SSU-align User’s Guide

Structural alignment of small subunit ribosomal RNA sequences

http://infernal.janelia.org/
Version @RELEASE@; @RELEASEDATE@

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1 Introduction

INFERNAL is a software package that allows you to make consensus RNA secondary structure profiles, and use them to search nucleic acid sequence databases for homologous RNAs, or to create new structure-based multiple sequence alignments.

To make a profile, you need to have a multiple sequence alignment of an RNA sequence family, and the alignment must be annotated with a consensus RNA secondary structure. The program **`cmbuild`** takes an annotated multiple alignment as input, and outputs a profile.

You can then use that profile to search a sequence database for homologs, using the program **`cmsearch`**.

You can also use the profile to align a set of unaligned sequences to the profile, producing a structural alignment, using the program **`cmalign`**. This allows you to build hand-curated representative alignments of RNA sequence families, then use a profile to automatically align any number of sequences to that profile. This seed alignment/full alignment strategy combines the strength of stable, carefully human-curated alignments with the power of automated updating of complete alignments as sequence databases grow. This is the strategy used to maintain the RFAM database of RNA multiple alignments and profiles.

INFERNAL is comparable to HMMER (http://hmmer.janelia.org). The HMMER software package builds profile hidden Markov models (profile HMMs) of multiple sequence alignments. Profile HMMs capture only primary sequence consensus features. INFERNAL models are profile stochastic context-free grammars (profile SCFGs). Profile SCFGs include both sequence and RNA secondary structure consensus information.

INFERNAL is slow and CPU-intensive. You will probably need a large number of CPUs in order to use it for serious work.

2 Installation

Quick installation instructions

Download the source tarball (**`infernal.tar.gz`**) from ftp://selab.janelia.org/pub/software/infernal/ or http://infernal.janelia.org

Unpack the software:

```
> tar xvf infernal.tar.gz
```

Go into the newly created top-level directory (named either **`infernal`**, or **`infernal-xx`** where **`xx`** is a release number:

```
> cd infernal
```

Configure for your system, and build the programs:

```
> ./configure
> make
```

Run the automated testsuite. This is optional. All these tests should pass:

```
> make check
```

The programs are now in the **`src/`** subdirectory. The user's guide (this document) is in the **`documentation/userguide`** subdirectory. The man pages are in the **`documentation/manpages`** subdirectory. You can manually move or copy all of these to appropriate locations if you want. You will want the programs to be in your $PATH.

Optionally, you can install the man pages and programs in system-wide directories. If you are happy with the default (programs in **`/usr/local/bin/`** and man pages in **`/usr/local/man/man1`**), do:

```
> make install
```

That's all. More complete instructions follow, including how to change the default installation directories for **`make install`**.

More detailed installation notes

INFERNAL is distributed as ANSI C source code. It is designed to be built and used on UNIX platforms. It is developed on Intel GNU/Linux systems, and intermittently tested on a variety of other UNIX platforms. It is not currently tested on either Microsoft Windows or Apple OS/X, but it should work there; it should be possible to build it on any platform with an ANSI C compiler. The software itself is vanilla POSIX-compliant ANSI C. You may need to work around the configuration scripts and Makefiles to get it built on a non-UNIX platform.

The GNU configure script that comes with INFERNAL has a number of options. You can see them all by doing:

```
> ./configure --help
```

All customizations can and should be done at the **`./configure`** command line, unless you're a guru delving into the details of the source code.

setting installation targets

The most important options are those that let you set the installation directories for **`make install`** to be appropriate to your system. What you need to know is that INFERNAL installs only two types of files: programs and man pages. It installs the programs in **`--bindir`** (which defaults to **`/usr/local/bin`**), and the man pages in the **`man1`** subdirectory of **`--mandir`** (default **`/usr/local/man`**). Thus, say you want **`make`**

install to install programs in **/usr/bioprogs/bin/** and man pages in **/usr/share/man/man1**; you would configure with:

```
> ./configure --mandir=/usr/share/man --bindir=/usr/bioprogs/bin
```

That's really all you need to know, since INFERNAL installs so few files. But just so you know; GNU configure is very flexible, and has shortcuts that accomodates several standard conventions for where programs get installed. One common strategy is to install all files under one directory, like the default **/usr/local**. To change this prefix to something else, say **/usr/mylocal/** (so that programs go in **/usr/mylocal/bin** and man pages in **/usr/mylocal/man/man1**, you can use the **--prefix** option:

```
> ./configure --prefix=/usr/mylocal
```

Another common strategy (especially in multiplatform environments) is to put programs in an architecture-specific directory like **/usr/share/Linux/bin** while keeping man pages in a shared, architecture-independent directory like **/usr/share/man/man1**. GNU configure uses **--exec-prefix** to set the path to architecture dependent files; normally it defaults to being the same as **--prefix**. You could change this, for example, by:

```
> ./configure --prefix=/usr/share --exec-prefix=/usr/share/Linux/
```

In summary, a complete list of the **./configure** installation options that affect INFERNAL:

Option	Meaning	Default
--prefix=PREFIX	architecture independent files	**/usr/local/**
--exec-prefix=EPREFIX	architecture dependent files	EPREFIX
--bindir=DIR	programs	PREFIX/bin/
--mandir=DIR	man pages	PREFIX/man/

setting compiler and compiler flags

By default, **configure** searches first for the GNU C compiler **gcc**, and if that is not found, for a compiler called **cc**. This can be overridden by specifying your compiler with the **CC** environment variable.

By default, the compiler's optimization flags are set to **-g -O2** for **gcc**, or **-g** for other compilers. This can be overridden by specifying optimization flags with the **CFLAGS** environment variable.

For example, to use an Intel C compiler in **/usr/intel/ia32/bin/icc** with optimization flags **-O3 -ipo**, you would do:

```
> env CC=/usr/intel/ia32/bin/icc CFLAGS="-O3 -ipo" ./configure
```

which is the one-line shorthand for:

```
> setenv CC /usr/intel/ia32/bin/icc
> setenv CFLAGS "-O3 -ipo"
> ./configure
```

If you are using a non-GNU compiler, you will almost certainly want to set **CFLAGS** to some sensible optimization flags for your platform and compiler. The **-g** default generated unoptimized code. At a minimum, turn on your compiler's default optimizations with **CFLAGS=-O**.

turning on Message Passing Interface (MPI) support

INFERNAL includes four programs **cmsearch**, **cmcalibrate**, **cmalign** and **cmscore** that optionally use MPI parallelization by invoking the **--mpi** option. To enable the option to use MPI in these four executables, add **--enable-mpi** to the configuration command:

```
> ./configure --enable-mpi
```

To run a program in MPI mode, you must run them in an MPI environment with **`mpirun`** or **`mpiexec`**, with the **`--mpi`** option enabled. For example, in our LAM environment:

```
> mpirun C cmsearch --mpi query.cm target.fa
```

Other environments besides LAM MPI should work also, but may require different command syntax.

No longer supported: rigorous filters

Previous versions of INFERNAL included programs by Zasha Weinberg that implement rigorous filtering. The 1.0 release does not include these programs. If you'd like to use them you can either download Zasha's own implementation in RAVENNA from http://bliss.biology.yale.edu/ zasha/ravenna/ download an older 0.x version of INFERNAL, or try to modify this version to work with rigorous filters (the code is still included in `rigfilters/`).

Example configuration

The Intel GNU/Linux version installed at Janelia Farm is configured as follows:

```
> env CFLAGS="-O3" ./configure --enable-mpi --enable-lfs --prefix=/usr/local/infernal-1
```

3 Tutorial

Here's a tutorial walk-through of some small projects with INFERNAL. This section should be sufficient to get you started on work of your own, and you can (at least temporarily) skip the rest of the Guide.

The programs in INFERNAL

There are seven programs supported in the INFERNAL 1.0 package:

`cmalign`	Align sequences to an existing model.
`cmbuild`	Build a model from a multiple sequence alignment.
`cmcalibrate`	Determine expectation value scores (E-values) for more sensitive searches and appropriate HMM filter score cutoffs for faster searches.
`cmemit`	Emit sequences probabilistically from a model.
`cmscore`	Test the efficacy of different alignment algorithms. (Mainly useful for development and testing).
`cmsearch`	Search a sequence database for matches to a model.
`cmstat`	Report statistics on a model.

Files used in the tutorial

The subdirectory **`/tutorial`** in the INFERNAL distribution contains the files used in the tutorial, as well as a number of examples of various file formats that INFERNAL reads. The important files for the tutorial are:

`trna.5.sto` A multiple alignment of five tRNA sequences. This file is a simple example of *Stockholm format* that INFERNAL uses for structurally-annotated alignments.

`tosearch.300Kb.db` A 300,000 nt sequence "database" that contains a tRNA. The file is in FASTA format, which INFERNAL uses for unaligned sequence data.

`toalign.3.fa` Three tRNA sequences in unaligned FASTA format.

`toalign.1trunc.fa` A truncated tRNA sequence, actually the first sequence from **`toalign.3.fa`** with some 5' and 3' residues removed to demonstrate alignment of truncated sequences.

`toalign.1.fa` The first tRNA sequence from toalign.3.fa.

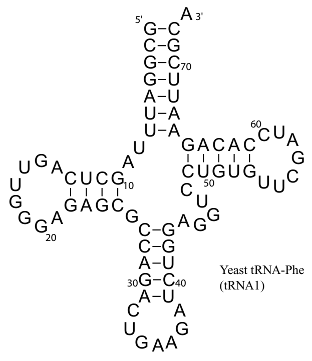
`my.c.cm` A calibrated version of a model built from **`trna.5.sto`**, included to save time.

Create a new directory that you can work in, and copy all the files in **`tutorial`** there. I'll assume for the following examples that you've installed the INFERNAL programs in your path; if not, you'll need to give a complete path name to the programs (e.g. something like **`/usr/people/nawrocki/infernal-1/src/cmbuild`** instead of just **`cmbuild`**).

Format of a simple input RNA alignment file

Look at the alignment file **`trna.5.sto`** in the **`intro/`** subdirectory of the INFERNAL distribution. It is shown below, with a secondary structure of the first sequence shown to the right for reference (yeast Phe tRNA, labeled as "tRNA1" in the file):

```
# STOCKHOLM 1.0

tRNA1            GCGGAUUUAGCUCAGUUGGG.AGAGCGCCAGACUGAAGAUCUGGAGGUCC
tRNA2            UCCGAUAUAGUGUAAC.GGCUAUCACAUCACGCUUUCACCGUGGAGA.CC
tRNA3            UCCGUGAUAGUUUAAU.GGUCAGAAUGGGCGCUUGUCGCGUGCCAGA.UC
tRNA4            GCUCGUAUGGCGCAGU.GGU.AGCGCAGCAGAUUGCAAAUCUGUUGGUCC
tRNA5            GGGCACAUGGCGCAGUUGGU.AGCGCGCUUCCCUUGCAAGGAAGAGGUCA
#=GC SS_cons     <<<<<<<..<<<<.........>>>>.<<<<<.......>>>>>.....<

tRNA1            UGUGUUCGAUCCACAGAAUUCGCA
tRNA2            GGGGUUCGACUCCCCGUAUCGGAG
tRNA3            GGGGUUCAAUUCCCCGUCGCGGAG
tRNA4            UUAGUUCGAUCCUGAGUGCGAGCU
tRNA5            UCGGUUCGAUUCCGGUUGCGUCCA
#=GC SS_cons     <<<<.......>>>>>>>>>>>>.
//
```



This is a simple example of a multiple RNA sequence alignment with secondary structure annotation, in *Stockholm format*. Stockholm format, the native alignment format used by HMMER and INFERNAL and the PFAM and RFAM databases, is documented in detail later in the guide.

For now, what you need to know about the key features of the input file is:

- The alignment is in an interleaved format, like other common alignment file formats such as CLUSTALW. Lines consist of a name, followed by an aligned sequence; long alignments are split into blocks separated by blank lines.
- Each sequence must have a unique name that has zero spaces in it. (This is important!)
- For residues, any one-letter IUPAC nucleotide code is accepted, including ambiguous nucleotides. Case is ignored; residues may be either upper or lower case.
- Gaps are indicated by the characters ., _, -, or ~. (Blank space is not allowed.)
- A special line starting with `#=GC SS_cons` indicates the secondary structure consensus. Gap characters annotate unpaired (single-stranded) columns. Base pairs are indicated by any of the following pairs: `<>`, `()`, `[]`, or `[]`. No pseudoknots are allowed; the open/close-brackets notation is only unambiguous for strictly nested base-pairing interactions.
- The file begins with the special tag line `# STOCKHOLM 1.0`, and ends with `//`.

Building a model with `cmbuild`

To build a model from this alignment, do:

```
    > cmbuild my.cm trna.5.sto
```

Almost instantly, **`cmbuild`** reads in the alignment, constructs a model, and saves that model to the new file **`my.cm`**. It is a convention to use the **`.cm`** suffix for model files; CM stands for "covariance model", another name for the profile SCFG architecture used by INFERNAL (Eddy and Durbin, 1994).

The output from **`cmbuild`** contains information about the size of your input alignment (in aligned columns and # of sequences), and some statistics describing the model that was constructed. You don't need to understand this to use the model, so for now we'll skip describing the output, and revisit it in the "Profile SCFG construction" section.

The result, the model file in **`my.cm`** is a text file. You can look at it (e.g. **`> more my.cm`**) if you like, but it isn't really designed to be human-interpretable. You can treat **`.cm`** files as compiled models of your RNA alignment.

> ▷ ***Can I build a model from a single sequence?*** *Yes. But a structure for the sequence must still be supplied. With single sequences, you can also build a RSEARCH (Klein and Eddy, 2003) CM using the* **`--rsearch`** *option to* **`cmbuild`***. There's more on this in a later section.*

> ▷ ***Can I build a model from unaligned sequences?*** *In principle, CMs can be trained from unaligned sequences; however, this functionality is not yet implemented in* INFERNAL*. I recommend CLUSTALW as an excellent, freely available multiple sequence alignment program. The original* **`covet`** *CM training program from COVE, the predecessor of* INFERNAL *is also still available by ftp.*

Calibrate the model with `cmcalibrate`

This step is optional, but we strongly recommend it because it will increase the sensitivity of your database search and potentially make it much faster.

When you search a sequence database, it is useful to get "E-values" (expectation values) in addition to raw scores. When you see a database hit that scores x, an E-value tells you the number of hits you would've expected to score x or more just by chance in a sequence database of this size.

Additionally, for some searches with some models it is possible to use an HMM filter to accelerate the search at a very small (theoretical) cost to sensitivity. Besides calibrating E-values, the **`cmcalibrate`** program determines when this acceleration is possible, and a "calibrated" model will automatically employ the HMM filter during search.

If a non-calibrated model is used to search a database, E-values will not be calculated, and a default HMM filter will be used at an unknown cost to sensitivity. There's an example of this in section 6.

Importantly, if you're not going to use a model for searching, there is no need to calibrate it. For example, if you are only going to build alignments with a model of a large family like small subunit ribosomal RNA, don't waste time calibrating it. **`cmsearch`** is the only INFERNAL program that uses E-values and HMM filters, so if you won't use it, don't calibrate your model.

> ▷ ***Do I really have to calibrate my model?*** *No, but we recommend it in most situations. Importantly, if you're not going to search with your model, then don't calibrate it (see above). If you are going to search with your model, you still are not required to calibrate your model. If you choose not to calibrate, you'll have to settle for default filter threshold cutoffs which will compromise sensitivity to an unknown degree and possibly not accelerate your search as much as possible. An example of searching with non-calibrated models is in section 6.*

CM calibration takes a long time, but it only has to be done once per model, and can potentially save a lot of time during database searches. The amount of time the calibration takes varies widely, but depends mainly on the size of the RNA family being modeled. So you can know what kind of a wait you're in for, the **`cmcalibrate`** has a **`--forecast <n>`** option which reports an estimate of the running time (**`<n>`** is the number of processors you'll use for calibration, for now **`<n>`** is 1, but if you're using MPI it could be higher). To estimate the time required for calibration of your tRNA model, type:

```
> cmcalibrate --forecast 1 my.cm
```

The program will dump about fifty lines to standard output but don't worry about any of it now except the line with "all" in the "stage" column towards the end:

```
# all         -    -    -          -      -       01:24:22
```

This line gives the total predicted time of this run of **`cmcalibrate`**, about 1 hour and twenty minutes. So that you don't have to wait an hour to do this step, we've included the file **`my.c.cm`**, a calibrated version of **`my.cm`**. The **`my.c.cm`** file was created with the same **`cmbuild`** command you just performed (except **`my.c.cm`** was used as the output file instead of **`my.cm`**) and then calibrated with:

```
> cmcalibrate my.c.cm
```

When **`cmcalibrate`** finished, the **`my.c.cm`** file had been updated to include information about E-values and HMM filter thresholds. More detail on what **`cmcalibrate`** does can be found in sections 5 and 6. To make things simpler for our tutorial, copy over the **`my.cm`** file you just made with the calibrated version:

`> cp my.c.cm my.cm`.

Searching a sequence database with `cmsearch`

You can use your model to search for new homologues of your RNA family. The file **`tosearch.300Kb.db`** contains an example sequence "database": one 300,000 nt sequence, with yeast tRNA-Phe embedded at position 101…173. The **`cmsearch`** also has a **`--forecast`** option to predict running times, which is useful if you're searching large database files. Since this database is relatively small, we'll just do the search:

```
> cmsearch my.cm tosearch.300Kb.db
```

First, the program will print a header and "Pre-search info" to the screen. This output is explained in detail later in section 6, don't worry about it right now. The search should take about 2 minutes.

`cmsearch` now searches both strands of each sequence in the target database, and returns alignments for high scoring hits. In this case 3 hits are returned. Look at the first hit:

```
CM: trna.5-1
>example

  Plus strand results:

 Query = 1 - 72, Target = 101 - 173
 Score = 78.06, E = 3.133e-21, P = 2.906e-26, GC =  53

          (((((((,,<<<<___.____>>>>,<<<<<_______>>>>>,,,,,<<<<<_______
        1 gCcgacAUaGcgcAgU.GGuAgcgCgccagccUgucAagcuggAGgUCCgggGUUCGAUu 59
          GC:+A::UAGC:CAGU GG AG:GCGCCAG:CUG+++A:CUGGAGGUCC:G:GUUCGAU
      101 GCGGAUUUAGCUCAGUuGGGAGAGCGCCAGACUGAAGAUCUGGAGGUCCUGUGUUCGAUC 160

          >>>>>))))))):
       60 CcccGUgucgGca 72
          C:C:G::U+:GCA
      161 CACAGAAUUCGCA 173
```

The first line gives the name of the CM (this can be defined in the input Stockholm alignment file or as an option to **`cmbuild`**, as described later). Next comes the results section, the name of each target sequence in the target database is given starting with a $>$, in this case there is only one: **`example`**. Next, all the hits to the top (Watson) strand of **`example`** are given, in this example there is a single hit from position 101 to 173 with a score of 78.06 bits. The E-value of this hit is $3.133e - 21$, this is the number hits we expect to find with a bit score of 78.06 or better if we were searching a database of random sequences of total length 300,000. So this is a really good hit. Bit scores and E-values are discussed in more detail in section 5.

The alignment is shown in a BLAST-like format, augmented by secondary structure annotation.

The top line shows the predicted secondary structure of the target sequence. The format is a little fancier and more informative than the simple least-common-denominator format we used in the input alignment file. It's designed to make it easier to see the secondary structure by eye. The format is described in detail later; for now, here's all you need to know. Base pairs in simple stem loops are annotated with `<>` characters. Base pairs enclosing multifurcations (multiple stem loops) are annotated with `()`, such as the tRNA acceptor stem in this example. In more complicated structures, `[]` and `{}` annotations also show up, to reflect deeper nestings of multifurcations. For single stranded residues, `_` characters mark hairpin loops; `-` characters mark interior loops and bulges; `,` characters mark single-stranded residues in multifurcation loops; and `:` characters mark single stranded residues external to any secondary structure. Insertions relative to this consensus are annotated by a `.` character.

The second line shows that consensus of the query model. The highest scoring residue sequence is shown. Upper case residues are highly conserved. Lower case residues are weakly conserved or unconserved.

The third line shows where the alignment score is coming from. For a consensus base pair, if the observed pair is the highest-scoring possible pair according to the consensus, both residues are shown in upper case; if a pair has a score of ≥ 0, both residues are annotated by : characters (indicating an acceptable compensatory base pair); else, there is a space, indicating that a negative contribution of this pair to the alignment score. For a single-stranded consensus residue, if the observed residue is the highest scoring possibility, the residue is shown in upper case; if the observed residue has a score of ≥ 0, a `+` character is shown; else there is a space, indicating a negative contribution to the alignment score.

Finally, the fourth line is the target sequence.

After the alignment is post-search information. This is explained later in section 6.

> ▷ ***How come the dbsize reported by `cmsearch` is twice the length of my database?*** *This is because by default **`cmsearch`** searches both strands of the database, so according to the program your database is twice the size you might think it is. You may have noticed this in the tutorial example, the 300 Kb database is reported as 600 Kb in the beginning of the **`cmsearch`** output. You can optionally search only the top strand or the bottom strand of your target database with the **`--toponly`** and **`--bottomonly`** options to **`cmsearch`** respectively.*

Creating new multiple alignments with `cmalign`

You can also use a model to structurally align any number of new RNA sequences to your consensus structure. This is how the RFAM database is constructed: we start with a "seed" alignment, build a CM of it, and use that CM to align all known members of the sequence family and create a "full" alignment. This allows us to maintain representative seed alignments that are stable and small enough to be human-curated, while still being able to automatically incorporate and align all homologues detected in the rapidly growing public sequence databases.

An example of three unaligned tRNA sequences are in the file **toalign.3.fa**. The first two sequences are real tRNAs. The third sequence, tRNA8 was created by deleting some residues out of the middle of the tRNA1 sequence from the file **trna.5.sto**. (tRNA8 will be used a little later to demonstrate local alignment.)

To align these sequences to the model we made in **my.cm**, do:

```
> cmalign my.cm toalign.3.fa
```

The output of cmalign is described later in detail. For now let's only look at the alignment:

```
# STOCKHOLM 1.0
#=GF AU Infernal 1.0

tRNA6        GUCCCGCUGGUGUAAU.GGAuAGCAUACGAUCCUUCUAAGUUUGCGG-UC
tRNA7        ACUUUUAAAGGAUAGU.AGUuUAUCCGUUGGUCUUAGGAACCAAAAA--A
tRNA8        GCGGAUUUAGCUCAGUuGGG.AGAGCGC------CAGAC----GAGGUCC
#=GC SS_cons (((((((,,<<<<___.___._>>>>,<<<<<_______>>>>>,,,,,<
#=GC RF      gCcgacAUaGcgcAgU.GGu.AgcgCgccagccUgucAagcuggAGgUCC

tRNA6        CUGGUUCGAUCCCAGGGCGGGAUA
tRNA7        UUGGUGCAACUCCAAAUAAAAGUA
tRNA8        UGUGUUCGAUCCACAGAAUUCGCA
#=GC SS_cons <<<<_______>>>>>))))))):
#=GC RF      gggGUUCGAUuCcccGUgucgGca
//
```

In the aligned sequences, a `.` character indicates an inserted column relative to consensus; the `.` character is an alignment pad. A `-` character is a deletion relative to consensus.

The symbols in the consensus secondary structure annotation line have the same meaning that they did in a pairwise alignment from **cmsearch**.

The `#=GC RF` line is *reference annotation*. Non-gap characters in this line mark consensus columns; **cmalign** uses the residues of the consensus sequence here, with upper case denoting strongly conserved residues, and lower case denoting weakly conserved residues. Gap characters (specifically, the `.` pads) mark insertions relative to consensus. As described below, **cmbuild** is capable of reading these RF lines, so you can specify which columns are consensus and which are inserts (otherwise, **cmbuild** makes an automated guess, based on the frequency of gaps in each column).

If you want to save the alignment to a file, you can use the **-o** option:

```
> cmalign -o my.sto my.cm toalign.3.fa
```

Local versus glocal alignment in `cmsearch` and `cmalign`

The programs **cmsearch** and **cmalign** can be run in two different modes. Glocal alignment requires that the entire model match a subsequence of the target (global with respect to the query model, local with respect to the target sequence). Local alignment allows only part of the model to match a subsequence of the target. Local alignment is useful when a homologous RNA structure has undergone enough changes that parts of the its structure cannot be aligned to the full consensus model. Empirically, local alignment is often a more sensitive search strategy so by default **cmsearch** uses local alignment. Glocal mode can be turned on in **cmsearch** using the **-g** option. Conversely, **cmalign** uses glocal alignment by default, and the local alignment mode can be switched on using the the **-l** option.

First let's look at an example of local alignment in **cmsearch**:

```
> cmsearch my.cm toalign.3.fa
```

Look at the alignment for the target sequence tRNA8 (the last alignment in the output):

```
>tRNA8

  Plus strand results:

 Query = 1 - 72, Target = 1 - 63
 Score = 54.39, E = 4.664e-17, P = 6.303e-19, GC =  56

           (((((((,,<<<<___.____>>>>,<~~~~~~>,,,,,<<<<<_______>>>>>))))
         1 gCcgacAUaGcgcAgU.GGuAgcgCgc*[15]*gAGgUCCgggGUUCGAUuCcccGUguc 68
           GC:+A::UAGC:CAGU GG AG:GCGC      GAGGUCC:G:GUUCGAU C:C:G::U+
         1 GCGGAUUUAGCUCAGUuGGGAGAGCGC*[ 5]*GAGGUCCUGUGUUCGAUCCACAGAAUU 59

           ))):
        69 gGca 72
           :GCA
        60 CGCA 63
```

The `*[15]*` and `*[5]*` in the query and target, respectively, indicate that 15 consensus residues and 5 target residues were left unaligned; the target does not appear to have the consensus structure in this region. (Not surprising, since I made the tRNA8 example sequence by deleting part of the anticodon stem.) The structure annotation line is marked with `~~~~~~` to indicate the gap in the alignment, and to distinguish local alignment induced gaps from normal insertions (which are marked with **.** characters).

You can activate local alignment in **cmalign** with the **-l** option:

```
> cmalign -l my.cm toalign.3.fa
```

This results in the following alignment: [1]

```
# STOCKHOLM 1.0
#=GF AU Infernal 1.0

tRNA6          GUCCCGCUGGUGUAAU.GGAuAGCAUACGAUCCUUCUAA.....GUUUGC
tRNA7          ACUUUUAAAGGAUAGU.AGUuUAUCCGUUGGUCUUAGGA.....ACCAAA
tRNA8          GCGGAUUUAGCUCAGUuGGG.AGAGCGC-----------cagac----GA
#=GC SS_cons   (((((((,,<<<<___.___._>>>>,<<<<<_______~~~~~>>>>>,
#=GC RF        gCcgacAUaGcgcAgU.GGu.AgcgCgccagccUgucAa~~~~~gcuggA

tRNA6          GG-UCCUGGUUCGAUCCCAGGGCGGGAUA
tRNA7          AA--AUUGGUGCAACUCCAAAUAAAAGUA
tRNA8          GGUCCUGUGUUCGAUCCACAGAAUUCGCA
#=GC SS_cons   ,,,,<<<<<_______>>>>>))))))):
#=GC RF        GgUCCgggGUUCGAUuCcccGUgucgGca
//
```

Note how the local alignment is represented for tRNA8. The deleted consensus columns are marked by - characters. The unaligned "insertion" is shown in its own columns; those columns are again marked with `~` characters in the consensus secondary structure annotation and the reference (RF) annotation lines.

[1]The discontinuity of structural local alignment presents a quandary for representing multiple alignments. On the one hand, you might not want to even show the unaligned target residues in the gap (e.g., cagac) – they aren't aligned to the model. On the other hand, you sort of expect that if you pull an RNA sequence out of a multiple alignment, it represents a true subsequence of a larger sequence, not a concatenation of disjoint subsequences – you'd at least like some indication of where some residues have gone missing. One option would be to leave a *[5]* in the gap, as in the pairwise representation; but one of the nice properties of Stockholm format is that it's easy to interconvert it to other alignment formats just by stripping off everything by the name/sequence part of the alignment, and sticking non-sequence characters like *[5]* in the alignment would prevent that.

Now you have successfully built a CM, calibrated it and used it search for and align new sequences using INFERNAL programs. These programs have some useful options that you haven't seen yet, which we discuss and in some cases demonstrate next. Before you start using INFERNAL, we strongly urge you to at least skim this part.

Important options to `cmbuild`

using optional annotation to completely specify model architecture

`cmbuild` needs to know two things to convert your alignment into a profile SCFG.

First, it needs to know the consensus secondary structure. It reads this from the `#=GC SS_cons` line, as described above. This annotation is mandatory.

It also needs to know which columns are consensus, and which columns are insertions relative to consensus. By default, it will determine this by a simple rule: if a column contains more than a certain fraction of gap characters (by default $>$50%, but this can be changed with the **`--gapthresh`** option), the column is called an insertion. This may not be what you want; for instance, maybe you are trying to iteratively build models based on larger and larger numbers of sequences (based on an RFAM seed, say), but you don't want the curated consensus model architecture to change just because you added some new sequences to the alignment.

You can optionally override that default and specify the complete architecture of the model, using both a `#=GC SS_cons` structure annotation line and a `#=GC RF` reference column annotation line. To do this, you use the **`--rf`** option to **`cmbuild`**.

For example, if **`trna.5.sto`** had `#=GC RF` annotation, to build a model from it called **`second.cm`** ith architecture dictated by the RF annotation, you would do:

```
> cmbuild --rf second.cm trna.5.sto
```

Since **`cmalign`** leaves an RF line on the alignments it generates, the **`--rf`** option allows you to propagate your consensus structure into new, larger alignments. The RF line is also handy when you want the model's coordinate system to be the same as a canonical, well-studied single sequence: you can simply use that sequence as the RF line, or manually create any consensus coordinate system you like. (This is the origin of RF as the "reference line", e.g. giving a reference coordinate system.) The only thing that matters in the RF line is nongap versus gap characters: the line can be as simple as x's marking consensus columns, `.`'s for insert columns.

creating multiple models from a single alignment

`cmbuild` has the ability to cluster and partition the input training alignment into several alignments based on sequence identity and build a separate CM from each of those alignments. This can be viewed as splitting the input alignment of a single RNA family into "subfamilies" based on sequence identity, each cluster being a subfamily of sequences more similar to each other than to those in other clusters. Performing homology search with these several models collectively may be a more sensitive search strategy than a single search with one model built from the entire alignment (although it will take longer). The most important options affecting this behavior are the **`--cmaxid <f>`** and **`--ctarget <n>`** options. With **`--cmaxid <f>`** the clusters are defined such that no two sequences in *different* clusters are more than **`<f>`** fractionally identical. With **`--ctarget <n>`**, a fractional identity cutoff is found that partitions the alignment into exactly **`<n>`** clusters. The **`--cdump <f>`** option can be used in combination with either of these options to cause **`cmbuild`** to dump each cluster alignment to the file **`<f>`**. Let's try the **`--cmaxid`** option:

```
> cmbuild --cmaxid 0.6 --cdump my.cmaxid60.sto my.cmaxid60.cm trna.5.sto
```

```
# Alignment split into 3 clusters; each will be used to train a CM.
# Maximum identity b/t any 2 seqs in different clusters: 0.60
#
#                                                                     rel entropy
#                                                                    ------------
#  aln  cm idx  name            nseq  eff_nseq    alen   clen   bps  bifs     CM    HMM
# ----  ------  ----------  --------  --------  ------  -----  ----  ----  -----  -----
     1       1  trna.5-1.1         1      1.00      74     73    21     2  0.693  0.409
     1       2  trna.5-1.2         2      1.31      74     73    21     2  0.696  0.395
     1       3  trna.5-1.3         2      1.41      74     72    21     2  0.704  0.407
```

In this case, the input alignment was split into three clusters, of 1 sequence, 2 sequences and 2 sequences. The file **`my.cmaxid60.sto`** includes these three alignments. The CM file **`my.cmaxid60.cm`** is a database of three CMs, one built from each of these three alignments.

The **`--corig`** option in combination with the **`--ctarget`** or **`--cmaxid`** options will cause **`cmbuild`** to build one extra model, the original default model from the entire alignment, and add it to the end of the cluster models. The **`--call`** option can be used instead of the **`--ctarget`** or **`--cmaxid`** options to build a separate model for each sequence in the training alignment.

refining the training alignment prior to building a model

Constructing structural RNA alignments is not easy. **`cmbuild`** offers one experimental option to potentially help automate this procedure. The **`--refine <f>`** option will cause **`cmbuild`** to go through a two step iterative procedure to realign the sequences in the input alignment before building the model. First, a model is built from the initial given alignment. This model is then used to optimally align all the sequences, giving a new alignment. The new alignment is used to build a new model and the sequences are realigned to the new model. This two step build/align procedure repeats until convergence, when two successive iterations yield identical (or very nearly identical) alignments. The final alignment is used to build a model which is saved to the CM file and saved to file **`<f>`**. Importantly, the **`--refine`** option will not change the consensus structure of the initial alignment, so this is not a structure prediction tool. The **`--gibbs`** option can be used in combination with **`--refine`** to modify it's behavior. Instead of choosing the optimal alignment during alignment refinement, with **`--gibbs`** an alignment is sampled from the posterior distribution of alignments given the current model.

building RSEARCH models

The RSEARCH program (Klein and Eddy, 2003) implements a special case of homology search with covariance models in which only one training sequence/structure is known. RSEARCH had forked from INFERNAL a few years ago, but has been reintegrated. The main difference between RSEARCH and INFERNAL is the determination of the model scoring parameters. Whereas INFERNAL uses mean posterior estimates (see the "parameterization" subsection of section 4), RSEARCH uses a RIBOSUM scoring matrix to determine emission scores. You can build CMs parameterized with RIBOSUM scores using the **`--rsearch <f>`** option where **`<f>`** is the RIBOSUM matrix file to use . (The matrix files are in the **`/matrices`** subdirectory of INFERNAL). For more on how these matrices were created see (Klein and Eddy, 2003). Importantly, you can only use the **`--rsearch`** option if the input training alignment has only 1 sequence, or if you use it in combination with the **`--call`** option which causes **`cmbuild`** to build a separate model from each sequence in the input alignment file.

Here's an example of building five **`rsearch`** CMs for tRNA:

```
    > cmbuild -F --rsearch  /infernal/matrices/RIBOSUM85-60.mat --call my.rsearch.cm
trna.5.sto
```

```
# Alignment split into 5 clusters; each comprised of exactly 1 sequence
#
#                                                                      rel entropy
#                                                                     ------------
#  aln  cm idx  name           nseq  eff_nseq    alen   clen   bps  bifs     CM    HMM
# ----  ------  ----------  --------  --------  ------  -----  ----  ----  -----  -----
     1       1  trna.5-1.1         1      1.00      74     73    21     2  1.022  0.739
     1       2  trna.5-1.2         1      1.00      74     72    21     2  1.033  0.758
     1       3  trna.5-1.3         1      1.00      74     72    21     2  1.035  0.777
     1       4  trna.5-1.4         1      1.00      74     72    21     2  1.016  0.741
     1       5  trna.5-1.5         1      1.00      74     73    21     2  1.014  0.752
```

▷ ***I used the `cmbuild --rsearch` option and tested my results against the RSEARCH program, and the scores didn't match. Why?*** *INFERNAL can't build models quite exactly the same as RSEARCH does. The main reason is because INFERNAL uses probabilistic transition scores while RSEARCH does not. This difference leads to large differences in the bit scores from the programs, but the E-values of those scores should be similar (see section 5 for more on bit scores and E-values.)*

Important options to `cmalign`

including a fixed alignment within the output alignment

When aligning sequences to a model, **`cmalign`** allows you to include the initial training alignment used to build the model within its output alignment. This could be useful if you're updating the training alignment with new homologs, or just to easily see how new sequences align in the context of the original alignment. To turn on this behavior, use the **`--withali <f>`** option to **`cmalign`**, where **`<f>`** is the existing fixed alignment to include. Note, if you used the **`--rf`** or **`--gapthresh <x>`** options to **`cmbuild`** when you built the model, you must use those same options to **`cmalign`**. Here is an example:

```
> cmalign --withali trna.5.sto my.cm toalign.3.fa
```

```
# STOCKHOLM 1.0
#=GF AU Infernal 1.0

tRNA1        GCGGAUUUAGCUCAGUuGGG.AGAGCGCCAGACUGAAGAUCUGGAGGUCC
tRNA2        UCCGAUAUAGUGUAAC.GGCuAUCACAUCACGCUUUCACCGUGGAGA-CC
tRNA3        UCCGUGAUAGUUUAAU.GGUcAGAAUGGGCGCUUGUCGCGUGCCAGA-UC
tRNA4        GCUCGUAUGGCGCAGU.GGU.AGCGCAGCAGAUUGCAAAUCUGUUGGUCC
tRNA5        GGGCACAUGGCGCAGUuGGU.AGCGCGCUUCCCUUGCAAGGAAGAGGUCA
tRNA6        GUCCCGCUGGUGUAAU.GGAuAGCAUACGAUCCUUCUAAGUUUGCGG-UC
tRNA7        ACUUUUAAAGGAUAGU.AGUuUAUCCGUUGGUCUUAGGAACCAAAAA--A
tRNA8        GCGGAUUUAGCUCAGUuGGG.AGAGCGC------CAGAC----GAGGUCC
#=GC SS_cons (((((((,,<<<<___.___._>>>>,<<<<<_______>>>>>,,,,,<
#=GC RF      gCcgacAUaGcgcAgU.GGu.AgcgCgccagccUgucAagcuggAGgUCC

tRNA1        UGUGUUCGAUCCACAGAAUUCGCA
tRNA2        GGGGUUCGACUCCCCGUAUCGGAG
tRNA3        GGGGUUCAAUUCCCCGUCGCGGAG
tRNA4        UUAGUUCGAUCCUGAGUGCGAGCU
tRNA5        UCGGUUCGAUUCCGGUUGCGUCCA
tRNA6        CUGGUUCGAUCCCAGGGCGGGAUA
tRNA7        UUGGUGCAACUCCAAAUAAAAGUA
tRNA8        UGUGUUCGAUCCACAGAAUUCGCA
#=GC SS_cons <<<<_______>>>>>))))))):
#=GC RF      gggGUUCGAUuCcccGUgucgGca
//
```

The top five sequences are from the training alignment **`trna.5.sto`** and the bottom three sequence are from **`toalign.3.fa`**.

▷ ***How come my fixed alignment didn't stay fixed in the `cmalign` output?*** *It is possible for the **`--withali`** alignment to change slightly when it is output from **`cmalign`**, but only residues that are insertions (i.e. present in non-consensus columns) should move around. This is a feature, not a bug, of the program; it is impossible for **`cmalign`** to always keep inserted residues in one sequence fixed with respect to inserted residues in another sequence. For more information on insertion columns versus consensus columns see the **`cmbuild`** section.*

▷ ***I'd like to use the `--withali` option to `cmalign` but my training alignment is hundreds of sequences deep which makes the cmalign output difficult to read, is there a way to include a subset of the training alignment in the output alignment?*** *Yes. You can remove as many sequences as you like from the training alignment if it you add `#=GC RF` annotation that defines the consensus columns the same way they were defined in **`cmbuild`** using the full alignment. Also, in this case you must specify the **`--rf`** option to **`cmalign`**.*

aligning truncated sequences

By default, the **`cmalign`** program assumes that the target sequences it is aligning are full length as they appear in their genomic context. However some sequences you want to align may be truncated, i.e. have missing residues at the 5’ and/or 3’ end . For example, sequences from shotgun sequencing projects or 16S SSU ribosomal RNA sequences from PCR based environmental surveys are often truncated. If you think the sequences you’re aligning are potentially truncated, you should use the **`--sub`** option to **`cmalign`**. Without this option, truncated sequences are often terribly misaligned.

Here is an example with a single, artificially truncated tRNA sequence. The file **`toalign.1trunc.fa`** includes a truncated version the sequence in **`toalign.1.fa`** (residues 1-20 and 57-72 were removed) that was aligned as the example above for the **`--withali`** option. If we align the truncated sequence **without** the **`--sub`** option:

```
> cmalign --withali trna.5.sto my.cm toalign.1trunc.fa
```

```
# STOCKHOLM 1.0
#=GF AU Infernal 1.0

tRNA1        GCGGAUUUAGCUCAGUuGGG.AGAGCGCCAGACUGAAGAUCUGGAGGUCC
tRNA2        UCCGAUAUAGUGUAAC.GGCuAUCACAUCACGCUUUCACCGUGGAGA-CC
tRNA3        UCCGUGAUAGUUUAAU.GGUcAGAAUGGGCGCUUGUCGCGUGCCAGA-UC
tRNA4        GCUCGUAUGGCGCAGU.GGU.AGCGCAGCAGAUUGCAAAUCUGUUGGUCC
tRNA5        GGGCACAUGGCGCAGUuGGU.AGCGCGCUUCCCUUGCAAGGAAGAGGUCA
tRNA6-trunc  AG--------------.--C.A---UACGAUCCUUCUAAGUUUGCGGUCC
#=GC SS_cons (((((((,,<<<<___.___._>>>>,<<<<<_______>>>>>,,,,,<
#=GC RF      gCcgacAUaGcgcAgU.GGu.AgcgCgccagccUgucAagcuggAGgUCC

tRNA1        UGUGUUCGAUCCACAGAAUUCGCA
tRNA2        GGGGUUCGACUCCCCGUAUCGGAG
tRNA3        GGGGUUCAAUUCCCCGUCGCGGAG
tRNA4        UUAGUUCGAUCCUGAGUGCGAGCU
tRNA5        UCGGUUCGAUUCCGGUUGCGUCCA
tRNA6-trunc  U-------------GG-----UUC
#=GC SS_cons <<<<_______>>>>>))))))):
#=GC RF      gggGUUCGAUuCcccGUgucgGca
//
```

We know the alignment of the truncated sequence **`tRNA6-trunc`** is wrong because we saw the correct alignment of the full tRNA6 sequence in the example above. Particularly striking is the misalignment of the 3’ end (**`UGGUUC`**) which you’d think based on the other sequences would be easily alignable.

Here’s what happens with the **`--sub`** option turned on:

```
> cmalign --sub --withali trna.5.sto my.cm toalign.1trunc.fa
```

```
# STOCKHOLM 1.0
#=GF AU Infernal 1.0

tRNA1        GCGGAUUUAGCUCAGUuGGG.AGAGCGCCAGACUGAAGAUCUGGAGGUCC
tRNA2        UCCGAUAUAGUGUAAC.GGCuAUCACAUCACGCUUUCACCGUGGAGA-CC
tRNA3        UCCGUGAUAGUUUAAU.GGUcAGAAUGGGCGCUUGUCGCGUGCCAGA-UC
tRNA4        GCUCGUAUGGCGCAGU.GGU.AGCGCAGCAGAUUGCAAAUCUGUUGGUCC
tRNA5        GGGCACAUGGCGCAGUuGGU.AGCGCGCUUCCCUUGCAAGGAAGAGGUCA
tRNA6-trunc  ----------------.---.AGCAUACGAUCCUUCUAAGUUUGCGG-UC
#=GC SS_cons (((((((,,<<<<___.___._>>>>,<<<<<_______>>>>>,,,,,<
#=GC RF      gCcgacAUaGcgcAgU.GGu.AgcgCgccagccUgucAagcuggAGgUCC

tRNA1        UGUGUUCGAUCCACAGAAUUCGCA
tRNA2        GGGGUUCGACUCCCCGUAUCGGAG
tRNA3        GGGGUUCAAUUCCCCGUCGCGGAG
tRNA4        UUAGUUCGAUCCUGAGUGCGAGCU
tRNA5        UCGGUUCGAUUCCGGUUGCGUCCA
tRNA6-trunc  CUGGUUC-----------------
#=GC SS_cons <<<<_______>>>>>))))))):
#=GC RF      gggGUUCGAUuCcccGUgucgGca
//
```

This is much better. Briefly, the **--sub** option uses an HMM to predict where the start and end points of the alignment are and then restructures the model to only align between that start and end. This is a hack that usually works, but there's more well-principled ways of doing this, which we're currently working on.

alignment confidence estimates

cmalign has the ability to estimate the confidence of the alignment at each residue for each sequence. These confidence estimates are actually posterior probabilities, which can be added to the output alignment using the **-p** option. We'll demonstrate this with alignment of a single sequence:

```
> cmalign -p my.cm toalign.1.fa
```

```
# STOCKHOLM 1.0
#=GF AU Infernal 1.0

tRNA6              GUCCCGCUGGUGUAAUGGAuAGCAUACGAUCCUUCUAAGUUUGCGG-UCC
#=GR tRNA6 POSTX.  9999999999999999887799999999999999999999999999-899
#=GR tRNA6 POST.X  9999999999999999119999999999999999999999999999-977
#=GC SS_cons       (((((((,,<<<<______._>>>>,<<<<<_______>>>>>,,,,,<<
#=GC RF            gCcgacAUaGcgcAgUGGu.AgcgCgccagccUgucAagcuggAGgUCCg

tRNA6              UGGUUCGAUCCCAGGGCGGGAUA
#=GR tRNA6 POSTX.  99999999999999999999999
#=GR tRNA6 POST.X  89999999999998779999999
#=GC SS_cons       <<<_______>>>>>))))))):
#=GC RF            ggGUUCGAUuCcccGUgucgGca
//
```

The **-p** option adds #=GR `tRNA6 POSTX.` and #=GR `tRNA6 POST.X` markup to the output stockholm alignment. The `POSTX.` row indicates the 'tens' place of the confidence estimate while `POST.X` row indicates the 'ones' place. So the confidence estimate for the final **C** in the first row of the alignment to two significant digits is 97%. This means that if you sampled alignments from the posterior distribution of all possible alignments of this sequence to the model, about 97% of the time that **C** would appear in that position of the alignment. One special case: if the posterior probability is “very nearly” 100% (it’s difficult to be more precise on the exact percentage due to numerical precision issues) the annotated posterior values will be “*” characters in both the tens and one places.

Putting it all together: an example of iterative search

Now that you've seen some useful features of **`cmbuild`** and **`cmalign`**, we're ready to go through an example of an iterative search with a CM with real sequence data. Iterative search is a powerful technique for finding homologs consisting of three main steps:

1. Build and calibrate a model from current alignment of homologs.
2. Search genomes (or databases) for new homologs.
3. Add the new homologs to the alignment.
4. Go back to step 1.

You can iterate over these steps as long as you'd like or until until you stop finding new homologs in step 2. The example we present here starts with only 1 training sequence/structure in the first iteration. In this case, with few sequences in the training alignment, it's smart to carefully select the genomes you search in step 2, picking ones that are rather closely related to the organisms represented in your alignment. Then, if you're able to find homologs in these closely related genomes, you can build a new alignment in step 3 which, because it's built from a deeper alignment, will have a more knowledge of the sequence divergence of the family than the initial model. You can then search genomes a bit further away on the tree of life in the next iteration. Then realign any new homologs you find and build a new model, and so on.

files used in the iterative search example

Let's work through an example of iterative search with a Purine riboswitch model. Here's a list of the files we'll use, from the **`/tutorial`** subdirectory of INFERNAL:

`purine.1.sto` A Stockholm alignment file with a single Purine riboswitch sequence and structure.

`purine.1.c.cm` A calibrated version of a model built from **`purine.1.sto`**, included to save time.

`T.tengcongensis.genome.fa` : the 2.5 Mb genome of the bacteria *Thermoanaerobacter tengcongensis*, in FASTA format, downloaded from the NCBI CoreNucleotide database (accession: NC_003869).

`C.psychrerythraea.genome.fa` : the 5 Mb genome of the bacteria *Colwellia psychrereythraea*, in FASTA format, downloaded from the NCBI CoreNucleotide database (accession: CP000083).

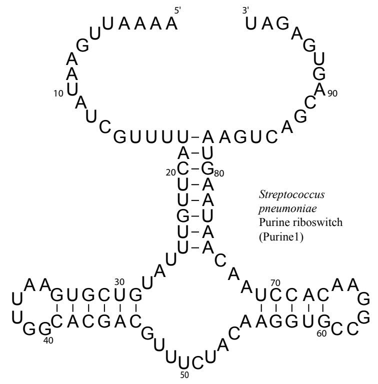
`purine.teng.fa` A putative Purine riboswitch in FASTA format.

`purine.psych.fa` A different putative Purine riboswitch in FASTA format.

iteration 1, step 1: build and calibrate a model

Look at the **`purine.1.sto`** stockholm file. It contains exactly one sequence and predicted structure of a Purine riboswitch from the genome of *Streptococcus pneumoniae*, a member of the Firmicutes division of the Bacterial domain. The structure from the `#=GC SS_cons` annotation for the sequence is shown on the right. (This sequence is part of the Rfam 8.1 Purine RF00167 "full" alignment).

```
# STOCKHOLM 1.0
#=GF ID    Purine
#=GF AU    Boese B, Barrick JE, Breaker RR

Purine1          AAAAUUGAAUAUCGUUUUACUUGUUUAUGUCGUGAAUUGGCACGACGUUU
#=GC SS_cons     .................<<<<<<<<...<<<<<<......>>>>>>....

Purine1          CUACAAGGUGCCGGAACACCUAACAAUAAGUAAGUCAGCAGUGAGAU
#=GC SS_cons     ....<<<<<<......>>>>>>..>>>>>>>>..............
//
```



First we build the model:

```
> cmbuild purine.1.cm purine.1.sto
```

Now we want to calibrate it. As before, we can use **--forecast** to see the predicted running time:

```
> cmcalibrate --forecast 1 purine.1.cm
```

It should take about two hours. Feel free to calibrate the model yourself if you want to, but to save time we've included **purine.1.c.cm** a calibrated version of the single sequence Purine model. To use our calibrated model, copy it over the model you just built with:

```
> cp purine.1.c.cm purine.1.cm
```

Now we're ready to search genomes. As mentioned earlier, at early stages of iterative search if you've built a model from very few training sequences you should carefully pick target genomes to search that are not too evolutionarily distant from the genomes of your training sequences. In this case let's search another Firmicutes bacteria, *Thermoanerobacteria tengcongensis*, the genome of which is in **T.tengcongensis.genome.fa**:

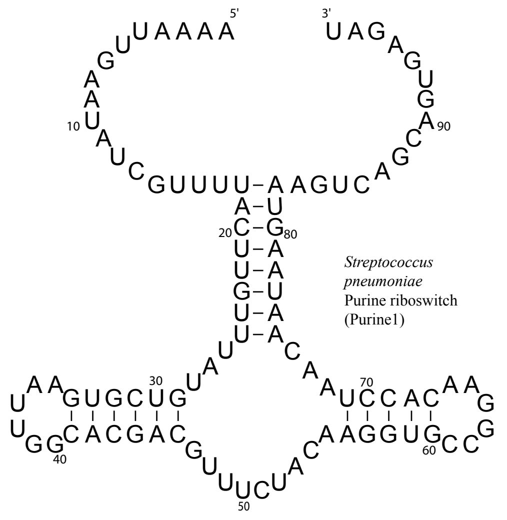
iteration 1, step 2: search a genome

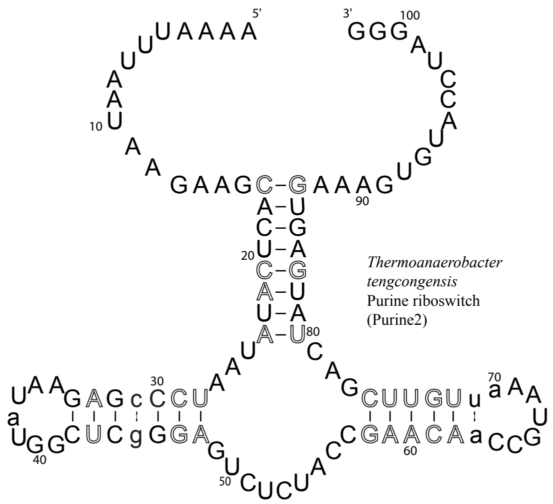
```
> cmsearch purine.1.cm T.tengcongensis.genome.fa
```

This will take about twenty minutes for **cmsearch** to search the 5 Mb genome. (If you don't want to wait, please continue reading). Let's look at the first hit:

```
>gi|20806542|ref|NC_003869.1|

  Plus strand results:

 Query = 1 - 97, Target = 586366 - 586467
 Score = 35.67, E = 3.979e-06, P = 3.121e-12, GC =  40

          :::::::::::::::::((((((((,,,<<<<<<.______..>>>>>>,,,,,,,,<<<
        1 AAAAUUGAAUAUCGUUUuaCuuguuUAUGuCGuG.AAUUGG..CaCGaCGUUUCUACaaG 57
          AAAAUU AAUA  G   :ACU::U:UA ::C::: AAU  G  :::G::GU UCUAC:::
   586366 AAAAUUUAAUAA-GAAGCACUCAUAUAAUCCCGAgAAUAUGgcUCGGGAGUCUCUACCGA 586424

          <<<.______..>>>>>>,,))))))))::::::::::::::::
       58 GuG.CCGGAA..CaCCuaACaauaaGuaAGUCAGCAGUGAGAU 97
          ::: CCG AA  ::::::AC:A::AGU:A    G A   AG
   586425 ACAaCCGUAAauUGUUCGACUAUGAGUGAAAGUGUACCUAGGG 586467
```

The E-value of this hit is 3.979e-06, which means we'd expect about 0.000003979 hits of this score in searching a 5 Mb database of random sequence. Obviously an E-value by itself doesn't prove this is a homologous sequence, but this is a good hit, and it warrants closer scrutiny. Let's look at the structure more closely and how it relates to our initial training sequence. As we saw earlier in the tutorial, the **cmsearch** output is showing us where the high score is coming from. Included below is another view of the secondary structures. The figure on the left is the structure of the *Streptococcus pneumoniae* Purine riboswitch that we built our model from (this exact figure is also shown above). The figure on the right is our putative homolog from *Thermoanaerobacter tengcongensis*. Base-paired residues that are different from the training sequence are indicated as hollow outlined letters. Insertions relative to the consensus model are in lowercase (these residues are also lower case in the **cmsearch** alignment above). Notice that all the base-paired residues that are different between the sequences are putative *compensatory mutations* from one Watson-Crick (A-U, U-A, C-G, G-C) or U-G/G-U base-pair to another. You can also see this above in the **cmsearch** output. This is very strong evidence that these two sequences are homologous as they share strong structural similarity despite weak conservation at the primary sequence level (55% identity).





iteration 1, step 3: add new homolog to training alignment

Let's assume that you've convinced yourself our putative homolog is a real Purine riboswitch. Now we can use knowledge of this new homolog to increase our power in the search for new ones. First we need to add our new sequence to our initial training alignment. This requires extracting the hit from the genome. We've already done this for you, the single hit from the *Thermoanerobacteria* genome is in unaligned FASTA format in the file **`purine.2.fa`**. Let's align it to our training sequence, and output both sequences aligned together using the **`--withali`** option. We'll save the output alignment to **`purine.2.sto`**:

```
cmalign -o purine.2.sto --withali purine.1.sto purine.1.cm purine.teng.fa
```

Now we've completed one round of the iterative search strategy listed above and we're ready to do another round, armed with an alignment of two examples of the Purine riboswitch. First, we build a new model:

iteration 2, step 1: build and calibrate a new model

```
> cmbuild purine.2.cm purine.2.sto
```

Once again the calibration step is a bottleneck. We've provided a calibrated file so you can proceed with this tutorial in **`purine.2.c.cm`**, copy this file over your own model:

```
> cp purine.2.c.cm purine.2.cm
```

iteration 2, step 2: search a genome

Now we're at the search stage. In the previous iteration we searched a Firmicutes genome mainly because we only had one training sequence, from Firmicutes. Now we have two training sequences, both from Firmicutes, but actually they're pretty divergent at the sequence level. Let's do a more ambitious search this time, and look for Purine riboswitch homologs in the genome of *Colwellia psychrerythraea*, a member of the Bacterial γ-proteobacteria division:

```
> cmsearch purine.2.cm C.psychrerythraea.genome.fa
```

This search will take about 50 minutes. Two hits are found. Let's look at the first, highest-scoring hit:

```
>gi|71143482|gb|CP000083.1|

  Plus strand results:

 Query = 19 - 87, Target = 1401709 - 1401775
 Score = 39.23, E = 2.291e-06, P = 8.005e-13, GC =  45

           (((((((,,,<<<-<<<_______>>>->>>,,,,,,,<<<<<<_________>>>>>>
        19 ACucauaUAagcCcGaGAAUAUGGCuCgGgcGUuUCUACcgggcgACCGuAAAucgcccg 78
           ::UC:UAUAA:CCC: : AUAUGG: :GGG:GU+UCUACC:GG:  C  UAA   :CC:G
   1401709 CUUCGUAUAACCCCAGUGAUAUGGAUUGGGGGUCUCUACCAGGAACCAAUAA--AUCCUG 1401766

           ,,)))))))
        79 ACuaugaGU 87
           A UA:GA::
   1401767 AUUACGAAG 1401775
```

This hit looks very promising. As before, even with a good E-value (this one is 2.291e-06), a hit is not necessarily a real homolog, but this hit is definitely worth looking at more closely. The **`cmsearch`** alignment is showing us that the loop regions have high primary sequence conservation and the stems exhibit compensatory mutations. The figure below on the left shows the sequence and structure of the putative homolog. To me (and hopefully to you) this hit is convincing.

iteration 2, step 3: add new homolog to training alignment

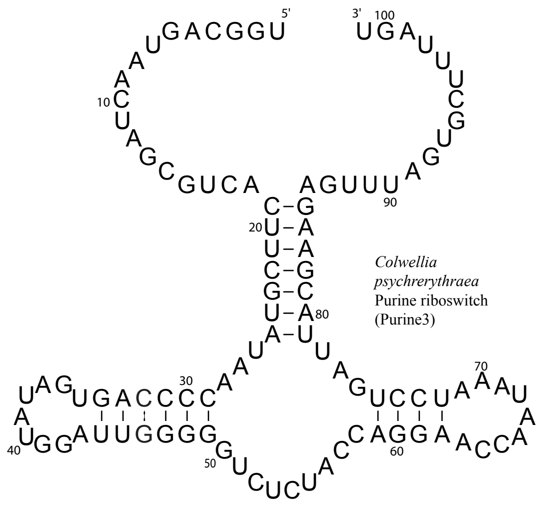
To save time we've extracted this sequence from the genome and saved it as **`purine.psych.fa`**. Let's align it our model:

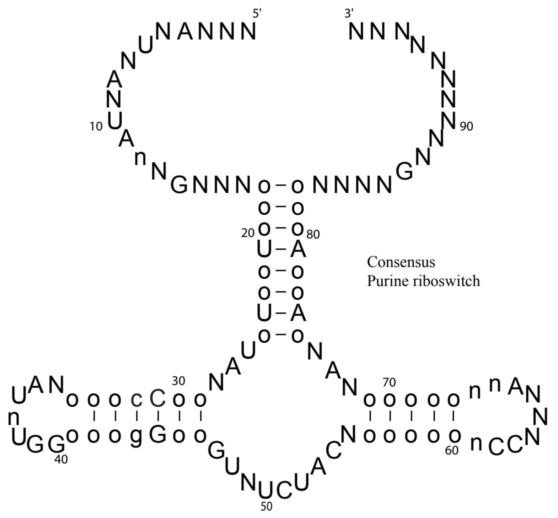
```
> cmalign -o purine.3.sto --withali purine.2.sto purine.2.cm purine.psych.fa
```

This alignment is depicted in the structure on the right below. Each column of the alignment is represented by a residue. Lowercase residues indicate positions where are least one sequence is a gap. For single-stranded residues: Ns denote any column that does not have identical residues in all three sequences, and non-Ns indicate all three sequence are identical. Any base-pair for which at least two distinct Watson-Crick or U-G/G-U pairs are present are indicated by open circles. Notice that 16 of the 20 base pairs show compensatory mutations between at least two of our three sequences.

Now, in theory we're ready for another round of iterative search. However, this is as far as we'll go with the guided tutorial.

This example was contrived to showcase the power of CMs to detect homologous structural RNAs with very little primary sequence conservation. The iterative approach is a powerful one, and we'd love to be able to automate it, but we haven't tried. Mainly because we're seriously impeded by the incredibly slow calibration step. As E-values for CMs become more completely understood, we hope to be able to streamline calibration and implement automated iterative search routines.





Parallelizing search, alignment and calibration with Message Passing Interface (MPI)

As mentioned in the Installation section, four of the seven INFERNAL programs can be run in parallel using MPI: **`cmalign`**, **`cmcalibrate`**, **`cmscore`** and **`cmsearch`**. These programs must be run using **`mpirun`**. These MPI programs are under current development, and we have only tested them using the LAM implementation of MPI. Here are example runs using LAM:

```
> mpirun C cmsearch --mpi query.cm target.fa

> mpirun C cmcalibrate --mpi query.cm

> mpirun C cmalign --mpi query.cm target.fa

> mpirun C cmscore --mpi query.cm
```

Getting more information

For a quick refresher on the command line usage of any program and its commonly used options, just type the name of the program with no other arguments: e.g.

```
> cmemit
```

and you'll get a brief help:

```
> cmemit
Incorrect number of command line arguments.
Usage: cmemit [-options] <cmfile> <sequence output file>

  where basic options are:
  -h        : show brief help on version and usage
  -n <n>    : generate <n> sequences  [10]  (n>0)
  -u        : write generated sequences as unaligned FASTA  [default]
  -a        : write generated sequences as a STOCKHOLM alignment
  -c        : generate a single "consensus" sequence only
  -l        : local; emit from a locally configured model
  -s <n>    : set random number generator seed to <n>  (n>0)
  --devhelp : show list of otherwise undocumented developer options

To see more help on other available options, do cmemit -h
```

To see more help on other available options, do **`cmemit -h`**

```
> cmemit -h


# cmemit :: generate sequences from a covariance model
# INFERNAL 1.0 (June 2008)
# Copyright (C) 2001-2008 HHMI Janelia Farm Research Campus
# Freely distributed under the GNU General Public License (GPL)
# - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Usage: cmemit [-options] <cmfile> <sequence output file>

where general options are:
  -h        : show brief help on version and usage
  -n <n>    : generate <n> sequences  [10]  (n>0)
  -u        : write generated sequences as unaligned FASTA  [default]
  -a        : write generated sequences as a STOCKHOLM alignment
  -c        : generate a single "consensus" sequence only
  -l        : local; emit from a locally configured model
  -s <n>    : set random number generator seed to <n>  (n>0)
  --devhelp : show list of otherwise undocumented developer options

miscellaneous output options are:
  --rna       : output alignment as RNA sequence data  [default]
  --dna       : output alignment as DNA (not RNA) sequence data
  --tfile <f> : dump parsetrees to file <f>

expert options:
  --exp <x>   : exponentiate CM probabilities by <x> before emitting  (x>0)
  --begin <n> : truncate alignment, begin at match column <n>  (n>=1)
  --end <n>   : truncate alignment,   end at match column <n>  (n>=1)
```

More detailed information on usage and command line options is available in UNIX manual pages. If they have been installed for your system, you can see this information with, e.g.:

```
> man cmalign
```

Copies of the man pages are also provided at the end of this guide.

References

Eddy, S. R. and Durbin, R. (1994). RNA sequence analysis using covariance models. *Nucl. Acids Res.*, 22:2079–2088.

Klein, R. J. and Eddy, S. R. (2003). RSEARCH: finding homologs of single structured RNA sequences. *BMC Bioinformatics*, 4:44.