RNA Structure Framework (v1.1.0)

User Manual

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Date released: 4th June 2016

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1. Introduction

The recent advent of high-throughput methods for probing RNA secondary structures has enabled transcriptome-scale analysis of the *RNA structurome*. Despite the establishment of several methods for querying RNA secondary structures on a genome-wide scale (CIRS-seq, SHAPE-seq, Structure-seq, DMS-seq, PARS), no tool has been developed to date to enable the rapid analysis and interpretation of these data.

The **RNA Structure Framework** is a modular toolkit developed to deal with RNA structure probing high-throughput data, from reads mapping to structure inference.
Its main features are:

- Automatic reference transcriptome creation
- Automatic reads preprocessing (adapter clipping and trimming) and mapping
- Scoring and data normalization
- Accurate RNA folding prediction by incorporating structural probing data

2. Requirements

- Linux/Mac system
- Bowtie v1.0.0 (http://bowtie-bio.sourceforge.net/index.shtml)
- SAMTools v1.2 or greater (http://www.htslib.org/)
- BEDTools v2.0 or greater (https://github.com/arq5x/bedtools2/)
- FASTX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/)
- ViennaRNA Package v2.2.0 or greater (http://www.tbi.univie.ac.at/RNA/)
- RNAstructure v5.6 or greater (http://rna.urmc.rochester.edu/RNAstructure.html)
- Perl v5.12 (or greater), with ithreads support
- Perl non-CORE modules (http://search.cpan.org/):

    - DBD::MySQL
    - LWP::UserAgent
    - RNA (part of the ViennaRNA package)
    - XML::LibXML::Simple

3. List of toolkit components

CORE modules	
rsf-index	Automatically queries UCSC genome database and builds the transcriptome Bowtie reference index for the RT Count module
rt-count	Performs reads pre-processing and mapping (where needed), and calculates per-base RT-stops and coverage
rsf-norm	Performs whole-transcriptome normalization of structure probing data
rsf-fold	Produces secondary structures for the analyzed transcripts using structure probing data to guide folding

Utilities	
rsf-combine	Allows combining SPD files from multiple experiments into a single structure profile
rsf-compare	Compares inferred secondary structures with a set of reference structures, computing PPV and sensitivity
rsf-silico	Produces SPD files by calculating the partition function folding for a given RNA, and reporting the probability of each base of being unpaired

4. Usage

4.1. rsf-index

The RSF Index tool is designed to automatically generate a Bowtie reference index, that will be used by the RT Count module for reads mapping.
This tool requires an internet connection, since it relies on querying the UCSC Genome database to obtain transcripts annotation and reference genome's sequence.
To list the required parameters, simply type:

$ *rsf-index -h* (or *--help)*

Parameter	Description
-o *or* --output-dir	Bowtie index output directory (Default: <assembly>_<annotation>, e.g. "mm9_refFlat/")
-ow *or* --overwrite	Overwrites the output directory if already exists
-g *or* --genome-assembly	Genome assembly for the species of interest (Default: mm9). For a complete list of UCSC available assemblies, please refer to Appendix A, or to the UCSC website (https://genome.ucsc.edu/FAQ/FAQreleases.html)
-a *or* --annotation	Name of the UCSC table containing the genes annotation (Default: refFlat). For a complete list of tables available for the chosen assembly, please refer to the UCSC website (https://genome.ucsc.edu/cgi-bin/hgTables)
-n *or* --gene-name	When possible, gene names will be used instead of gene IDs/accessions
-t *or* --timeout	Connection's timeout in seconds (Default: 180)
-r *or* --reference	Path to a FASTA file containing chromosome (or scaffold) sequences for the chosen genome assembly. **Note:** if no file is specified, RSF Index will try to obtain sequences from the UCSC DAS server. This process may take up to hours, depending on your connection's speed.
-b *or* --bowtie-build	Path to bowtie-build executable (Default: assumes bowtie-build is in PATH)
-e *or* --bedtools	Path to BEDTools executable (Default: assumes BEDTools is in PATH)

Note: For RNA structure probing experiments conducted over synthetic RNAs (or custom pools of RNAs), a reference can be generated by invoking directly the *bowtie-build* command, however it is necessary to first sort lexicographically the FASTA file by sequence IDs:

$** *awk 'BEGIN{RS=">"} NR>1 {gsub("\n", "\t"); print ">"$0}'* ***reference_unsorted.fa* *| *
LC_ALL=C sort -T "" -t ' ' -k2,2 | awk '{sub("\t", "\n"); gsub("\t", ""); print $0}' > ***reference_sorted.fa***

$ *bowtie-build reference_sorted.fa reference_sorted*

$ *ls -l*

```
-rwxrwxrwx 1 danny epigenetics  96041105  5 mar 10.50 reference_sorted.1.ebwt
-rwxrwxrwx 1 danny epigenetics  37313744  5 mar 10.50 reference_sorted.2.ebwt
-rwxrwxrwx 1 danny epigenetics   1844468  5 mar 10.28 reference_sorted.3.ebwt
-rwxrwxrwx 1 danny epigenetics  74627475  5 mar 10.28 reference_sorted.4.ebwt
-rwxrwxrwx 1 danny epigenetics 302198817  5 mar 10.28 reference_sorted.fa
-rwxrwxrwx 1 danny epigenetics  96041105  5 mar 11.11 reference_sorted.rev.1.ebwt
-rwxrwxrwx 1 danny epigenetics  37313744  5 mar 11.11 reference_sorted.rev.2.ebwt
-rwxrwxrwx 1 danny epigenetics 302198817  5 mar 10.28 reference_unsorted.fa
```

4.2. rt-count

The RT Count module is the core component of the toolkit. It can process any number of both FastQ or SAM/BAM files, also mixed. In case FastQ files are passed, reads are pre-processed (trimming and clipping), and mapped to the reference transcriptome. Each SAM/BAM file is then processed to calculate per-base RT-stops and reads coverage on each transcript.
To list the required parameters, simply type:

$ *rt-count -h* (or *--help)*

Parameter	Description
-p *or* --processors	Number of processors (threads) to use (Default: 1)
-t *or* --tmp-dir	Path to a directory for temporary files creation (Default: /tmp) **Note:** If the provided directory does not exist, it will be created
-o *or* --output-dir	Output directory for writing counts in RTC (RT Count) format (Default: rt_count/)
-ow *or* --overwrite	Overwrites the output directory if already exists
-k *or* --keep	Keeps SAM/BAM files after reads mapping (in case FastQ files are passed) **Note:** If unsorted SAM/BAM files are passed, this option will cause RT Count to keep the sorted SAM/BAM file
-n *or* --no-bam	Disables conversion of SAM files to BAM format (requires -k)
-b *or* --bowtie	Path to Bowtie v1 executable (Default: assumes Bowtie is in PATH)
-fx *or* --fastx	Path to FASTX Clipper executable (Default: assumes FASTX Clipper is in PATH)
-s *or* --samtools	Path to SAMTools executable (Default: assumes SAMTools is in PATH)
-r *or* --sorted	In case SAM/BAM files are passed, assumes that they are already sorted lexicographically by transcript ID, and numerically by position
-t5 *or* --trim-5prime	In case SAM/BAM files are passed, allows to specify a comma separated list (no spaces) of values indicating the number of bases trimmed from the 5'-end of reads in the respective sample SAM/BAM files (Default: 0) **Note #1:** Values must be provided in the same order as the input files (e.g. *rt-counter -t5 0,5 file1.bam file2.bam*, will consider 0 bases trimmed from *file1* reads, and 5 bases trimmed from *file2* reads). **Note #2:** If a single value is specified along with multiple SAM/BAM files, it will be used for all files.

-f *or* --fasta	Path to a FASTA file containing the reference transcripts **Note #1:** Transcripts in this file must match transcripts in SAM/BAM file headers. **Note #2:** This can be omitted if a Bowtie index is specified by -bi (or --bowtie-index)

FASTX Clipper options	
-fa *or* --fastx-adapter	Sequence of 3' adapter for clipping (Default: TGGAATTCTCGGGTGCCAAGG, Illumina TruSeq Small RNA 3' Adapter)
-fq *or* --fastx-qual	FastQ files quality scale (33 [Default], or 64)
-fl *or* --fastx-len	Minimum length to keep reads after clipping (>=35, Default: 35)
-c *or* --clipped	Assumes that reads have been already clipped

Bowtie options	
-bn *or* --bowtie-n	Use Bowtie mapper in -n mode (0-3 mismatches, Default: 2)
-bv *or* --bowtie-v	Use Bowtie mapper in -v mode (0-3 mismatches, Default: disabled). If both --bowtie-v and --bowtie-n parameters are passed, the -n mode will be overriden by the -v mode.
-ba *or* --bowtie-all	Report all equally scoring positions for multi-mapping reads (Default: disabled, reports only uniquely mapped reads)
-bc *or* --bowtie-chunkmbs	Maximum MB of RAM for best-first search frames (Default: 128)
-bp *or* --bowtie-threads	Number of threads to use for each instance of Bowtie (Default: 1) **Note:** RT Count executes 1 instance of Bowtie for each processor specified by -p. At least -p <processors> * bowtie-threads processors are required.
-bi *or* --bowtie-index	Path to transcriptome reference index (see paragraph 4.1)

4.2.1. RTC format

RT Count produces a RTC (RT Count) file for each analyzed sample. RTC files are proprietary binary files, that store transcript's sequence, per-base RT-stop counts, and per-base reads coverage. These files can be indexed for fast random access.
Each entry in a RTC file is structured as follows:

Field	Description	Type
len_transcript_id	Length of the transcript ID (plus 1, including NULL)	uint32_t
transcript_id	Transcript ID (NULL terminated)	char[len_transcript_id]
len_seq	Length of sequence	uint32_t
seq	4-bit encoded sequence: 'ACGTN' -> [0,4] (High nybble first)	uint8_t[(len_seq+1)/2]
stops	RT-stops at each base of transcript	uint32_t[len_seq]
cov	Coverage at each base of transcript	uint32_t[len_seq]

The RTC file EOF marker (last 8 bytes of file) is "\x5b\x65\x6f\x66\x72\x74\x63\x5d".
If the marker is absent, then the file is truncated or corrupted.

RTI index files are binary files, structured as follows:

Field	Description	Type
len_transcript_id	Length of the transcript ID (plus 1, including NULL)	uint32_t
transcript_id	Transcript ID (NULL terminated)	char[len_transcript_id]
offset	Offset position of transcript in the RTC file	uint32_t

4.3. rsf-norm

The RSF Norm tool takes one (Rouskin method), or two (Ding method) RTC files generated by the RT Count module, and performs normalization to obtain a per-base reactivity score for each transcript.
Reactivity scores can be computed using two methods:

[1] Ding *et al.*, 2014
In this scoring approach, the signal per-base is calculated as the natural log (ln) of the ratio between the raw count of RT-stops/Nuclease cuts at a given position of a transcript, and the average of the ln of RT-stops/Nuclease cuts along the whole transcript's length:

$$U_i = \frac{\ln\left(n_{1i}+p\right)}{\left(\sum_{j=o}^{l}\frac{\ln\left(n_{1j}+p\right)}{l}\right)} \qquad \textit{and} \qquad T_i = \frac{\ln\left(n_{2i}+p\right)}{\left(\sum_{j=o}^{l}\frac{\ln\left(n_{2j}+p\right)}{l}\right)}$$

where n_{1i} and n_{2i} are respectively the raw read counts in the untreated (or RNase V1) and treated (DMS, CMCT, SHAPE, or Nuclease S1) experiments at position *i* of the transcript, *l* is transcript's length, and *p* is a pseudocount added to deal with non-covered regions. U_i and T_i are respectively the normalized number of RT-stops at position *i* in the untreated and treated samples.
Score at position *i* is then calculated as:

$$S_i = max(0, (T_i - U_i))$$

Note: Since version 1.1.0, RSF Norm allows Ding scoring scheme to be applied within sliding windows

[2] Rouskin *et al.*, 2014
In this scoring approach, the untreated sample is not considered. Signal per-base is calculated within fixed size windows, by dividing the number of RT-stops on each residue, by the number of RT-stops on the most reactive residue within the same window after removing the outliers.

Once computed, reactivity scores are normalized. Three normalization methods are actually provided:

Parameter	Description
2-8% Normalization	From the top 10% of values, the top 2% is ignored, then any reactivity value along the entire transcript is divided by the average of the remaining 8%
90% Winsorising	Each reactivity value above the 95th percentile is set to the 95th percentile, and the reactivity at each position of the transcript is divided by the 95th percentile
Box-plot Normalization	Values greater than 1.5x the interquartile range (numerical distance between the 25th and 75th percentiles) above the 75th percentile are removed. After excluding these outliers, the next 10% of reactivities are averaged, and all reactivities (including outliers) are divided by this value.

Since Box-plot normalization will return values ranging from 0 to 1.5-2.2, normalized reactivities can be further remapped to values ranging from 0 to 1 according to Zarringhalam *et al.*, 2012.
In this approach, values < 0.25 are linearly mapped to [0-0.35[, values ≥ 0.25 and < 0.3 are linearly mapped to [0.35-0.55[, values ≥ 0.3 and < 0.7 are linearly mapped to [0.55-0.85[, and values ≥ 0.7 are linearly mapped to [0.85-1].

To list the paramaters required to the RSF Norm tool, simply type:

$ *rsf-norm -h* (or *--help)*

Parameter	Description
-u *or* --untreated	Path to the RTC file for the non-treated (or RNase V1) sample
-t *or* --treated	Path to the RTC file for the treated (DMS, SHAPE, or Nuclease S1) sample
-i *or* --index	A comma separated (no spaces) list of RTI index files for the provided RTC files **Note #1:** RTI files must be provided in the order 1. Untreated, 2. Treated **Note #2:** If a single RTI file is specified along with both untreated and treated samples, it will be used for both samples **Note #3:** If no RTI index is provided, it will be generated at runtime, and stored in the same folder of the untreated/treated samples
-p *or* --processors	Number of processors (threads) to use (Default: 1)
-o *or* --output-dir	Output directory for writing normalized data in SPD (Structure Probing Data file) format (Default: <treated>_vs_<untreated>_norm/ for Ding method, <treated>_norm/ for Rouskin method)
-ow *or* --overwrite	Overwrites the output directory if already exists
-c *or* --config-file	Path to a configuration file with normalization parameters (see paragraph 4.2.1) **Note #1:** If the provided file exists, the loaded configuration will override any command-line specified parameter **Note #2:** If the provided file doesn't exist, it will be generated using the command-line specified (or the default) parameters
-sm *or* --scoring-method	Score calculation method (1-2, Default: 1), where: **1.** Ding *et al.,* 2014; **2.** Rouskin *et al.,* 2014
-nm *or* --norm-method	Score normalization method (1-3, Default: 1), where: **1.** 2-8% normalization; **2.** 90% Winsorising; **3.** Box-plot normalization
-rb *or* --reactive-bases	Reactive bases to consider for signal normalization (Default: N [ACGT]) **Note:** This parameter accepts any IUPAC code, or combinations of them (e.g. *-rb M*, or *-rb AC*). Reactivity for any other base will be reported as NaN.
-ni *or* --norm-independent	Each one of the reactive bases will be normalized independently (e.g. *-rb AC -ni* will normalize independently A and C residues)

-mc *or* --mean-coverage	Discards any transcript with mean coverage below this threshold (≥0, Default: 1)
-ec *or* --median-coverage	Discards any transcript with median coverage below this threshold (≥0, Default: 1)
-nw *or* --norm-window	Window size (in nt) for signal normalization (≥3, Default: whole transcript [Ding], 50 [Rouskin])
-wo *or* --window-offset	Offset for sliding window during normalization (Default: none [Ding], 50 (non-overlapping windows) [Rouskin])
-d *or* --decimals	Number of decimals for reporting reactivities (1-10, Default: 3)
-n *or* --nan	Non-covered transcript positions will be reported as NaN in the reactivity profile

Scoring method #1 options (Ding *et al.*, 2014)	
-pc *or* --pseudocount	Pseudocount added to reactivities to avoid division by 0 (>0, Default: 1)
-s *or* --max-score	Score threshold for capping raw reactivities (>1, Default: 10)

Normalization method #3 options (Box-plot normalization)	
-rm *or* --remap-reactivities	Remaps reactivities to values ranging from 0 to 1 according to Zarringhalam *et al.*, 2012

4.3.1. Configuration files

RSF Norm configuration files are used to provide normalization parameters for the analysis, without the need to manually specify them from the command-line.
Configuration files are composed of a list of key/value pairs, separated by the equal sign (=), or by the colon punctuation mark (:). Keys and values are *case-insensitive*.

Accepted key/value pairs are:

Parameter	Accepted values	Default value
scoreMethod	"Ding" (or 1); "Rouskin" (or 2)	Ding
normMethod	"2-8%" (or 1); "90% Winsorising" (or 2); "Box-plot" (or 3)	2-8%
reactiveBases	[ACGTURYSWKMBDHVN] (or "all")	all
normIndependent	TRUE/FALSE; Yes/No; 1/0	FALSE
normWindow	Positive integer ≥ 3	1e9 [Ding] 50 [Rouskin]
windowOffset	Positive integer ≥ 0	1e9 [Ding] 50 [Rouskin]
meanCoverage	Positive integer ≥ 0	1
medianCoverage	Positive integer ≥ 0	1

Scoring method #1 parameters (Ding *et al.*, 2014)		
maxScore	Positive integer ≥ 1	10
pseudoCount	Positive integer ≥ 1	1

Normalization method #3 parameters (Box-plot normalization)		
remapReactivities	TRUE/FALSE; Yes/No; 1/0	FALSE

e.g. **A typical configuration file**

```
scoreMethod=Ding
normMethod=2-8%
maxScore=50
pseudoCount=1
reactiveBases=ACGT
normIndependent=no
normWindow=1e9
windowOffset=1e9
meanCoverage=1
```

4.3.2. Structure Probing Data (SPD) files

RSF Norm produces a set of SPD files, one for each transcript being analyzed.
This files are essentially XML files, and therefore can be parsed using any standard XML parsing library.
SPD files tree structure is the following:

```
<?xml version="1.0" encoding="UTF-8"?>
<data [attributes]>
   <transcript id="Transcript ID" length="Transcript length">
      <sequence>
        Transcript sequence
      </sequence>
      <reactivity>
        Comma-separated list of reactivity values
      </reactivity>
      <error>
        Comma-separated list of standard deviations from multiple experiments
      </error>
   </transcript>
</data>
```

The “error” tag is optional. It is introduced by RSF Combine when multiple experiments are combined into a single SPD profile, and stores the per-base standard deviation of the normalized reactivity.

The “data” tag’s attributes allow keeping track of the analysis performed to obtain the SPD file:

Attribute	Description
combined	Whether multiple experiments have been combined into a single profile (TRUE or FALSE, see paragraph 5.1)
scoring	Scoring method (Ding or Rouskin)
norm	Normalization method (2-8%, Winsorising 90%, or Box-plot)
reactive	Reactive bases
win	Normalization window’s size (in nt)
offset	Offset for normalization window sliding

Scoring method #1 attributes (Ding *et al.*, 2014)	
max	Score threshold for reactivity capping
pseudo	Pseudocount added during score calculation

Normalization method #3 attributes (Box-plot normalization)	
remap	Whether box-plot normalized reactivities have been remapped according to Zarringhalam *et al.*, 2012

4.3. rsf-fold

The RSF Fold tool is designed to allow the transcriptome-wide reconstruction of RNA structures, starting from SPD files generated using the RSF Norm tool.
This tool can process a single, or an entire directory of SPD files, and produces the inferred secondary structures (either in dot-bracket notation, or CT format) and their graphical representation (either in Postscript, or SVG format).
To allow higher analysis flexibility, the tool incorporates two different prediction methods:

[1] ViennaRNA
[2] RNAstructure

Note #1: Only the new v2.2.0 ViennaRNA soft-constraint approach is provided, since hard-constraint predictions are in most cases totally unreliable.
Note #2: The Iterative Cluster Refinement method (Incarnato *et al.*, 2016) has been temporary removed due to a change in the ViennaRNA APIs, and will be added back in a future release.

To list the parameters required to the RSF Fold tool, simply type:

$ *rsf-fold -h* (or *--help)*

Parameter	Description
-o *or* --output-dir	Output directory for writing structural data (Default: structurome/)
-ow *or* --overwrite	Overwrites output directory (if the specified path already exists)
-ct *or* --connectivity-table	Writes predicted structures in CT format (Default: Dot-bracket notation)
-m *or* --folding-method	Specifies the folding method (1-2, Default: 1): **1.** ViennaRNA; **2.** RNAstructure
-p *or* --processors	Number of processors to use for the analysis (Default: 1)
-g *or* --img	Enables generation of structure representations (Default: Postscript format)
-s *or* --svg	Structure representations are generated in SVG format (requires -g)
-sl *or* --slope	Sets slope used with structure probing data restraints (Default: 1.8 [kcal/mol])
-in *or* --intercept	Sets intercept used with structure probing data restraints (Default: -0.6 [kcal/mol])
-m *or* --maximum-distance	Sets the maximum pairing distance in nucleotides between transcript's residues (Default: 0, [no limit])

Folding method #1 options (ViennaRNA)	
-v *or* --viennarna	Path to ViennaRNA RNAfold executable (Default: assumes RNAfold is in PATH)
-nlp *or* --no-lonely-pairs	Disallows lonely (unstacked) base-pairs inside predicted structure
-ngu *or* --no-closing-gu	Disallows G:U wobbles at the end of helices
-cm *or* --constraint-method	Method for converting SPD reactivities into pseudo-energies (1-2, Default: 1): **1.** Zarringhalam *et al.*, 2012; **2.** Deigan *et al.*, 2009

Zarringhalam *et al.*, 2012 method options	
-cc *or* --constraint-conversion	Method for converting SPD reactivities to pairing probabilities (1-5, Default: 1): **1.** Skip normalization step (SPD reactivities are treated as pairing probabilities) **2.** Linear mapping according to Zarringhalam et al., 2012 **3.** Use a cutoff to divide into paired and unpaired nucleotides **4.** Linear model for converting SPD reactivities into probabilities of being unpaired **5.** Linear model for converting the logarithm of SPD reactivities into probabilities of being unpaired
-bf *or* --beta-factor	Sets the magnitude of penalities for deviations from the observed pairing probabilities (Default: 0.5)
-f *or* --cutoff	Cutoff for constraining a position as unpaired (0-1, Default: 0.7) **Note:** This option requires constraint conversion method #3
-ms *or* --model-slope	Sets the slope used by the linear model (Default: 0.68 [Method #4], or 1.6 [Method #5]) **Note:** This option requires constraint conversion methods #4 or #5
-mi *or* --model-intercept	Sets the intercept used by the linear model (Default: 0.2 [Method #4], or -2.29 [Method #5]) **Note:** This option requires constraint conversion methods #4 or #5

Folding method #2 options (RNAstructure)	
-r *or* --rnastructure	Path to RNAstructure Fold executable (Default: assumes RNAstructure is in PATH)
-dp *or* --data-path	Path to RNAstructure data tables (Default: assumes DATAPATH environment variable is already set)

Note: ViennaRNA constraint method #2 (Deigan *et al.*, 2009) is essentially the same employed by RNAstructure, therefore the two approaches should yield approximately the same results.

For additional details relatively to ViennaRNA soft-constraint prediction methods, please refer to the ViennaRNA manual, or to Lorenz *et al.*, 2016.

5. Utilities

5.1. rsf-combine

RSF Combine allows combining multiple experiments into a single reactivity profile. This is useful for example when performing CIRS-seq experiments, to combine into a single profile both the reactivity of A/C residues probed with DMS, and of G/U residues probed with CMCT. Alternatively, RSF Combine is able to combine into a single profile multiple replicates of the same probing experiment. In these cases, the resulting SPD file may contain the optional "error" tag, in which the per-base standard deviation of the reactivity is reported.
When combining SPD files generated using the *-n* (or *--nan*) option of RSF Norm, only positions covered in all experiments will be combined, while the others will be reported as NaN.
There's no limit to the number of experiments that RSF Combine can handle. Moreover, it can be used both on individual SPD files, or on whole SPD folders generated by RSF Norm.

Note: RSF Combine does not allow combining SPD files generated using different scoring/normalization methods, since this will produce inconsistent data

To list the parameters required to the RSF Combine tool, simply type:

$ *rsf-combine -h* (or *--help)*

Parameter	Description
-p *or* --processors	Number of processors (threads) to use (Default: 1)
-o *or* --output-dir	Output directory for writing combined SPD (Structure Probing Data) files (Default: combined/)
-ow *or* --overwrite	Overwrites the output directory if already exists
-s *or* --stdev	When multiple replicates are combined, an optional "error" tag will be reported within the output SPD files, containing the per-base reactivity's standard deviation
-d *or* --decimals	Number of decimals for reporting reactivities (1-10, Default: 3)

5.2. rsf-compare

RSF Compare allows comparing inferred secondary structures from RSF Fold, with a reference of known secondary structures, reporting for each comparison the PPV (Positive Predictive Value, the fraction of base-pairs present in the predicted structure that are also present in the reference structure) and the sensitivity (the fraction of base-pairs present in the reference structure that are also in the predicted structure).
Reference structures must be provided in Vienna format:

```
>Transcript_1
AAAAAAAAAAAAAAAAAAAAUUUUUUUUUUUUUUUUUUUU
.((((((((((((((((((((....))))))))))))))))))))
>Transcript_2
CCCCCCCCCCCCCCCCCGGGGGGGGGGGGGGGGGGG
((((((((((((((((((...))))))))))))))))))

-- cut --

>Transcript_n
GCUAGCUAGCUAGCUAGCUAGUCAAGACGAGUCGAUGCU
(((((((((....)))))))))..................
```

RSF Compare can be invoked both on a single structure, or on an entire folder of RSF Fold predicted structure files. Structures can be provided either in CT or Vienna (dot-bracket) format.

To list the parameters required to the RSF Combine tool, simply type:

$ *rsf-compare -h* (or *--help)*

Parameter	Description
-r *or* --reference	A file containing reference structures in Vienna format (dot-bracket)

5.3. rsf-silico

RSF Silico calculates partition function folding for a set of given RNAs, using either ViennaRNA, RNAstructure, or their combination. The probability of each base of being unpaired is then reported in the form of a SPD file. To keep compatibility with RSF Norm, it is possible to specify which bases should be excluded from the analysis. Those bases are reported as NaN in the resulting SPD file.

To list the parameters required to the RSF Silico tool, simply type:

$ *rsf-silico -h* (or *--help)*

Parameter	Description
-p *or* --processors	Number of processors (threads) to use (Default: 1)
-o *or* --output-dir	Output directory for writing combined SPD (Structure Probing Data) files (Default: combined/)
-ow *or* --overwrite	Overwrites the output directory if already exists
-t *or* --tmp-dir	Path to a directory for temporary files creation (Default: /tmp) **Note:** If the provided directory does not exist, it will be created
-m *or* --method	Partition function calculation method (1-3, Default: 1), where: **1.** ViennaRNA; **2.** RNAstructure; **3.** Combined **Note:** Method #3 (Combined) calculates base-pair probabilities using both ViennaRNA and RNAstructure, and produces a SPD file containing the per-base average of the two methods
-e *or* --temperature	Temperature in Celsius degrees (Default: 37)
-md *or* --maximum-distance	Sets the maximum pairing distance in nucleotides between transcript's residues (Default: 0 [No limit])
-v *or* --viennarna	Path to ViennaRNA RNAfold executable (Default: assumes RNAfold is in PATH)
-pr *or* --partition	Path to RNAstructure Partition executable (Default: assumes Partition is in PATH)
-pp *or* --probability-plot	Path to RNAstructure ProbabilityPlot executable (Default: assumes ProbabilityPlot is in PATH)
-dp *or* --data-path	Path to RNAstructure data tables (Default: assumes DATAPATH environment variable is already set)

-w *or* -- window-size	Window size in nt for base-pair probability calculation (>=3, Default: full transcript)
-wo *or* --window-offset	Offset for window sliding (Default: none)
-kb *or* --keep-bases	Bases to report in the SPD file (Default: N [ACGT]) **Note:** This parameter accepts any IUPAC code, or combinations of them (e.g. *-kb M*, or *-kb AC*). Reactivity for any other base will be reported as NaN.
-d *or* --decimals	Number of decimals for reporting reactivities (1-10, Default: 3)

6. Application case

Analysis of PARS data on GM12878 native deproteinized RNA structures

Reference: Wan,Y. *et al.* (2014) Landscape and variation of RNA secondary structure across the human transcriptome. *Nature*, **505**, 706–709.
GEO Dataset: GSE50676
Description: In this work, Wan and colleagues used PARS to probe transcriptome-wide the structures of RNA from GM12878 cells in native deproteinized conformation, following phenol-chloroform extraction.

First of all, data in Sequence Read Archive (SRA) format should be downloaded from the Gene Expression Omnibus database:

$ *wget 'ftp://ftp-trace.ncbi.nlm.nih.gov/sra/sra-instant/reads/ByExp/sra/SRX%2FSRX346%2FSRX346863/SRR972714/SRR972714.sra' -O S1.sra*
$ *wget 'ftp://ftp-trace.ncbi.nlm.nih.gov/sra/sra-instant/reads/ByExp/sra/SRX%2FSRX346%2FSRX346864/SRR972715/SRR972715.sra' -O V1.sra*

These commands will download the SRA files for the Nuclease S1 and RNase V1 treatments. Once downloaded, SRA files should be converted to FastQ format. To perform conversion, it is necessary first to download and install the NCBI SRA Toolkit (http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=software). Issue the following commands to convert the SRA files into FastQ files:

$ *fastq-dump S1.sra*
$ *fastq-dump V1.sra*

To build the reference index for the *Homo sapiens* genome (hg19 assembly), using the Ensembl gene annotation, run the *RSF Reference Builder* module with the following parameters:

$ *rsf-index -g hg19 -a ensGene*

```
[+] Making output directory...
[+] Connecting to UCSC genome database (genome-mysql.cse.ucsc.edu:3306)...
[+] Connected. Searching annotation...
[+] Annotation found. Validating columns...
[+] Downloading annotation data. Please wait...

  [!] Warning: No reference multi-FASTA file has been provided.
               RSF Index will now try to download reference genome sequence
               from UCSC DAS server.
               This may take up to hours, depending on your connection's speed.

[+] Downloading sequence data for 52 chromosomes. Please wait...

  [*] Chromosome chr1                 [DONE]
  [*] Chromosome chr10                [DONE]
  [*] Chromosome chr11                [DONE]
  [*] Chromosome chr12                [DONE]
  [*] Chromosome chr13                [DONE]
  [*] Chromosome chr14                [DONE]
  [*] Chromosome chr15                [DONE]
  [*] Chromosome chr16                [DONE]
  [*] Chromosome chr17                [DONE]
  [*] Chromosome chr17_ctg5_hap1      [DONE]
  [*] Chromosome chr17_gl000205_random [DONE]
  [*] Chromosome chr18                [DONE]
  [*] Chromosome chr19                [DONE]
  [*] Chromosome chr19_gl000209_random [DONE]
  [*] Chromosome chr1_gl000191_random [DONE]
  [*] Chromosome chr1_gl000192_random [DONE]
  [*] Chromosome chr2                 [DONE]
  [*] Chromosome chr20                [DONE]
  [*] Chromosome chr21                [DONE]
  [*] Chromosome chr22                [DONE]
  [*] Chromosome chr3                 [DONE]
  [*] Chromosome chr4                 [DONE]
  [*] Chromosome chr4_ctg9_hap1       [DONE]
  [*] Chromosome chr4_gl000193_random [DONE]
  [*] Chromosome chr4_gl000194_random [DONE]
  [*] Chromosome chr5                 [DONE]
```

```
  [*] Chromosome chr6                  [DONE]
  [*] Chromosome chr6_apd_hap1         [DONE]
  [*] Chromosome chr6_cox_hap2         [DONE]
  [*] Chromosome chr6_dbb_hap3         [DONE]
  [*] Chromosome chr6_mann_hap4        [DONE]
  [*] Chromosome chr6_mcf_hap5         [DONE]
  [*] Chromosome chr6_qbl_hap6         [DONE]
  [*] Chromosome chr6_ssto_hap7        [DONE]
  [*] Chromosome chr7                  [DONE]
  [*] Chromosome chr7_gl000195_random  [DONE]
  [*] Chromosome chr8                  [DONE]
  [*] Chromosome chr9                  [DONE]
  [*] Chromosome chrUn_gl000211        [DONE]
  [*] Chromosome chrUn_gl000212        [DONE]
  [*] Chromosome chrUn_gl000213        [DONE]
  [*] Chromosome chrUn_gl000215        [DONE]
  [*] Chromosome chrUn_gl000218        [DONE]
  [*] Chromosome chrUn_gl000219        [DONE]
  [*] Chromosome chrUn_gl000220        [DONE]
  [*] Chromosome chrUn_gl000222        [DONE]
  [*] Chromosome chrUn_gl000223        [DONE]
  [*] Chromosome chrUn_gl000227        [DONE]
  [*] Chromosome chrUn_gl000228        [DONE]
  [*] Chromosome chrUn_gl000241        [DONE]
  [*] Chromosome chrX                  [DONE]
  [*] Chromosome chrY                  [DONE]

[+] Extracting transcript sequences...
[+] Building Bowtie transcriptome index from sequences. Please wait...
[+] All done.
```

Once the reference index has been prepared, the "*hg19_ensGene*" folder should appear in the current path, containing all the relevant index files. The FastQ files can now be passed to the RT Count module that will perform reads mapping, and compute normalized reactivity scores for all covered transcripts. The module will also perform all the necessary pre-processing steps on the FastQ files (trimming and adapter clipping). These steps are not mandatory, and can be skipped by simply setting "*-t5 0*" or "*-t3 0*" to either disable trimming from the 5'- or 3'-end of the reads, and "*--clipped*" to disable adapter clipping. According to the GEO datasets page, the last 51 nt of each read should be trimmed (moreover, following analysis of FastQ files with FASTX Toolkit, we also decided to trim the first 3 nt of each read). To perform reads mapping, and data normalization, run the RT Counter module with the following parameters:

$ *rt-counter -t tmp/ -k -b5 3 -b3 51 -c -bm 20 -bc 3200000 -bi hg19_ensGene/hg19_ensGene S1.fq V1.fq*

```
[+] Reference FASTA file is present. Skipping FASTA regeneration...
[+] Making output directory...
[+] Guessing file types:

  Sample    Type     5'-end trimming

  S1        FastQ    3 nt
  V1        FastQ    3 nt

[+] Processing FastQ files...
[+] Input FastQ files are already clipped. Skipping adapter clipping...
[+] Mapping reads to transcriptome...

  [-] Mapping sample "S1" (PID: 19759)
  [-] Mapping sample "V1" (PID: 19760)

[+] Mapping statistics:

  [*] Sample "S1" [Mapped: 68.82%; Failed: 29.08%; Suppressed: 2.11%]
  [*] Sample "V1" [Mapped: 65.15%; Failed: 29.81%; Suppressed: 5.04%]

[+] Sorting BAM files...

  [-] Sorting sample "S1.bam" (PID: 19879)
  [-] Sorting sample "V1.bam" (PID: 19880)

[+] Getting transcripts from reference, and building count table base structure...
[+] Validating SAM/BAM file headers...
[+] Calculating per-base RT-stops and coverage. This may take a while...

  [-] Processing sample "S1" (Thread #2)...
  [-] Processing sample "V1" (Thread #1)...
```

```
[+] Statistics:

  [*] Sample "S1": 58725 transcripts covered
  [*] Sample "V1": 98452 transcripts covered

[+] Cleaning up temporary files...
[+] All done.
```

The program reports mapping statistics, and the number of covered transcripts. RT Count has generated a folder named "*rt_counter/*", which contains two folders:
1. a "*BAM/*" folder containing mapped reads for both samples
2. a "*counts/*" folder containing per-base transcript RT-stops in RTC (RT Count) format (see paragraph 4.2.1), and a RTI index file for fast RTC files random access:

$ *ls -l*

```
rt_counter/BAM:
-rw-r--r-- 1 danny epigenetics 136030227  8 feb 12.04 S1.bam
-rw-r--r-- 1 danny epigenetics 130537439  8 feb 12.07 V1.bam

rt_counter/counts:
-rw-r--r-- 1 danny epigenetics   102  8 feb 12.02 index.rti
-rw-r--r-- 1 danny epigenetics 63658  8 feb 12.17 S1.rtc
-rw-r--r-- 1 danny epigenetics 63658  8 feb 12.22 V1.rtc
```

The RTC files can now be used as input for the RSF Norm module, that will normalize per-base signals. In this case, the V1 sample will be used as the untreated sample, while the S1 sample as the treated sample:

$ *rsf-norm -u rt_counter/counts/V1.rtc -t rt_counter/counts/S1.rtc -c parameters.conf*

```
[+] Parsing configuration...

  [!] Warning: Provided configuration file doesn't exist. Will be created...

[+] Configuration summary:

  Parameter                          Value

  Scoring method                     Ding
  Normalization method               2-8%
  Pseudocount                        1
  Maximum score                      10
  Reactive bases                     ACGT
  Normalize each base independently  No
  Minimum mean coverage              1
  Minimum median coverage            1

[+] Making output directory...
[+] Regenerating RTI index files...
[+] Loading transcript IDs... 204940 transcripts loaded.
[+] Normalizing reactivities [Last: ENST00000610125]
[+] Normalization statistics:

  [*] Covered transcripts:   3359
  [*] Discarded transcripts: 201581 total
                             201581 insufficient coverage
                             0 mismatch between treated and untreated sample sequence
                             0 absent in untreated sample reference

[+] All done.
```

RSF Norm has generated a folder containing one SPD (Structure Probing Data) file (see paragraph 4.3.2) for each transcript being analyzed, named "*S1_vs_V1_norm/*", and a configuration file with the parameters used for the analysis named "*parameters.conf*":

$ *ls -l*

```
-rw-r--r-- 1 danny epigenetics        129 12 feb 17.51 parameters.conf
drwxr-xr-x 3 danny epigenetics       4096 13 feb 10.36 rt_counter
drwxr-xr-x 2 danny epigenetics     135168 13 feb 12.52 S1_vs_V1_norm
```

SPD files can now be used as input for the RSF Fold module to perform data-guided folding prediction. In the following example, we will pass the RSF-normalized reactivity profile for the U1 snRNA (Ensembl ID: ENST00000383861) to the RSF Fold module to perform prediction using the ViennaRNA package:

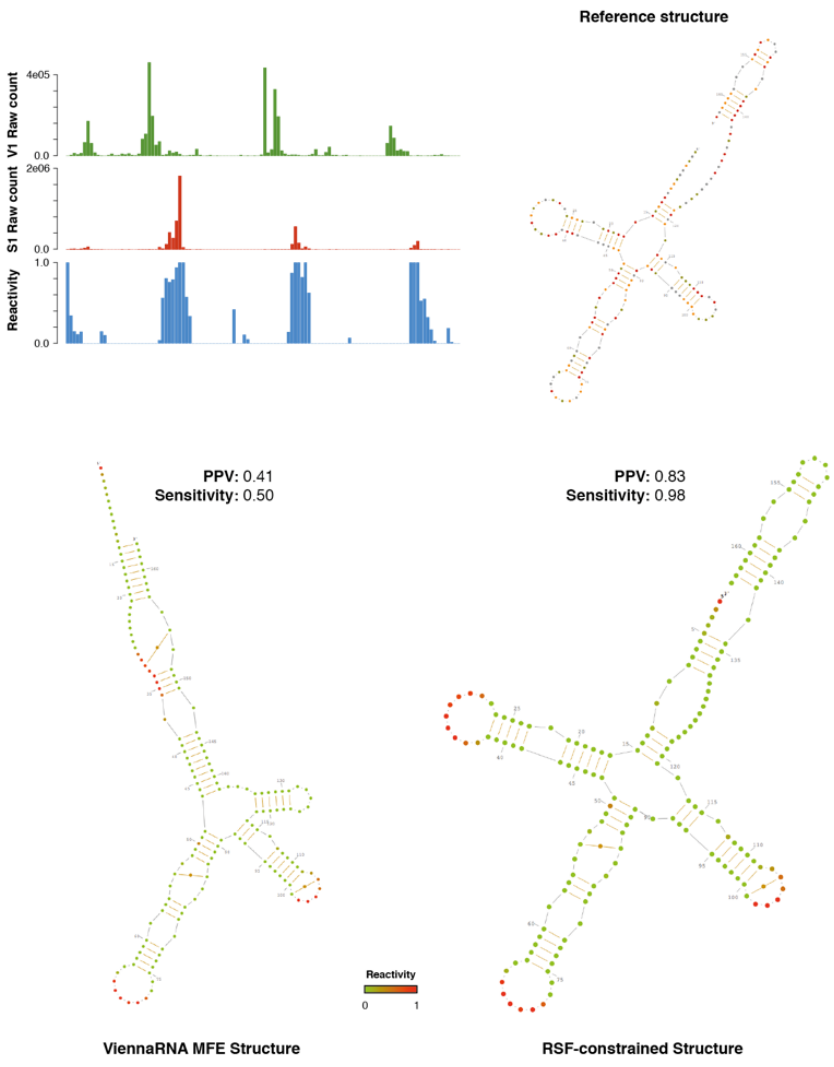
$ *rsf-fold -m 1 -cm 1 -cc 3 -f 0.7 -g S1_vs_V1_norm/ENST00000383861.spd*

```
[+] Checking method's requirements...
[+] Making output directory tree...
[+] Importing SPD file(s) [1 imported]
[+] Building RNA structurome [Last: ENST00000383861]
[+] Folding statistics:

  [*] Folded transcripts:    1
  [*] Discarded transcripts: 0 total
                             0 SPD parsing failed
                             0 constraint file generation failed
                             0 folding failed
                             0 I/O error

[+] All done.
```

As shown in the following figure, the RSF-constrained structure for the U1 snRNA (bottom right) better recapitulates the known reference structure (upper right) in terms of both Sensitivity and Positive Predictive Value (PPV) than the unconstrained MFE structure does (bottom left).



(Structure plots in this figure have been generated using the Assemble2 software, http://bioinformatics.org/s2s/)

Appendix A. List of UCSC genome assembly releases (https://genome.ucsc.edu/FAQ/FAQreleases.html)

Species	UCSC Version	Release date	Release name	Status
			Mammals	
Human	hg38	Dec. 2013	Genome Reference Consortium GRCh38	**Available**
	hg19	Feb. 2009	Genome Reference Consortium GRCh37	**Available**
	hg18	Mar. 2006	NCBI Build 36.1	**Available**
	hg17	May 2004	NCBI Build 35	**Available**
	hg16	Jul. 2003	NCBI Build 34	**Available**
	hg15	Apr. 2003	NCBI Build 33	Archived
	hg13	Nov. 2002	NCBI Build 31	Archived
	hg12	Jun. 2002	NCBI Build 30	Archived
	hg11	Apr. 2002	NCBI Build 29	Archived (data only)
	hg10	Dec. 2001	NCBI Build 28	Archived (data only)
	hg8	Aug. 2001	UCSC-assembled	Archived (data only)
	hg7	Apr. 2001	UCSC-assembled	Archived (data only)
	hg6	Dec. 2000	UCSC-assembled	Archived (data only)
	hg5	Oct. 2000	UCSC-assembled	Archived (data only)
	hg4	Sep. 2000	UCSC-assembled	Archived (data only)
	hg3	Jul. 2000	UCSC-assembled	Archived (data only)
	hg2	Jun. 2000	UCSC-assembled	Archived (data only)
	hg1	May 2000	UCSC-assembled	Archived (data only)
Alpaca	vicPac2	Mar. 2013	Broad Institute Vicugna_pacos-2.0.1	**Available**
	vicPac1	Jul. 2008	Broad Institute VicPac1.0	**Available**
Armadillo	dasNov3	Dec. 2011	Broad Institute DasNov3	**Available**
Bushbaby	otoGar3	Mar. 2011	Broad Institute OtoGar3	**Available**
Baboon	papHam1	Nov. 2008	Baylor College of Medicine HGSC Pham_1.0	**Available**
	papAnu2	Mar. 2012	Baylor College of Medicine Panu_2.0	**Available**
Cat	felCat5	Sep. 2011	ICGSC Felis_catus-6.2	**Available**
	felCat4	Dec. 2008	NHGRI catChrV17e	**Available**
	felCat3	Mar. 2006	Broad Institute Release 3	**Available**
Chimp	panTro4	Feb. 2011	CGSC Build 2.1.4	**Available**
	panTro3	Oct. 2010	CGSC Build 2.1.3	**Available**
	panTro2	Mar. 2006	CGSC Build 2.1	**Available**
	panTro1	Nov. 2003	CGSC Build 1.1	**Available**
Chinese hamster	criGri1	Jul. 2013	Beijing Genomics Institution-Shenzhen C_griseus_v1.0	**Available**
Cow	bosTau7	Oct. 2011	Baylor College of Medicine HGSC Btau_4.6.1	**Available**
	bosTau6	Nov. 2009	University of Maryland v3.1	**Available**
	bosTau4	Oct. 2007	Baylor College of Medicine HGSC Btau_4.0	**Available**
	bosTau3	Aug. 2006	Baylor College of Medicine HGSC Btau_3.1	**Available**
	bosTau2	Mar. 2005	Baylor College of Medicine HGSC Btau_2.0	**Available**
	bosTau1	Sep. 2004	Baylor College of Medicine HGSC Btau_1.0	Archived
Dog	canFam3	Sep. 2011	Broad Institute v3.1	**Available**
	canFam2	May 2005	Broad Institute v2.0	**Available**
	canFam1	Jul. 2004	Broad Institute v1.0	**Available**
Dolphin	turTru2	Oct. 2011	Baylor College of Medicine Ttru_1.4	**Available**
Elephant	loxAfr3	Jul. 2009	Broad Institute LoxAfr3	**Available**
Ferret	musFur1	Apr. 2011	Ferret Genome Sequencing Consortium MusPutFur1.0	**Available**
Gibbon	nomLeu3	Oct. 2012	Gibbon Genome Sequencing Consortium Nleu3.0	**Available**

	nomLeu2	Jun. 2011	Gibbon Genome Sequencing Consortium Nleu1.1	Available
	nomLeu1	Jan. 2010	Gibbon Genome Sequencing Consortium Nleu1.0	Available
Gorilla	gorGor3	May 2011	Wellcome Trust Sanger Institute gorGor3.1	Available
Guinea pig	cavPor3	Feb. 2008	Broad Institute cavPor3	Available
Hedgehog	eriEur2	May 2012	Broad Institute EriEur2.0	Available
	eriEur1	Jun. 2006	Broad Institute Draft_v1	Available
Horse	equCab2	Sep. 2007	Broad Institute EquCab2	Available
	equCab1	Jan. 2007	Broad Institute EquCab1	Available
Kangaroo rat	dipOrd1	Jul. 2008	Baylor/Broad Institute DipOrd1.0	Available
Manatee	triMan1	Oct. 2011	Broad Institute TriManLat1.0	Available
Marmoset	calJac3	Mar. 2009	WUSTL Callithrix_jacchus-v3.2	Available
	calJac1	Jun. 2007	WUSTL Callithrix_jacchus-v2.0.2	Available
Megabat	pteVam1	Jul. 2008	Broad Institute Ptevap1.0	Available
Microbat	myoLuc2	Jul. 2010	Broad Institute MyoLuc2.0	Available
Minke whale	balAcu1	Oct. 2013	KORDI BalAcu1.0	Available
Mouse	mm10	Dec. 2011	Genome Reference Consortium GRCm38	Available
	mm9	Jul. 2007	NCBI Build 37	Available
	mm8	Feb. 2006	NCBI Build 36	Available
	mm7	Aug. 2005	NCBI Build 35	Available
	mm6	Mar. 2005	NCBI Build 34	Archived
	mm5	May 2004	NCBI Build 33	Archived
	mm4	Oct. 2003	NCBI Build 32	Archived
	mm3	Feb. 2003	NCBI Build 30	Archived
	mm2	Feb. 2002	MGSCv3	Archived
	mm1	Nov. 2001	MGSCv2	Archived (data only)
Mouse lemur	micMur1	Jul. 2007	Broad Institute MicMur1.0	Available
Naked mole-rat	hetGla2	Jan. 2012	Broad Institute HetGla_female_1.0	Available
	hetGla1	Jul. 2011	Beijing Genomics Institute HetGla_1.0	Available
Opossum	monDom5	Oct. 2006	Broad Institute release MonDom5	Available
	monDom4	Jan. 2006	Broad Institute release MonDom4	Available
	monDom1	Oct. 2004	Broad Institute release MonDom1	Available
Orangutan	ponAbe2	Jul. 2007	WUSTL Pongo_albelii-2.0.2	Available
Panda	ailMel1	Dec. 2009	BGI-Shenzhen AilMel 1.0	Available
Pig	susScr3	Aug. 2011	Swine Genome Sequencing Consortium Sscrofa10.2	Available
	susScr2	Nov. 2009	Swine Genome Sequencing Consortium Sscrofa9.2	Available
Pika	ochPri2	Jul. 2008	Broad Institute release OchPri2	Available
Platypus	ornAna1	Mar. 2007	WUSTL v5.0.1	Available
Rabbit	oryCun2	Apr. 2009	Broad Institute release OryCun2	Available
Rat	rn5	Oct. 2011	RGSC Rnor_5.0	Available
	rn4	Nov. 2004	Baylor College of Medicine HGSC v3.4	Available
	rn3	Jun. 2003	Baylor College of Medicine HGSC v3.1	Available
	rn2	Jan. 2003	Baylor College of Medicine HGSC v2.1	Archived
	rn1	Nov. 2002	Baylor College of Medicine HGSC v1.0	Archived
Rhesus	rheMac3	Oct. 2010	Beijing Genomics Institute CR_1.0	Available
	rheMac2	Jan. 2006	Baylor College of Medicine HGSC v1.0 Mmul_051212	Available
	rheMac1	Jan. 2005	Baylor College of Medicine HGSC Mmul_0.1	Archived
Rock hyrax	proCap1	Jul. 2008	Baylor College of Medicine HGSC Procap1.0	Available
Sheep	oviAri3	Aug. 2012	ISGC Oar_v3.1	Available
	oviAri1	Feb. 2010	ISGC Ovis aries 1.0	Available
Shrew	sorAra1	Jun. 2006	Broad Institute SorAra1.0	Available
Sloth	choHof1	Jul. 2008	Broad Institute ChoHof1.0	Available

Squirrel	speTri2	Nov. 2011	Broad Institute SpeTri2.0	**Available**
Squirrel monkey	saiBol1	Oct. 2011	Broad Institute SaiBol1.0	**Available**
Tarsier	tarSyr1	Aug. 2008	WUSTL/Broad Institute Tarsyr1.0	**Available**
Tasmanian devil	sarHar1	Feb. 2011	Wellcome Trust Sanger Institute Devil_refv7.0	**Available**
Tenrec	echTel2	Nov. 2012	Broad Institute EchTel2.0	**Available**
	echTel1	Jul. 2005	Broad Institute echTel1	**Available**
Tree shrew	tupBel1	Dec. 2006	Broad Institute Tupbel1.0	**Available**
Wallaby	macEug2	Sep. 2009	Tammar Wallaby Genome Sequencing Consortium Meug_1.1	**Available**
White rhinoceros	cerSim1	May 2012	Broad Institute CerSimSim1.0	**Available**
American alligator	allMis1	Aug. 2012	Int. Crocodilian Genomes Working Group allMis0.2	**Available**
Atlantic cod	gadMor1	May 2010	Genofisk GadMor_May2010	**Available**
Budgerigar	melUnd1	Sep. 2011	WUSTL v6.3	**Available**
Chicken	galGal4	Nov. 2011	ICGC Gallus-gallus-4.0	**Available**
	galGal3	May 2006	WUSTL Gallus-gallus-2.1	**Available**
	galGal2	Feb. 2004	WUSTL Gallus-gallus-1.0	**Available**
Coelacanth	latCha1	Aug. 2011	Broad Institute LatCha1	**Available**
Elephant shark	calMil1	Dec. 2013	IMCB Callorhinchus_milli_6.1.3	**Available**
Fugu	fr3	Oct. 2011	JGI v5.0	**Available**
	fr2	Oct. 2004	JGI v4.0	**Available**
	fr1	Aug. 2002	JGI v3.0	**Available**
Lamprey	petMar2	Sep. 2010	WUGSC 7.0	**Available**
	petMar1	Mar. 2007	WUSTL v3.0	**Available**
Lizard	anoCar2	May 2010	Broad Institute AnoCar2	**Available**
	anoCar1	Feb. 2007	Broad Institute AnoCar1	**Available**
Medaka	oryLat2	Oct. 2005	NIG v1.0	**Available**
Medium ground finch	geoFor1	Apr. 2012	BGI GeoFor_1.0 / NCBI 13302	**Available**
Nile tilapia	oreNil2	Jan. 2011	Broad Institute Release OreNil1.1	**Available**
Painted turtle	chrPic1	Dec. 2011	IPTGSC Chrysemys_picta_bellii-3.0.1	**Available**
Stickleback	gasAcu1	Feb. 2006	Broad Institute Release 1.0	**Available**
Tetraodon	tetNig2	Mar. 2007	Genoscope v7	**Available**
	tetNig1	Feb. 2004	Genoscope v7	**Available**
Turkey	melGal1	Dec. 2009	Turkey Genome Consortium v2.01	**Available**
X. tropicalis	xenTro3	Nov. 2009	JGI v.4.2	**Available**
	xenTro2	Aug. 2005	JGI v.4.1	**Available**
	xenTro1	Oct. 2004	JGI v.3.0	**Available**
Zebra finch	taeGut2	Feb. 2013	WUSTL v3.2.4	**Available**
	taeGut1	Jul. 2008	WUSTL v3.2.4	**Available**
Zebrafish	danRer7	Jul. 2010	Sanger Institute Zv9	**Available**
	danRer6	Dec. 2008	Sanger Institute Zv8	**Available**
	danRer5	Jul. 2007	Sanger Institute Zv7	**Available**
	danRer4	Mar. 2006	Sanger Institute Zv6	**Available**
	danRer3	May 2005	Sanger Institute Zv5	**Available**
	danRer2	Jun. 2004	Sanger Institute Zv4	Archived
	danRer1	Nov. 2003	Sanger Institute Zv3	Archived
			Deuterostomes	
C. intestinalis	ci2	Mar. 2005	JGI v2.0	**Available**
	ci1	Dec. 2002	JGI v1.0	**Available**
Lancelet	braFlo1	Mar. 2006	JGI v1.0	**Available**
S. purpuratus	strPur2	Sep. 2006	Baylor College of Medicine HGSC v. Spur 2.1	**Available**
	strPur1	Apr. 2005	Baylor College of Medicine HGSC v. Spur_0.5	**Available**
			Insects	
A. mellifera	apiMel2	Jan. 2005	Baylor College of Medicine HGSC v.Amel_2.0	**Available**

	apiMel1	Jul. 2004	Baylor College of Medicine HGSC v.Amel_1.2	**Available**
A. gambiae	anoGam1	Feb. 2003	IAGP v.MOZ2	**Available**
D. ananassae	droAna2	Aug. 2005	Agencourt Arachne release	**Available**
	droAna1	Jul. 2004	TIGR Celera release	**Available**
D. erecta	droEre1	Aug. 2005	Agencourt Arachne release	**Available**
D. grimshawi	droGri1	Aug. 2005	Agencourt Arachne release	**Available**
D. melanogaster	dm3	Apr. 2006	BDGP Release 5	**Available**
D. melanogaster	dm2	Apr. 2004	BDGP Release 4	**Available**
	dm1	Jan. 2003	BDGP Release 3	**Available**
D. mojavensis	droMoj2	Aug. 2005	Agencourt Arachne release	**Available**
	droMoj1	Aug. 2004	Agencourt Arachne release	**Available**
D. persimilis	droPer1	Oct. 2005	Broad Institute release	**Available**
D. pseudoobscura	dp3	Nov. 2004	Flybase Release 1.0	**Available**
	dp2	Aug. 2003	Baylor College of Medicine HGSC Freeze 1	**Available**
D. sechellia	droSec1	Oct. 2005	Broad Institute Release 1.0	**Available**
D. simulans	droSim1	Apr. 2005	WUSTL Release 1.0	**Available**
D. virilis	droVir2	Aug. 2005	Agencourt Arachne release	**Available**
	droVir1	Jul. 2004	Agencourt Arachne release	**Available**
D. yakuba	droYak2	Nov. 2005	WUSTL Release 2.0	**Available**
	droYak1	Apr. 2004	WUSTL Release 1.0	**Available**
			Nematodes	
C. brenneri	caePb2	Feb. 2008	WUSTL 6.0.1	**Available**
	caePb1	Jan. 2007	WUSTL 4.0	**Available**
C. briggsae	cb3	Jan. 2007	WUSTL Cb3	**Available**
	cb1	Jul. 2002	WormBase v. cb25.agp8	**Available**
C. elegans	ce10	Oct. 2010	WormBase v. WS220	**Available**
	ce6	May 2008	WormBase v. WS190	**Available**
	ce4	Jan. 2007	WormBase v. WS170	**Available**
	ce2	Mar. 2004	WormBase v. WS120	**Available**
	ce1	May 2003	WormBase v. WS100	Archived
C. japonica	caeJap1	Mar. 2008	WUSTL 3.0.2	**Available**
C. remanei	caeRem3	May 2007	WUSTL 15.0.1	**Available**
	caeRem2	Mar. 2006	WUSTL 1.0	**Available**
P. pacificus	priPac1	Feb. 2007	WUSTL 5.0	**Available**
			Other	
Sea Hare	aplCal1	Sep. 2008	Broad Release Aplcal2.0	**Available**
Yeast	sacCer3	apr-11	SGD April 2011 sequence	**Available**
	sacCer2	June 2008	SGD June 2008 sequence	**Available**

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