ViennaRNA Tutorial

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Software ViennaRNA Tutorial

A short Tutorial on RNA Bioinformatics
The ViennaRNA Package and related Programs

http://www.tbi.univie.ac.at/RNA/

This tutorial aims to give a basic introduction to using the programs in the ViennaRNA Package.

Get started

What's in the `ViennaRNA Package`

What's in the ViennaRNA Package

The core of the `ViennaRNA Package` is formed by a collection of routines for the prediction and comparison of RNA secondary structures. These routines can be accessed through stand-alone programs, such as `RNAfold`, `RNAdistance` etc., which should be sufficient for most users. For those who wish to develop their own programs a library which can be linked to your own code is provided.

Which programs are available?

- `RNA2Dfold` Compute coarse grained energy landscape of representative sample structures
- `RNAaliduplex` Predict conserved RNA-RNA interactions between two alignments
- `RNAalifold` Calculate secondary structures for a set of aligned RNA sequences
- `RNAcofold` Calculate secondary structures of two RNAs with dimerization
- `RNAdistance` Calculate distances between RNA secondary structures
- `RNAduplex` Compute the structure upon hybridization of two RNA strands
- `RNAeval` Evaluate free energy of RNA sequences with given secondary structure
- `RNAfold` Calculate minimum free energy secondary structures and partition function of RNAs
- `RNAheat` Calculate the specific heat (melting curve) of an RNA sequence
- `RNAinverse` Find RNA sequences with given secondary structure (sequence design)
- `RNALalifold` Calculate locally stable secondary structures for a set of aligned RNAs
- `RNALfold` Calculate locally stable secondary structures of long RNAs

- `RNApaln` RNA alignment based on sequence base pairing propensities
- `RNApdist` Calculate distances between thermodynamic RNA secondary structures ensembles
- `RNAparconv` Convert energy parameter files from ViennaRNA 1.8 to 2 format
- `RNAPKplex` Predict RNA secondary structures including pseudoknots
- `RNAplex` Find targets of a query RNA
- `RNAplfold` Calculate average pair probabilities for locally stable secondary structures
- `RNAplot` Draw and markup RNA secondary structures in PostScript, SVG, or GML
- `RNApvmin` Find a vector of perturbation energies which may further be used to constrain folding
- `RNAsnoop` Find targets of a query H/ACA snoRNA
- `RNAsubopt` Calculate suboptimal secondary structures of RNAs
- `RNAup` Calculate the thermodynamics of RNA-RNA interactions
- `Kinfold` simulates the stochastic folding kinetics of RNA sequences into secondary structures
- `RNAforester` compare RNA secondary structures via forest alignment

The Input File Format

The Input File Format

RNA sequences come in a variety of formats. The sequence format used throughout the ViennaRNA Package is very simple. A sequence file contains one or more sequences. Each sequence must appear as a single line in the file without embedded white spaces. A sequence may be preceded by a special line starting with the '>' character followed by a sequence name. This name will be used by the programs in the `ViennaRNA Package` as basename for the PostScript output files for this sequence. Note that this is almost the `fasta` sequence format, except that no line-breaks are allowed within a sequence while the header line is optional. Following programs provide full fasta support: `RNAfold, RNAsubopt, RNAcofold, RNAKplex, RNALfold, RNAplfold, RNAeval, RNAplot, RNAheat`

Structure Prediction on single Sequences

Structure Prediction on single Sequences

The Program `RNAfold`

The Program RNAfold

Our first task will be to do a structure prediction using `RNAfold`. This should get you familiar with the input and output format as well as the graphical output produced. `RNAfold` reads RNA sequences from *stdin*, calculates their minimum free energy (`MFE`) structure, prints the `MFE` structure in dot-bracket notation and its free energy to *stdout*. If the `-p` option is set it also computes the partition function, the base pairing probability matrix and additionally prints the free energy of the thermodynamic ensemble, the frequency of the `MFE` structure in the ensemble and the ensemble diversity to *stdout*. Another useful option is the `--MEA` option, which also shows the maximum expected accuracy, but remember that this also needs more CPU time than without `--MEA`.

MFE structure of a single sequence
1. Use a text editor (emacs, vi, nano, gedit) to prepare an input file by pasting the text below and save it under the name `test.seq` in your `Data` folder.

```
``` {.commands}
> test
CUACGGCGCGGCGCCCUUGGCGA
```

</div>
```

2. Compute the best (MFE) structure for this sequence

```
$ RNAfold < test.seq
CUACGGCGCGGCGCCCUUGGCGA
...........((((...)))). ( -5.00)
```

The last line of the text output contains the predicted MFE structure as dot-bracket notation and its free energy in `kcal/mol`. A dot in the dot-bracket notation represents an unpaired position, while a base pair (i, j) is represented by a pair of matching parentheses at position i and j.

`RNAfold` created a file named `test_ss.eps`. The filename is taken from the fasta header; if there's no header the output is simply called `rna.eps`.
Let's take a look at the output file with `gv`, a PostScript[^2^](https://www.tbi.univie.ac.at/RNA/tutorial/tutorial3
viewer. The & at the end starts the program in the background.

```
$ gv test_ss.ps &
```

Compare the dot-bracket notation to the PostScript drawing shown in the file `test_ss.eps`.

The calculation above does not tell us whether the predicted structure is the only possibility or not, so let's look at the equilibrium ensemble instead.

Predicting equilibrium pair probabilities
1. Run `RNAfold -p --MEA` to compute the partition function and pair probabilities as well as the maximum expected accurarcy. 2. Look at the generated PostScript files `test_ss.eps` and `test_dp.eps`

```
``` {.commands}
$ RNAfold -p --MEA < test.seq
  CUACGGCGCGGCGCCCUUGGCGA
  ...........((((...)))). ( -5.00)
  ....{,{{...||||...)}}}. [ -5.72]
  ........................ {  0.00 d=4.66}
  ......((...))((...))... {  2.90 MEA=14.79}
  frequency of mfe structure in ensemble 0.311796; ensemble diversity 6.36
```

</div>
```

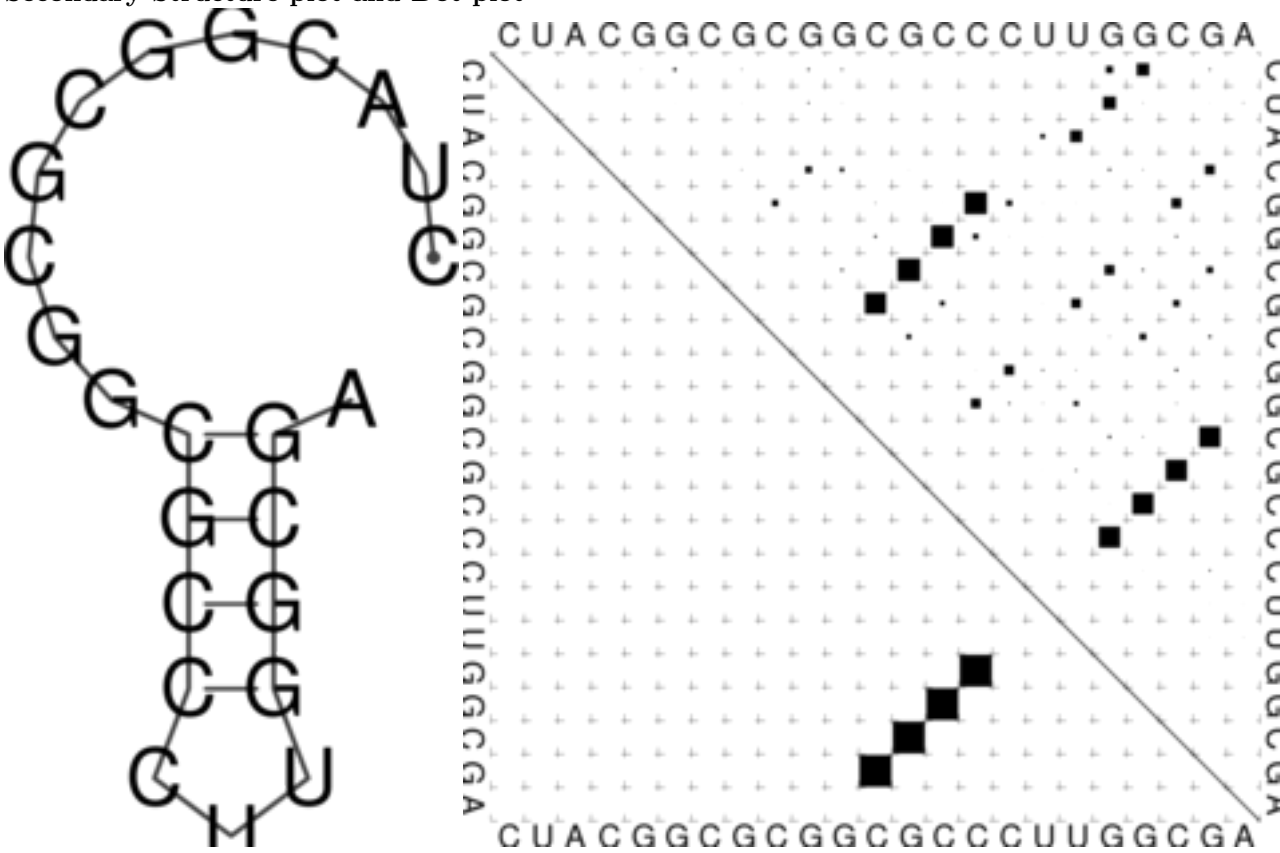
Here the last four lines are new compared to the text output without the `-p --MEA` options. The partition function is a rough measure for the well-definedness of the `MFE` structure. The third line shows a condensed representation of the pair probabilities of each nucleotide, similar to the dot-bracket notation, followed by the ensemble free energy (-kT ln(Z)) in `kcal/mol`. The next two lines represent the centroid structure with its free energy, its distance to the ensemble and the MEA. The last line shows the frequency of the MFE structure in the ensemble of secondary structures and the diversity of the ensemble.

"." denotes bases that are essentially unpaired, "," weakly paired, "|"strongly paired without preference, "{},()" weakly (>33%) upstream (downstream) paired or strongly (>66%) up-/downstream paired bases, respectively.

Note that the MFE structure is adopted only with 31% probability, also the diversity is very high for such a short sequence.
For rotating the secondary structure plot there is a usefull tool called `rotate_ss.pl` included in the ViennaRNA Package. Just read the `perldoc` for this tool to know how to handle the rotation and use the information to get your secondary structure in a vertical position.

```
$ perldoc rotate_ss.pl
```

Secondary Structure plot and Dot plot

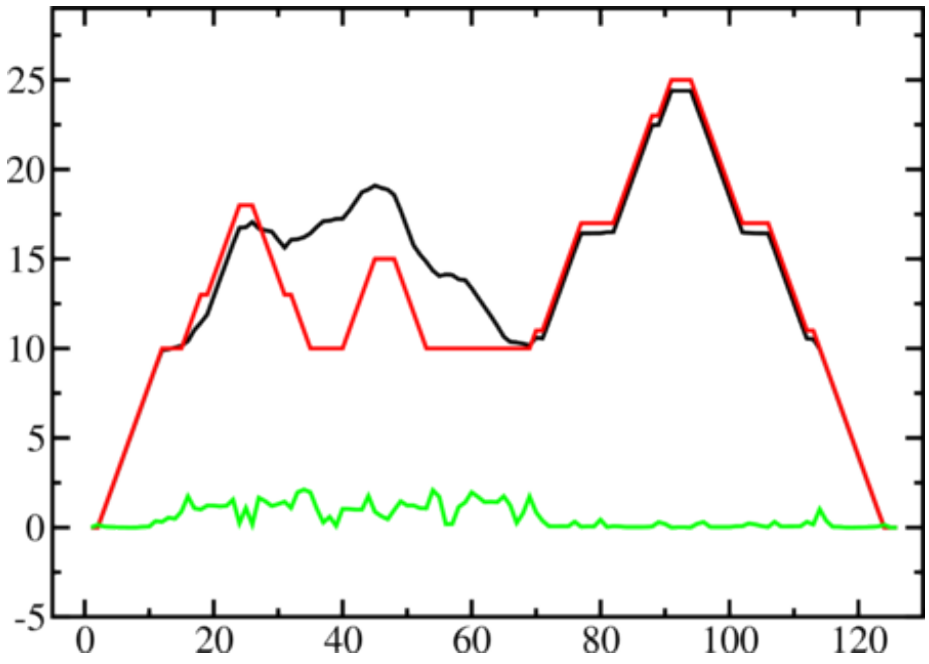


The "dot plot" in `test_dp.eps` shows the pair probabilities within the equilibrium ensemble as $n \times n$ matrix, and is an excellent way to visualize structural alternatives. A square at row i and column j indicates a base pair. The area of a square in the upper right half of the matrix is proportional to the probability of the base pair (i, j) within the equilibrium ensemble. The lower left half shows all pairs belonging to the `MFE` structure. While the MFE consists of a single helix, several different helices are visualized in the pair probabilities.
Next, let's use the `relplot` utility to visualize which parts of a predicted MFE are well-defined and thus more reliable. Also let's use a real example for a

change and produce yet another representation of the predicted structure, the *mountain plot*.

Mountain and Reliability plot
Fold the 5S rRNA sequence and visualize the structure. (The `5S.seq` is shipped with the tutorial)

```
$ RNAfold -p < 5S.seq
$ mountain.pl 5S_dp.ps | xmgrace -pipe
$ relplot.pl 5S_ss.ps 5S_dp.ps > 5S_rss.ps
```



A mountain plot is especially useful for long sequences where conventional structure drawings become terribly cluttered. It is a xy-diagram plotting the number of base pairs enclosing a sequence position *versus* the position. The `Perl` script `mountain.pl` transforms a dot plot into the mountain plot coordinates which can be visualized with any xy-plotting program, e.g. `xmgrace`.

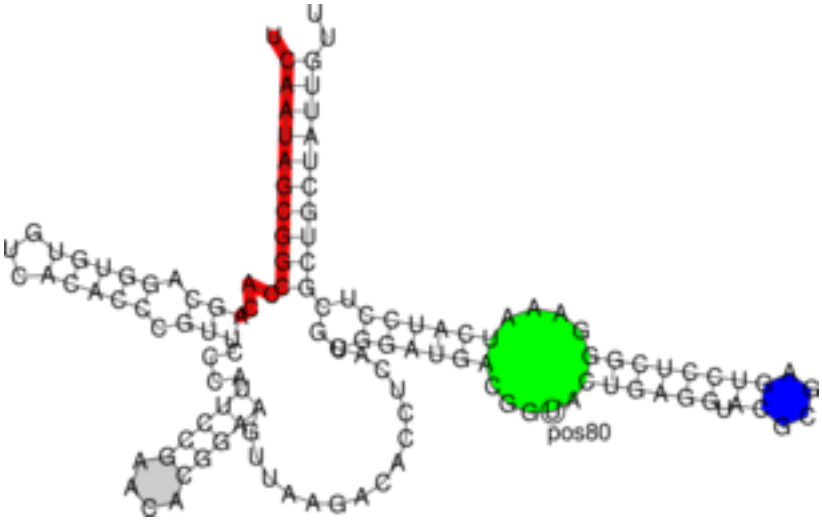
The resulting plot shows three curves, two mountain plots derived from the `MFE` structure (red) and the pairing probabilities (black) and a positional entropy curve (green). Well-defined regions are identified by low entropy. By superimposing several mountain plots structures can easily be compared.
The perl script `relplot.pl` adds reliability information to a RNA secondary structure plot in the form of color annotation. The script computes a well-definedness measure we call "positional entropy" ($S(i) = -\sum p_{ij} \log(p_{ij})$ for those who want to know the details) and encodes it as color hue, ranging from red (low entropy, well-defined) via green to blue and violet (high entropy, ill-defined). In the example above two helices of the 5S RNA are well-defined (red) and indeed predicted correctly, the left arm is not quite correct and disordered.
For the figure above we had to rotate and mirror the structure plot, e.g.

```
$ rotate_ss.pl -a 180 -m 5S_rss.ps > 5S_rot.ps
```

You can manually add annotation to structure drawings using the RNAplot program (for information see the `man` page). Here's a somewhat complicated example:

```
$ RNAfold < 5S.seq > 5S.fold
$ RNAplot --pre "76 107 82 102 GREEN BFmark 44 49 0.8 0.8 0.8 Fomark \
```

```
   1 15 8 RED omark 80 cmark 80 -0.23 -1.2 (pos80) Label 90 95 BLUE Fomark" < 5S.fold
$ gv 5S_ss.ps
```



`RNAplot` is a very useful tool to color plots. The `--pre` tag adds PostScript code to color distinct regions of your molecule. There are some predefined statements with different options for annotations listed below:

`i cmark` draws circle around base i `i j c gmark` draw basepair i,j with c counter examples in grey `i j lw rgb omark` stroke segment i...j with linewidth lw and color (rgb) `i j rgb Fomark` fill segment i...j with color (rgb) `i j k l rgb BFmark` fill block between pairs i,j and k,l with color (rgb) `i dx dy (text) Label` adds a textlabel with an offset dx and dy relative to base i

Predefined color options are `BLACK, RED, GREEN, BLUE, WHITE` but you can also replace the value to some standard RGB code (e.g. 0 5 8 for lightblue).
To see what exactly the alternative structures of our sequence are, we need to predict *suboptimal* structures.

SHAPE directed RNA folding
In order to further improve the quality of secondary structure predictions, mapping experiments like SHAPE (selective 2'-hydroxyl acylation analyzed by primer extension) can be used to exerimentally determine the pairing status for each nucleotide. In addition to thermodynamic based secondary structure predictions, RNAfold supports the incorporation of this additional experimental data as soft constraints.

If you want to use SHAPE data to guide the folding process, please make sure that your experimental data is present in a text file, where each line stores three white space separated columns containing the position, the abbreviation and the normalized SHAPE reactivity for a certain nucleotide.

```
1 G 0.134
2 C 0.044
3 C 0.057
4 G 0.114
5 U 0.094
  ...
  ...
  ...
71 C 0.035
72 G 0.909
73 C 0.224
74 C 0.529
75 A 1.475
```

The second column, which holds the nucleotide abbreviation, is optional. If it is present, the data will be used to perform a cross check against the provided input sequence. Missing SHAPE reactivities for certain positions can be indicated by omitting the reactivity column or the whole line. Negative reactivities will be treated as missing. Once the SHAPE file is ready, it can be used to constrain folding:

```
$ RNAfold --shape=rna.shape --shapeMethod=D < rna.seq
```

The Program `RNApvmin`

The Program RNApvmin

The program `RNApvmin` reads a RNA sequence from *stdin* and uses an iterative minimization process to calculate a perturbation vector that minimizes the discripancies between predicted pairing probabilites and observed pairing probabilities (deduced from given shape reactivities). The experimental SHAPE data has to be present in the file format described above. The application will write the calculated vector of perturbation energies to *stdout*, while the progress of the minimization process is written to *stderr*. The resulting perturbation vector can be interpreted directly and gives usefull insights into the discrepancies between thermodynamic prediction and experimentally determined pairing status. In addition the perturbation energies can be used to constrain folding with `RNAfold`:

```
$ RNApvmin rna.shape < rna.seq >vector.csv
$ RNAfold --shape=vector.csv --shapeMethod=W < rna.seq
```

The perturbation vector file uses the same file format as the SHAPE data file. Instead of SHAPE reactivities the raw perturbation energies will be storred in the last column. Since the energy model is only adjusted when necessary, the calculated perturbation energies may be used for the interpretation of the

secondary structure prediction, since they indicate which positions require major energy model adjustments in order to yield a prediction result close to the experimental data. High perturbation energies for just a few nucleotides may indicate the occurrence of features, which are not explicitly handled by the energy model, such as posttranscriptional modifications and intermolecular interactions.

The Program `RNAsubopt`

The Program RNAsubopt

`RNAsubopt` calculates all suboptimal secondary structures within a given energy range above the `MFE` structure. Be careful, the number of structures returned grows exponentially with both sequence length and energy range.

Suboptimal folding
- Generate all suboptimal structures within a certain energy range from the `MFE` specified by the `-e` option.

```
``` {.commands}
$ RNAsubopt -e 1 -s < test.seq
CUACGGCGCGGCGCCCUUGGCGA   -500    100
...........((((...)))).  -5.00
....((((...))))........  -4.80
(((.((((...))))..)))...  -4.20
...((.((.((...)).)).)).  -4.10
```
```

The text output shows an energy sorted list (option `-s`) of all secondary structures within 1 kcal/mol of the `MFE` structure. Our sequence actually has a ground state structure (-5.70) and three structures within 1 kcal/mol range.

`MFE` folding alone gives no indication that there are actually a number of plausible structures. Remember that `RNAsubopt` cannot automatically plot structures, therefore you can use the tool `RNAplot`. Note that you CANNOT simply pipe the output of `RNAsubopt` to `RNAplot` using

```
$ RNAsubopt < test.seq | RNAplot
```

You need to manually create a file for each structure you want to plot. Here, for example we created a new file named suboptstructure.txt:

```
> suboptstructure-4.20
CUACGGCGCGGCGCCCUUGGCGA
(((.((((...))))..)))...
```

The fasta header is optional, but useful (without it the outputfile will be named rna.ps). The next two lines contain the sequence and the suboptimal structure you want to plot; in this case we plotted the structure with the folding energy of -4.20. Then plot it with

```
$ RNAplot < suboptstructure.txt
```

Note that the number of suboptimal structures grows exponentially with sequence length and therefore this approach is only tractable for sequences with less than 100 nt. To keep the number of suboptimal structures manageable the option `--noLP` can be used, forcing `RNAsubopt` to produce only structures without isolated base pairs. While `RNAsubopt` produces *all* structures within an energy range, `mfold` produces only a few, hopefully representative, structures. Try folding the sequence on the mfold server at
http://mfold.rna.albany.edu/?q=mfold.
Sometimes you want to get information about unusual properties of the Boltzmann ensemble (the sum of all RNA structures possible) for which no specialized program exists. For example you want to know all fractions of a bacterial mRNA in the Boltzmann ensemble where the Shine-Dalgarno (SD) sequence is unpaired. If the SD sequence is concealed by secondary structure the translation efficiency is reduced.

In such cases you can resort to drawing a representative sample of structures from the Boltzmann ensemble by using the option `-p`. Now you can simply count how many structures in the sample possess the feature you are looking for. This number divided by the size of your sample gives you the desired fraction.
The following example calculates the fraction of structures in the ensemble that have bases 6 to 8 unpaired.

Sampling the Boltzmann Ensemble
1. Draw a sample of size 10,000 from the Boltzmann ensemble 2. Calculate the desired property by using a perl script

```
$ RNAsubopt -p 10000 < test.seq > tt
$ perl -nle '$h++ if substr($_,5,3) eq "...";
     END {print $h/$.}' tt
     0.391960803919608
```

A far better way to calculate this property is to use `RNAfold -p` to get the ensemble free energy, which is related to the partition function via $F = -RT \ln(Q)$, for the unconstrained (F_u) and the constrained case (F_c), where the three bases are not allowed to form base pairs (use option `-C`), and evaluate $p_c = \exp\left(\left(F_u - F_c\right)/RT\right)$ to get the desired probability.
So let's do the calculation using `RNAfold`.

```
$RNAfold -p
```

```
Input string (upper or lower case); @ to quit
....,....1....,....2....,....3....,....4....,....5....,....6....,....7....,....8
CUACGGCGCGGCGCCCUUGGCGA
length = 23
CUACGGCGCGGCGCCCUUGGCGA
...........((((...)))).
 minimum free energy =  -5.00 kcal/mol
....{,{{...||||...)}}}.
 free energy of ensemble =  -5.72 kcal/mol
....................... {  0.00 d=4.66}
 frequency of mfe structure in ensemble 0.311796; ensemble diversity 6.36
```

Now we have calculated the free ensemble energy of the ensemble over all structures (F_u), in the next step we have to calculate it for the structures using a constraint(F_c).
Following notation has to be used for defining the constraint:

1. | : paired with another base
2. . : no constraint at all
3. x : base must not pair
4. < : base i is paired with a base j¡i
5. > : base i is paired with a base j¿i
6. matching brackets ( ): base i pairs base j

So our constraint should look like this:

```
.....xxx.............
```

Next call the application with following command and provide the sequence and constraint we just created.

```
$ RNAfold -p -C
```

The output should look like this

```
length = 23
CUACGGCGCGGCGCCCUUGGCGA
...........((((...)))).
 minimum free energy =  -5.00 kcal/mol
...........((((...)))).
 free energy of ensemble =  -5.14 kcal/mol
...........((((...)))). { -5.00 d=0.42}
 frequency of mfe structure in ensemble 0.792925; ensemble diversity 0.79
```

Afterwards evaluate the desired probability according to the formula given before e.g. with a simple perl script.

```
$ perl -e 'print exp(-(5.72-5.14)/(0.00198*310.15))."\n"'
```

You can see that there is a slight difference between the `RNAsubopt` run with 10,000 samples and the `RNAfold` run including all structures.

RNA folding kinetics

RNA folding kinetics

RNA folding kinetics describes the dynamical process of how a RNA molecule approaches to its unique folded biological active conformation (often referred to as the native state) starting from an initial ensemble of disordered conformations e.g. the unfolded open chain. The key for resolving the dynamical behavior of a folding RNA chain lies in the understanding of the ways in which the molecule explores its astronomically large free energy landscape, a rugged and complex hyper-surface established by all the feasible base pairing patterns a RNA sequence can form. The challenge is to understand how the interplay of formation and break up of base pairing interactions along the RNA chain can lead to an efficient search in the energy landscape which reaches the native state of the molecule on a biologically meaningful time scale.

RNA2Dfold

RNA2Dfold

RNA2Dfold is a tool for computing the MFE structure, partition function and representative sample structures of κ, λ neighborhoods and projects an high dimensional energy landscape of RNA into two dimensions. Therefore a sequence and two user-defined reference structures are expected by the program. For each of the resulting distance class, the MFE representative, the Boltzmann probabilities and the Gibbs free energy is computed. Additionally, representative suboptimal secondary structures from each partition can be calculated.

```
$ RNA2Dfold -p < 2dfold.inp > 2dfold.out
```

The outputfile `2dfold.out` should look like below, check it out using `less`.

```
CGUCAGCUGGGAUGCCAGCCUGCCCCGAAAGGGGCUUGGCGUUUUGGUUGUUGAUUCAACGAUCAC
((((((((((....))))))..(((((....))))).))))).((((((((((...))))))))). (-30.40)
((((((((((....))))))..(((((....))))).))))).((((((((((...))))))))). (-30.40)
............................................................ (  0.00)
```

```
free energy of ensemble = -31.15 kcal/mol
k       l       P(neighborhood) P(MFE in neighborhood)  P(MFE in ensemble)      MFE     E_gi
0       24      0.29435909      1.00000000      0.29435892      -30.40  -30.40  (((((((((..
1       23      0.17076902      0.47069889      0.08038083      -29.60  -30.06  (((((((((..
2       22      0.03575448      0.37731068      0.01349056      -28.50  -29.10  ((((.(((((..
2       24      0.00531223      0.42621709      0.00226416      -27.40  -27.93  (((((((((..
3       21      0.00398349      0.29701636      0.00118316      -27.00  -27.75  .(((.(((((..
3       23      0.00233909      0.26432372      0.00061828      -26.60  -27.42  (((((((((..
[...]
```

For visualizing the output the ViennaRNA Package includes two scripts `2Dlandscape_pf.gri, 2Dlandscape_mfe.gri` located in `VRP/share/ViennaRNA/`. gri (a language for scientific graphics programing) is needed to create a colored postscript plot. We use the partition function script to show the free energies of the distance classes (graph below, left):

```
$ gri ../Progs/VRP/share/ViennaRNA/2Dlandscape_pf.gri 2dfold.out
```

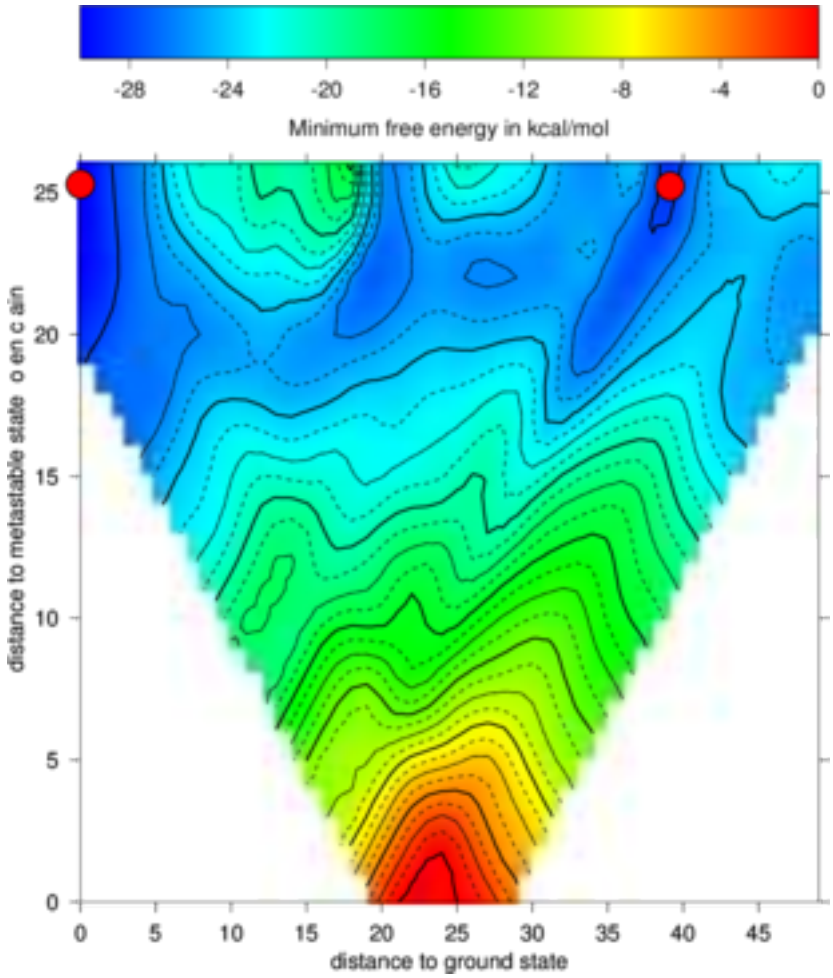
Compare the output file with the colored plot and determine the MFE minima with corresponding distance classes. For easier comparision the outputfile of `RNA2Dfold` can be sorted by a simple sort command. For further information regarding sort use the `--help` option.

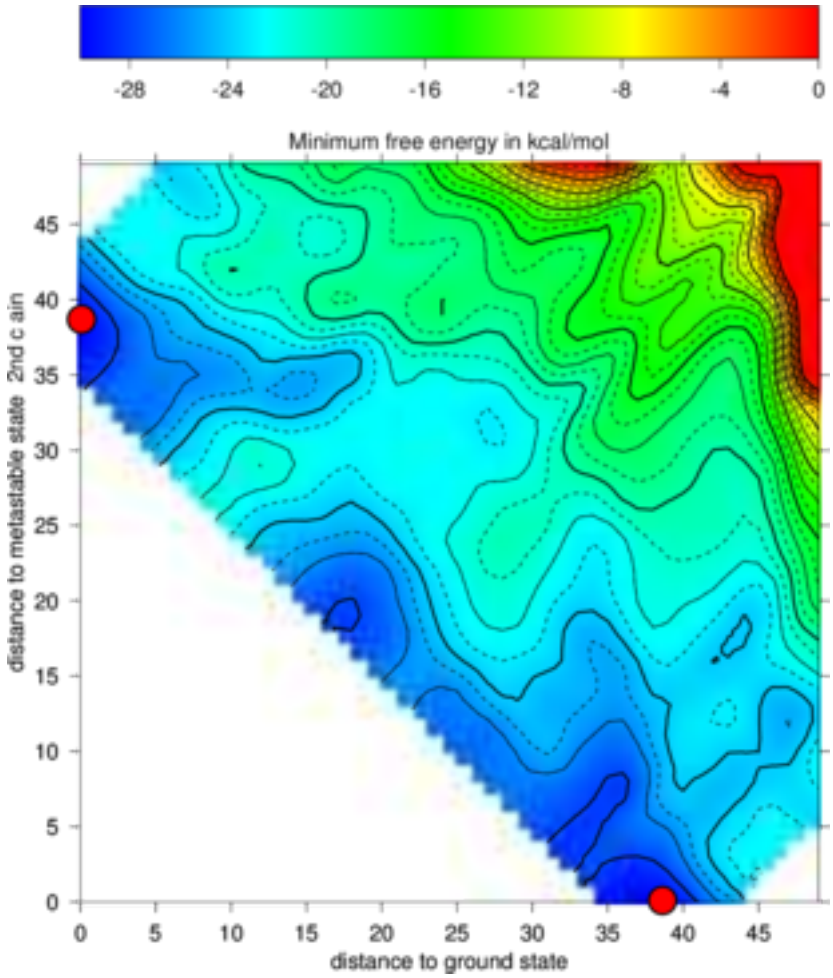
```
$ sort -k6 -n 2dfold.out > sort.out
```

Now we choose the structure with the lowest energy besides our startstructure, replace the open chain structure from our old input with that structure and repeat the steps above with our new values

- run `RNA2Dfold`
- plot it using `2Dlandscape_pf.gri`

The new projection (right graph) shows the two major local minima which are separated by 39 bp (red dots in figure below) and both are likely to be populated with high probability. The landscape gives an estimate of the energy barrier separating the two minima (about -20 kcal/mol).
The red dots mark the distance from open chain to the MFE structure respectively the distance from the 2nd best structure to the MFE. Note that the red dots were manually added to the image afterwards so don't panic if you don't see them in your gri output.


-28
-24
-20
-16
-12
-8
-4
0
Minimum free energy in kcal/mol
25
20
15
10
5
0
distance to metastable state
0
5
10
15
20
25
30
35
40
45
distance to ground state



barriers & treekin

barriers & treekin

The following assumes you have the barriers and treekin programs installed. If not, the current release can be found at http://www.tbi.univie.ac.at/RNA/Barriers/. Installation proceeds as shown for the ViennaRNA Package in section 2.4. One problem that often occurs during treekin installation is the dependency on `blas` and `lapack` packages which is not carefully checked. For further information according to the barriers and treekin program also see the website.

A short recall on howto install/compile a program
1. Get the barriers source from http://www.tbi.univie.ac.at/RNA/Barriers/ 2. extract the archive and go to the directory

```
``` {.commands}
$ tar -xzf Barriers-1.5.2.tar.gz
$ cd Barriers-1.5.2
```

</div>
```

3. use the `--prefix` option to install in your `Progs` directory

   ```
   $ ./configure --prefix=$HOME/Tutorial/Progs/barriers-1.5.2
   ```

4. make install

   ```
   $ make
   $ make install
   ```

Now barriers is ready to use. Apply the same steps to install treekin. `Note:` Copy the barriers and treekin binaries to your `bin` folder or add the path to your `PATH` variable.

Calculate the Barrier Tree

```
$ echo UCCACGGCUGUUAGUGGAUAACGGC | RNAsubopt --noLP -s -e 10 > barseq.sub
$ barriers -G RNA-noLP --bsize --rates < barseq.sub > barseq.bar
```

You can restrict the number of local minima using the `barriers` command-line option `--max` followed by a number. The option `-G RNA-noLP` instructs barriers that the input consists of RNA secondary structures without isolated basepairs. `--bsize` adds size of the gradient basins and `--rates` tells barriers to compute rates between macro states/basins for use with treekin. Another useful options is `--minh` to print only minima with a barrier $> dE$. Look at the output file `less -S barseq.bar`. Use the arrow keys to navigate.

```
  UCCACGGCUGUUAGUGGAUAACGGC
1 (((((........)))))........  -6.90    0  10.00    115      0  -7.354207     23  -7.012023
2 ......(((((((.....)))))))  -6.80    1   9.30     32     58  -6.828221     38  -6.828218
3 (((...(((...)))))).......  -0.80    1   0.90      1     10  -0.800000      9  -1.075516
4 ....((..((((....)))).))..  -0.80    1   2.70      5     37  -0.973593     11  -0.996226
5 ..........................   0.00    1   0.40      1     14  -0.000000     26  -0.612908
6 ......(((....((.....)))))   0.60    2   0.40      1     22   0.600000      3   0.573278
7 ......((((((....)))...)))   1.00    1   1.50      1     95   1.000000      2   0.948187
8 .((....((......))).....)).  1.40    1   0.30      1     30   1.400000      2   1.228342
```

The first row holds the input sequence, the successive list the local minima ascending in energy. The meaning of the first 5 columns is as follows

1. label (number) of the local minima (1=MFE)
2. structure of the minimum
3. free energy of the minimum
4. label of deeper local minimum the current minimum merges with (note that the `MFE` has no deeper local minimum to merge with)
5. height of the energy barrier to the local minimum to merge with
6. numbers of structures in the basin we merge with
7. number of basin which we merge to
8. free energy of the basin
9. number of structures in this basin using gradient walk
10. gradient basin (consisting of all structures where gradientwalk ends in the minimum)

aCalculate The Barrier Tree

`barriers` produced two additional files, the `PostScript` file `tree.eps` which represents the basic information of the `barseq.bar` file visually (look at the file e.g. `gv tree.eps`) and a text file `rates.out` which holds the matrix of transition probabilities between the local minima.

Simulating the Folding Kinetics
The program `treekin` is used to simulate the evolution over time of the population densities of local minima starting from an initial population density distribution $p0$ (given on the command-line) and the transition rate matrix in the file `rates.out`.

```
$ treekin -m I --p0 5=1 < barseq.bar | xmgrace -log x -nxy -
```

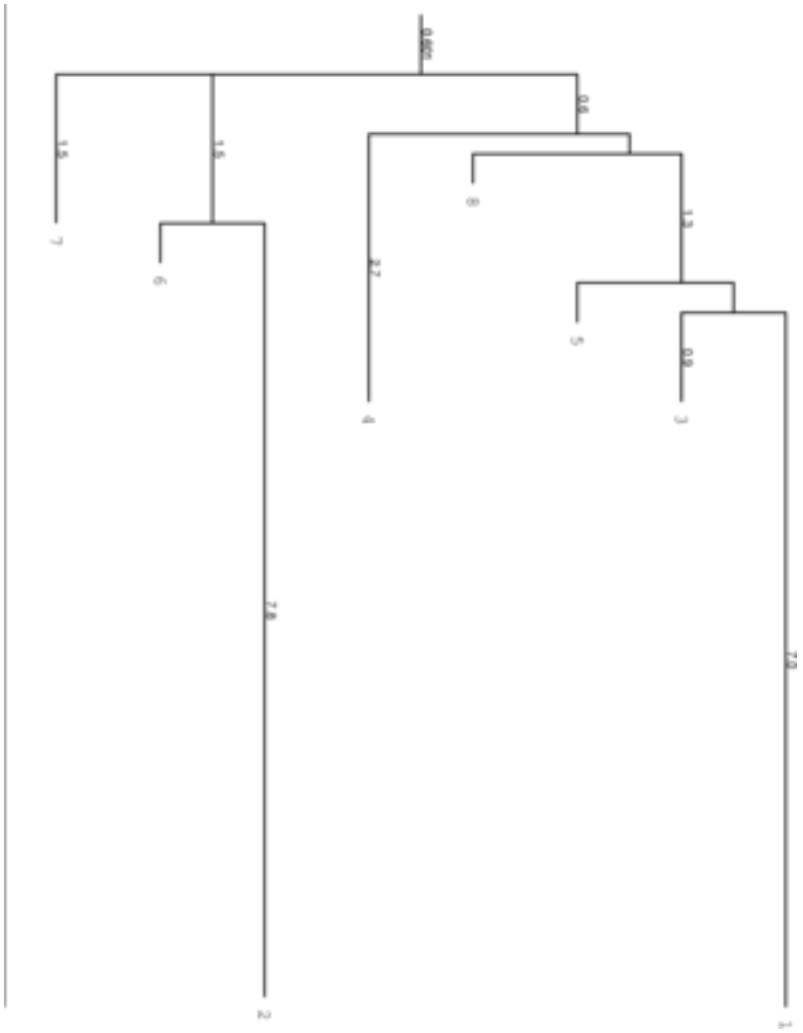
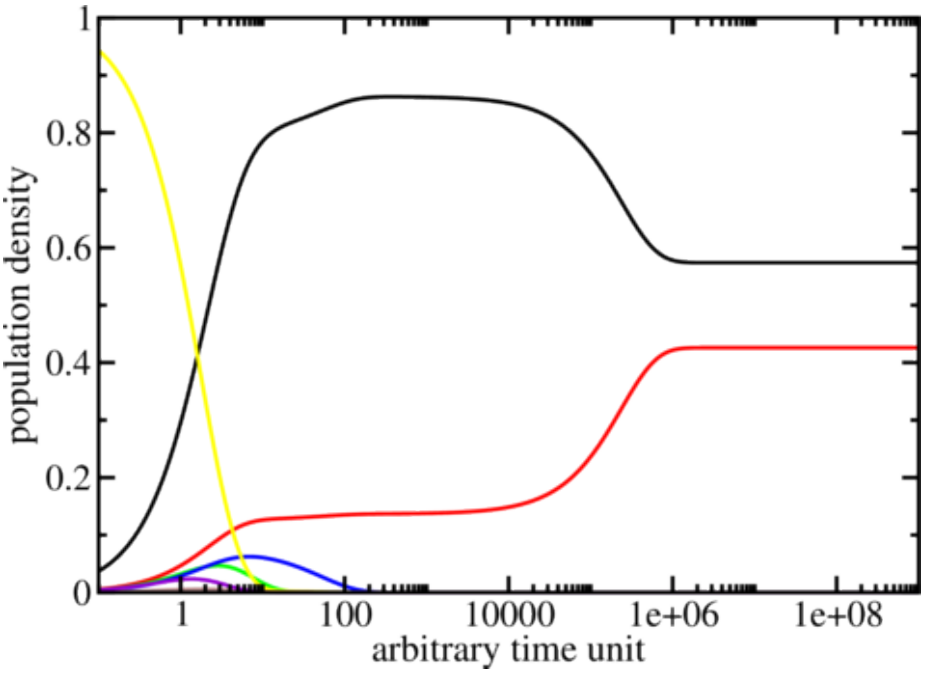
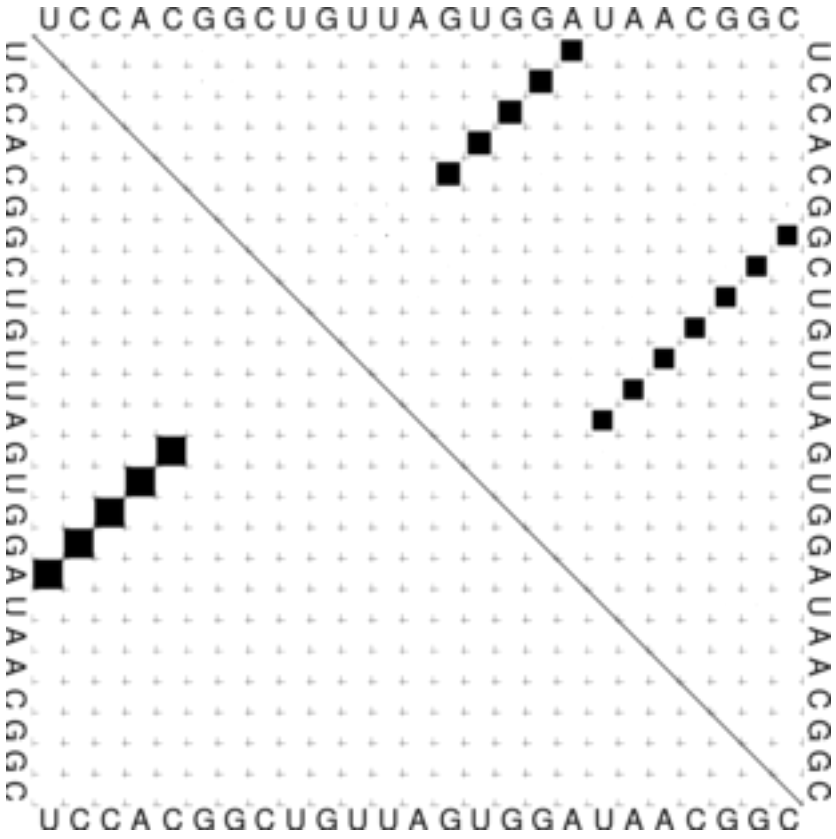


Figure 1: pict


population density
1
0.8
0.6
0.4
0.2
0
1
100
10000
1e+06
1e+08
arbitrary time unit



The simulation starts with all the population density in the open chain (local minimum 5, see `barseq.bar`). Over time the population density of this state decays (yellow curve) and other local minima get populated. The simulation ends with the population densities of the thermodynamic equilibrium in which the MFE (black curve) and local minimum 2 (red curve) are the only ones populated. (Look at the dot plot of the sequence created with `RNAsubopt` and `RNAfold`!)

Sequence Design

Sequence Design

The Program `RNAinverse`

The Program RNAinverse

`RNAinverse` searches for sequences folding into a predefined structure, thereby inverting the folding algorithm. Input consists of the target structures (in dot-bracket notation) and a starting sequence, which is optional.

Lower case characters in the start sequence indicate fixed positions, i.e. they can be used to add sequence constraints. 'N's in the starting sequence will be replaced by a random nucleotide. For each search the best sequence found and its Hamming distance to the start sequence are printed to *stdout*. If the the search was unsuccessful a structure distance to the target is appended.
By default the program stops as soon as it finds a sequence that has the target as MFE structure. The option `-Fp` switches `RNAinverse` to the partition function mode where the probability of the target structure $\exp\left(-E\left(S\right)/RT\right)/Q$ is maximized. This tends to produce sequences with a more well-defined structure. This probability is written in dot-brackets after the found sequence and Hamming distance. With the option `-R` you can specify how often the search should be repeated.

Sequence Design
1. Prepare an input file `inv.in` containing the target structure and sequence constraints

```
``` {.commands}
  (((.(((....))).)))
  NNNgNNNNNNNNNaNNN
```
```

2. Design sequences using RNAinverse

```
$ RNAinverse < inv.in
      GGUgUUGGAUCCGAaACC    5

$ RNAinverse -R5 -Fp < inv.in
      GGUgUGAACCCUCGaACC    5
      GGCgCCCUUUUGGGaGCC   12  (0.967418)
      CUCgAUCUCACGAUaGGG    6
      GGCgCCCGAAAGGGaGCC   13  (0.967548)
      GUUgAGCCCAUGCUaAGC    6
      GGCgCCCUUAUGGGaGCC   10  (0.967418)
      CGGgUGUUGUGACAaCCG    5
      GCGgGUCGAAAGGCaCGC   12  (0.925482)
      GCCgUAUCCGGGUGaGGC    6
      GGCgCCCUUUUGGGaGCC   13  (0.967418)
```

The output consists of the calculated sequence and the number of mutations needed to get the MFE-structure from the start sequence (start sequence not shown). Additionaly, with the partition function folding (`-Fp`) set, the second output is another refinement so that the ensemble preferes the MFE and folds into your given structure with a distinct probability, shown in brackets.
Another useful program for inverse folding is `RNA designer`, see http://www.

rnasoft.ca/. RNA Designer takes a secondary structure description as input and returns an RNA strand that is likely to fold in the given secondary structure.

The `sequence design application` of the `ViennaRNA Design Webservices`, see http://nibiru.tbi.univie.ac.at/rnadesign/index.html uses a different approach, allowing for more than one secondary structure as input. For more detail read the online Documentation and the next section of this tutorial.

switch.pl

switch.pl

The `switch.pl` script can be used to design bi-stable structures, i.e. structures with two almost equally good foldings. For two given structures there are always a lot of sequences compatible with both structures. If both structures are reasonably stable you can find sequences where both target structures have almost equal energy and all other structures have much higher energies. Combined with RNAsubopt, barriers and treekin, this is a very useful tool for designing RNAswitches.
The input requires two structures in dot-bracket notation and additionally you can add a sequence. It is also possible to calculate the switching function at two different temperatures with option `-T` and `-T2`.

Designing a Switch
Now we try to create an RNA switch using `switch.pl`. First we create our inputfile, then invoke the program using ten optimization runs (`-n 10`) and do not allow lonely pairs. Write it out to `switch.out`

```
switch.in
    (((((((.....)))))))....(((((((.....)))))))
    ((((((((((((((((((.....))))))))))))))))).....

$ switch.pl -n 10 --noLP < switch.in > switch.out
```

`switch.out` should look similar like this, the first block represents our bi-stable structures in random order, the second block shows the resulting sequences ordered by their score.

```
$ less switch.out

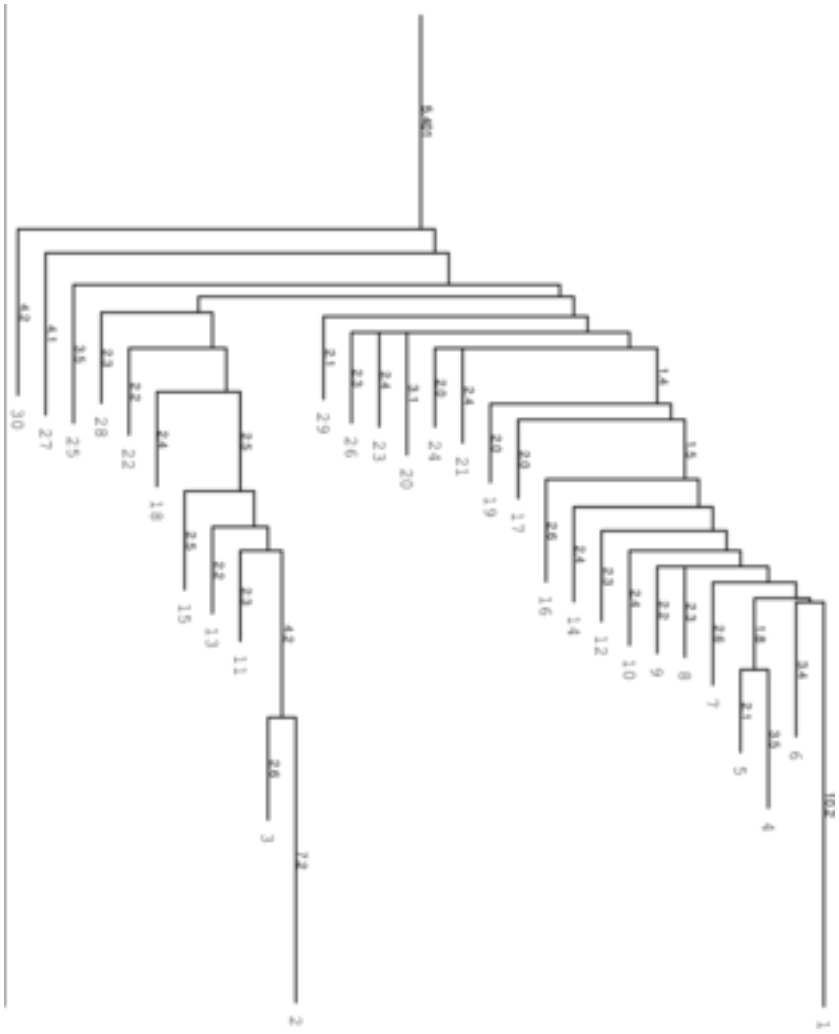
GGGUGGACGUUUCGGUCCAUCCUUACGGACUGGGGCGUUUACCUAGUCC   0.9656
CAUUUGGCUUGUGUGUCGAAUGGCCCCGGUACGUAGGCUAAAUGUACCG   1.2319
GGGGGGUGCGUUCACACCCCUCAUUUGGUGUGGAUGUGCUUUCUACACU   1.1554
[...]
the resulting sequences are:
CAUUUGGCUUGUGUGUCGAAUGGCCCCGGUACGUAGGCUAAAUGUACCG   1.2319
```

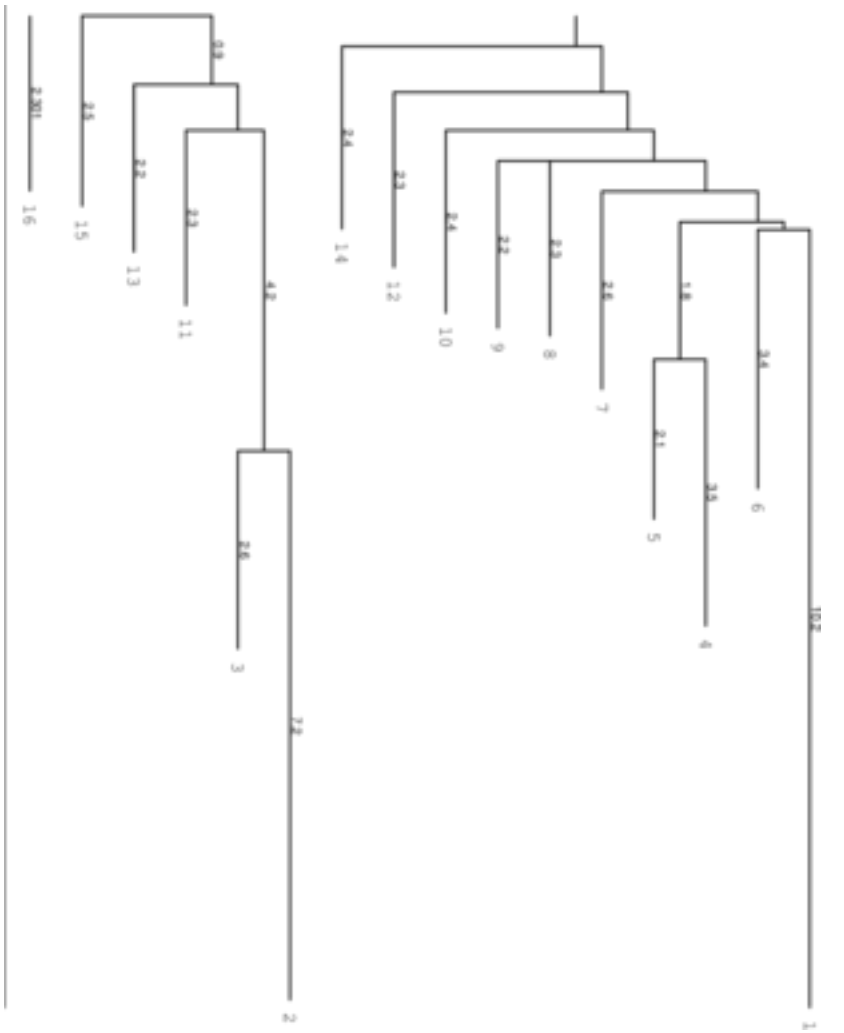
```
GGGGGGUGCGUUCACACCCCUCAUUUGGUGUGGAUGUGCUUUCUACACU   1.1554
CGGGUUGUAACUGGAUAGCCUGGAAACUGUUUGGUUGUAAUCCGAACAG   1.0956
[...]
```

Given all 10 suggestions in our `switch.out`, we select the one with the best score with some command line tools to use it as an `RNAsubopt` input file and build up the barriers tree.

```
$ tail -10 switch.out | awk '{print($1)}'  | head -n 1 > subopt.in
$ RNAsubopt --noLP -s -e 25 < subopt.in > subopt.out
$ barriers -G RNA-noLP --bsize --rates --minh 2 --max 30 < subopt.out > barriers.out
```

`tail -10` cuts the last 10 lines from the `switch.out` file and pipes them into an `awk` script. The function `print($1)` echoes only the first column and this is piped into the `head` program where the first line, which equals the best scored sequence, is taken and written into `subopt.in`. Then `RNAsubopt` is called to process our sequence and write the output to another file which is the input for the barriers calculation.
Below you find an example of the barriertree calculation above done with the right settings (connected root) on the left side and the wrong `RNAsubobt -e` value on the right. Keep in mind that `switch.pl` performs an stochastic search and the output sequences are different every time because there are a lot of sequences which fit the structure and switch calculates a new one everytime. Simply try to make sure.



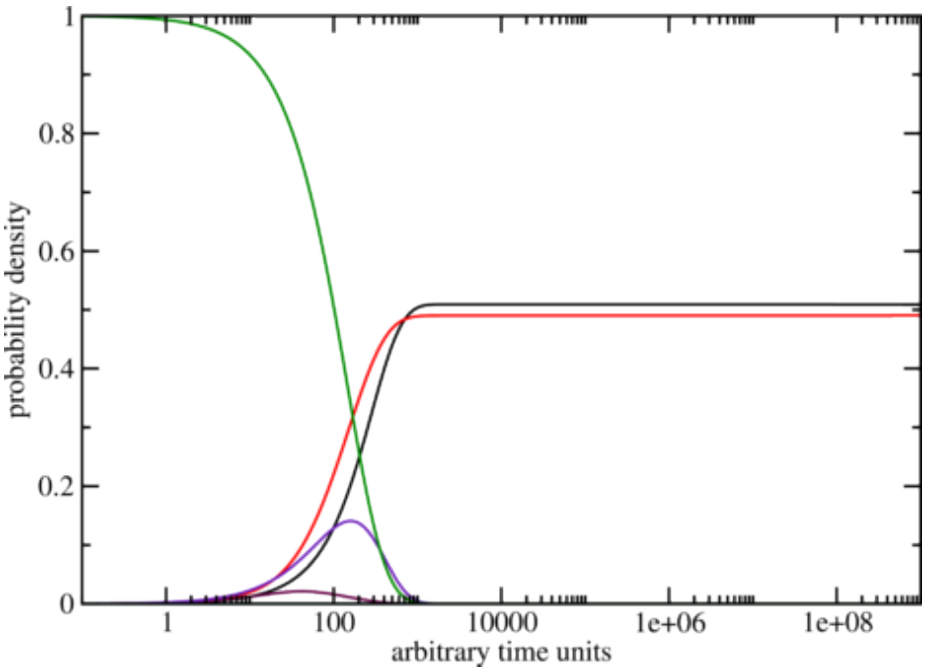


left: Barriers tree as it should look like, all branches connected to the main root right: disconnected tree due to a too low `energy range (-e)` parameter set in `RNAsubopt`.

Be careful to set the range -e high enough, otherwise we get a problem when calculation the kinetics using treekin. Every branch should be somehow connected to the main root of the tree. Try `-e 20` and `-e 30` to see the difference in the trees and choose the optimal value. By using `--max 30` we shorten our tree to focus only on the lowest minima. We then select a branch preferably outside of the two main branches, here branch 30 (may differ from your own calculation). Look at the barrier tree to find the best branch to start and replace `30` by the branch you would choose. Now use treekin to plot concentration kinetics and think about the graph you just created.

```
$ treekin -m I --p0 30=1  < barriers.out > treekin.out
$ xmgrace -log x -nxy treekin.out
```

The graph could look like the one below, remember everytime you use `switch.pl` it can give you different sequences so the output varies too. Here the one from the example.



RNA-RNA Interactions

RNA-RNA Interactions

A common problem is the prediction of binding sites between two RNAs, as in the case of miRNA-mRNA interactions. Following tools of the `ViennaRNA Package` can be used to calculate base pairing probabilities.

The Program `RNAcofold`

The Program RNAcofold

`RNAcofold` works much like `RNAfold` but uses two RNA sequences as input which are then allowed to form a dimer structure. In the input the two RNA sequences should be concatenated using the '`&`' character as separator. As in

RNAfold the -p option can be used to compute partition function and base pairing probabilities.
Since dimer formation is concentration dependent, RNAcofold can be used to compute equilibrium concentrations for all five monomer and (homo/hetero)-dimer species, given input concentrations for the monomers (see the man page for details).

Two Sequences one Structure
1. Prepare a sequence file (t.seq) for input that looks like this

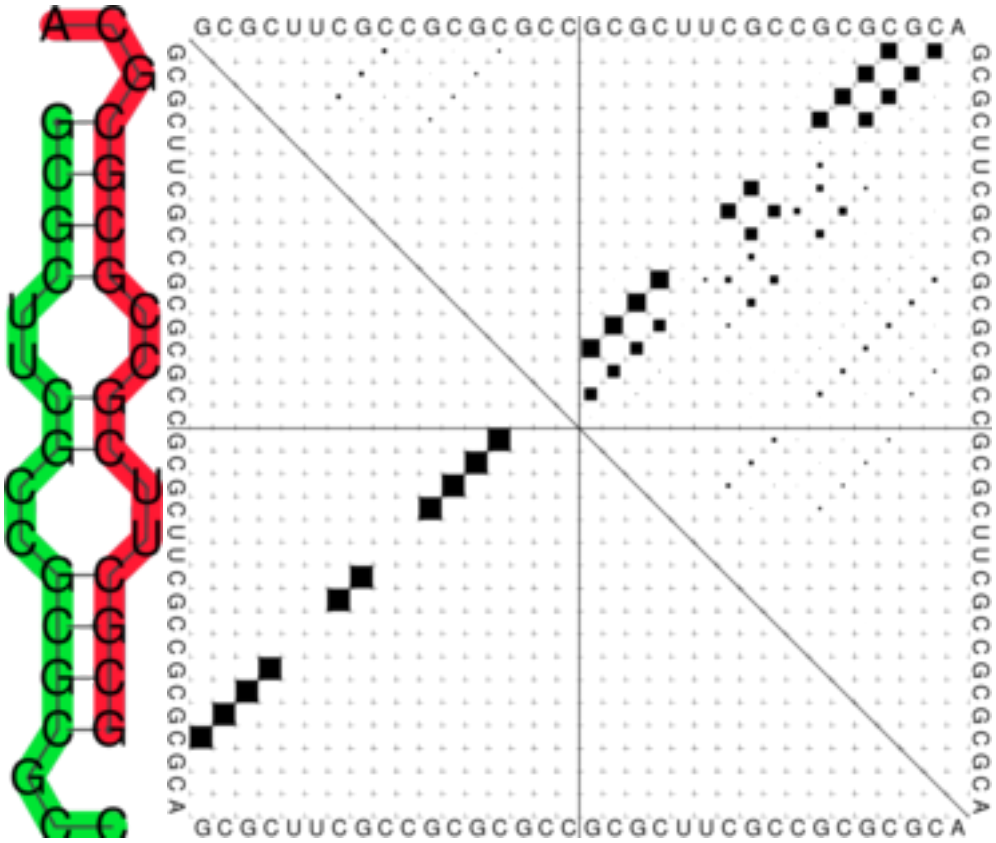
```
``` {.commands}
>t
GCGCUUCGCCGCGCGCC&GCGCUUCGCCGCGCGCA
```

</div>
```

2. Compute the MFE and the ensemble properties
3. Look at the generated PostScript files t_ss.ps and t_dp.ps

```
$ RNAcofold -p < t.seq
>t
GCGCUUCGCCGCGCGCC&GCGCUUCGCCGCGCGCA
((((..((..((((...&))))..))..))))... (-17.70)
((((..{(,.((((,,.&))))..}),.)))),,. [-18.26]
frequency of mfe structure in ensemble 0.401754 , delta G binding= -3.95
```

Secondary Structure Plot and Dot Plot



In the dot plot a cross marks the chain break between the two concatenated sequences.

Concentration Dependency

Concentration Dependency

Cofolding is an intermolecular process, therefore whether duplex formation will actually occur is concentration dependent. Trivially, if one of the molecules is not present, no dimers are going to be formed. The partition functions of the molecules give us the equilibrium constants:

$$K_{AB} = \frac{[AB]}{[A]\,[B]} = \frac{Z_{AB}}{Z_A Z_B}$$

with these and mass conservation, the equilibrium concentration of homodimers, heterodimers and monomers can be computed in dependence of the start

concentrations of the two molecules. This is most easily done by creating a file with the initial concentrations of molecules A and B in two columns:

$$\begin{array}{ll} [a_1]\,([mol/l]) & [b_1]\,([mol/l]) \\ [a_2]\,([mol/l]) & [b_2]\,([mol/l]) \\ \vdots & \\ [a_n]\,([mol/l]) & [b_n]\,([mol/l]) \end{array}$$

Concentration Dependency

1. Prepare a concentration file for input with this little perl script

```
``` {.commands}
$ perl -e '$c=1e-07; do {print "$c\t$c\n"; $c*=1.71;} while $c<0.2' > concfile
```

This script creates a file displaying values from 1e-07 to just below
0.2, with 1.71-fold steps in between. For convenience, concentration
of molecule A is the same as concentration of molecule B in
each row. This will facilitate visualization of the results.
```

2. Compute the `MFE`, the ensemble properties and the concentration dependency of hybridization.

   ```
   $ RNAcofold -f concfile < t.seq > cofold.out
   ```

3. Look at the generated output with

   ```
   $ less cofold.out
   ```

```
[...]
Free Energies:
AB              AA              BB              A               B
-18.261023      -17.562553      -18.274376      -7.017902       -7.290237
Initial concentrations          relative Equilibrium concentrations
A               B               AB              AA              BB              A
1e-07           1e-07           0.00003         0.00002         0.00002         0.49994
[...]
```

The five different free energies were printed out first, followed by a list of all the equilibrium concentrations, where the first two columns denote the initial (absolute) concentrations of molecules A and B, respectively. The next five columns denote the equilibrium concentrations of dimers and monomers, relative to the total particle number. (Hence, the concentrations don't add up to one, except in the case where no dimers are built – if you want to know the fraction of particles in a dimer, you have to take the relative dimer concentrations times

2).
Since relative concentrations of species depend on two independent values - initial concentration of A as well as initial concentration of B - it is not trivial to visualize the results. For this reason we used the same concentration for A and for B. Another possibility would be to keep the initial concentration of one molecule constant. As an example we show the following plot of *t.seq*. Now we use some commandline tools to render our plot. We use `tail -n +11` to show all lines starting with line 11 (1-10 are cut) and pipe it into an `awk` command, which prints every column but the first from our input. This is then piped to `xmgrace`. With `-log x -nxy -` we tell it to plot the x axis in logarithmic scale and to read data file in X Y1 Y2 ... format.

```
$ tail -n +11 cofold.out | awk '{print $2, $3, $4, $5, $6, $7}' | xmgrace -log x -nxy -
```

Concentration Dependency plot

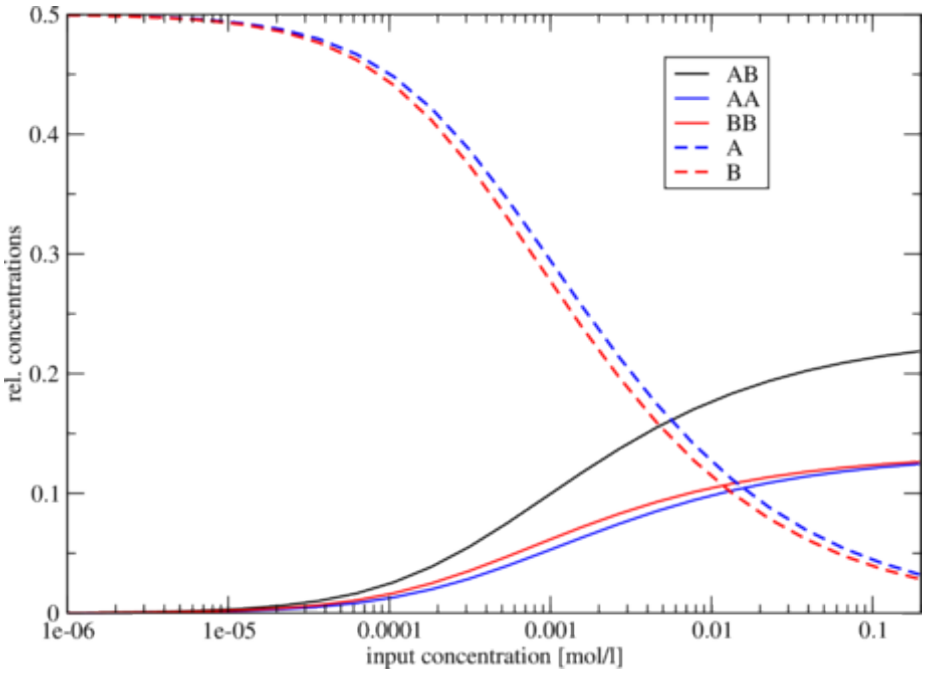


Figure 2: pict

$\Delta G_{\text{binding}} = -5.01$ kcal/mol

```
sequences:GCGCUUCGCCGCGCGCG&GCGCUUCGCCGCGCGCG
```

Since the two sequences are almost identical, the monomer and homo-dimer concentrations behave very similarly. In this example, at a concentration of about 1 mmol 50% of the molecule is still in monomer form.

Finding potential binding sites with RNAduplex

Finding potential binding sites with RNAduplex

If the sequences are very long (many kb)`RNAcofold` is too slow to be useful. The `RNAduplex` program is a fast alternative, that works by predicting *only* intermolecular base pairs. It's almost as fast as simple sequence alignment, but much more accurate than a `BLAST` search.

The example below searches the 3' UTR of an mRNA for a miRNA binding site.

Binding site prediction with RNAduplex
The file `duplex.seq` contains the 3'UTR of NM_024615 and the microRNA mir-145.

```
$ RNAduplex < duplex.seq
>NM_024615
>hsa-miR-145
.(((((.(((...((((((((((.&))))))))))))))))).  34,57  :   1,19  (-21.90)
```

Most favorable binding has an interaction energy of -21.90 kcal/mol and pairs up on positions 34-57 of the UTR with positions 1-22 of the miRNA.
`RNAduplex` can also produce alternative binding sites, e.g. running `RNAduplex -e 10` would list all binding sites within 10 kcal/mol of the best one.

Since `RNAduplex` forms only intermolecular pairs, it neglects the competition between intramolecular folding and hybridization. Thus, it is recommended to use `RNAduplex` as a pre-filter and analyse good `RNAduplex` hits additionally with `RNAcofold` or `RNAup`. Using the example above, running `RNAup` will yield:

```
$ RNAup -b < duplex.seq

>NM_024615
>hsa-miR-145
(((((((&)))))))  50,56  :   1,7   (-8.41 = -9.50 + 0.69 + 0.40)
GCUGGAU&GUCCAGU
RNAup output in file: hsa-miR-145_NM_024615_w25_u1.out
```

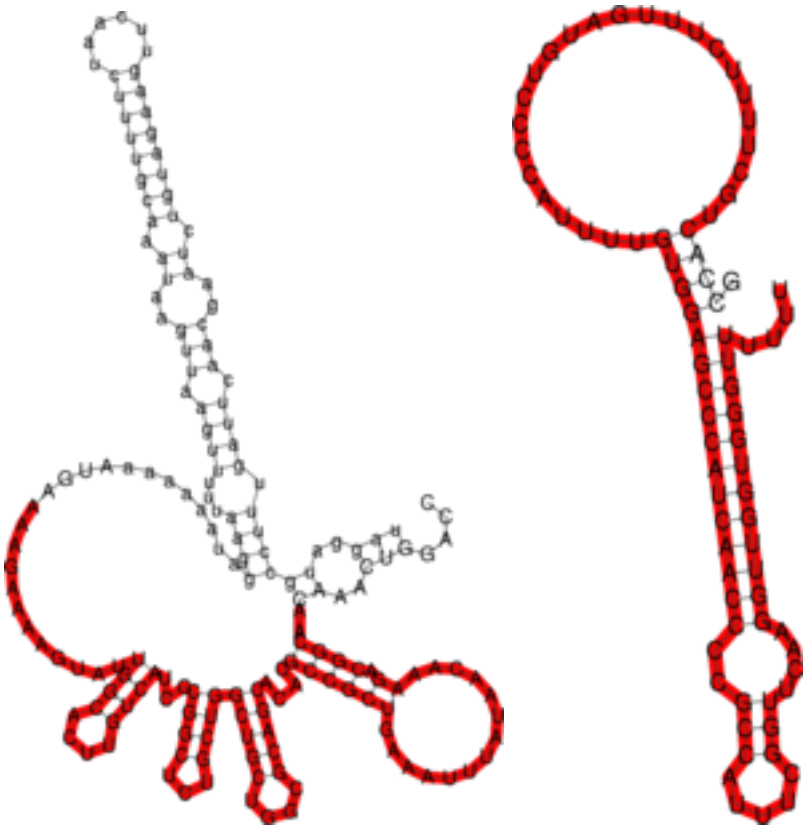
The free energy of the duplex is -9.50 kcal/mol and shows a discrepancy to the structure and energy value computed by `RNAduplex` (differences may arise from the fact that RNAup computes partition functions rather than optimal structures). However, the total free energy of binding is less favorable (-8.41 kcal/mol), since it includes the energetic penalty for opening the binding site on the mRNA (0.69 kcal/mol) and miRNA (0.40 kcal/mol). The `-b` option includes the probability of unpaired regions in both RNAs.

You can also run `RNAcofold` on the example to see the complete structure after hybridization (neither `RNAduplex` nor `RNAup` produce structure drawings). Note however, that the input format for `RNAcofold` is different. An input file suitable for `RNAcofold` has to be created from the `duplex.seq` file first (use any text editor).
As a more difficult example, let's look at the interaction of the bacterial smallRNA RybB and its target mRNA ompN. First we'll try predicting the binding site using RNAduplex:

```
$ RNAduplex < RybB.seq
>RybB
>ompN
.((((..((((((.(((....(((((((..(((((.((..((.((....((((..((((((((((..((((((&
.))))))..)))))))..))))....))))....)).)).)).))).))..))))........))))..))).))))))..))))).
 5,79  :  80,164 (-34.60)
```

Note, that the predicted structure spans almost the full length of the RybB small RNA. Compare the predicted interaction to the structures predicted for RybB and ompN alone, and ask yourself whether the predicted interaction is indeed plausible.

Below the structure of ompN on the left and RybB on the right side. The respective binding regions predicted by RNAduplex are marked in red.



```
GCCAC-----TGCTTTTCTTTGATGTCCCCATTTT-GTGGA-------GC-CCATCAACCCCGCCATTTCGGTT---CAAG-GTTGGTGGGT
 |||      ||||  |||||| |||    |||||  ||||        || ||| || ||  ||    ||||     |||| ||  |||  |
AGGTCAAACAACGGC-AGAAACAATATT--TAAAGTCGCCGCACACGACGCGGTCGTCGGT-CGTCTCGGCCCTACTGTTCACGGTTATGAA
```

Compare the `RNAduplex` prediction with the interaction predicted by `RNAcofold`, `RNAup` and the handcrafted prediction you see above.

Consensus Structure Prediction

Consensus Structure Prediction

Sequence co-variations are a direct consequence of RNA base pairing rules and can be deduced to alignments. RNA helices normally contain only 6 out of the 16 possible combinations: the Watson-Crick pairs GC, CG, AU, UA, and the somewhat weaker wobble pairs GU and UG. Mutations in helical regions therefore have to be correlated. In particular we often find “compensatory mutations” where a mutation on one side of the helix is compensated by a second mutation on the other side, e.g. a C·G pair changes into a U·A pair. Mutations where

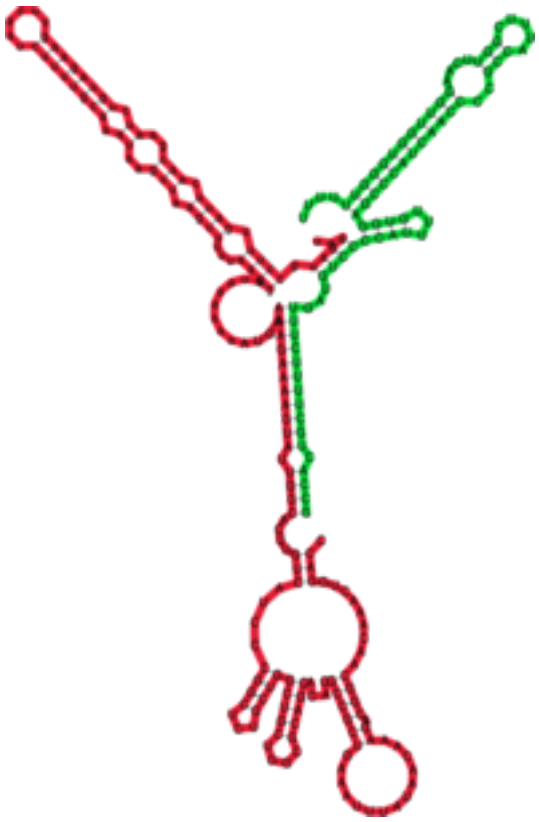


Figure 3: pict

only one pairing partner changes (such as C·G to U·G) are termed "consistent mutations".

The Program `RNAalifold`

The Program RNAalifold

`RNAalifold` generalizes the folding algorithm for sequence alignments, treating the entire alignment as a single "generalized sequence". To assign an energy to a structure on such a generalized sequence, the energy is simply averaged over all sequences in the alignment. This average energy is augmented by a covariance term, that assigns a bonus or penalty to every possible base pair (i, j) based on the sequence variation in columns i and j of the alignment.
Compensatory mutations are a strong indication of structural conservation, while consistent mutations provide a weaker signal. The covariance term used by `RNAalifold` therefore assigns a bonus of 1 kcal/mol to each consistent and 2 kcal/mol for each compensatory mutation. Sequences that cannot form a standard base pair incur a penalty of -1 kcal/mol. Thus, for every possible consensus pair between two columns i and j of the alignment a covariance score C_{ij} is computed by counting the fraction of sequence pairs exhibiting consistent and compensatory mutations, as well as the fraction of sequences that are inconsistent with the pair. The weight of the covariance term relative to the normal energy function, as well as the penalty for inconsistent mutations can be changed via command line parameters.
Apart from the covariance term, the folding algorithm in `RNAalifold` is essentially the same as for single sequence folding. In particular, folding an alignment containing just one sequence will give the same result as single sequence folding using `RNAfold`. For N sequences of length n the required CPU time scales as $\mathcal{O}\left(N \cdot n^2 + n^3\right)$ while memory requirements grow as the square of the sequence length. Thus `RNAalifold` is in general faster than folding each sequence individually. The main advantage, however, is that the accuracy of consensus structure predictions is generally much higher than for single sequence folding, where typically only between 40% and 70% of the base pairs are predicted correctly.
Apart from prediction of `MFE` structures `RNAalifold` also implements an algorithm to compute the partition function over all possible (consensus) structures and the thermodynamic equilibrium probability for each possible pair. These base pairing probabilities are useful to see structural alternatives, and to distinguish well defined regions, where the predicted structure is most likely correct, from ambiguous regions.
As a first example we'll produce a consensus structure prediction for the following four tRNA sequences.

```
$ cat four.seq

>M10740 Yeast-PHE
```

```
GCGGAUUUAGCUCAGUUGGGAGAGCGCCAGACUGAAGAUUUGGAGGUCCUGUGUUCGAUCCACAGAAUUCGCA
>K00349 Drosophila-PHE
GCCGAAAUAGCUCAGUUGGGAGAGCGUUAGACUGAAGAUCUAAAGGUCCCCGGUUCAAUCCCGGGUUUCGGCA
>K00283 Halobacterium volcanii Lys-tRNA-1
GGGCCGGUAGCUCAUUUAGGCAGAGCGUCUGACUCUUAAUCAGACGGUCGCGUGUUCGAAUCGCGUCCGGCCCA
>AF346993
CAGAGUGUAGCUUAACACAAAGCACCCAACUUACACUUAGGAGAUUUCAACUUAACUUGACCGCUCUGA
```

`RNAalifold` uses aligned sequences as input. Thus, our first step will be to align the sequences. We use `clustalw2` in this example, since it's one of the most widely used alignment programs and has been shown to work well on structural RNAs. Other alignment programs can be used (including programs that attempt to do structural alignment of RNAs), but the resulting multiple sequence alignment must be in `Clustal` format. Get `clustalw2` and install it as you have done it with the other packages: http://www.clustal.org/clustal2

Consensus Structure from related Sequences
1. Prepare a sequence file (use file `four.seq` and copy it to your working directory) 2. Align the sequences 3. Compute the consensus structure from the alignment 4. Inspect the output files `alifold.out`, `alirna.ps`, `alidot.ps` 5. For comparison fold the sequences individually using `RNAfold`

```
$ clustalw2 four.seq > four.out
```

`Clustalw2` creates two more output files, `four.aln` and `four.dnd`. For `RNAalifold` you need the `.aln` file.

```
$ RNAalifold -p four.aln
$ RNAfold -p < four.seq
```

`RNAalifold` output:

```
__GCCGAUGUAGCUCAGUUGGG_AGAGCGCCAGACUGAAAAUCAGAAGGUCCCGUGUUCAAUCCACGGAUCCGGCA__
..(((((((..((((.........)))).(((((.......))))).....(((((.......)))))))))))...
 minimum free energy = -15.12 kcal/mol (-13.70 +  -1.43)
..((((((..((((.........)))).(((((.......))))).....(((((.......)))))))))))...
 free energy of ensemble = -15.75 kcal/mol
 frequency of mfe structure in ensemble 0.361603
..(((((((..((((.........)))).(((((.......))))).....(((((.......)))))))))))... -15.20 {-13.7
```

`RNAfold` output:

```
>M10740 Yeast-PHE
GCGGAUUUAGCUCAGUUGGGAGAGCGCCAGACUGAAGAUUUGGAGGUCCUGUGUUCGAUCCACAGAAUUCGCA
```

```
(((((((.......((((.((((((..((((..........))))..))))))..))))..)))))))). (-21.60)
(((((((.{...,,.{,((((.((((((..((((..........))))..))))))..)))))),)))))). [-23.20]
(((((((.........(((.((((((..((((..........))))..))))))..)))...)))))))). {-20.00 d=9.63}
 frequency of mfe structure in ensemble 0.0744065; ensemble diversity 15.35
>K00349 Drosophila-PHE
[...]
```

The output contains a consensus sequence and the consensus structure in dot-bracket notation. The consensus structure has an energy of −15.12 kcal/mol, which in turn consists of the average free energy of the structure −13.70 kcal/mol and the covariance term −1.43 kcal/mol. The strongly negative covariance term shows that there must be a fair number of consistent and compensatory mutations, but in contrast to the average free energy it's not meaningful in the biophysical sense.

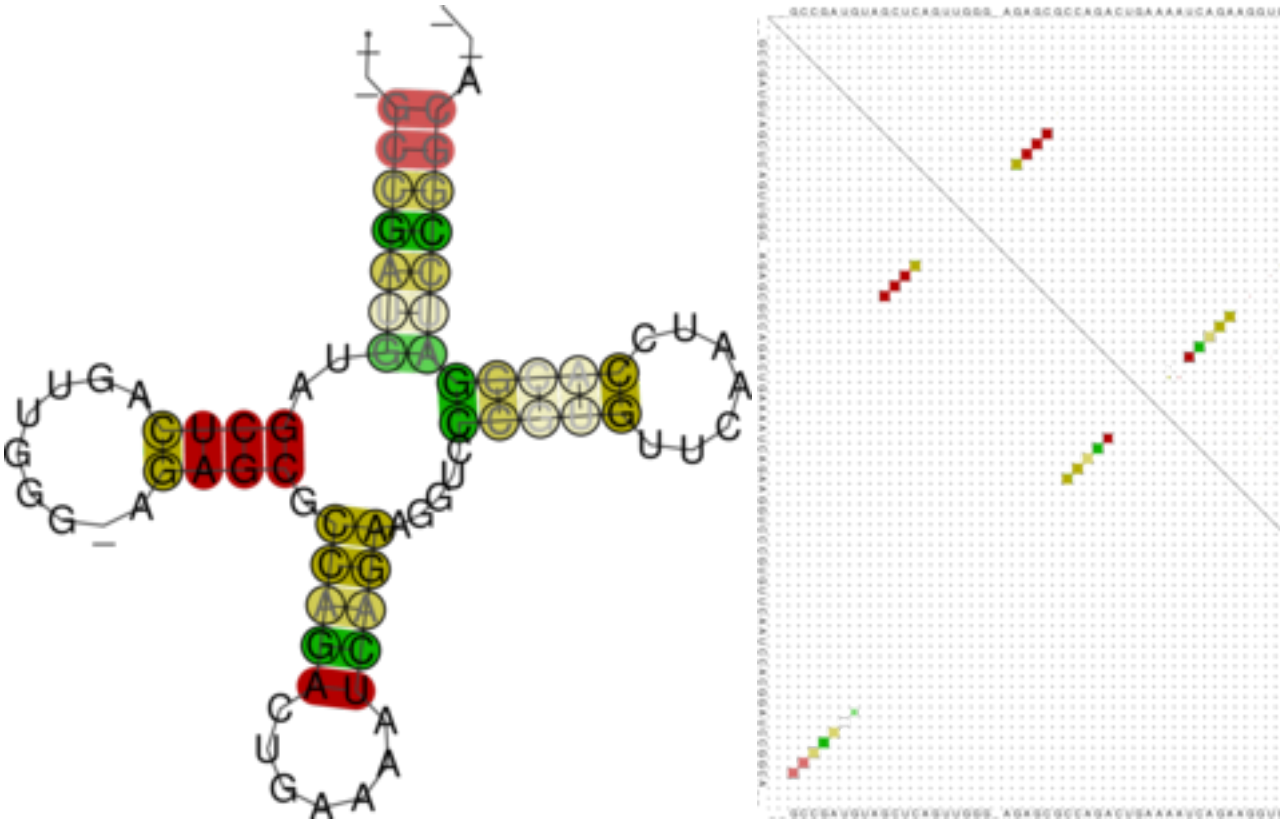
Compare the predicted consensus structure with the structures predicted for the individual sequences using `RNAfold`. How often is the correct "clover-leaf" shape predicted?

For better visualization, a structure annotated alignment or color annotated structure drawing can be generated by using the `--aln` and `--color` options of `RNAalifold`.

```
$ RNAalifold --color --aln four.aln
$ gv aln.ps &
$ gv alirna.ps &
```

`RNAalifold` Output Files

```
4 sequence; length of alignment 78
alifold output
   6    72  0  99.8%   0.007 GC:2    GU:1    AU:1
  33    43  0  98.9%   0.033 GC:2    GU:1    AU:1
  31    45  0  99.0%   0.030 CG:3    UA:1
  15    25  0  98.9%   0.045 CG:3    UA:1
   5    73  1  99.7%   0.008 CG:2    GC:1
  13    27  0  99.1%   0.042 CG:4
  14    26  0  99.1%   0.042 UA:4
   4    74  1  99.5%   0.015 CG:3
[...]
```



The last output file produced by `RNAalifold -p`, named `alifold.out`, is a plain text file with detailed information on all plausible base pairs sorted by the likelihood of the pair. In the example above we see that the pair $(6, 72)$ has no inconsistent sequences, is predicted almost with probability 1, and occurs as a GC pair in two sequences, a GU pair in one, and a AU pair in another.

`RNAalifold` automatically produces a drawing of the consensus structure in Postscript format and writes it to the file "`alirna.ps`". In the structure graph consistent and compensatory mutations are marked by a circle around the variable base(s), i.e. pairs where one pairing partner is encircled exhibit consistent mutations, whereas pairs supported by compensatory mutations have both bases marked. Pairs that cannot be formed by some of the sequences are shown gray instead of black. In the example given, many pairs show such inconsistencies. This is because one of the sequences (AF346993) is not aligned well by `clustalw`.

Note, that subsequent calls to `RNAalifold` will overwrite any existing output `alirna.ps` (`alidot.ps`, `alifold.out`) files in the current directory. Be sure to rename any files you want to keep.

Structure predictions for the individual sequences

The consensus structure computed by `RNAalifold` will contain only pairs that

can be formed by most of the sequences. The structures of the individual sequences will typically have additional base pairs that are not part of the consensus structure. Moreover, ncRNA may exhibit a highly conserved core structure while other regions are more variable. It may therefore be desirable to produce structure predictions for one particular sequence, while still using covariance information from other sequences.

This can be accomplished by first computing the consensus structure for all sequences using `RNAalifold`, then folding individual sequences using `RNAfold -C` with the consensus structure as a constraint. In constraint folding mode `RNAfold -C` allows only base pairs to form which are compatible with the constraint structure. This resulting structure typically contains most of the constraint (the consensus structure) plus some additional pairs that are specific for this sequence.

Refolding Individual Sequences
The `refold.pl` (find it in the Progs folder) script removes gaps and maps the consensus structure to each individual sequence.

```
$ RNAalifold  RNaseP.aln > RNaseP.alifold
$ gv alirna.ps
$ refold.pl RNaseP.aln RNaseP.alifold | head -3 > RNaseP.cfold
$ RNAfold -C --noLP < RNaseP.cfold > RNaseP.refold
$ gv E-coli_ss.ps
```

If you compare the refolded structure (E-coli_ss.ps) with the structure you get by simply folding the E.coli sequence in the RNaseP.seq file (RNAfold –noLP) you find a clear rearrangement.

In cases where constrained folding results in a structure that is very different from the consensus, or if the energy from constrained folding is much worse than from unconstrained folding, this may indicate that the sequence in question does not really share a common structure with the rest of the alignment or is misaligned. One should then either remove or re-align that sequence and recompute the consensus structure.

Note that since RNase P forms sizable pseudo-knots, a perfect prediction is impossible in this case.

Structural Alignments

Structural Alignments

Manually correcting Alignments

Manually correcting Alignments

As the tRNA example above demonstrates, sequence alignments are often unsuitable as a basis for determining consensus structures. As a last resort, one may always try manually correcting an alignment. Sequence editors that are structure-aware may help in this task. In particular the SARSE http://sarse.kvl.dk/ editor, and the `ralee-mode` for emacs http://personalpages.manchester.ac.uk/staff/sam.griffiths-jones/software/ralee/ are useful. After downloading the `ralee`-files extract them and put them in a folder called `~/Tutorial/Progs/ralee`. Now read the `00README` file and follow the instructions. If you don't find an ".emacs" file in your home directory execute the following command to copy it from the Data directory.

```
$ cp Data/dot.emacs ~/
```

Next try correcting the `ClustalW` generated alignment (`four.aln`) from the example above. For this we first have to convert it to the Stockholm format. Fortunately the formats are similar. Make a copy of the file add the correct header line and the consensus structure from RNAalifold:

```
$ cp four.aln four.stk
$ emacs four.stk
  .....
$ cat four.stk
```

The final alignment should look like:

```
# STOCKHOLM 1.0

K00349          --GCCGAAAUAGCUCAGUUGGG-AGAGCGUUAGACUGAAGAUCUAAAGGUCCCCGGUUCAAUCCCGGGUUUCGGCA
K00283          GGGCCG--GUAGCUCAUUUAGGCAGAGCGUCUGACUCUUAAUCAGACGGUCGCGUGUUCGAAUC--GCGUCCGGCC
M10740          --GCGGAUUUAGCUCAGUUGGG-AGAGCGCCAGACUGAAGAUUUGGAGGUCCUGUGUUCGAUCCACAGAAUUCGCA
AF346993        --CAGAGUGUAGCUUAAC---ACAAAGCACCCAACUUACACUUAGGAGAUUUCAACUUAACUUGACCGCUCUGA--
#=GC SS_cons    ..(((((((..((((.........)))).(((((.......)))))).....(((((.......)))))))))))
//
```

Now use the functions under the edit menu to improve the alignment, the coloring by structure should help to highlight misaligned positions.

Automatic structural alignments

Automatic structural alignments

Next, we'll compute alignments using two structural alignment programs: `LocARNA` and `T-Coffee`. `LocARNA` is an implementation of the Sankoff algorithm

for simultaneous folding and alignment (i.e. it will generate both alignment and consensus structure). `T-Coffee` uses a progressive alignment algorithm.

Download `LocARNA` from http://www.bioinf.uni-freiburg.de/Software/LocARNA/, extract and install it in your `Progs` folder and eventually add it to your path variable or copy it into the corresponding directory.

Both programs can read the fasta file `four.seq`.

```
$ mlocarna --alifold-consensus-dp four.seq
[...]
M10740              GCGGAUUUAGCUCAGUUGGG-AGAGCGCCAGACUGAAGAUUUGGAGGUCCUGUGUUCGAUCCACAGAAUUCGC
K00349              GCCGAAAUAGCUCAGUUGGG-AGAGCGUUAGACUGAAGAUCUAAAGGUCCCCGGUUCAAUCCCGGGUUUCGGC
K00283              GGGCCGGUAGCUCAUUUAGGCAGAGCGUCUGACUCUUAAUCAGACGGUCGCGUGUUCGAAUCGCGUCCGGCCC
AF346993            CAGAGUGUAGCUUAAC---ACAAAGCACCCAACUUACACUUAGGAGAUU-UCAACUUAA-CUUGACCGCUCUC
alifold             (((((((..((((.........)))).(((((.......)))))).....(((((.......)))))))))))
      (-52.53 = -21.58 + -30.95)
```

Install T-Coffee

Get `T-Coffee` from the github page https://github.com/cbcrg/tcoffee. There is a detailed information how you should download and install the software in the given README.md.

Go to the `downloads` directory and use the provided installer by typing

```
$ cd Tutorial/downloads
$ git clone git@github.com:cbcrg/tcoffee.git tcoffee
$ cd tcoffee/compile/
$ make t_coffee
$ cp t_coffee ~/Tutorial/Progs/
```

Afterwards align the `four.seq` using `t_coffee` and compare the output with the one given by LocARNA.

```
$ t_coffee four.seq > t_coffee.out
```

```
 [t_coffee.out]
 CLUSTAL FORMAT for T-COFFEE 20150925_14:18 [http://www.tcoffee.org] [MODE:  ],
 CPU=0.00 sec, SCORE=739, Nseq=4, Len=74

 M10740          GCGGAUUUAGCUCAGUU-GGGAGAGCGCCAGACUGAAGAUUUGGAGGUCC
 K00349          GCCGAAAUAGCUCAGUU-GGGAGAGCGUUAGACUGAAGAUCUAAAGGUCC
 K00283          GGGCCGGUAGCUCAUUUAGGCAGAGCGUCUGACUCUUAAUCAGACGGUCG
 AF346993        CAGAGUGUAGCUUAAC---ACAAAGCACCCAACUUACACUUAGGAGAUUU
                   ***** *       * ***     ***     *     * *
```

```
M10740          UGUGUUCGAUCCACAGAAUUCGCA
K00349          CCGGUUCAAUCCCGGGUUUCGGCA
K00283          CGUGUUCGAAUCGCGUCCGGCCCA
AF346993        CAACUUAACUUGACCG--CUCUGA
                **                  *
```

Use RNAalifold to predict structures for all your alignments (ClustalW, handcrafted, T-Coffee, and LocARNA) and compare them. The handcrafted and LocARNA alignments should be essentially perfect.

Other interesting approaches to structural alignment include `CMfinder`, `dynalign`, and `stemloc`.

Noncoding RNA gene prediction

Noncoding RNA gene prediction

Prediction of ncRNAs is still a challenging problem in bioinformatics. Unlike protein coding genes, ncRNAs do not have any statistically significant features in primary sequences that could be used for reliable prediction. A large class of ncRNAs, however, depend on a defined secondary structure for their function. As a consequence, evolutionarily conserved secondary structures can be used as characteristic signal to detect ncRNAs. All currently available programs for *de novo* prediction make use of this principle and are therefore, by construction, limited to structured RNAs.

Programs to predict structural RNAs
- `QRNA` (Eddy & Rivas, 2001) - `ddbRNA` (di Bernardo, Down & Hubbard, 2003) - `MSARi` (Coventry, Kleitman & Berger, 2004) - `AlifoldZ` (Washietl & Hofacker, 2004) - `RNAz` (Washietl, Hofacker & Stadler, 2005) - `EvoFold` (Pedersen et al, 2006)

QRNA

QRNA

`QRNA` analyzes pairwise alignments for characteristic patterns of evolution. An alignment is scored by three probabilistic models: (i) Position independent, (ii) coding, (iii) RNA. The independent and the coding model is a pair hidden Markov model. The RNA model is a pair stochastic context-free grammar. First, it calculates the *prior probability* that, given a model, the alignment is observed. Second, it calculates the *posterior probability* that, given an alignment, it has been generated by one of the three models. The posterior probabilities are compared to the position independent background model and a "winner" is found. `QRNA` reads pairwise alignments in MFASTA format (i.e. FASTA format with gaps)

Three competing models in QRNA

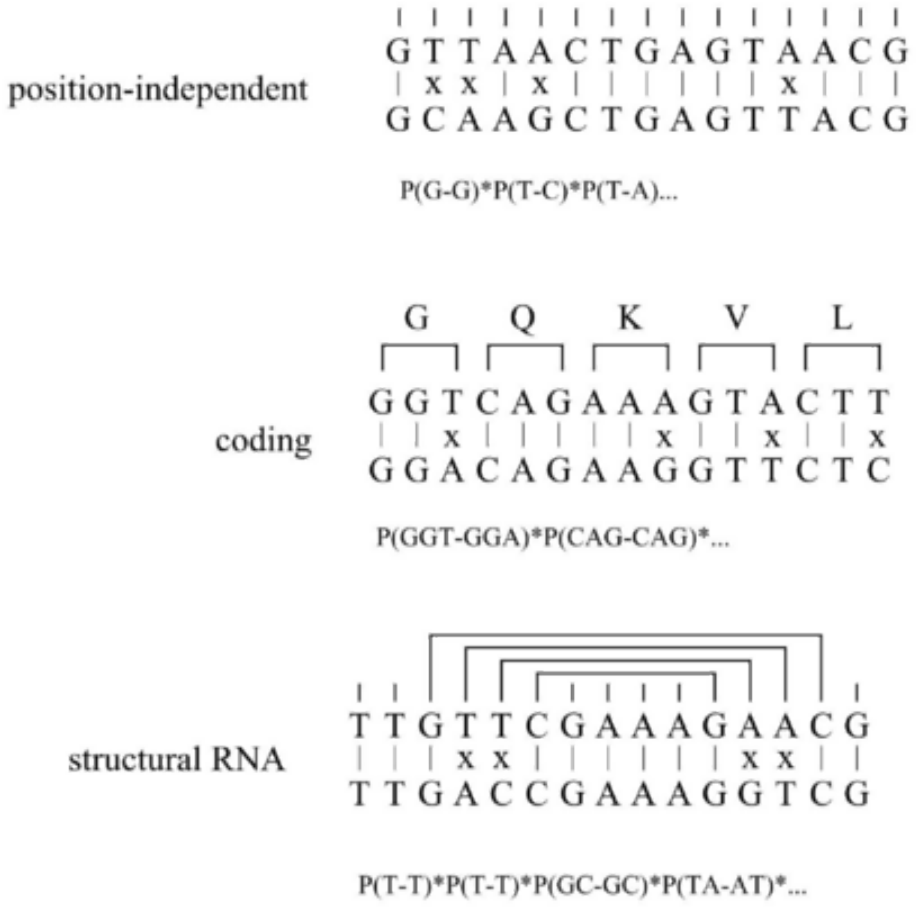


Figure 4: pict

Installing and basic usage of QRNA

- Use the files in `qrna-2.0.3d.tar.gz` located in the `Data/programs`-folder shipped with the tutorial - don't forget to set the `QRNADB` environment variable (e.g. export QRNADB=$HOME/Tutorial/Data/programs/qrna-2.0.3d/lib/) and add it to your `.bashrc` - follow the instructions in the `INSTALL` document and make the binaries - create the directory `~/Tutorial/Progs/qrna` and move the binaries located in the `src/` sub-directory into this folder and add it to your `.bashrc`
(e.g. export PATH=${HOME}/Tutorial/Progs:${PATH}:${HOME}/Tutorial/Progs/qrna)
- first read the help text (option `-h`). - for advanced use of `QRNA` read the `userguide.pdf` shipped with the package (in the `documentation` folder - `-a`

tells `QRNA` to print the alignment

```
$ eqrna -h
$ eqrna -a Data/qrna/tRNA.fa
$ eqrna -a Data/qrna/coding.fa

[...]
Divergence time (variable): 0.214132 0.208107 0.203995
[alignment ID = 72.37 MUT = 23.68 GAP = 3.95]

length alignment: 76 (id=72.37) (mut=23.68) (gap=3.95)
posX: 0-75 [0-72](73) -- (0.18 0.30 0.36 0.16)
posY: 0-75 [0-75](76) -- (0.14 0.34 0.37 0.14)


          DA0780 GGGCTCGTAGCTCAGCT.GGAAGAGCGCGGCGTTTGCAACGCCGAGGCCT
          DA0940 GGGCCGGTAGCTCAGCCTGGGAGAGCGTCGGCTTTGCAAGCCGAAGGCCC

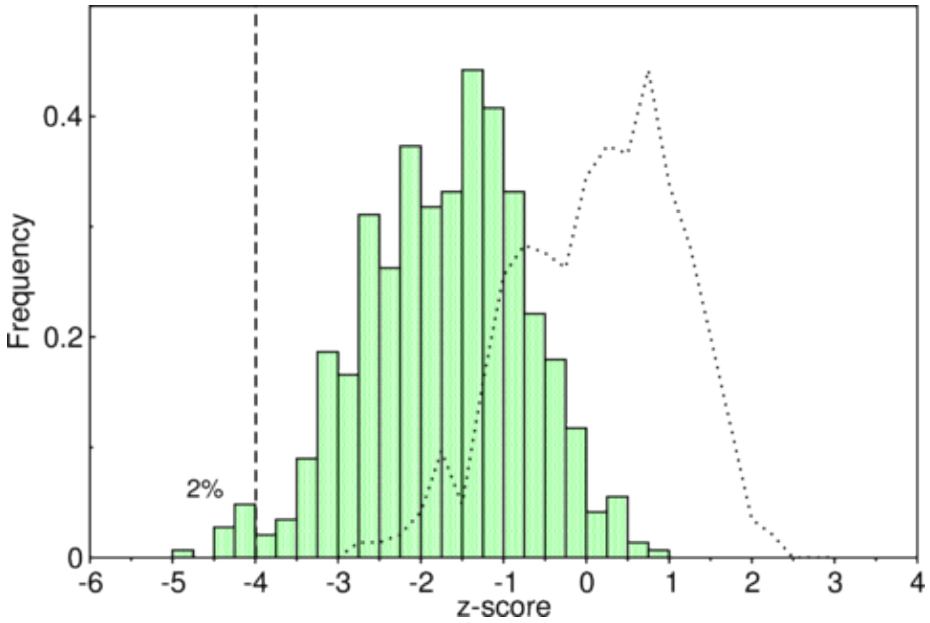
          DA0780 GGGGTTCAAATCCCCACGGGTCCA..
          DA0940 CGGGTTCGAATCCCGGCCGGTCCACC
[..]
```

AlifoldZ

AlifoldZ

`AlifoldZ` is based on an old hypothesis: functional RNAs are thermodynamically more stable than expected by chance. This hypothesis can be statistically tested by calculating z-scores: Calculate the MFE m of the native RNA and the mean μ and standard deviation σ of the background distribution of a large number of random (shuffled) RNAs. The normalized z-score $z = (m - \mu)/\sigma$ expresses how many standard deviations the native RNA is more stable than random sequences. Unfortunately, most ncRNAs are not significantly more stable than the background. See for example the distribution of z-scores of some tRNAs, where the overlap of real (green bars) and shuffled (dashed line) tRNAs is relatively high.

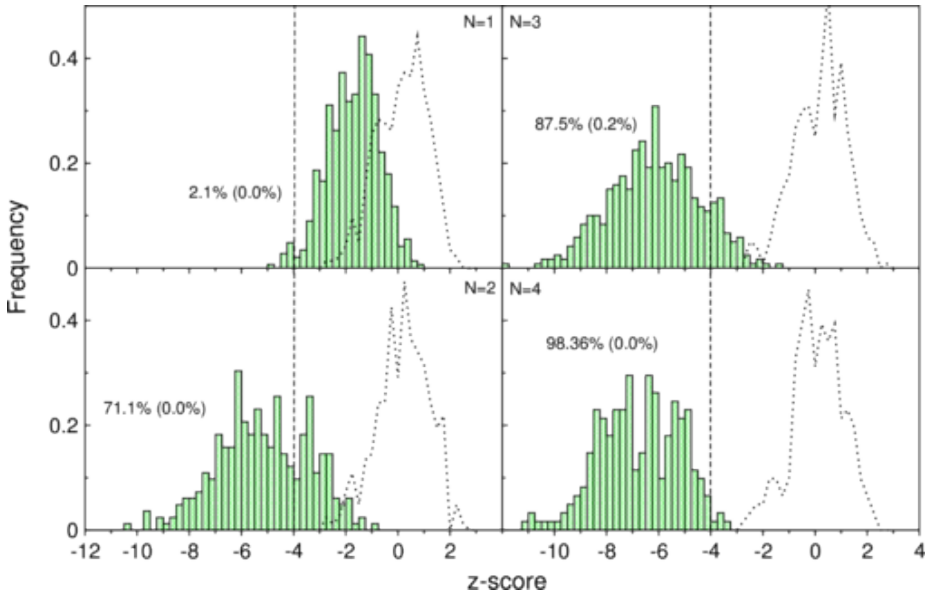
z-score distribution of tRNAs



The overlap of real (bars) and shuffled (dashed line) tRNAs is relatively high.

`AlifoldZ` calculates z-scores for consensus structures folded by `RNAalifold`. This significantly improves the detection performance compared to single sequence folding.

z-score distribution of tRNA consensus folds



The separation of real and shuffled tRNAs gets evident with more sequences in the alignment.

Installation and basic usage of AlifoldZ

- Use the tarball `alifoldz_adopted.tar.gz` located in the `Data/programs/`-folder shipped with the tutorial - Copy the files into your `Progs` directory (It's just one single Perl script which needs `RNAfold` and `RNAalifold` and an important perl module located in the Math subdirectory)

$ cp -r alifoldz.pl Math/ ~/Tutorial/Progs/ - add the perl module to your `PERL5LIB` variable in the `.bashrc`

$ export PERL5LIB=$HOME/Tutorial/Progs/:$PERL5LIB - test the tool

```
$ alifoldz.pl -h
$ alifoldz.pl < Data/alifoldz/miRNA.aln
$ alifoldz.pl -w 120 -x 100 < Data/alifoldz/unknown.aln
```

RNAz

RNAz

* New version by Someone who loves RNAz. This part of the tutorial is based on the RNAz 1.0 version which is obsolete quite a while already!!! *

`AlifoldZ` has some shortcomings that limits its usefulness in practice: The z-scores are not deterministic, i.e. you get a different score each time you run

`AlifoldZ`. To get stable z-scores you need to sample a large number of random alignments which is computationally expensive. Moreover, `AlifoldZ` is extremely sensitive to alignment errors.

The program `RNAz` overcomes these problems by using a different approach to asses a multiple sequence alignment for significant RNA structures. It is based on two key innovations: (i) The structure conservation index (SCI) to measure structural conservation in an alignment and (ii) z-scores that are calculated by regression without sampling. Both measures are combined to an overall score that is used to classify an alignment as "structured RNA" or "other".

The structure conservation index

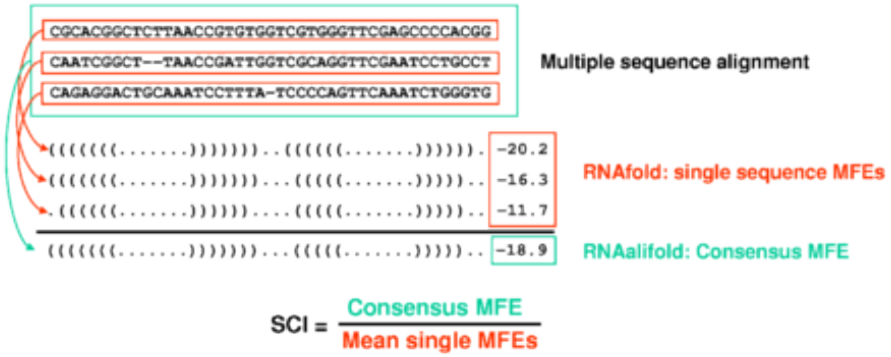


Figure 5: pict

- The structure conservation index is an easy way to normalize an `RNAalifold` consensus MFE.

z-score regression

- The mean μ and standard deviation σ of random samples of a given sequence are functions of the length and the base composition:

$$\mu, \sigma \left(length, \frac{GC}{AT}, \frac{G}{C}, \frac{A}{T} \right)$$

- It is therefore be possible to *calculate* z-scores by solving this 5 dimensional regression problem.

SVM Classification

- A support vector machine learning algorithm is used to classify an alignment based on z-score and structure conservation index.

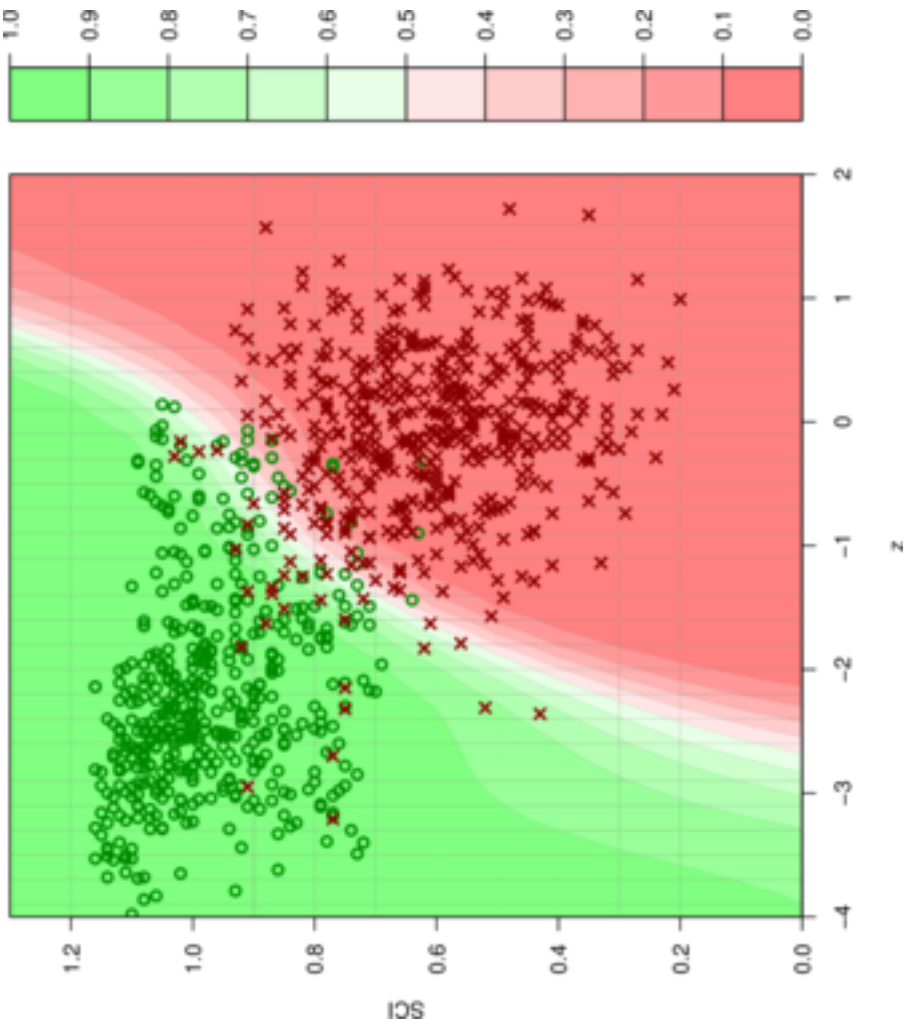


Figure 6: pict

Installation of RNAz
Installation is done according to the instructions used by the `ViennaRNA Package`. Just use the `--prefix` option as mentioned earlier and add the PATH to `.bashrc`

- RNAz is available at: http://www.tbi.univie.ac.at/~wash/RNAz
- Package includes the core program `RNAz` in ISO `C`, a set of helper programs in Perl, and an extensive manual.

Basic usage of RNAz
* where to get examples from (RNAz install package) - commands work with v2 but txt needs to be adopted * - `RNAz` reads one or more multiple sequence alignments in `clustalw` or MAF format.

```
$ RNAz --help
$ RNAz tRNA.aln
$ RNAz --both-strands --predict-strand tRNA.maf
```

Advanced usage of RNAz
- `RNAz` is limited to a maximum alignment length of 400 columns and a maximum number of 6 sequences. To process larger alignments a set of Perl helper scripts are used. - Selecting one or more subsets of sequences from an alignment with more than 6 sequences:

```
$ rnazSelectSeqs.pl miRNA.maf |RNAz
$ rnazSelectSeqs.pl --num-seqs=4 --num-samples=3 miRNA.maf |RNAz
```

- Scoring long alignments in overlapping windows:

```
$ rnazWindow.pl --window=120 --slide=40 unknown.aln \
              | RNAz --both-strands
```

Large scale screens

Large scale screens

The `RNAz` package provides a set of Perl scripts that implement a complete analysis pipeline suitable for medium to large scale screens of genomic data.

General procedure
1. Obtain or create multiple sequence alignments in MAF format 2. Run through the `RNAz` pipeline:

Examples in this tutorial
1. Align Epstein Barr Virus genome (Acc.no: NC_007605) to two related primate viruses (Acc.nos: NC_004367, NC_006146) using `multiz` and run it through

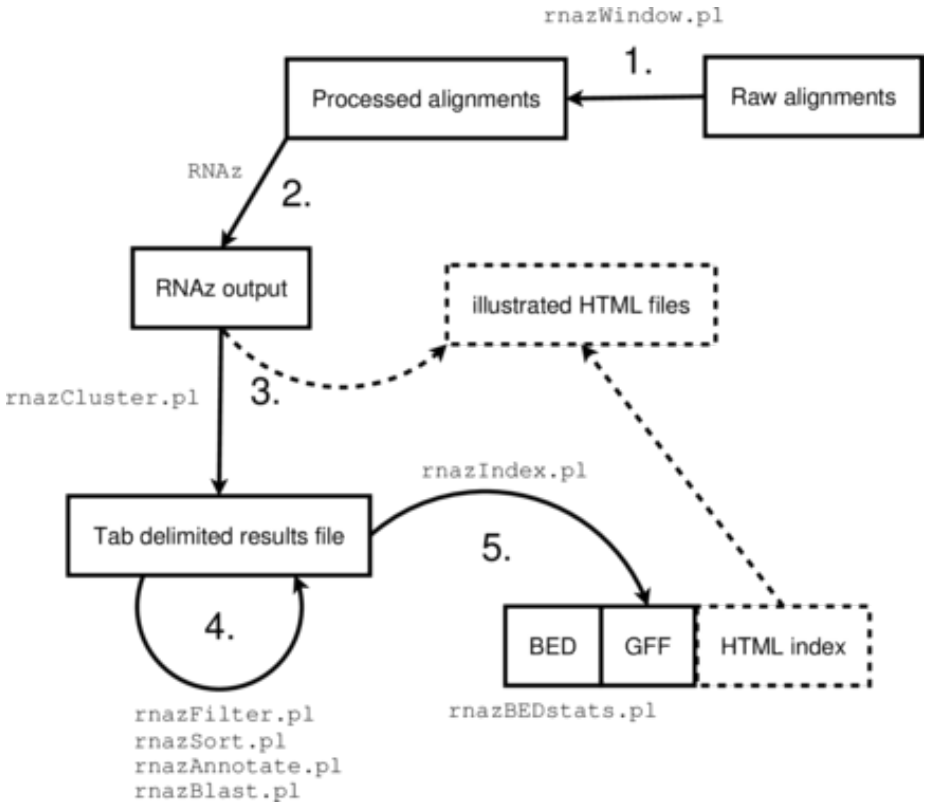


Figure 7: pict

the RNAz pipeline. * where are this data comeing from? file from NCBI differs from those hidden in genefinding/rnaz/herpes * 2. Analyze snoRNA cluster in the human genome for conserved RNA structures: download pre-computed alignments from the UCSC genome browser and run it through the RNAz pipeline

Example a: Preparation of data
- multiz and blastz are available here: http://www.bx.psu.edu/miller_lab/ - Download the viral genomes in FASTA format and reformat the header strictly according to the rules given in the multiz documentation (http://www.bx.psu.edu/miller_lab/dist/tba_howto.pdf), e.g.: - You have to edit the "multiz " Makefile and replace

```
<div id="verbatim-88" class="verbatim">

``` {.commands}
CFLAGS = -Wall -Wextra -Werror
```

</div>

with

<div id="verbatim-89" class="verbatim">

``` {.commands}
CFLAGS = -Wall -Wextra #-Werror
```

</div>

and then simply use the `make` command to compile both programs.
```

* dont understand what to do *

```
>NC_007605:genome:1:+:149696
AGAATTCGTCTTGCTCTATTCACCCTTACTTTTCTTCTTGCCCGTTCTCTTTCTTAGTAT
GAATCCAGTATGCCTGCCTGTAATTGTTGCGCCCTACCTCTTTTGGCTGGCGGCTATTGC
CGCCTCGTGTTTCACGGCCTCAGTTAGTACCGTTGTGACCGCCACCGGCTTGGCCCTCTC
ACTTCTACTCTTGGCAGCAGTGGCCAGCTCATATGCCGCTGCACAAAGGAAACTGCTGAC
ACCGGTGACAGTGCTTACTGCGGTTGTCACTTGTGAGTACACACGCACCATTTACAATGC
ATGATGTTCGTGAGATTGATCTGTCTCTAACAGTTCACTTCCTCTGCTTTTCTCCTCAGT
CTTTGCAATTTGCCTAACATGGAGGATTGAGGACCCACCTTTTAATTCTCTTCTGTTTGC
[...]
```

Example a: Aligning viral genomes
- To get a multiple alignment a phylogenetic tree and the following three steps

are necessary: 1. Run `blastz` each vs. each 2. Combine blastz results to multiple sequence alignments 3. Project raw alignments to a reference sequence. - The corresponding commands:

```
all_bz - "((NC_007605 NC_006146) NC_004367)" | bash
tba "((NC_007605 NC_006146) NC_004367)" \
*.sing.maf raw-tba.maf
maf_project raw-tba.maf NC_007605 > final.maf
```

- Note: The tree is given in NEWICK like format with blanks instead of commas. The sequence data files must be named exactly like the names in this tree and in the FASTA headers.

Example a: Running the pipeline I
- First the alignments are filtered and sliced in overlapping windows:

```
$ rnazWindow.pl < final.maf > windows.maf
```

- `RNAz` is run on these windows:

```
$ RNAz --both-strands --show-gaps --cutoff=0.5 windows.maf \
       > rnaz.out
```

- Overlapping hits are combined to "loci" and visualized on a web-site:

```
$ rnazCluster.pl --html rnaz.out > results.dat
```

Example a: Running the pipeline II
- The predicted hits are compared with available annotation of the genome:

```
$ rnazAnnotate.pl --bed annotation.bed results.dat \
         > results_annotated.dat
```

- The results file is formatted in a HTML overview page:

```
$ rnazIndex.pl --html results_annotated.dat \
         > results/index.html
```

Example a: Statistics on the results
- `rnazIndex.pl` can be used to generate a BED formatted annotation file which can be analyzed using `rnazBEDstats.pl` (after sorting, for the case the input alignments were unsorted)"

```
$ rnazIndex.pl --bed results.dat | \
        rnazBEDsort.pl | rnazBEDstats.pl
```

- RNAzfilter.pl can be used to filter the results by different criteria. In this case it gives us all loci with P$>$0.9":

```
$ rnazFilter.pl "P>0.9" results.dat | \
        rnazIndex.pl --bed | \
        rnazBEDsort.pl | rnazBEDstats.pl
```

- To get an estimate on the (statistical) false positives one can repeat the complete screen with randomized alignments:

```
$ rnazRandomizeAln final.maf > random.maf
```

Example b: Obtaining pre-computed alignments from UCSC
- Go to the UCSC genome browser (http://genome.ucsc.edu) and go to "Tables". Download "multiz17" alignments in MAF format for the region: chr11:93103000-93108000

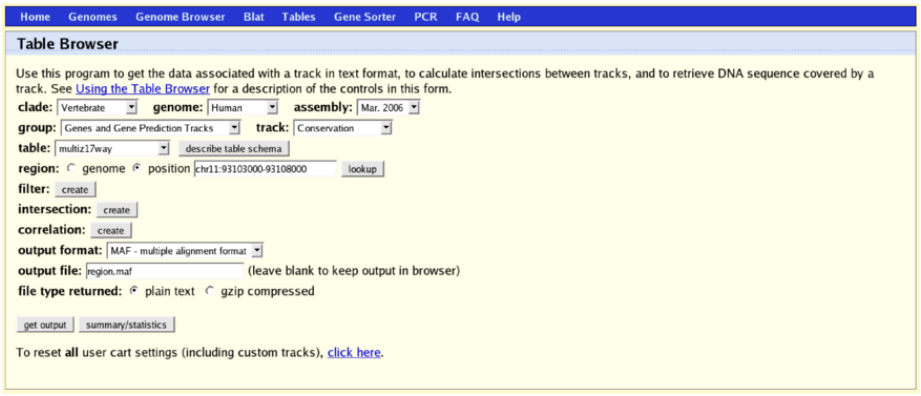


Figure 8: pict

Example b: Running the pipeline
- The Perl scripts are run in the same order as in Example 1:

```
$ rnazWindow.pl --min-seqs=4 region.maf > windows.maf
$ RNAz --both-strands --show-gaps --cutoff=0.5 windows.maf \
        > rnaz.out
$ rnazCluster.pl --html rnaz.out > results.dat
$ rnazAnnotate.pl --bed annotation.bed results.dat \
        > results_annotated.dat
$ rnazIndex.pl --html results_annotated.dat \
        > results/index.html
```

- The results can be exported as UCSC BED file which can be displayed in the genome browser:

```
$ rnazIndex.pl --bed --ucsc results.dat > prediction.bed
```

Example b: Visualizing the results on the genome browser
- Upload the BED file as "Custom Track"...

Figure 9: pict

- ...and have a look at the results:

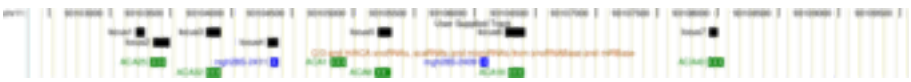


Figure 10: pict

\let \prOteCt \relax \Protect \gl:nopartrue

- RNA Web Services
  - Useful Web Services
- Get started
  - Typographical Conventions
  - Data Files
  - Terminal, Command line and Editor
  - Installing Software from Source
  - Build the `ViennaRNA Package`
  - What's in the `ViennaRNA Package`
  - The Input File Format

- Structure Prediction on single Sequences
  - The Program `RNAfold`
  - The Program `RNApvmin`
  - The Program `RNAsubopt`
- RNA folding kinetics
  - RNA2Dfold
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Legal Details