Stitch-seq-tools Documentation

Release 1.0.1

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ONE

STITCH-SEQ-TOOLS 1.0 DOCUMENTATION

1.1 Installation

1.1.1 step 1: Install the dependent prerequisites:

1. Python libraries [for python 2.x]:
   - Biopython
   - Pysam
   - BAM2X
   - Numpy, Scipy
   - Parallel python (Only for `Select_strongInteraction_pp.py`)
   - PyCogent (for annotation of RNA types) [see note]
2. The Boost.Python C++ library
3. Other softwares needed:
   - Bowtie (not Bowtie 2)
   - samtools
   - NCBI blast+ (use blastn)

Note: the Annotation feature need the development version of PyCogent (install instruction). Since we need the getTranscriptByStableId function which is described here.

1.1.2 Step 2: Download the package

Clone the package from GitHub:

```
git clone http://github.com/yu68/stitch-seq.git
```

1.1.3 Step 3: Add library source to your python path

Add these lines into your ~/.bash_profile or ~/.profile

```
Location="/path/of/Stitch-seq-tools" # change accordingly
export PYTHONPATH="$Location/src:$PYTHONPATH"
export PATH="$PATH:$Location/bin"
```

1.2 Overview

Stitch-seq-tools is a set of bioinformatic tools for analysis of a novel DNA sequencing based technology to detect RNA-RNA interactome and RNA-chromatin interactome.

1.3 Support

For issues related to the use of Stitch-seq-tools, or if you want to **report a bug or request a feature**, please contact Pengfei Yu <p3yu at ucsd dot edu>

CHAPTER

TWO

ANALYSIS PIPELINE

2.1 Overview

The next generation DNA sequencing based technology utilize RNA proximity ligation to transfrom RNA-RNA interactions into chimeric DNAs. Through sequencing and mapping these chimeric DNAs, it is able to achieve high-throughput mapping of nearly entire interaction networks. RNA linkers were introduced to mark the junction of the ligation and help to split the chimeric RNAs into two interacting RNAs. This bioinformatic pipeline is trying to obtain the strong interactions from raw fastq sequencing data. The major steps are:

- *Step 1: Remove PCR duplicates.*
- *Step 2: Split library based on barcode.txt.*
- *Step 3: Recover fragments for each library.*
- *Step 4: Split partners and classify different types of fragments.*
- *Step 5: Align both parts of "Paired" fragment to the genome.*
- *Step 6: Determine strong interactions.*

Other functions:

1. *Determine the RNA types of different parts within fragments.*
2. *Find linker sequences within the library.*

2.2 Pipeline

2.2.1 Step 1: Remove PCR duplicates.

Starting from the raw pair-end sequencing data, PCR duplicates should be removed as the first step if both the 10nt random indexes and the remaining sequences are exactly the same for two pairs. It is achieved by `remove_dup_PE.py`

```
usage: remove_dup_PE.py [-h] reads1 reads2

Remove duplicated reads which have same sequences for both forward and reverse
reads. Choose the one appears first.

positional arguments:
  reads1      forward input fastq/fasta file
  reads2      reverse input fastq/fasta file

optional arguments:
  -h, --help  show this help message and exit
```

```
Library dependency: Bio, itertools
```

The program will generate two fastq/fasta files after removind PCR duplicates and report how many read pairs has been removed. The output are prefixed with 'Rm_dupPE'

Note: One pair is considered as a PCR duplicate only when the sequences of both two ends (including the 10nt random index) are the exactly same as any of other pairs.

2.2.2 Step 2: Split library based on barcode.txt.

After removing PCR duplicates, the libraries from different samples are separated based on 4nt barcodes in the middle of random indexes ("RRRBBBBRRR"; R: random, B: barcode). It is implemented by `split_library_pairend.py`

```
usage: split_library_pairend.py [-h] [-f | -q] [-v] [-b BARCODE]
                                [-r RANGE [RANGE ...]] [-t] [-m MAX_SCORE]
                                input1 input2

Example: split_library_pairend.py -q Rm_dupPE_example.F1.fastq
         Rm_dupPE_example.R1.fastq -b barcode.txt

positional arguments:
  input1                input fastq/fasta file 1 for pairend data (contain
                        barcodes)
  input2                input fastq/fasta file 2 for pairend data

optional arguments:
  -h, --help            show this help message and exit
  -f, --fasta           add this option for fasta input file
  -q, --fastq           add this option for fastq input file
  -v, --version         show program's version number and exit
  -b BARCODE, --barcode BARCODE
                        barcode file
  -r RANGE [RANGE ...], --range RANGE [RANGE ...]
                        set range for barcode location within reads,default is
                        full read
  -t, --trim            trim sequence of 10nt index
  -m MAX_SCORE, --max_score MAX_SCORE
                        max(mismatch+indel) allowed for barcode match,
                        otherwise move reads into 'unassigned' file
                        default: 2.

Library dependency: Bio
```

Here is a example for barcode.txt

```
ACCT
CCGG
GGCG
```

The output of this script are several pairs of fastq/fasta files prefixed with the 4nt barcode sequences, together with another pair of fastq/fasta files prefixed with 'unassigned'.

For example, if the input fastq/fasta files are `Rm_dupPE_example.F1.fastq` and `Rm_dupPE_example.R1.fastq`, and the barcode file is the same as above, then the output files are:

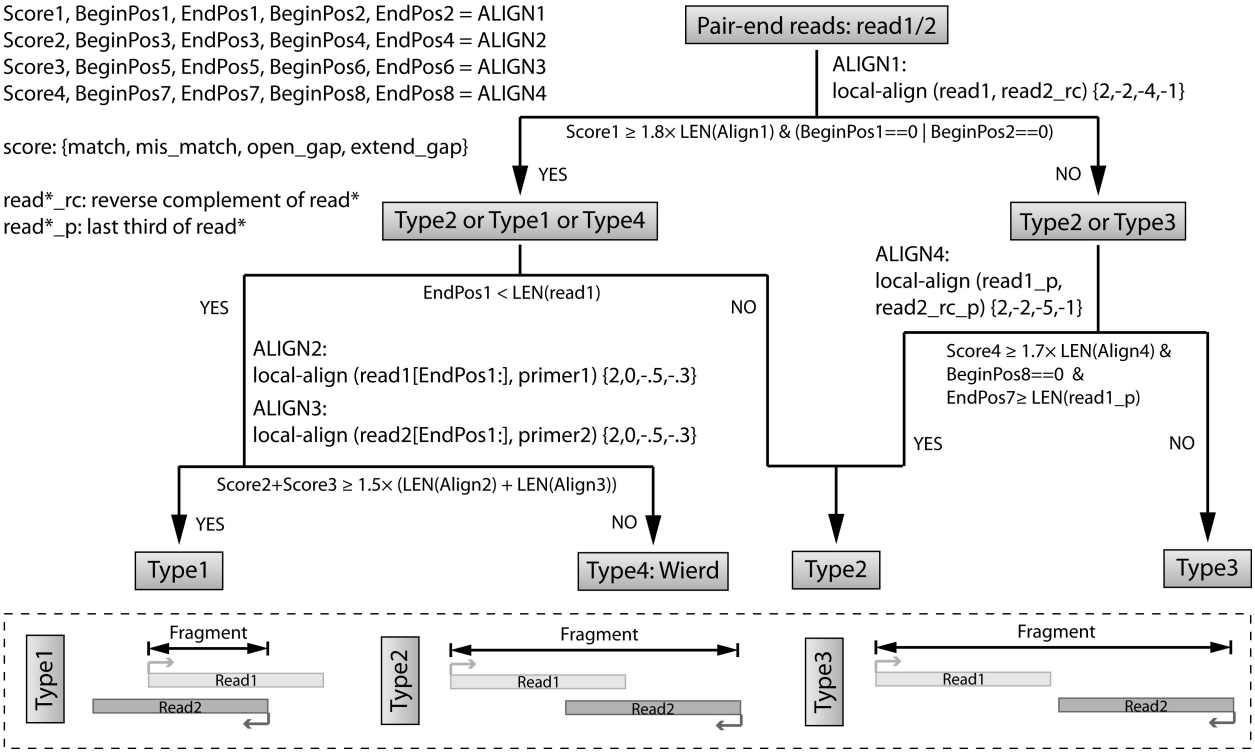
- ACCT_Rm_dupPE_example.F1.fastq

- ACCT_Rm_dupPE_example.R1.fastq
- CCGG_Rm_dupPE_example.F1.fastq
- CCGG_Rm_dupPE_example.R1.fastq
- GGCG_Rm_dupPE_example.F1.fastq
- GGCG_Rm_dupPE_example.R1.fastq
- unassigned_Rm_dupPE_example.F1.fastq
- unassigned_Rm_dupPE_example.R1.fastq

2.2.3 Step 3: Recover fragments for each library.

After splitting the libraries, the later steps from here (Step 3-6) are executed parallelly for each sample.

In this step, we are trying to recover the fragments based on local alignment. The fragments are classifed as several different types as shown in the figure below. The flow chart is also clarified at the top.



We will use a complied program `recoverFragment` to do that

```
recoverFragment - recover fragment into 4 different categories from pair-end seq data
=======================================================================================

SYNOPSIS

DESCRIPTION
    -h, --help
          Displays this help message.
    --version
          Display version information
    -I, --inputs STR
```

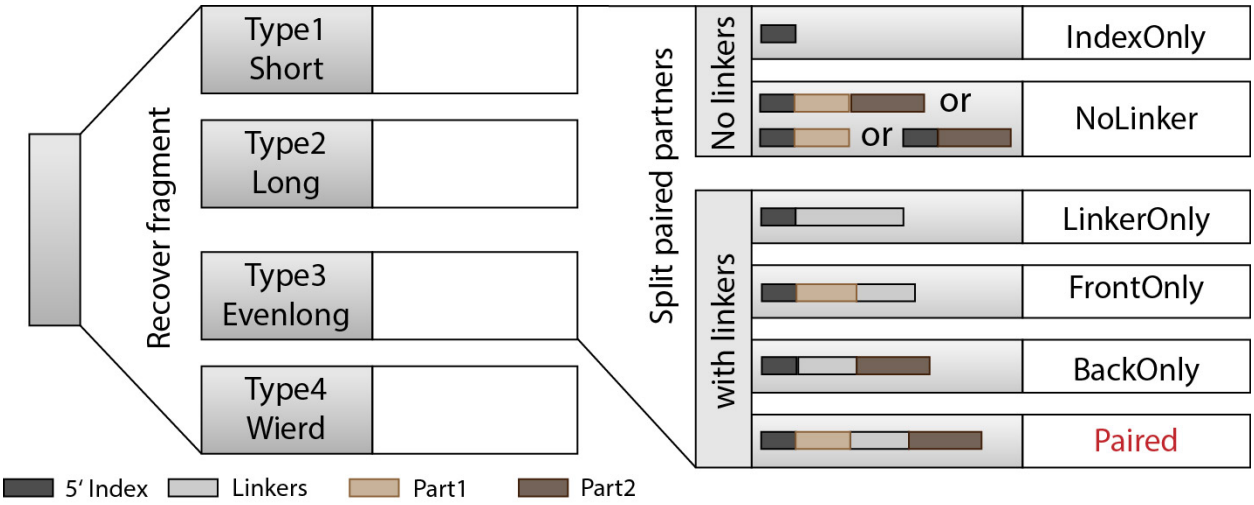
```
          input of forward and reverse fastq file, path of two files separated by SPACE
    -p, --primer STR
          fasta file contianing two primer sequences
    -v, --verbose
          print alignment information for each alignment

EXAMPLES
    recoverFragment -I read_1.fastq read_2.fastq -p primer.fasta
          store fragment using fasta/fastq into 4 output files
          'short_*', 'long_*','evenlong_*','wierd_*'

VERSION
    recoverFragment version: 0.1
    Last update August 2013
```

2.2.4 Step 4: Split partners and classify different types of fragments.

When we recovered the fragments, the next we are goting to do is to find parts that are seprarated by the linkers, and from here, we will be able to classify the fragments into different types: "IndexOnly", "NoLinker", "LinkerOnly", "BackOnly", "FrontOnly", "Paired". (see the figure below).



This will be done by `split_partner.py`

```
usage: split_partner.py [-h] [-e EVALUE] [--linker_db LINKER_DB]
                        [--blast_path BLAST_PATH] [-o OUTPUT] [-t TRIM]
                        [-b BATCH] [-l LENGTH]
                        input type3_1 type3_2

DESCRIPTION: Run BLAST, find linker sequences and split two parts connected by
linkers

positional arguments:
  input                 the input fasta file containing fragment sequences of
                        type1 and type2
  type3_1               read_1 for evenlong (type3) fastq file
  type3_2               read_2 for evenlong (type3) fastq file

optional arguments:
  -h, --help            show this help message and exit
```

```
  -e EVALUE, --evalue EVALUE
                        cutoff evalues, only choose alignment with evalue less
                        than this cutoffs (default: 1e-5).
  --linker_db LINKER_DB
                        BLAST database of linker sequences
  --blast_path BLAST_PATH
                        path for the local blast program
  -o OUTPUT, --output OUTPUT
                        output file containing sequences of two sepatated
                        parts
  -t TRIM, --trim TRIM  trim off the first this number of nt as index,
                        default:10
  -b BATCH, --batch BATCH
                        batch this number of fragments for BLAST at a time.
                        default: 100000
  -l LENGTH, --length LENGTH
                        shortest length to be considered for each part of the
                        pair, default: 15

Library dependency: Bio, itertools
```

The linker fasta file contain sequences of all linkers

```
>L1
CTAGTAGCCCATGCAATGCGAGGA
>L2
AGGAGCGTAACGTACCCGATGATC
```

The output fasta files will be the input file name with different prefix ("NoLinker", "LinkerOnly", "BackOnly", "FrontOnly", "Paired") for different types. The other output file specified by `-o` contains information of aligned linker sequences for each Type1/2 fragment.

For example, if the commend is

```
split_partner.py fragment_ACCT.fasta evenlong_ACCTRm_dupPE_stitch_seq_1.fastq
    evenlong_ACCTRm_dupPE_stitch_seq_2.fastq
    -o fragment_ACCT_detail.txt --linker_db linker.fa
```

Then, the output files will be:

- backOnly_fragment_ACCT.fasta
- NoLinker_fragment_ACCT.fasta
- frontOnly_fragment_ACCT.fasta
- Paired1_fragment_ACCT.fasta
- Paired2_fragment_ACCT.fasta
- fragment_ACCT_detail.txt

The format of the last output file `fragment_ACCT_detail.txt` will be "Name | linker_num | linker_loc | Type | linker_order". Here are two examples:

```
HWI-ST1001:238:H0NYEADXX:1:1101:10221:1918      L1:2;L2:1  19,41;42,67;68,97       None    L2;L1;L1
HWI-ST1001:238:H0NYEADXX:1:1101:4620:2609       L1:2 28,46;47,79     Paired  L1;L1
```

In the **first** fragment, there are three regions can be aligned to linkers, 2 for L1 and 1 for L2, the order is L2, L1, L1. And they are aligned in region [19,41], [42,67], [68,97] of the fragment. "None" means this fragment is either 'LinkerOnly' or 'IndexOnly' (in this case it is 'LinkerOnly'). This fragment won't be written to any of the output fasta files.

In the **second** fragment, two regions can be aligned to linkers, and they are both aligned to L1. The two regions are in [28,46], [47,79] of the fragment. the fragment is "Paired" because on both two sides flanking the linker aligned regions, the length is larger than 15nt. The left part will be writen in `Paired1_fragment_ACCT.fasta` and the right part in `Paired2_fragment_ACCT.fasta`

2.2.5 Step 5: Align both parts of "Paired" fragment to the genome.

In this step, we will use the Paired1* and Paired2* fasta files output from the previous step. The sequences of part1 and part2 are aligned to the mouse genome mm9 with Bowtie and the pairs with both part1 and part2 mappable are selected as output. We also annotate the RNA types of each part in this step. All of these are implemented using script `Stitch-seq_Aligner.py`.

```
usage: Stitch-seq_Aligner.py [-h] [-s samtool_path] [-a ANNOTATION]
                             [-A DB_DETAIL]
                             miRNA_reads mRNA_reads bowtie_path miRNA_ref
                             mRNA_ref

Align miRNA-mRNA pairs for Stitch-seq. print the alignable miRNA-mRNA pairs
with coordinates

positional arguments:
  part1_reads           paired part1 fasta file
  part2_reads           paired part2 fasta file
  bowtie_path           path for the bowtie program
  part1_ref             reference genomic seq for part1
  part2_ref             reference genomic seq for part2

optional arguments:
  -h, --help            show this help message and exit
  -s samtool_path, --samtool_path samtool_path
                        path for the samtool program
  -a ANNOTATION, --annotation ANNOTATION
                        If specified, include the RNA type annotation for each
                        aligned pair, need to give bed annotation RNA file
  -A DB_DETAIL, --annotationGenebed DB_DETAIL
                        annotation bed12 file for lincRNA and mRNA with intron
                        and exon

Library dependency: Bio, pysam, itertools
```

An annotation file for different types of RNAs in mm9 genome (bed format, 'all_RNAs-rRNA_repeat.txt.gz') was included in Data folder. The annotation bed12 file for lincRNA and mRNA ('Ensembl_mm9.genebed.gz') was also included in Data folder. One can use the option `-a ../Data/all_RNAs-rRNA_repeat.txt.gz -A ../Data/Ensembl_mm9.genebed.gz` for annotation.

Here is a example:

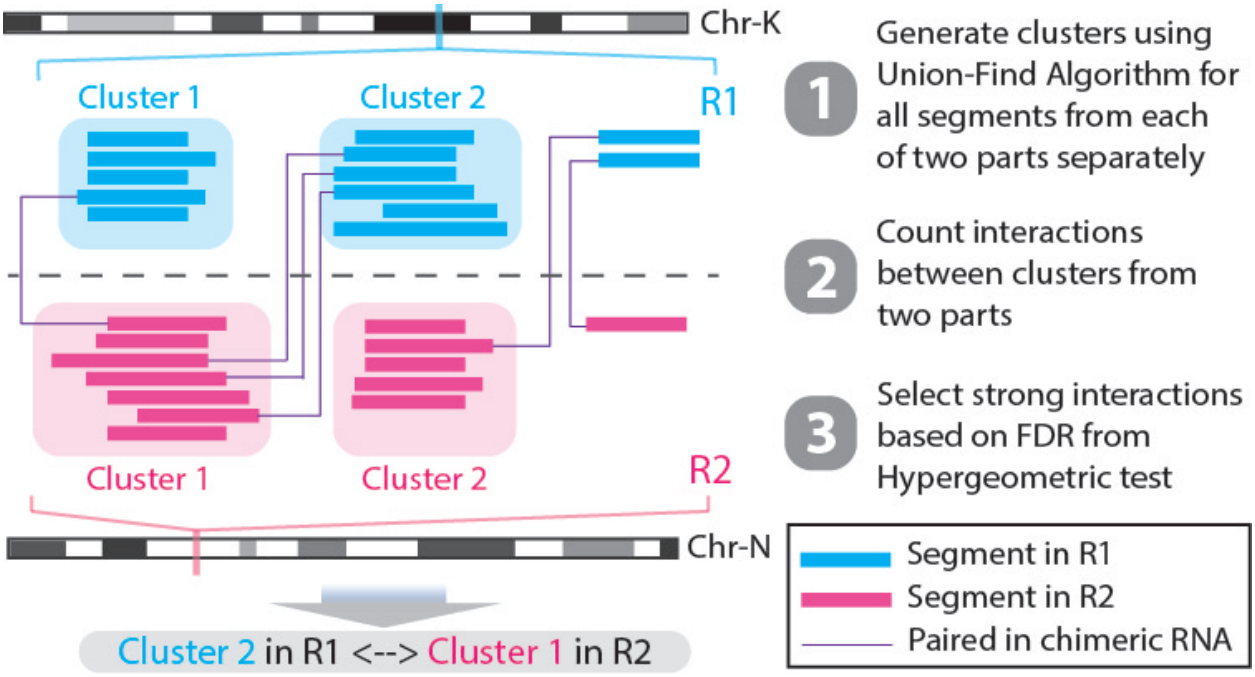
```
Stitch-seq_Aligner.py Paired1_fragment_ACCT.fasta Paired2_fragment_ACCT.fasta
    ~/Software/bowtie-0.12.7/bowtie mm9 mm9 -s samtools
    -a ../Data/all_RNAs-rRNA_repeat.txt.gz -A ../Data/Ensembl_mm9.genebed.gz
    > ACCT_fragment_paired_align.txt
```

The format for the output file `ACCT_fragment_paired_align.txt` will be:

Column [1]	Description
1	chromosome name of part1
2,3	start/end position of part1
4	sequence of part1
5	RNA type for part1
6	RNA name for part1
7	RNA subtype [2] for part1
8	name of the pair

2.2.6 Step 6: Determine strong interactions.

In this step, we will generate clusters with high coverage separately for all part1 (R1) an part2 (R2) segments. Then based on the pairing information, we count the interactions between clusters from part1 and part2. The strong interactions can be selected by applying a p-value cutoff from hypergeometric test. (See figure below)



We will use the script `Select_strongInteraction_pp.py`, parallel computing are implemented for clustering parallelly on different chromosomes:

```
usage: Select_strongInteraction_pp.py [-h] -i INPUT [-M MIN_CLUSTERS]
                                      [-m MIN_INTERACTION] [-p P_VALUE]
                                      [-o OUTPUT] [-P PARALLEL] [-F]

find strong interactions from paired genomic location data

optional arguments:
  -h, --help            show this help message and exit
  -i INPUT, --input INPUT
                        input file which is the output file of Stitch-seq-
                        Aligner.py
  -M MIN_CLUSTERS, --min_clusterS MIN_CLUSTERS
                        minimum number of segments allowed in each cluster,
```

[1] column 9-15 are the same as column 1-7 except they are for part2 instead of part1.
[2] subtype can be intron/exon/utr5/utr3 for lincRNA and mRNA (protein-coding), '.' for others

```
                        default:5
  -m MIN_INTERACTION, --min_interaction MIN_INTERACTION
                        minimum number of interactions to support a strong
                        interaction, default:3
  -p P_VALUE, --p_value P_VALUE
                        the p-value based on hypergeometric distribution to
                        call strong interactions, default: 0.05
  -o OUTPUT, --output OUTPUT
                        specify output file
  -P PARALLEL, --parallel PARALLEL
                        number of workers for parallel computing, default: 5
  -F, --FDR             Compute FDR if specified

need Scipy for hypergeometric distribution
```

The input of the script is the output of Step 5 (`ACCT_fragment_paired_align.txt` in the example). "annotated_bed" class is utilized in this script.

Here is a example:

```
Select_strongInteraction.py -i ACCT_fragment_paired_align.txt -o ACCT_interaction_clusters.txt
```

The column description for output file `ACCT_interaction_clusters.txt` is:

Column	Description
1	chromosome name of cluster in part1
2,3	start/end position of cluster in part1
4	RNA type for cluster in part1
5	RNA name for cluster in part1
6	RNA subtype for cluster in part1
7	# of counts for cluster in part1
8-14	Same as 1-7, but for cluster in part2
15	# of interactions between these two clusters
16	p-value of the hypergeometric testing

2.3 Other functions

2.3.1 Determine the RNA types of different parts within fragments.

2.3.2 Find linker sequences within the library.

CHAPTER

THREE

PYTHON APIS CREATED FOR THIS PROJECT

3.1 Annotation module

For the purpose of annotating RNA types for genomic regions.

`Annotation.`**`overlap`**(*bed1*, *bed2*)

This function compares overlap of two Bed object from same chromosome

Parameters

- **bed1** – A Bed object from xplib.Annotation.Bed (BAM2X)
- **bed2** – A Bed object from xplib.Annotation.Bed (BAM2X)

Returns boolean – True or False

Example:

```
>>> from xplib.Annotation import Bed
>>> bed1=Bed(["chr1",10000,12000])
>>> bed2=Bed(["chr1",9000,13000])
>>> print overlap(bed1,bed2)
True
```

`Annotation.`**`Subtype`**(*bed1*, *genebed*)

This function determines intron or exon or utr from a BED12 file.

Parameters

- **bed1** – A Bed object defined by xplib.Annotation.Bed (BAM2X)
- **genebed** – A Bed12 object representing a transcript defined by xplib Annotaton.Bed with information of exon/intron/utr from an BED12 file

Returns str – RNA subtype. "intron"/"exon"/"utr3"/"utr5"/"."

Example:

```
>>> from xplib.Annotation import Bed
>>> from xplib import DBI
>>> bed1=Bed(["chr13",40975747,40975770])
>>> a=DBI.init("../../Data/Ensembl_mm9.genebed.gz","bed")
>>> genebed=a.query(bed1).next()
>>> print Subtype(bed1,genebed)
"Intron"
```

`Annotation.`**`annotation`**(*bed*, *ref_allRNA*, *ref_detail*)

This function is based on `overlap()` and `Subtype()` functions to annotate RNA type/name/subtype for any genomic region.

Parameters

- **bed** – A Bed object defined by xplib.Annotation.Bed (in BAM2X).
- **ref_allRNA** – the DBI.init file (from BAM2X) for bed6 file of all kinds of RNA
- **ref_detail** – the DBI.init file for bed12 file of lincRNA and mRNA with intron, exon, UTR

Returns list of str – [type,name,subtype]

Example:

```
>>> from xplib.Annotation import Bed
>>> from xplib import DBI
>>> bed=Bed(["chr13",40975747,40975770])
>>> ref_allRNA=DBI.init("../../Data/all_RNAs-rRNA_repeat.txt.gz","bed")
>>> ref_detail=DBI.init("../../Data/Ensembl_mm9.genebed.gz","bed")
>>> print annotation(bed,ref_allRNA,ref_detail)
["protein_coding","gcnt2","intron"]
```

3.2 "annotated_bed" data class

class data_structure.**annotated_bed**(*x=None*, ***kwargs*)

To store, compare, cluster for the genomic regions with RNA annotation information. Utlized in the program *Select_stronginteraction_pp.py*

Cluster(*c*)

Store cluster information of self object

Parameters **c** – cluster index

Example:

```
>>> a=annotated_bed(chr="chr13",start=40975747,end=40975770)
>>> a.Cluster(3)
>>> print a.cluster
3
```

Note: a.cluster will be the count information when a become a cluster object in *Select_stronginteraction_pp.py*

Update(*S*, *E*)

Update the upper and lower bound of the cluster after adding segments using Union-Find.

Parameters

- **S** – start loc of the newly added genomic segment
- **E** – end loc of the newly added genomic segment

Example:

```
>>> a=annotated_bed(chr="chr13",start=40975747,end=40975770)
>>> a.Update(40975700,40975800)
>>> print a.start, a.end
40975700 40975800
```

__init__(*x=None*, ***kwargs*)

Initiation example:

```
>>> str="chr13  40975747        40975770        ATTAAG...TGA    protein_coding  gcnt2   intr
>>> a=annotated_bed(str)
or
>>> a=annotated_bed(chr="chr13",start=40975747,end=40975770,type="protein_coding",)
```

__lt__(*other*)

Compare two objects self and other when they are not **overlapped**

Parameters **other** – another `annotated_bed` object

Returns boolean – "None" if overlapped.

Example:

```
>>> a=annotated_bed(chr="chr13",start=40975747,end=40975770)
>>> b=annotated_bed(chr="chr13",start=10003212,end=10005400)
>>> print a>b
False
```

__str__()

Use print function to output the cluster information (chr, start, end, type, name, subtype,cluster)

Example:

```
>>> str="chr13  40975747        40975770        ATTAAG...TGA    protein_coding  gcnt2   intr
>>> a=annotated_bed(str)
>>> a.Cluster(3)
>>> a.Update(40975700,40975800)
>>> print a
"chr13  40975700        40975800        protein_coding  gcnt2   intron  3"
```

overlap(*other*)

Find overlap between regions

Parameters **other** – another `annotated_bed` object

Returns boolean

CHAPTER

FOUR

INDICES AND TABLES

- *genindex*
- *modindex*
- *search*

a

Symbols

A

C

O

R

S

U