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User guide

To develop matrices for ESTScan

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Contents

1. Introduction

ESTScan is a bioinformatic tool that permits the analysis of ESTs (expressed sequence tags). The program scans the ESTs, detects their coding part even if they are of low quality, and corrects the frameshift errors [6, 8, 9].

ESTScan takes advantages of the known associated bias in hexanucleotide composition, imposed by species dependent codon usage biases and amino acid composition inhomogeneities. This bias, which is used as a component in many gene prediction algorithms [2,3], was formalized as an inhomogeneous 3-periodic fifth-order Hidden Markov Model (HMM) in the ESTScan program [5]. This HMM has been extended to allow for various types of sequencing errors: (1) frameshift errors that would destroy the periodicity of the Markov chain; (2) sequencing errors that would introduce erroneous stop codons; (3) the presence of a considerable number of ambiguous nucleotides. It has also been normalized to correct biases introduced by the length of the sequence and its G+C isochore group.

In order to function correctly, the program needs some scores matrices which are normally specific for each species. These matrices reflect the codon preferences in the studied organism and can be obtained by using three scripts: extract_mrna, prepare_data and build_model.

The first script, **extract_mrna** extracts the data from files previously downloaded from FTP sites. The extraction is done by taking the sequences, contained in files, that correspond to a species or to a higher taxonomic level according to what has been chosen by the user.

The second script, **prepare_data**, classifies the mRNAs extracted previously. First, it separates the mRNAs in two groups: training and test data, if the user chooses to separate them. If he does not, the same data will appear in both files. Second, the training data is divided according to the mRNAs' GC-content. Third, the redundancy is masked. Fourth, the coding and non-coding parts of mRNAs are classified in two different files.

Finally, the third program called **build_model** creates the matrices using the data prepared by the previous scripts.

Following this, it could be useful to evaluate the efficiency of the matrices. This step is done by using two different scripts: extract_UG_EST and evaluate_model.

The first script, **extract_UG_EST**, uses UniGene clusters produced by the NCBI [12, 15]. A cluster is composed of ESTs that match a genomic sequence with an annotation making reference to RefSeq or EMBL mRNA sequence. This script searches for the clusters that correspond to the mRNAs of the test file. All clustered ESTs are aligned with their respective mRNA with megablast. The alignment enables the annotation of the ESTs and their classification into two main groups: coding or non-coding sequences.

The second program called **evaluate_model** evaluates the matrices. It launches ESTScan on data that have been obtained previously in order to predict the coding parts.It then calculates the false negative rate (sensitivity): the percentage of coding sequences classified as non-coding, the false positive rate (specificity): the percentage of non-coding sequences classified as coding and the detection accuracy of the start and stop sites.

The use of the different programs is not difficult. Nevertheless, this user guide will assist people using ESTScan during the first stages: the writing of the configuration file, which contains information required for the programs and the execution thereof.

2. Required configuration

The use of ESTScan requires:

-The use of a computer that has a C compiler and manage to use the Perl language.

-The download of the programs from http://sourceforge.net/projects/estscan/:

- Download all the files from the BTLib package
- Download all the files from the ESTScan package, version 3.0.

-The download of megaBLAST [13] distributed from the NCBI FTP site: ftp://ftp.ncbi.nlm.nih.gov/blast/ under the /blast/executables/ directory. Some information is available on the blast/documents/ directory of this FTP site.

-The download of R and gnuplot; in most cases, they are in the Unix distribution.

-The download of files containing sequences from the RefSeq, EMBL and UniGene FTP sites. One should bear in mind that the creation of the matrices implies the use of **mRNAs** that must be **annotated** and must contain the **entire coding region**. Without these two conditions being met, it is impossible to create reliable matrices.

Note: the explanations in this user guide consider that the user has chosen a UNIX environment, for example Fedora [19].

3. Content of the ESTScan package

Some information, reports and links are available at http://estscan.sourceforge.net/.

The programs that must be downloaded are available from http://sourceforge.net/projects/estscan/.

There are fives groups of files:

- The BTLib files that are necessary for the partitioning of the EMBL or RefSeq files.
- The ESTscan package that contains all the programs required to run the program on sequences (version 3.0).
- The ESTscan1 package
- The ESTscan2 package
- Some matrices with their evaluation.

All the scripts needed to create and evaluate the matrices are available in files in the ESTScan package version 3.0:

- estscan-devel-3.0-0.i386.rpm
- estscan-devel-3.0-0.ia64.rpm
- estscan-devel-3.0-0.x86_64.rpm

4. Configuration file

The use of ESTScan implies the creation of matrices that reflect the codon preferences of the species of interest. All the scripts used to create or evaluate these matrices need a **configuration file** that contains the main piece of information.

4.1. Description of the configuration file

The configuration file must be in Perl syntax; it looks as follows.

```
################################################################################
#
#   Parameters for the mouse
#   (use PERL syntax!)
#

$organism       = "Mus musculus";
$hightaxo       = "Rodentia";
$dbfiles        ="/db/refseq/release/mus*.gbff        /db/refseq/new/mus*.gbff        /db/embl/86/mus*.dat
                /db/embl/new/mus*.dat";
$ugdata         = "/db/unigene/Mm.data";
$estdata        = "/db/dbest/est_mus-??.seq";

$datadir        = "/ESTScan/Results/Mm";
$nb_isochores   = 2;
$tuplesize      = 6;
$minmask        = 30;

#
#   End of File
#
################################################################################
```

The first variable **$organism** contains in quotation marks the name of the species you are interested in. This variable is used to select entries in files containing information for various species.

$hightaxo is a variable that can be used to consider sequences from a group of species instead of only involving those from one species. It contains a taxonomic level that has been chosen by the user (see section 5).

$dbfiles specifies the local files, in EMBL or RefSeq format, from which full-length mRNA sequences are extracted.

$ugdata specifies the files containing the UniGene clusters.

$estdata specifies the files containing ESTs.

$datadir is the base directory where all the files are located and the temporary results are stored.

$nb_isochores or **@isochore_borders** allows the separation of the training data according to their GC content (see section 6).

$tuplesize indicates the order of the Hidden Markov Model; its default value is 6.

Finally **$minmask** is a parameter used during masking redundancy. It indicates the threshold length above which redundant pieces of sequences will be masked. Its default value is 30.

4.2. Creation of the configuration file

4.2.1. Opening a configuration file

For the generation of matrices for a specific species one needs to follow a series of steps in order to create the configuration file which allows the use of the scripts.

The first step is to copy a configuration file (“.conf” ending) available from http://estscan.sourceforge.net/.

Save this file with a name that reminds you of the content of the file, for example: hs.conf for the configuration file for human sequences.

4.2.2. Changing the species’ name

One configuration file per species has to be written for it to be used during the execution of the scripts. Thus it is necessary to replace the name of the species by the name of the species you are interested in.

Changing the name on the first line

You have to change the name written in the first line after “parameter for” in order to inform users that this file corresponds to a specific species.

Changing the name following the variable $organism

You also have to change the name of the species that is stored in the variable **$organism**.

Pay attention to the spelling and the capital letters, because they are essential for the program to find the entries corresponding to the species of interest (e.g.: Homo sapiens). In case of doubt, check for species names on the NCBI website [14].

4.2.3. The variable $hightaxo

If there is not enough data available in databases for the species of interest, you need to add the variable **$hightaxo** below $organism. Choose the taxonomic level from which the data will be extracted on the taxonomic part of the NCBI’s website [21] and write this level after $hightaxo. Do not forget to use quotation marks and to put a semicolon at the end of the line.

If you want to use the sequences of one organism only, do not add the $hightaxo variable or leave a space between the quotation marks. Remember that $hightaxo has priority over $organism, thus the files will be processed according to this $hightaxo variable and not according to $organism (see section 5).

4.2.4. Location of the downloaded files

The mRNA or ESTs sequences can be provided as several files. The name of these files must be contained in the configuration file.

There are two methods for indicating the files to the program: -globbing
-listing of all files

In order to refer to several sequences with similar file names, use an asterisk (*) to replace a part of the files name or use a question mark (?) to replace one character only. Remember that the Unix separation of directories is a slash (/).

RefSeq sequences

After **$dbfiles** you need to indicate the file names in quotation marks.

For example: $dbfiles = “/db/refseq/release/mus*.gbff”;

The asterisk replaces the number written after the abbreviation of the species (e.g. 01, 02, 03,... of mus01.gbff, mus02.gbff, mus03.gbff)

EMBL sequences

You have to indicate the location as previously described just after the information for the RefSeq sequences. Again, use the asterisk to indicate that you require multiple sequences.

For example: $dbfiles = “/db/refseq/release/mus*.gbff /db/embl/new/mus*.dat”;

UniGene sequences

Indicate the location of the requisite file after **$ugdata** in the same way as for the RefSeq and EMBL sequences files. This time you do not need to use the asterisk because there is only one file.

For example: $ugdata = “/db/unigene/Mm.data”;

DbEST sequences

The variable **$estdata** specifies the location of the ESTs that will be analyzed later. This variable is not necessary at the moment, however, we can specify the location of the files after **$estdata**.

For example: $estdata = “db/dbest/est_mus-??.seq”;

4.2.5. Location of new files

Add in quotation marks after **$datadir** where the results of the build_model program should be stored.

For example: $datadir   = “ /home/user/ESTScan/Results/Mm”;

To create some new directories use either the windows-like graphical interface or the terminal. If you use the shell, enter the directories with the order cd followed by the name of the directories and then use the order **mkdir** to create directories.

4.2.6. Number of isochores

The coding potential of sequences can change according to the GC percentage (reviewed in [1]). In order to obtain better matrices, one should separate the data according to their GC content.

The user can specify the desired number of isochores. The program will then calculate the delimitation of the different isochores in order to have the same number of sequences in each group.

It is also possible to specify the delimitation of the isochores. In this case, the program will apply the parameter specified by the user and the number of sequences in each group may vary a lot.

Number of isochores

If you have no specific idea of the GC percentage distribution, it is advisable to simply choose a number of isochores. The value can be chosen arbitrarily and then written beside **$nb_isochores** (see section 6).

Delimitation of the isochores

In the case that you have a publication that explains the content of the genome of interest, specify the delimitation of the isochores. Replace **$nb_isochores** with **@isochore_borders** and indicate the isochores' borders after it (e.g. @isochore_borders = (0.0, 43.0, 47.0, 51.0, 100.0)).

4.2.7. Number of tuplesize

The tuple size indicates the number of nucleotides used in the Hidden Markov Model. Its default value is 6.

The user has to indicate the number of tuples after the variable **$tuplesize**.

4.2.8. Number of minmask

In order to avoid biases in the training data, redundant pieces of sequences longer than a given threshold should be masked. The threshold can be set using the variable **$minmask**.

Its default value is 30. Write this number after **$minmask**.

5. Sequences to download

The amount of data available on FTP sites like EMBL or RefSeq can vary a lot depending on the species. For some organisms the amount of data will be sufficient to create reliable matrices.

For other organisms, not enough data is available. You may download files from other species which are phylogenetically closely related. In this case, the user has to choose a higher taxonomic level from which all the sequences will be extracted. The choice of this level can be done with the help of the taxonomic browser of the NCBI website [21]. This website provides the lineage of species and also the classification of some model organisms that can help understand the phylogeny.

For example:

If you want to analyze some ESTs of the beetle (Tribolium castaneum) :

You can find its abbreviated taxonomy (which is the same as that used in EMBL and RefSeq) in the taxonomy part of the website of the NCBI:

Eukaryota; Metazoa; Arthropoda; Hexapoda; Insecta; Pterygota; Neoptera; Endopterygota; Coleoptera; Polyphaga; Cuccjiformia; Tenebrionidae; Tribolium

If you have a look at the classification of model organisms, you will discover that the closest organism commonly used in molecular research projects is Drosophila melanogaster. In this case, one solution is to involve all the sequences of the Insecta class.

When we extract the data of Tribolium castaneum in RefSeq and Embl database (data available on 15th december 2006) we obtain only 239 sequences, whereas the extraction of the data for the insecta class provides 50'361 sequences. Thus we may suppose that the matrix obtained with 239 sequences of the beetle would not be reliable as there is not enough data to permit a significant training of the program. Using the data for all insects allows one to obtain matrices that are useable, and thus a better analysis of the ESTs using ESTScan.

To discover how many sequences are required to obtain reliable matrices, we build matrices with different numbers of sequences. We see that at least 2000 sequences are required.

In practice, the user has to add the variable **$hightaxo** in the configuration file to indicate the taxonomic level. The program will process all the sequences of organisms in this taxonomic level and use them to build the matrices (see section 4.2.3).

For example: $hightaxo = “Insecta”;

Moreover, there is sometimes one file per species in the databases, but commonly there are the sequences of many species in one file. Thus the user has to download the file that contains the sequences of the species of interest or taxonomic group, and then the program will use the sequences of interest only (species or the taxonomic group). The sequences of other species or groups will be excluded from the analysis.

6. Isochores

At the beginning of the 1970s, centrifugations in analytical CsCl density gradients of warm-blooded vertebrate DNAs showed a compositional heterogeneity, which is not the case for cold-blooded vertebrates.

The analysis of the DNA by centrifugation in Cs_2SO_4 density gradients in the presence of sequence-specific DNA ligands (e.g. Ag^+) then allowed the identification of families of DNA fragments. These families are characterized by different GC levels. Long DNA stretches (more than 300 kb) that contain local similarities in GC content are called **isochores**.

If this type of analysis is applied to human DNA preparations in the 30-100 Kilobase (Kb) size range, we can observe 5 families, two with GC-poor major components (L1, L2) representing about two thirds of the genome and three GC-rich components (H1, H2, H3). Furthermore, the gene distribution is not uniform in the genome. Most of the genes are localized in component H3, which is the most heterogeneous component and represents 3-5 % of the genome (reviewed in [1]).

Nowadays, if the genomic sequence is available, it is possible to use a computer to analyse the GC content and to discover the presence of isochores. The bioinformatics approach has mostly replaced the experimental studies using the CsCl density gradient.

The use of ESTScan implies the choice of some parameters, like the number of tuple or the number of isochores. Defining the isochores number can be quite difficult. We can analyse the GC content using a computer or refer to some articles that report the border for the different isochores present in the species and explain their significance [4], but this information does not exist for every species.

In order to show the effect of the number of isochores on the false positive and false negative rates of ESTScan, we performed the analysis on mRNAs and ESTs data, using different matrices created with a variable number of isochores. We can see that the number of isochores does not seem to be of major importance (Figure 1). There are no significant differences between the groups.

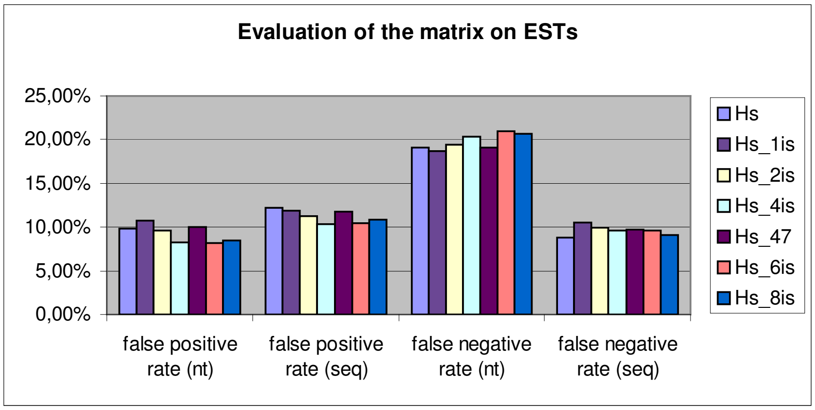


Figure 1: Representation of the false positive and negative rates on the nucleotide and sequence levels, using matrices created with different number of isochores. The Hs training data was split into four isochores, with the borders: 0.0, 43.0, 47.0, 51.0, 100.0. The data of Hs_47 was divided into two parts, choosing 47 as a boundary. The other groups are composed of a variable number of isochores indicated after Hs. The boundaries are chosen by the computer in order to have the same number of data in each group.

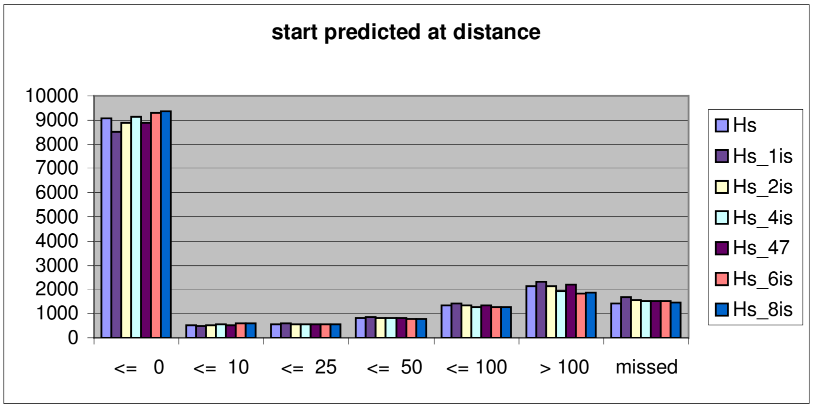


Figure 2: Number of start sites predicted at a specific distance from the annotation, using matrices created with different number of isochores.

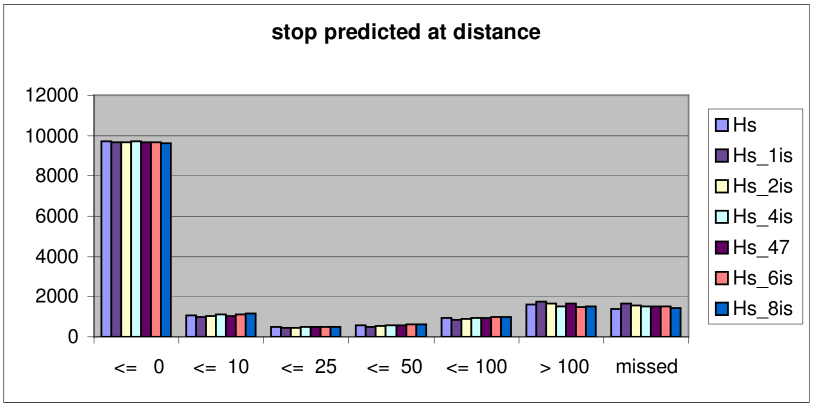


Figure 3: Number of stop sites predicted at a specific distance from the annotation, using matrices created with different number of isochores.

As one matrix is created for each isochore, the amount of data necessary to build the matrices increases with the number of isochores. It is advisable to use only one isochore to create the matrix and, in case of trouble, to retry with a higher number of isochores.

7. Obtaining the matrices

Once the file has been written and saved, the three programs that calculate the matrices can be executed.

The first program *extract_mrna* extracts the data from the files previously downloaded from the FTP sites. The extraction is done taking the sequences that correspond to a species or to a higher taxonomic level. Moreover, the program checks if the sequence is an mRNA and if the entire coding sequence is contained in the sequence.

To launch the program, type into a terminal:

```
$ cd ESTScan                  #  where the scripts are

$ ./ extract_mrna sp.conf.    # sp.conf correspond to the configuration file you created
                              previously
```

The program extract_mrna will be executed using the file sp.conf. The script build_model_utils.pl contained in the package enables the reading of the configuration files. The results will be stored in the directory indicated in $datadir.

When the processing of the program is finished, “ sp.conf done” will appear in the terminal. This information will appear after the execution of any script.

Then you have to use the second program which classifies the sequences into several groups. It is possible to use the option **-e** with this script. This option is useful in cases where the matrices must be evaluated, because it allows the splitting of the data into two groups : a training and a test set. The splitting is necessary for the evaluation, because it is not particulary relevant to test the discrimination power of the matrix on the same data as the data used for the training. If no option is used, the training and test data will be the same. In addition the program splits the data from the training set into several isochores and masks the redundant pieces of sequences. Finally, the mRNAs are split into two files, one containing the coding part and the second containing the non-coding part.

To launch the program write:

```
$ ./prepare_mrna -e sp.conf
```

If you not need to evaluate the matrices, write only:

```
$ ./prepare_mrna sp.conf
```

Finally, you have to launch the third program that creates the codon usage table.

```
$ ./build_model sp.conf
```

8. Evaluation of the matrices

Once the matrices have been built, the user can evaluate their accuracy with the help of two programs: extract_UG_est and evaluate_model.

Extract_UG_est searches UniGene clusters with an annotation making reference to the mRNA of the test file previously created. It then does a megablast to know where the sequences match. Finally based on the length of the match, its location and the mismatches, the program selects one coding and another non coding sequence that matches an mRNA of the test file. These sequences are used by the last script evaluate_model, which calculates the sensitivity and the specificity of the matrices and also the accuracy to detect the start and stop sites. Furthermore, it produces some histograms to illustrate the results.

```
$ cd ESTScan                    #  where the scripts are
```

```
$./extract_UG_est sp.conf
```

This step is time consuming.

Then you can launch the second program used for the evaluation: evaluate_model.

```
$ ./evaluate_model sp.conf.
```

The results appear in the shell and are also stored in your computer (see section 10).

9. Input and output of scripts

Each script has its own input and output files. In the following section, existing files will be described.

9.1. Input and output of extract_mRNA

The script extract_mRNA expects files in Genbank or EMBL format [7, 11] (see section 12.1 and 12.2).

The output of this script is a file containing mRNA sequences in FASTA format with headers containing: the accession number, annotation of coding sequence start and stop as two integers values following the tag 'CDS:' and a description.

Example of sequence in FASTA format:

```
>tem|NM_014580 CDS: 46 1479 Homo sapiens solute carrier family 2, (facilitated glucose transporter) member 8 (SLC2A8), mRNA

GGCGGTTCAGGCGCCAGAGCTGGCCGATCGGCGTTGGCCGCCGACATGACGCCCGAGGACCCAGAGGA
AACCCAGCCGCTTCTGGGGCCTCCTGGCGGCAGCGCGCCCCGCGGCCGCCGCGTCTTCCTCGCCGCCTTC
GCCGCTGCCCTGGGCCCACTCAGCTTCGGCTTCGCGCTCGGCTACAGCTCCCCGGCCATCCCTAGCCTGC
AGCGCGCCGCGCCCCCGGCCCCGCGCCTGGACGACGCCGCCGCCTCCTGGTTCGGGGCTGTCGTGACCC
```

Description of the FASTA format:

A sequence in FASTA format begins with a greater-than (">") symbol immediately followed by the identifier of the sequence and eventually followed by a description. After that, there are several lines of sequence data. The sequence ends when another line starts with the ">" symbol, indicating the start of another sequence, or at the end of the file.

9.2. Input and output of prepare_data

Prepare_data classifies mRNAs sequences, normally extracted from RefSeq or EMBL files by the script extract_mRNA. However, it is possible to use a particular collection of mRNAs. In this case, you must provide the data in FASTA format under the name of mRNA file: mrna.seq (where extract_mRNA would store the extracted data). The header must contain annotations of coding sequence start and stop in the header as two integer values following the tag 'CDS:'. The first integer points to the first and the second integer to the last nucleotide of the CDS. Thus the length of the CDS is <stop> - <start> + 1. The first nucleotide in the sequence has index 1 (see section 9.1).

The output of this script is mRNA data classified in several files: training and test set. Furthermore the data from the training set is split according to its GC content. These files in FASTA format are stored in the main directory and in the Isochores directory (see section 9.1). Two other files in FASTA format are created and stored in the Evaluate directory. The first file contains the coding part of the mRNAs (rnacds.seq) and the second contains the non-coding part (rnautr.seq).

9.3. Input and output of build_model

Build_model needs as input the mRNAs of the training set, always in FASTA format with the annotation of the coding region in the header (see section 9.1 and 9.2). The sequences of the training set can be taken from EMBL or RefSeq files if the scripts extract_mrna and prepare_data have been used or from a particular collection of mRNAs in FASTA format.The output consists of the matrices, one for each isochore, and which look like this:

```
FORMAT: hse_4is.conf CODING REGION 6 3 1 s C+G: 0 44

-1   0   2   -2

2    1   -8   0

1    0   1   -4

-1   -1   4   -3

0    -2   3   -2

3    0   -8   0

0    0   2   -1

-3   0   4   -2

2    -1   1   -3

3    1   -9   -1
```

9.4. Input and output of extract_UG_EST

The input of extract_UG_EST are the mRNA sequences of the test file (FASTA format) and the UniGene clusters taken from the UniGene FTP site [15] (see section 12.3).

The output of this script includes two files containing the UTR part or the coding part of ESTs (estutr.seq and estcds.seq respectively). These are in FASTA format. Three other files are created during the execution of the script: ug.data which contains the information about the UniGene clusters, clusters.lst which contains organized data of ug.data and matches.lst which contains the information about the megablast done between the ESTs and the mRNAs (see section 12.4, 12.5, 12.6).

If during the use of the first script you have provided your own mRNAs, you also have to provide ESTs with annotation in order to enable the evaluation. The annotation of the ESTs is possible doing like extract_UG_EST. You can blast the ESTs against an mRNA to benefit from the annotation of the mRNA in order to find out which part is coding or not, and divide the data into two groups in the files estcds.seq and estutr.seq. Estutr.seq only contains non-coding nucleotides whereas estcds.seq contains partially coding ESTs. For the sequences that are in the estcds.seq file, it is necessary to indicate the location of the coding part (see section 9.1).

9.5. Input and output of evaluate_model

The execution of evaluate_model requires the following files:

-test.seq

-rnautr.seq

-rnacds.seq

-estutr.seq

-estcds.seq

-The matrices

All of the files except for the matrices are in FASTA format (see section 9.1). Estutr.seq and nrautr.seq do not necessarily contain the tag 'CDS:' followed by the location of the coding sequence, because all nucleotides may be non-coding.

The output is composed of many files, some containing sequences (ending with .seq), some ending with ".dat", containing data necessary to build gnuplot graphs. Other files end with ".gplot" and are needed to create the graph easily and, finally, one file ends with ".R" containing the information to draw a pie chart representing the percentage of start or stop sites that have been found with a specific accuracy (see section 10). The most important file is stored in the report directory and contains all the false positive and negative rates.

10. Results

10.1. Description of the results

The results are stored in the location indicated in the configuration file ($datadir). When the user has launched the first script: extract_mrna, a directory is created for the species. This directory contains several sub-directories: **Evaluate**, **Isochores**, **Matrices**, **Report** and **Shuffled,** as well as a file called **mrna.seq**, which contains all the extracted mRNAs. Running prepare_data will add two more files to the main directory: **test.seq** and **training.seq**.

The Evaluate sub-directory contains:

- one file named 6_00030_0000001_4242_piecharts.R
- Files containing sequences used during the evaluation (files ending with ".seq").
- Files ending with ".dat", which contain the values to create some histograms.
- Files ending with "gplot" that permit an easy output of histograms, because they contain all the information needed (e.g. the title of the graph and the name of axes).

Examples of graphs that can be obtained with the files ending with gplot:

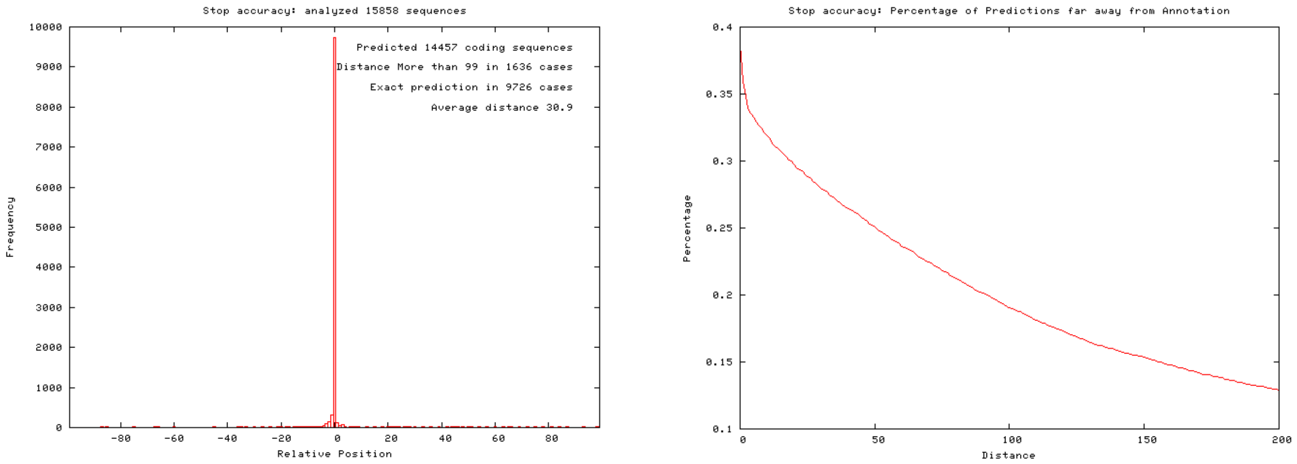


Figure 4: Distribution of distances between predicted and annotated start sites. Position zero is the predicted start/stop site. Number (left) and percentage (right) of start sites predicted with a particular accuracy.

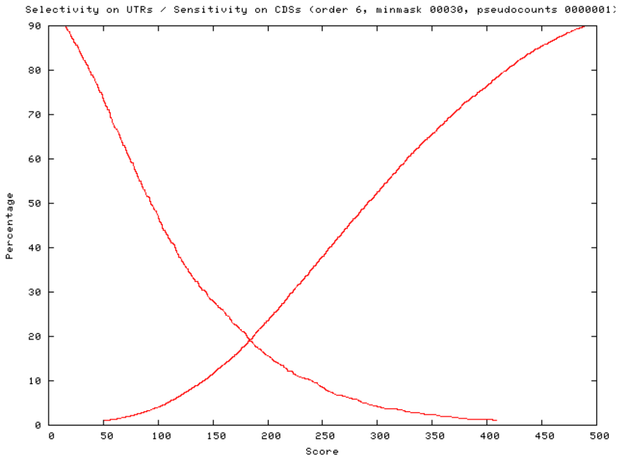


Figure 5: Sensitivity and specificity of models for untranslated regions and coding sequences.

The sub-directory Isochores contains all the mRNAs of the training set classified into several groups of isochores. For each isochore there are two files, one with complete sequences and another for which the redundancy has been deleted.

For example, we can find these file names:

mrna0-44_mr30.seq mrna0-44.seq mrna44-51_mr30.seq mrna44-51.seq mrna51-57_mr30.seq mrna51-57.seq mrna57-100_mr30.seq mrna57-100.seq

The files that do not contain "mr30" in their name contain the data from the training file that have been separated according to their GC percentage. In the other files, the redundancy has been removed.

The matrices are stored in the Matrices sub-directory with a complex name, which ends with ".smat". This file is created after the script build_model has finished processing the data.

The Report sub-directory is useful to obtain the text written in the terminal when the program is running. For each script that has been executed, there is one file in the report directory. Furthermore, there are two other files in the report directory: gc.dat, gc.gplot. The first file gc.dat contains the number of sequences for each CG percentage. The second file gc.gplot contains the information necessary to build a histogram with the GC percentage on the x axes and the frequencies of the sequences for each GC percentage on the y axes.

If you create the histogram with the data of gc.gplot, you will obtain a graph that looks as follows:

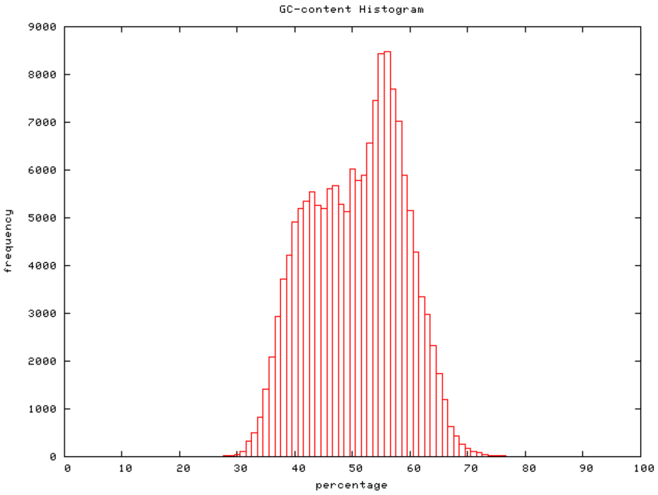


Figure 6: Histogram representing the GC content of human sequences.

The shuffled directory has no utility after the creation and the evaluation of the matrices, it is empty.

When the scripts are running, some information appears in the terminal and is then stocked in the report directory. The most important data that is written in the terminal is the one obtained during the evaluation.

For example:

```
Using estscan to scan EST/mRNA
Evaluating new model on mRNA data using -m -100 -d -50 -i -50 -N 0....      ⇐rate calculated on part
                                                                             of mRNAs or ESTs
 - predicting CDS for /export/scratch/ludi/ESTScan/Hse_4is/Evaluate/rnautr.seq...
   found 44471302 coding of 176493655 nucleotides in 69999 of 335386 sequences
   estimated false positive rate: 25.20% (nt) 20.87% (seq)
 - predicting CDS for /export/scratch/ludi/ESTScan/Hse_4is/Evaluate/rnacds.seq...
   found 180346838 coding of 182762167 nucleotides in 127695 of 167693 sequences
   estimated false negative rate: 1.32% (nt) 23.85% (seq)
 - predicting CDS on /export/scratch/ludi/ESTScan/Hse_4is/test.seq...
 - computing histograms from
/export/scratch/ludi/ESTScan/Hse_4is/Evaluate/rnaprc6_00030_0000001_4242m100d50i50N0.seq...
   predicted 159980 coding regions
   estimated false positive rate 17.73% (nt)      ⇐ rate calculated on complete mRNAs or ESTs
   estimated false negative rate 2.47% (nt)
 - writing data-files and gnuplot scripts...
   start predicted at distance
      <=   0:   45143
      <=  10:   34804
      <=  25:    8233
      <=  50:   13089
      <= 100:   19731
       > 100:   38980
      missed:    7713
   stop predicted at distance
      <=   0:  102436
      <=  10:    3400
      <=  25:    4445
      <=  50:    8196
      <= 100:   11503
       > 100:   30000
      missed:    7713
```

Four blocks like this one are written in the shell. The first two summarize the evaluation of the matrix obtained on mRNAs, and the last two describe the evaluation obtained on ESTs (from UniGene clusters). Moreover, if we focus on the results obtained on mRNAs or ESTs, we notice that the second block reflects the analysis scanning the data (mRNAs or ESTs of UniGene clusters) in one direction only, and the first block analyzing the data in both directions.

After the sentence: “computing histograms from” we have the false positive and negative rates calculated on complete mRNAs or ESTs.

Finally, we can see the number of start sites followed by the number of stop sites, predicted at a specific distance from the annotation.

This information is particulary important to get an idea of the precision for detecting the coding region (sensitivity and specificity) and the accuracy of prediction of the start and stop site. These numeric data help the user to determine if the matrix is good enough and then enable the results obtained using ESTscan to be interpreted. Some graphical data is available, too; it helps to visualize and understand the results (see section 10.2).

10.2. How to use the results

10.2.1. The report’s files

All the information that appears in the terminal when the programs are running are stored in the Report directory.

To see these files, go to the location you specified in $datadir and then enter the Report directory using the command **cd**. Use the command **ls** to see the content of the directory, and then the command **less** followed by the name of the file you are interested in to see the file.

Content of the report directory :

6_00030_0000001_4242_evaluate_model.log

6_00030_0000001_4242_extract_data.log

6_00030_0000001_4242_extract_UG_EST.log

6_00030_0000001_4242_generate_tables.log

6_00030_0000001_4242_prepare_data.log

6_00030_0000001_4242_readconfig.log

gc.gplot

gc.dat

The first six files represent the data obtained from the five scripts used to build the matrices and evaluate them, and the last two, gc.gplot and gc.dat, contain the information to create the histogram for the GC content.

To create the graph that shows the GC content use the gnuplot program (see section 10.2.2).

10.2.2. The files ending with “gplot”

The creation of the graphs is possible with gnuplot [20]. The files ending with “.gplot” contain the information necessary to draw the graph.

Write **gnuplot** in the terminal. At the end of the text which appears, just after “gnuplot >” write:

```
load “filename.gplot”           # Here, filename replaces the name of the file you want to visualize.
```

Saving the graph is possible using some commands in gnuplot, write:

```
> set terminal png

> set output “/tmp/gc.png”

> replot

> set output

> quit
```

10.2.3. The file ending with “.R”

The file ending with “ .R ” contains the information to draw a pie chart representing the percentage of start or stop sites that have been found with a specific accuracy. It draws the results stored in the evaluate file of the report directory.

To obtain these charts, type into the terminal:

```
Cat 6_00030_0000001_4242_piecharts.R ¦ R -- no-save
```

Then to see the graph, write:

```
gv  6_00030_0000001_4242_piecharts.R.
```

11. Conclusion

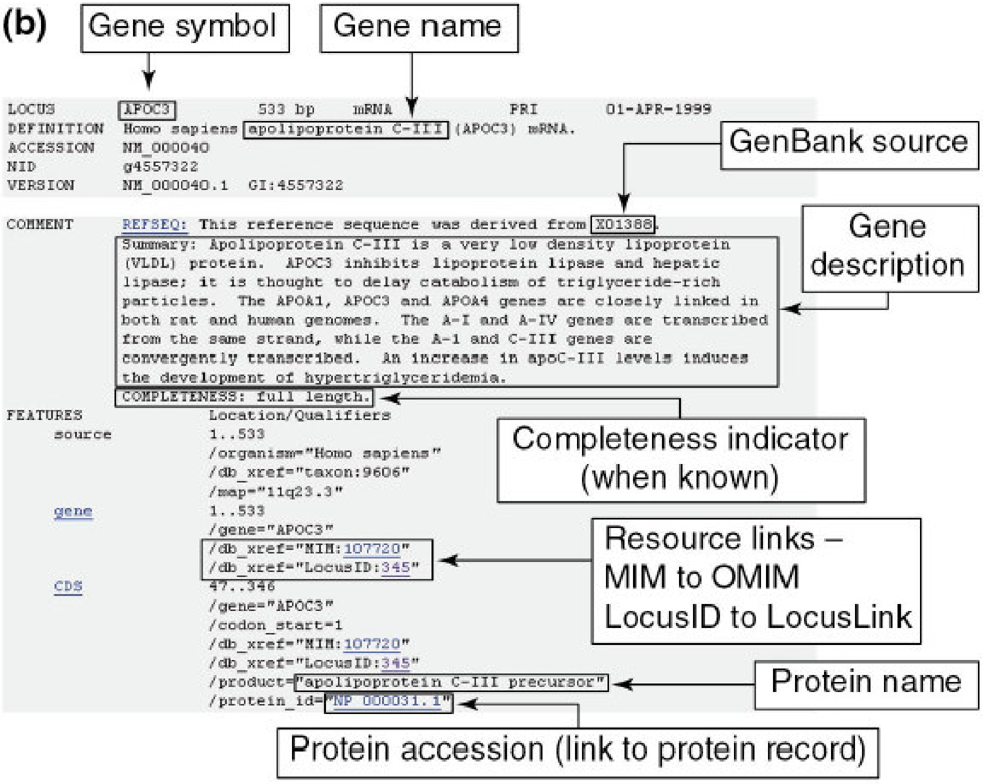
The creation of the matrices is one of the first steps if you want to use ESTScan. This step is really important since it determines all the results that will be obtained. Some parameters should be optimized in order to develop reliable matrices.

Once the matrices are available for analysis, it is necessary to have a look at the sensitivity and specificity of ESTScan using these matrices. These values reflect the capacity of ESTScan to correctly classify sequences into the two groups: coding and non-coding. Thus these values allow the results that will be obtained with ESTScan to be interpreted.

When we are able to use the matrices and understand their power, ESTScan can be launched on the ESTs that must be analyzed. This type of analysis enables a better utilization of the information contained in the ESTs. For example it allows the assessment of cDNA libraries that are subject to contamination with genomic contaminants (non-coding). It can also lead to exon detection and gene discovery.

12. Appendix

12.1. RefSeq format



```
LOCUS       APOC3         533 bp    mRNA            PRI       01-APR-1999
DEFINITION  Homo sapiens apolipoprotein C-III (APOC3) mRNA.
ACCESSION   NM_000040
NID         g4557322
VERSION     NM_000040.1  GI:4557322

COMMENT     REFSEQ: This reference sequence was derived from X01388.
            Summary: Apolipoprotein C-III is a very low density lipoprotein
            (VLDL) protein.  APOC3 inhibits lipoprotein lipase and hepatic
            lipase; it is thought to delay catabolism of triglyceride-rich
            particles.  The APOA1, APOC3 and APOA4 genes are closely linked in
            both rat and human genomes.  The A-I and A-IV genes are transcribed
            from the same strand, while the A-1 and C-III genes are
            convergently transcribed.  An increase in apoC-III levels induces
            the development of hypertriglyceridemia.
            COMPLETENESS: full length.
FEATURES             Location/Qualifiers
     source          1..533
                     /organism="Homo sapiens"
                     /db_xref="taxon:9606"
                     /map="11q23.3"
     gene            1..533
                     /gene="APOC3"
                     /db_xref="MIM:107720"
                     /db_xref="LocusID:345"
     CDS             47..346
                     /gene="APOC3"
                     /codon_start=1
                     /db_xref="MIM:107720"
                     /db_xref="LocusID:345"
                     /product="apolipoprotein C-III precursor"
                     /protein_id="NP_000031.1"
```

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(b) Excerpt of a reviewed RefSeq nucleotide record. Note some of the revisions in annotation: the official gene symbol and gene name defined by the Human Gene Nomenclature Committee; the primary source of this sequence, links to OMIM and LocusLink, the brief gene description (Summary), and the link to the RefSeq protein record [11].

12.2. EMBL format

```
ID   X56734; SV 1; linear; mRNA; STD; PLN; 1859 BP.
XX
AC   X56734; S46826;
XX
DT   12-SEP-1991 (Rel. 29, Created)
DT   25-NOV-2005 (Rel. 85, Last updated, Version 11)
XX
DE   Trifolium repens mRNA for non-cyanogenic beta-glucosidase
XX
KW   beta-glucosidase.
XX
OS   Trifolium repens (white clover)
OC   Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
OC   Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; rosids;
OC   eurosids I; Fabales; Fabaceae; Papilionoideae; Trifolieae; Trifolium.
XX
RN   [5]
RP   1-1859
RX   PUBMED; 1907511.
RA   Oxtoby E., Dunn M.A., Pancoro A., Hughes M.A.;
RT   "Nucleotide and derived amino acid sequence of the cyanogenic
RT   beta-glucosidase (linamarase) from white clover (Trifolium repens L.)";
RL   Plant Mol. Biol. 17(2):209-219(1991).
XX
RN   [6]
RP   1-1859
RA   Hughes M.A.;
RT   ;
RL   Submitted (19-NOV-1990) to the EMBL/GenBank/DDBJ databases.
RL   Hughes M.A., University of Newcastle Upon Tyne, Medical School, Newcastle
RL   Upon Tyne, NE2 4HH, UK
XX
FH   Key             Location/Qualifiers
FH
FT   source          1..1859
FT                   /organism="Trifolium repens"
FT                   /mol_type="mRNA"
FT                   /clone_lib="lambda gt10"
FT                   /clone="TRE361"
FT                   /tissue_type="leaves"
FT                   /db_xref="taxon:3899"
FT   CDS             14..1495
FT                   /product="beta-glucosidase"
FT                   /EC_number="3.2.1.21"
FT                   /note="non-cyanogenic"
FT                   /db_xref="GOA:P26204"
FT                   /db_xref="InterPro:IPR001360"
FT                   /db_xref="UniProtKB/Swiss-Prot:P26204"
FT                   /protein_id="CAA40058.1"
FT                   /translation="MDFIVAIFALFVISSFTITSTNAVEASTLLDIGNLSRSSFPRGFI
FT                   FGAGSSAYQFEGAVNEGGRGPSIWDTFTHKYPEKIRDGSNADITVDQYHRYKEDVGIMK
FT                   DQNMDSYRFSISWPRILPKGKLSGGINHEGIKYYNNLINELLANGIQPFVTLFHWDLPQ
FT                   VLEDEYGGFLNSGVINDFRDYTDLCFKEFGDRVRYWSTLNEPWVFSNSGYALGTNAPGR
FT                   CSASNVAKPGDSGTGPYIVTHNQILAHAEAVHVYKTKYQAYQKGKIGITLVSNWLMPLD
FT                    ..."
FT   mRNA            1..1859
FT                   /experiment="experimental evidence, no additional details
FT                   recorded"
XX
SQ   Sequence 1859 BP; 609 A; 314 C; 355 G; 581 T; 0 other;
     aaacaaacca aatatggatt ttattgtagc catatttgct ctgtttgtta ttagctcatt        60
     cacaattact tccacaaatg cagttgaagc ttctactctt cttgacatag gtaacctgag       120
     tcggagcagt tttcctcgtg gcttcatctt tggtgctgga …                           180

//
```

12.3. UniGene Format

ID Mm.1

TITLE S100 calcium binding protein A10 (calpactin)

GENE S100a10

CYTOBAND 3 F1-F2|3 41.7 cM

GENE_ID 20194

LOCUSLINK 20194

EXPRESS whole body; gastrointestinal tract; mixed; prostate; embryonic tissue; spleen; urinary; thymus; lymph node; mammary gland; muscle; whole brain; endocrine; pancreas; uncharacterized tissue; bone marrow; female genital; head and neck; extraembryonic tissue; eye; blood; brain; heart; liver; testis; limb; adipose tissue; lung; sympathetic ganglion; connective tissue; dorsal root ganglion;

skin; inner ear

CHROMOSOME 3

STS ACC=RH125510 UNISTS=162328

STS ACC=M16465 UNISTS=178878

STS ACC=RH124908 UNISTS=161730

STS ACC=RH128467 UNISTS=211775

STS ACC=S100a10 UNISTS=465493

PROTSIM ORG=Homo sapiens; PROTGI=107251; PROTID=pir:JC1139; PCT=91; ALN=97

PROTSIM ORG=Mus musculus; PROTGI=116487; PROTID=sp:P08207; PCT=100; ALN=97

PROTSIM ORG=Rattus norvegicus; PROTGI=116489; PROTID=sp:P05943; PCT=94; ALN=94

SCOUNT 340

SEQUENCE ACC=CA461262.1; NID=g24917614; CLONE=IMAGE:6754724; END=5'; LID=12110; MGC=6677832; SEQTYPE=EST; TRACE=158140953

SEQUENCE ACC=CB575716.1; NID=g29495246; CLONE=IMAGE:30295364; END=5'; LID=12733; SEQTYPE=EST; TRACE=196933136

SEQUENCE ACC=CB566164.1; NID=g29485694; CLONE=IMAGE:30294362; END=5'; LID=12615; SEQTYPE=EST; TRACE=196939979

SEQUENCE ACC=DV053483.1; NID=g76380766; CLONE=DLP01_06_N20; LID=18145; SEQTYPE=EST

SEQUENCE ACC=DV060885.1; NID=g76388183; CLONE=NEONATAL_04_M20; LID=18147; SEQTYPE=EST

SEQUENCE ACC=DV064402.1; NID=g76391700; CLONE=NEONATAL_22_E09; LID=18147; SEQTYPE=EST

SEQUENCE ACC=DV066639.1; NID=g76393937; CLONE=UGS01_04_L15; LID=18148; SEQTYPE=EST

SEQUENCE ACC=DV055636.1; NID=g76382938; CLONE=DLP01_15_A03; LID=18145; SEQTYPE=EST

//

12.4. Example of ug.data file:

```
ID          Hs.100043

TITLE       Coiled-coil domain containing 124

GENE        CCDC124

CYTOBAND    19p13.11

GENE_ID     115098

LOCUSLINK   115098

HOMOL       YES

EXPRESS     brain; lung; skin; colon; eye; mixed; whole body; placenta; embryonic tissue; connective tissue; larynx; uncharacterized tissue; bone; whole brain;

pharynx; salivary gland; lymph; muscle; uterus; spleen; ovary; blood; liver; parathyroid; heart; testis; mammary gland; lymph node; kidney; prostate; mouth; pancreas; cervix

CHROMOSOME  19

STS         ACC=RH93753 UNISTS=84827

STS         ACC=RH46130 UNISTS=88276

PROTSIM     ORG=Arabidopsis thaliana; PROTGI=18394335; PROTID=ref:NP_563993.1; PCT=33.04; ALN=216

PROTSIM     ORG=Caenorhabditis elegans; PROTGI=17510611; PROTID=ref:NP_490873.1; PCT=50.43; ALN=221

PROTSIM     ORG=Homo sapiens; PROTGI=1070603; PROTID=pir:CGHU7L; PCT=29.64; ALN=263

PROTSIM     ORG=Mus musculus; PROTGI=5921190; PROTID=sp:P08121; PCT=28.37; ALN=263

SCOUNT      261

SEQUENCE    ACC=BM554853.1; NID=g18794811; CLONE=IMAGE:5468925; END=5'; LID=8775; MGC=34147541; SEQTYPE=EST; TRACE=115579612

SEQUENCE    ACC=BU539378.1; NID=g22849819; CLONE=IMAGE:6570116; END=5'; LID=10554; MGC=34147541; SEQTYPE=EST; TRACE=158255138

SEQUENCE    ACC=BM558432.1; NID=g18801173; CLONE=IMAGE:5476590; END=5'; LID=8775; MGC=34147541; SEQTYPE=EST; TRACE=115580613

SEQUENCE    ACC=BQ883763.1; NID=g22275771; CLONE=IMAGE:6291297; END=5'; LID=7269; MGC=34147541; SEQTYPE=EST; TRACE=142965156
```

estcds.seq

```
>emb|BM912135|BM912135.1 CDS: 12 565  [Homo sapiens]AGENCOURT_6613231 NIH_MGC_41 Homo sapiens cDNA clone IMAGE:5473631 5', mRNA sequence. (first 565 nucleotides)

CCTGCTGAGGGATGCCCAAGAAGTTCCAGGGTGAGAACACCAAGTCGGCAGCGGCCCGGGCACGTAGG
GCAGAGGCCAAGGCGGCCGCTGATGCCAAGAAGCAGAAGGAGCTGGAGGATGCCTACTGGAAGGACGA
CGACAAACACGTCATGAGGAAGGAGCAGCGCAAGGAGGAGAAGGAGAAGCGGCGCCTCGACCAGCTG
GAACGTAAGAAGGAGACGCAGCGCCTACTGGAGGAGGAGGACTCCAAGCTCAAGGGCGGCAAGGCGCC
GCGGGTGGCCACGTCCAGCAAGGTCACCCGGGCCCAGATCGAGGACACGCTGCGCCGAGACCATCAGCT
CAGGGAGGCCCCGGACACAGCCGAGAAAGCCAAGAGCCATCTGGAGGTGCCGCTGGAGGAGAACGTGA
ACCGCCGCGTGCTGGAGGAGGGCAGCGTGGAGGCGCGCACCATCGAGGACGCCATTGCAGTGCTCAGC
GTGGCGGAGGAGGCGGCCGACCCGGCCCCAGAAAGACGCATGCGGGCACCCCTTCCCCGCTTTCAGGAA
CACCATCTGCCGCGGTTCAA
```

12.5. Example of cluster.lst file

```
rs:NM_138442 : embl:BM554853 embl:BU539378 embl:BM558432 embl:BQ883763
embl:BM912135 embl:BQ878175 embl:BM554613 embl:BM810865 embl:BM913379
embl:BE733510 embl:BM551615 embl:BQ071416 embl:BQ052057 embl:BM558385
embl:BQ889758 embl:BM811068 embl:BM915137 embl:BM914662 embl:BM915698
```

12.6. Example of matches.lst file

```
rs:NM_138442 108 779 embl:BM913379 97 948 11 31
rs:NM_138442 108 779 embl:BE733510 191 947 1 10
rs:NM_138442 108 779 embl:BQ071416 97 853 4 25
rs:NM_138442 108 779 embl:BQ052057 98 975 21 42
rs:NM_138442 108 779 embl:BM558385 97 1039 12 31
rs:NM_138442 108 779 embl:BQ889758 97 723 4 17
rs:NM_138442 108 779 embl:BM811068 97 773 24 33
rs:NM_138442 108 779 embl:BM915137 97 993 22 38
rs:NM_138442 108 779 embl:BM914662 97 1026 11 34
rs:NM_138442 108 779 embl:BM915698 97 985 22 36
rs:NM_138442 108 779 embl:BQ062920 97 975 17 39
```

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