A TOUR THROUGH HTSEQ

This is a one-chapter excerpt from the HTSeq documentation. For the full documentation, please see ***`http://www-huber.embl.de/users/anders/HTSeq/`.***

This tour demonstrates the functionality of HTSeq by performing a number of common analysis tasks:

- Getting statistical summaries about the base-call quality scores to study the data quality.
- Calculating a coverage vector and exporting it for visualization in a genome browser.
- Reading in annotation data from a GFF file.
- Assigning aligned reads from an RNA-Seq experiments to exons and genes.

The following description assumes that the reader is familiar with Python and with HTS data.

If you want to try out the examples on your own system, you can download the used example files here: HTSeq_example_data.tgz

Reading in reads

In the example data, a FASTQ file is provided with example reads from a yeast RNA-Seq experiment. The file `yeast_RNASeq_excerpt_sequence.txt` is an excerpt of the `_sequence.txt` file produced by the SolexaPipeline software. We can access it from HTSeq with

```
>>> import HTSeq
>>> fastq_file = HTSeq.FastqReader( "yeast_RNASeq_excerpt_sequence.txt", "solexa" )
```

The first argument is the file name, the optional second argument indicates that the quality values are encoded according to Solexa's specification. If you omit it, the default "phred" is assumed, which means the encoding originally suggested by the Sanger Institute. (A third option is "solexa_old", for data from the SolexaPipeline prior to version 1.3.)

The variable `fastq_file` is now an object of class `FastqReader`, which refers to the file:

```
>>> fastq_file
<FastqReader object, connected to file name 'yeast_RNASeq_excerpt_sequence.txt'>
```

When used in a `for` loop, it generates an iterator of objects representing the reads. Here, we use the `islice` function from `itertools` to cut after 10 reads.

```
>>> import itertools
>>> for read in itertools.islice( fastq_file, 10 ):
...     print read
CTTACGTTTTCTGTATCAATACTCGATTTATCATCT
```

```
AATTGGTTTCCCCGCCGAGACCGTACACTACCAGCC
TTTGGACTTGATTGTTGACGCTATCAAGGCTGCTGG
ATCTCATATACAATGTCTATCCCAGAAACTCAAAAA
AAAGTTCGAATTAGGCCGTCAACCAGCCAACACCAA
GGAGCAAATTGCCAACAAGGAAAGGCAATATAACGA
AGACAAGCTGCTGCTTCTGTTGTTCCATCTGCTTCC
AAGAGGTTTGAGATCTTTGACCACCGTCTGGGCTGA
GTCATCACTATCAGAGAAGGTAGAACATTGGAAGAT
ACTTTTAAAGATTGGCCAAGAATTGGGGATTGAAGA
```

Of course, there is more to a read than its sequence. The variable `read` still contains the tenth read, and we may examine it:

```
>>> read
<SequenceWithQualities object 'HWI-EAS225:1:10:1284:142#0/1'>
```

A `Sequence` object has two slots, called `seq` and `name`. This here is a `SequenceWithQualities`, and it also has a slot `qual`:

```
>>> read.name
'HWI-EAS225:1:10:1284:142#0/1'
>>> read.seq
'ACTTTTAAAGATTGGCCAAGAATTGGGGATTGAAGA'
>>> read.qual
array([33, 33, 33, 33, 33, 33, 29, 27, 29, 32, 29, 30, 30, 21, 22, 25, 25,
       25, 23, 28, 24, 24, 29, 29, 29, 25, 28, 24, 24, 26, 25, 25, 24, 24,
       24, 24])
```

The values in the quality array are, for each base in the sequence, the Phred score for the correctness of the base.

As a first simple example for the use of HTSeq, we now calculate the average quality score for each position in the reads by adding up the `qual` arrays from all reads and the dividing by the number of reads. We sum everything up in the variable `qualsum`, a `numpy` array of integers:

```
>>> import numpy
>>> len( read )
36
>>> qualsum = numpy.zeros( len(read), numpy.int )
```

Then we loop through the fastq file, adding up the quality scores and counting the reads:

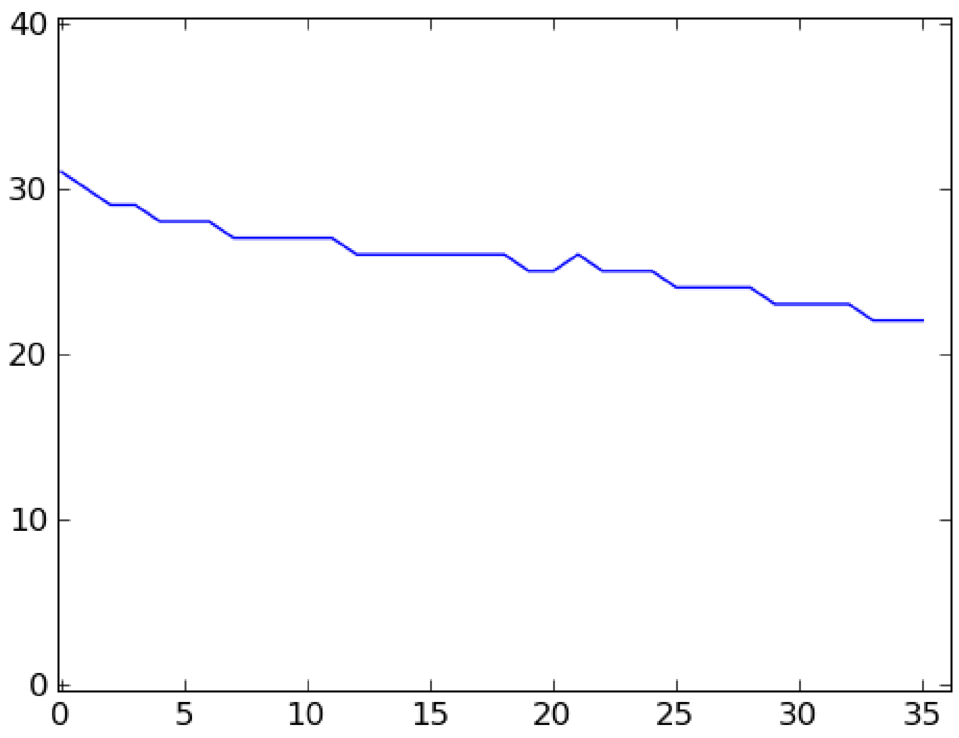
```
>>> nreads = 0
>>> for read in fastq_file:
...    qualsum += read.qual
...    nreads += 1
```

The average qualities are hence:

```
>>> qualsum / float(nreads)
array([ 31.56838274,  30.08288332,  29.4375375 ,  29.00432017,
        28.55290212,  28.26825073,  28.46681867,  27.59082363,
        27.34097364,  27.57330293,  27.11784471,  27.19432777,
        26.84023361,  26.76267051,  26.44885795,  26.79135165,
        26.42901716,  26.49849994,  26.13604544,  25.95823833,
        25.54922197,  26.20460818,  25.42333693,  25.72298892,
        25.04164167,  24.75151006,  24.48561942,  24.27061082,
        24.10720429,  23.68026721,  23.52034081,  23.49437978,
        23.11076443,  22.5576223 ,  22.43549742,  22.62354494])
```

If you have matplotlib installed, you can plot this.

```
>>> from matplotlib import pyplot
>>> pyplot.plot( qualsum / nreads )
[<matplotlib.lines.Line2D object at 0x...>]
>>> pyplot.show()
```



This is a very simple way of looking at the quality scores. For more sophisticated quality-control techniques, see the Chapter *Quality Assessment with htseq-qa*.

What if you did not get the `_sequence.txt` file from your core facility but instead the `export.txt` file? While the former contains only the reads and their quality, the latter also contains the alignment of the reads to a reference as found by Eland. To read it, simply use

```
>>> alignment_file = HTSeq.SolexaExportReader( "yeast_RNASeq_excerpt_export.txt" )
```

`HTSeq` can also use other alignment formats, e.g., SAM:

```
>>> alignment_file = HTSeq.SAM_Reader( "yeast_RNASeq_excerpt.sam" )
```

If we are only interested in the qualities, we can rewrite the commands from above to use the `alignment_file`:

```
>>> nreads = 0
>>> for aln in alignment_file:
...     qualsum += aln.read.qual
...     nreads += 1
```

We have simple replaced the `FastqReader` with a `SolexaExportReader`, which iterates, when used in a `for` loop, over `SolexaExportAlignment` objects. Each of these contain a field `read` that contains the `SequenceWithQualities` object, as before. There are more parses, for example the `SAM_Reader` that can read SAM files, and generates `SAM_Alignment` objects. As all `Alignment` objects contain a `read` slot with the `SequenceWithQualities`, we can use the same code with any alignment file for which a parser has been provided, and all we have to change is the name of the reader class in the first line.

The other fields that all `Alignment` objects contain, is a Boolean called `aligned` that tells us whether the read has been aligned at all, and a field called `iv` (for "interval") that shows where the read was aligned to. We use this information in the next section.

Calculating coverage vectors

By a "coverage vector", we mean a vector (one-dimensional array) of the length of a chromosome, where each element counts how many reads cover the corresponding base pair in their alignment. As chromosomes can be very long, it would be very inefficient to hold a coverage vector in memory by reserving space for each base pair. Rather, we take advantage of the fact that the value of the coverage vector usually stays constant (often it is just zero) over stretches of varying length, which we call steps. A `StepVector` is a data structure defined for this purpose.

It works as follows: Let's define a `StepVector` of length 30:

```
>>> sv = HTSeq.StepVector.StepVector( 30 )
```

Initially, it has value 0 everywhere. We set the positions 7 to 15 to the value 120:

```
>>> sv[ 7:15 ] = 120
```

Internally, `sv` now does not hold 30 numbers, but 3 steps, as follows:

```
>>> list( sv.get_steps() )
[(0, 7, 0.0), (7, 15, 120.0), (15, 30, 0.0)]
```

Each step is a triple, giving start, end and value of the step. If we now add the value 100 to the positions 10 to 20, the steps get split accordingly:

```
>>> sv.add_value( 100, 10, 20 )
>>> list( sv.get_steps() )
[(0, 7, 0.0), (7, 10, 120.0), (10, 15, 220.0), (15, 20, 100.0), (20, 30, 0.0)]
```

If you iterate over a **StepVector** object, it behaves like a list:

```
>>> list( sv )
[0.0, 0.0, 0.0, 0.0, 0.0, 0.0, 0.0, 120.0, 120.0, 120.0, 220.0, 220.0, 220.0,
220.0, 220.0, 100.0, 100.0, 100.0, 100.0, 100.0, 0.0, 0.0, 0.0, 0.0, 0.0,
0.0, 0.0, 0.0, 0.0, 0.0]
```

You can also take parts of a `StepVector`, which produces a new, shorter, `StepVector`.

```
>>> sv[6:12]
<StepVector object, type 'd', index range 6:12, 3 step(s)>
>>> sv[6:12].get_steps()
<generator object ...>
>>> list( sv[6:12].get_steps() )
[(6, 7, 0.0), (7, 10, 120.0), (10, 12, 220.0)]
>>> list( sv[6:12] )
[0.0, 120.0, 120.0, 120.0, 220.0, 220.0]
```

In practice, you will not work with **StepVector** objects directly, but rather with objects of class `GenomicArray`. These hold several step vectors, either one for each chromosome ("non-stranded genomic array") or one for each strand, i.e., two per chromosome ("stranded genomic array"). To specify the locations of steps on a `GenomicArray`, objects of class `GenomicInterval` are used, which are instantiated by specifying chromosome name, start, end, and position:

```
>>> iv = HTSeq.GenomicInterval( "II", 100234, 100789, "+" )
>>> iv
<GenomicInterval object 'II', [100234,100789), strand '+'>
>>> print iv
II:[100234,100789)/+
```

A `GenomicInterval` is a simple data structure with four slots:

```
>>> iv.chrom
'II'
>>> iv.start
100234
>>> iv.end
100789
>>> iv.strand
'+'
```

Two notes: `chrom` does not have to be chromosome, it could also be a contig name, or any other identifier. `strand` can be `+`, `-`, or `.`, where the latter means "no strand", to be used whenever specifying a strand would be meaning-less.

A `GenomicInterval` has some more features, e.g., to calculate overlaps etc. See the reference documentation for these.

In order to calculate the coverage vectors for our yeast RNA-Seq data, we first need a list of all the chromosomes in yeast:

```
>>> yeast_chroms = [ "2-micron", "MT", "I", "II", "III", "IV", "V", "VI", "VII",
...     "VIII", "IX", "X", "XI", "XII", "XIII", "XIV", "XV", "XVI" ]
```

Now, we define a `GenomicArray`:

```
>>> cvg = HTSeq.GenomicArray( yeast_chroms, stranded=True, typecode='i' )
```

As we specified `stranded=True`, there are now two `StepVector` objects for each chromosome, all holding integer values (`typecode='i'`). They all have an "infinte" length as we did not specify the actual lengths of the chromosomes.

```
>>> import pprint
>>> pprint.pprint( cvg.step_vectors )
 {'2-micron': {'+': <StepVector object, type 'i', index range 0:inf, 1 step(s)>,
               '-': <StepVector object, type 'i', index range 0:inf, 1 step(s)>},
  'I': {'+': <StepVector object, type 'i', index range 0:inf, 1 step(s)>,
        '-': <StepVector object, type 'i', index range 0:inf, 1 step(s)>},
  'II': {'+': <StepVector object, type 'i', index range 0:inf, 1 step(s)>,
         '-': <StepVector object, type 'i', index range 0:inf, 1 step(s)>},
  'III': {'+': <StepVector object, type 'i', index range 0:inf, 1 step(s)>,
          '-': <StepVector object, type 'i', index range 0:inf, 1 step(s)>},
  'IV': {'+': <StepVector object, type 'i', index range 0:inf, 1 step(s)>,
         '-': <StepVector object, type 'i', index range 0:inf, 1 step(s)>},
  'IX': {'+': <StepVector object, type 'i', index range 0:inf, 1 step(s)>,
         '-': <StepVector object, type 'i', index range 0:inf, 1 step(s)>},
  'MT': {'+': <StepVector object, type 'i', index range 0:inf, 1 step(s)>,
         '-': <StepVector object, type 'i', index range 0:inf, 1 step(s)>},
  'V': {'+': <StepVector object, type 'i', index range 0:inf, 1 step(s)>,
        '-': <StepVector object, type 'i', index range 0:inf, 1 step(s)>},
  'VI': {'+': <StepVector object, type 'i', index range 0:inf, 1 step(s)>,
         '-': <StepVector object, type 'i', index range 0:inf, 1 step(s)>},
  'VII': {'+': <StepVector object, type 'i', index range 0:inf, 1 step(s)>,
          '-': <StepVector object, type 'i', index range 0:inf, 1 step(s)>},
  'VIII': {'+': <StepVector object, type 'i', index range 0:inf, 1 step(s)>,
           '-': <StepVector object, type 'i', index range 0:inf, 1 step(s)>},
  'X': {'+': <StepVector object, type 'i', index range 0:inf, 1 step(s)>,
        '-': <StepVector object, type 'i', index range 0:inf, 1 step(s)>},
  'XI': {'+': <StepVector object, type 'i', index range 0:inf, 1 step(s)>,
         '-': <StepVector object, type 'i', index range 0:inf, 1 step(s)>},
  'XII': {'+': <StepVector object, type 'i', index range 0:inf, 1 step(s)>,
          '-': <StepVector object, type 'i', index range 0:inf, 1 step(s)>},
  'XIII': {'+': <StepVector object, type 'i', index range 0:inf, 1 step(s)>,
           '-': <StepVector object, type 'i', index range 0:inf, 1 step(s)>},
  'XIV': {'+': <StepVector object, type 'i', index range 0:inf, 1 step(s)>,
          '-': <StepVector object, type 'i', index range 0:inf, 1 step(s)>},
  'XV': {'+': <StepVector object, type 'i', index range 0:inf, 1 step(s)>,
         '-': <StepVector object, type 'i', index range 0:inf, 1 step(s)>},
  'XVI': {'+': <StepVector object, type 'i', index range 0:inf, 1 step(s)>,
          '-': <StepVector object, type 'i', index range 0:inf, 1 step(s)>}}
```

The integer values are all initialized to 0. We may put them to a value, say 100, at the genomic interval `iv` defined above:

```
>>> cvg[ iv ] = 100
```

If we want to add a value, we use:

```
>>> cvg.add_value( 50, iv )
```

To see the effect, let's make the interval slightly longer and then look at the steps:

```
>>> iv.start -= 30
>>> iv.end += 100
>>> pprint.pprint( list( cvg.get_steps( iv ) ) )
[(<GenomicInterval object 'II', [100204,100234), strand '+'>, 0),
 (<GenomicInterval object 'II', [100234,100789), strand '+'>, 150),
 (<GenomicInterval object 'II', [100789,100889), strand '+'>, 0)]
```

With these tools, we can now calculate the coverage vector very easily. We just iterate through all the reads and add the value 1 at the interval to which each read was aligned to:

```
>>> alignment_file = HTSeq.SAM_Reader( "yeast_RNASeq_excerpt.sam" )
>>> cvg = HTSeq.GenomicArray( yeast_chroms, stranded=True, typecode='i' )
>>> for alngt in alignment_file:
...    if alngt.aligned:
...       cvg.add_value( 1, alngt.iv )
```

We can plot an excerpt of this with:

```
>>> pyplot.plot( list( cvg[ HTSeq.GenomicInterval( "III", 200000, 500000, "+" ) ] ) )
[<matplotlib.lines.Line2D object at 0x...>]
```

However, a proper genome browser gives a better impression of the data. The following commands write two BedGraph (Wiggle) files, one for the plus and one for the minus strands:

```
>>> cvg.write_bedgraph_file( "plus.wig", "+" )
>>> cvg.write_bedgraph_file( "minus.wig", "-" )
```

These two files can then be viewed in a genome browser (e.g. IGB), alongside the annotation from a GFF file (see below).

Counting reads by genes

As the example data is from an RNA-Seq experiment, we want to know how many reads fall into the exonic regions of each gene. For this purpose we first need to read in information about the positions of the exons. A convenient source of such information are the GTF files from Ensembl (to be found here).

These file are in the GTF format, a tightening of the GFF format. HTSeq offers the `GFF_Reader` class to read in a GFF file:

```
>>> gtf_file = HTSeq.GFF_Reader( "Saccharomyces_cerevisiae.SGD1.01.56.gtf.gz" )
>>> for feature in itertools.islice( gtf_file, 10 ):
...    print feature
...
<GenomicFeature: exon 'R0010W' at 2-micron: 251 -> 1523 (strand '+')>
<GenomicFeature: CDS 'R0010W' at 2-micron: 251 -> 1520 (strand '+')>
<GenomicFeature: start_codon 'R0010W' at 2-micron: 251 -> 254 (strand '+')>
<GenomicFeature: stop_codon 'R0010W' at 2-micron: 1520 -> 1523 (strand '+')>
<GenomicFeature: exon 'R0020C' at 2-micron: 3007 -> 1887 (strand '-')>
<GenomicFeature: CDS 'R0020C' at 2-micron: 3007 -> 1890 (strand '-')>
<GenomicFeature: start_codon 'R0020C' at 2-micron: 3007 -> 3006 (strand '-')>
<GenomicFeature: stop_codon 'R0020C' at 2-micron: 1888 -> 1887 (strand '-')>
<GenomicFeature: exon 'R0030W' at 2-micron: 3270 -> 3816 (strand '+')>
<GenomicFeature: CDS 'R0030W' at 2-micron: 3270 -> 3813 (strand '+')>
```

The `feature` variable is filled with objects of class `GenomicFeature`. As with all Python objects, the **dir** function shows us its slots and functions:

```
>>> dir( feature )
['__class__', ..., '__weakref__', 'attr', 'frame', 'get_gff_line',
'iv', 'name', 'score', 'source', 'type']
```

Ignoring the attributes starting with an underscore, we can see now how to access the information stored in the GFF file. The information from the columns of the GFF table is accessible as follows:

```
>>> feature.iv
<GenomicInterval object '2-micron', [3270,3813), strand '+'>
>>> feature.source
'protein_coding'
>>> feature.type
'CDS'
>>> feature.score
'.'
```

The last column (the attributes) is parsed and presented as a dict:

```
>>> feature.attr
{'exon_number': '1', 'gene_id': 'R0030W', 'transcript_name': 'RAF1',
'transcript_id': 'R0030W', 'protein_id': 'R0030W', 'gene_name': 'RAF1'}
```

The very first attribute in this column is usually some kind of ID, hence it is stored in the slot `name`:

```
>>> feature.name
'R0030W'
```

To deal with this data, we will use a `GenomicArray`. A GenomicArray can store not only numerical data but also arbitrary Python objects (with *typecode 'O'*). Hence, we can assign those features that correspond to exons, to steps in the GenomicArray:

```
>>> exons = HTSeq.GenomicArray( yeast_chroms, stranded=False, typecode='O' )
>>> for feature in gtf_file:
...    if feature.type == "exon":
...       exons[ feature.iv ] = feature
```

Now, we can ask what exons occur in a certain interval:

```
>>> iv = HTSeq.GenomicInterval( "II", 120000, 125000, "." )
>>> pprint.pprint( list( exons.get_steps( iv ) ) )
[(<GenomicInterval object 'II', [120000,121877), strand '.'>,
  <GenomicFeature: exon 'YBL052C' at II: 121876 -> 119382 (strand '-')>),
 (<GenomicInterval object 'II', [121877,122755), strand '.'>, None),
 (<GenomicInterval object 'II', [122755,124762), strand '.'>,
  <GenomicFeature: exon 'YBL051C' at II: 124761 -> 122756 (strand '-')>),
 (<GenomicInterval object 'II', [124762,125000), strand '.'>, None)]
```

However, our RNA-Seq experiment was not strand-specific, i.e., we do not know whether the reads came from the plus or the minus strand. This is why we defined the GenomicArray as non-stranded (`stranded=False` in the instantiation of `exons` above), instructing it to ignore all strand information. An issue with this is that we now have many overlapping genes and the simple assignment `exons[ feature.iv ] = feature` is overwriting, so that it is not clear which feature we set.

The proper solution is to store not just single features at an interval but sets of all features which are present there. A specialization of `GenomicArray`, `GenomicArrayOfSets` is offered to simplify this:

```
>>> exons = HTSeq.GenomicArrayOfSets( yeast_chroms, stranded=False )
```

We populate the array again with the feature data. This time, we use the `add_value` method, which adds an object without overwriting what might already be there. Instead, it uses sets to deal with overlaps. (Also, we only store the gene name this time, as this will be more convenient later).

```
>>> for feature in gtf_file:
...    if feature.type == "exon":
...       exons.add_value( feature.name, feature.iv )
```

Assume we have a read covering this interval:

```
>>> iv = HTSeq.GenomicInterval( "III", 23850, 23950, "." )
```

Its left half covers two genes (YCL058C, YCL058W-A), but its right half only YCL058C because YCL058W-A end in the middle of the read:

```
>>> pprint.pprint( list( exons.get_steps( iv ) ) )
[(<GenomicInterval object 'III', [23850,23925), strand '.'>,
  set(['YCL058C', 'YCL058W-A'])),
 (<GenomicInterval object 'III', [23925,23950), strand '.'>, set(['YCL058C']))]
```

Assuming the transcription boundaries in our GTF file to be correct, we may conclude that this read is from the gene that appears in both steps and not from the one that appears in only one of the steps. More generally, whenever a read overlaps multiple steps (a new step starts wherever a feature starts or ends), we get a set of feature names for each step, and we have to find the intersection of all these. This can be coded as follows:

```
>>> intersection_set = None
>>> for step_set in exons.get_steps( iv, values_only=True ):
...    if intersection_set is None:
...       intersection_set = step_set.copy()
...    else:
...       intersection_set.intersection_update( step_set )
...
```

```
>>> print intersection_set
set(['YCL058C'])
```

Here, we have used the `values_only` option of `GenomicArray.get_steps()`, as we are not interested in the intervals, only in the sets. When we look at the first step, we make a copy of the step's (in order to not disturb the values stored in `exons`.) For the following steps, we use the **intersection_update** method Python's standard **set** class, which performs a set intersection in place. Afterwards, we have a set with precisely one element. Getting this one element is a tiny bit cumbersome; to access it, one needs to write:

```
>>> list(intersection_set)[0]
'YCL058C'
```

In this way, we can go through all our aligned reads, calculate the intersection set, and, if it contains a single gene name, add a count for this gene. For the counters, we use a dict, which we initialize with a zero for each gene name:

```
>>> counts = {}
>>> for feature in gtf_file:
...    if feature.type == "exon":
...       counts[ feature.name ] = 0
```

Now, we can finally count:

```
>>> sam_file = HTSeq.SAM_Reader( "yeast_RNASeq_excerpt.sam" )
>>> for alnmt in sam_file:
...    if alnmt.aligned:
...       intersection_set = None
...       for step_set in exons.get_steps( alnmt.iv, values_only=True ):
...           if intersection_set is None:
...              intersection_set = step_set.copy()
...           else:
...              intersection_set.intersection_update( step_set )
...       if len( intersection_set ) == 1:
...          counts[ list(intersection_set)[0] ] += 1
```

We can now conveniently print the result with:

```
>>> for name in sorted( counts.keys() ):
...    print name, counts[name]
15S_rRNA 0
21S_rRNA 0
HRA1 0
...
YPR048W 2
YPR049C 3
YPR050C 0
YPR051W 1
YPR052C 1
YPR053C 5
YPR054W 0
...
tY(GUA)M2 0
tY(GUA)O 0
tY(GUA)Q 0
```

Some aligners can output gapped or spliced alignments. In a SAM file, this in encoded in the CIGAR string. HTSeq has facilities to handle this conveniently, too, with the class `CigarOperation`. Chapter *Counting reads in features with htseq-count* describes a script which offers some further counting schemes.

And much more

This tour was only meant to give an overview. There are many more tasks that can be solved with HTSeq. Have a look at the reference documentation in the following pages to see what else is there.