the user through a non-encrypted connection which is the common case for similar research tools intended for the scientific community.

19 Technical Details of the Software and Hardware

The analysis software and the web server operates on a single computer featuring AMD Athlon II X2 240e processor and 4 GB of RAM installed with Debian 5.0 GNU Linux operating system. The server is physically located at King's College London with



ADVANCED DESIGNER (1)

Advanced designer searches for specific FISH probes within the provided DNA sequence using the settings from your uploaded configuration file. You may modify and use the configuration file obtained as a result of the standard designer query or the default configuration file can be downloaded in text format and PDF format. Note that the PDF version cannot be uploaded. It is only meant for viewing. Only unformatted raw text files can be uploaded.

Before proceeding to the next page, copy the captcha password protecting the server againgst automatic web attacks.

Upload Configuration File (.conf):

Type in the CAPTCHA password shown right:

Please copy the grainy captcha code which you see on the right.

It is important to use captcha to distinguish your genuine inquiry from automatic robots repeatedly submitting the forms and overloading the server.

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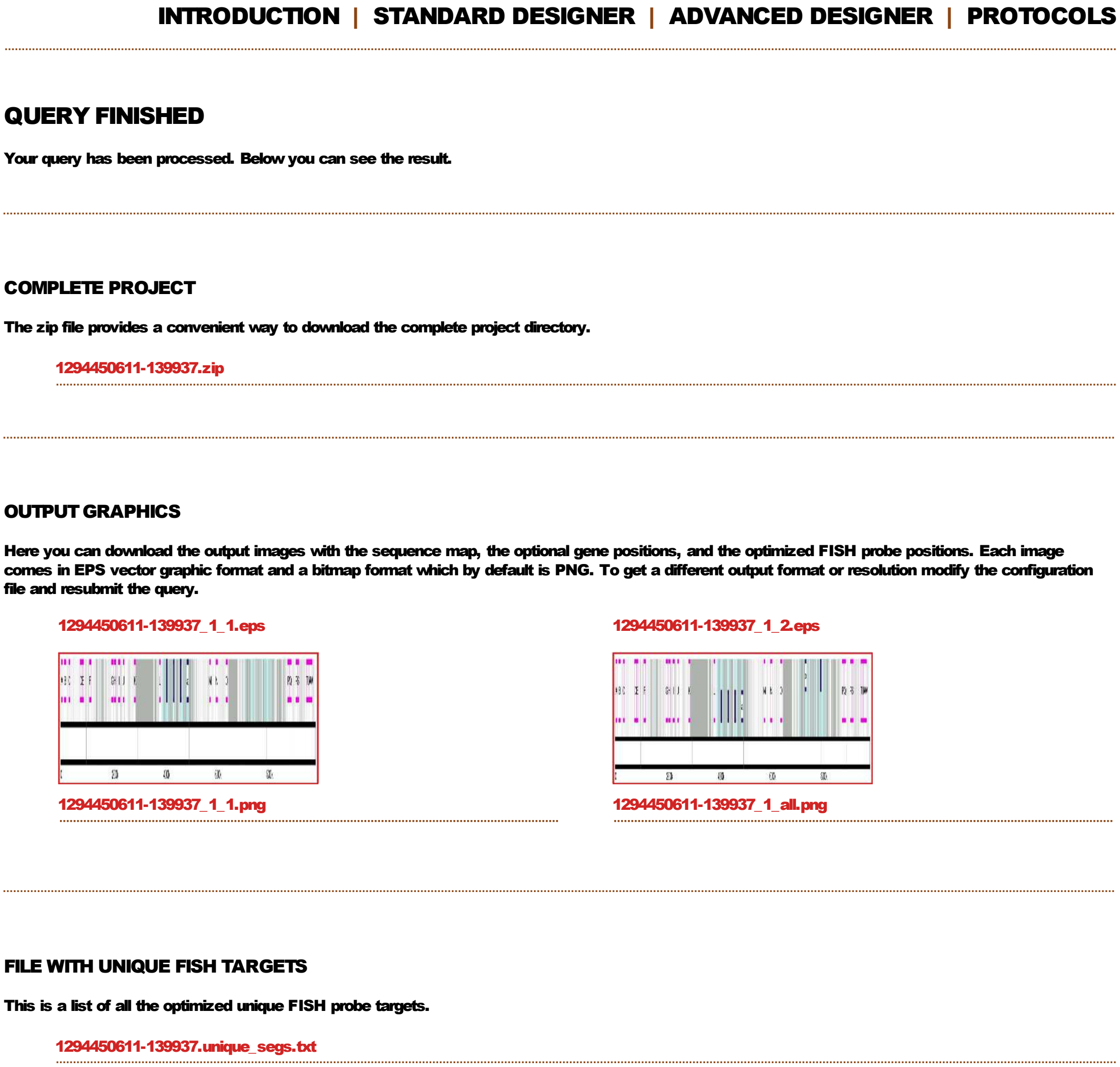
Figure 18.1: **Advanced Designer** shows typical layout used throughout the web site. The page is introduced by a short description of its content. It contains a file upload dialog button for the user to upload a configuration file. The safety password image at the bottom right to protect the page from automated attacks. At the bottom is a submission button leading to the next page. Hint buttons HINT reveal information dialogs to aid the user. The menu bar at the top simplifies the page navigation.



QUERY FINISHED

Your query has been processed. Below you can see the result.

COMPLETE PROJECT

The zip file provides a convenient way to download the complete project directory.

1294450611-139937.zip

OUTPUT GRAPHICS

Here you can download the output images with the sequence map, the optional gene positions, and the optimized FISH probe positions. Each image comes in EPS vector graphic format and a bitmap format which by default is PNG. To get a different output format or resolution modify the configuration file and resubmit the query.

1294450611-139937_1_1.eps

1294450611-139937_1_1.png

1294450611-139937_1_2.eps

1294450611-139937_1_all.png

FILE WITH UNIQUE FISH TARGETS

This is a list of all the optimized unique FISH probe targets.

1294450611-139937.unique_segs.txt

Figure 18.2: **Result output** consists of clearly laid out graphical outputs and textual reports for download and viewing. Only part of the result page is shown here for illustration.

the Internet traffic redirected from the http://www.nanoimaging.uni-jena.de/ domain server located at the University of Jena, Germany.

The core of the software runs in GNU Octave 3.2.4. It calls NCBI Blast 2.2.18.2 performing the sequence alignment, Primer3 2.2.2-beta searching for optimal primer pairs, Emboss 5.0.0 for restriction endonuclease site search, and Gnuplot 4.2 generating the graphical output. The website user interface is operated by Apache 2.2.9 web server with CGI scripts handling the user input values executed by Perl 5.10.0. The individual web pages are written in HTML with JavaScripts controlling their dynamic features such as the hint buttons HINT. Emails are sent through Exim 4.69 email server by mutt 1.5.18 email client. Other common programs supplied with Debian GNU Linux distribution are used for scheduling, data parsing, and file handling.

19.1 Task Scheduling

The script runs in an indefinite loop. Every 60 seconds, check for a new query submission is performed. In such case, the loop is temporarily interrupted and the query processing described throughout the Section **??** is initiated. At the start of each day, results older than one week are removed and the genome databases are updated if their new version is available at the Ensembl project FTP site[8].

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[8] ftp://ftp.ensembl.org/pub/current_fasta/, January 2011