RNA-SEQ

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CNR Naples

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Outline

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- Alignment, Gene Expression Quantification, Coverage,
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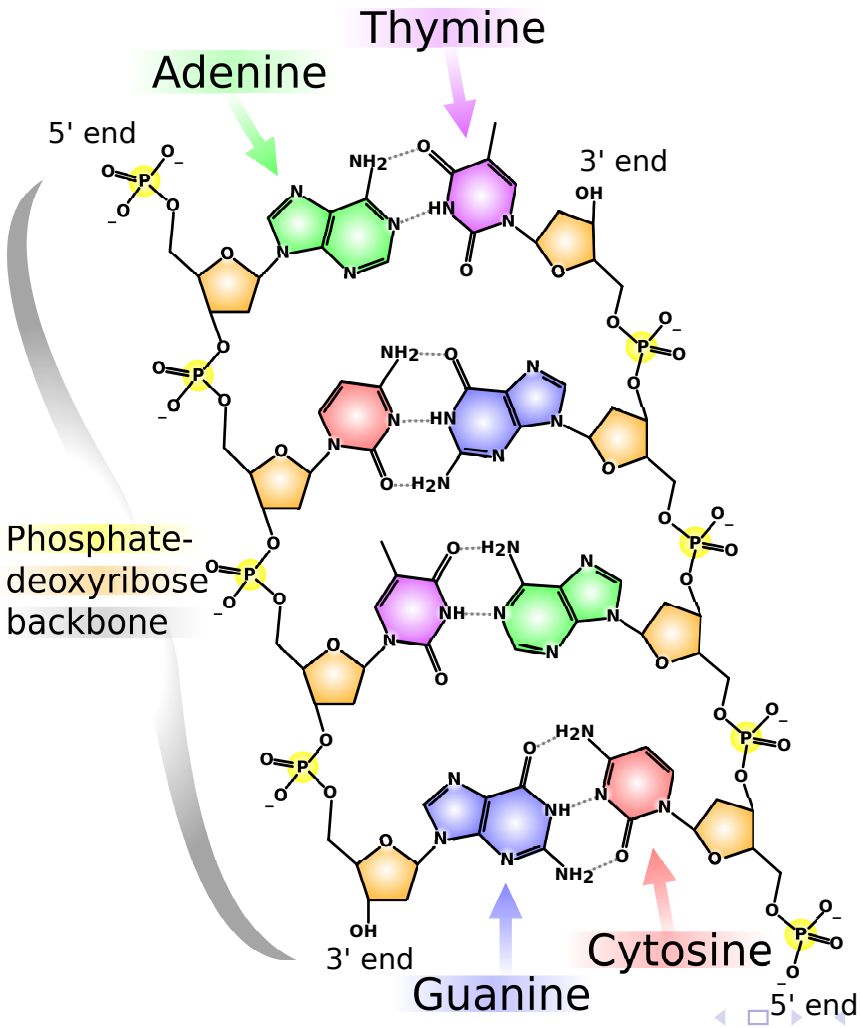
What is DNA?

DNA (Deoxy Ribonucleic Acid) is a informational molecule made from repeating units called *nucleotides*: Adenine, Guanine, Cytosine, Thymine.



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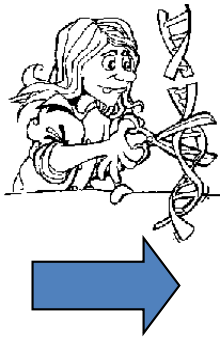
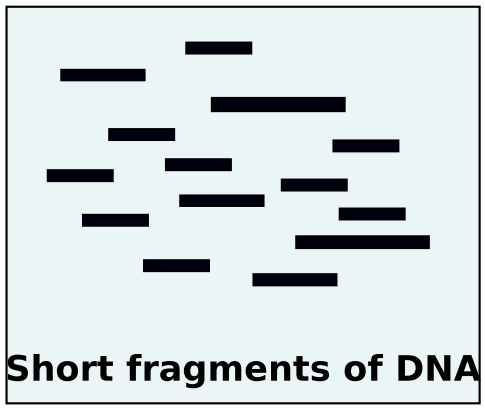
It can be used to determine the sequence of individual genes, chromosomes or entire genomes.

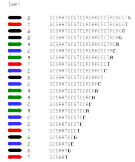
Sequencing process

Genome





Short fragments of DNA



AC..GC TG..GT TC..CC CG..CA TT..TC AC..GC GA..GC TG..AC CT..TG GT..GC AC..GC AC..GC AA..GC AT..AT TT..CC

Short DNA sequences

ACGTGGTAA CGTATACAC TAGGCCATA GTAATGGCG CACCCTTAG TGGCGTATA CATA...

ACGTGGTAATGGCGTATACACCCTTAGGCCATA

ACGTGACCGGTACTGGTAACGTACA
CCTACGTGACCGGTACTGGTAACGT
ACGCCTACGTGACCGGTACTGGTAA
CGTATACACGTGACCGGTACTGGTA
ACGTACACCTACGTGACCGGTACTG
GTAACGTACGCCTACGTGACCGGTA
CTGGTAACGTATACCTCT...

Sequenced genome

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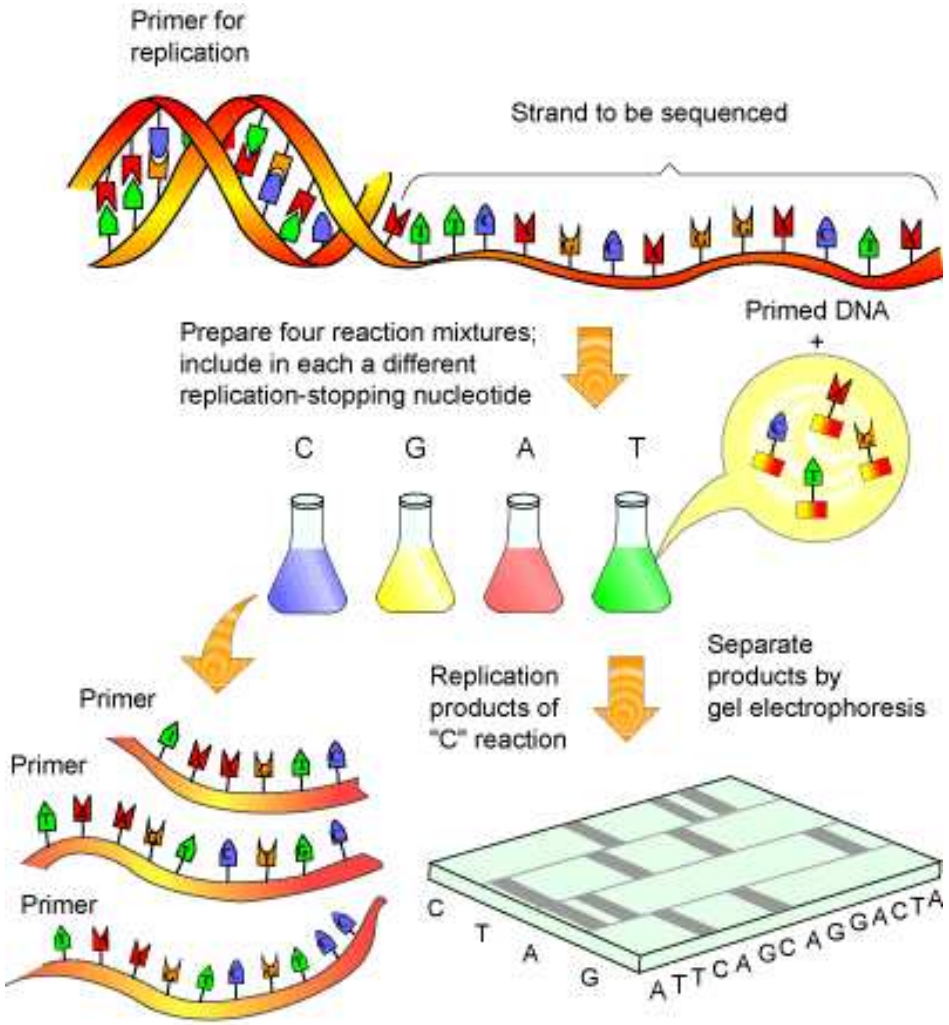
These fragments are then sorted according to size by placing them in a slab of polymeric gel and applying an electric field. This technique is called *electrophoresis*.
Because of DNA's negative charge, the fragments move across the gel toward the positive electrode. The shorter the fragment, the faster it moves.

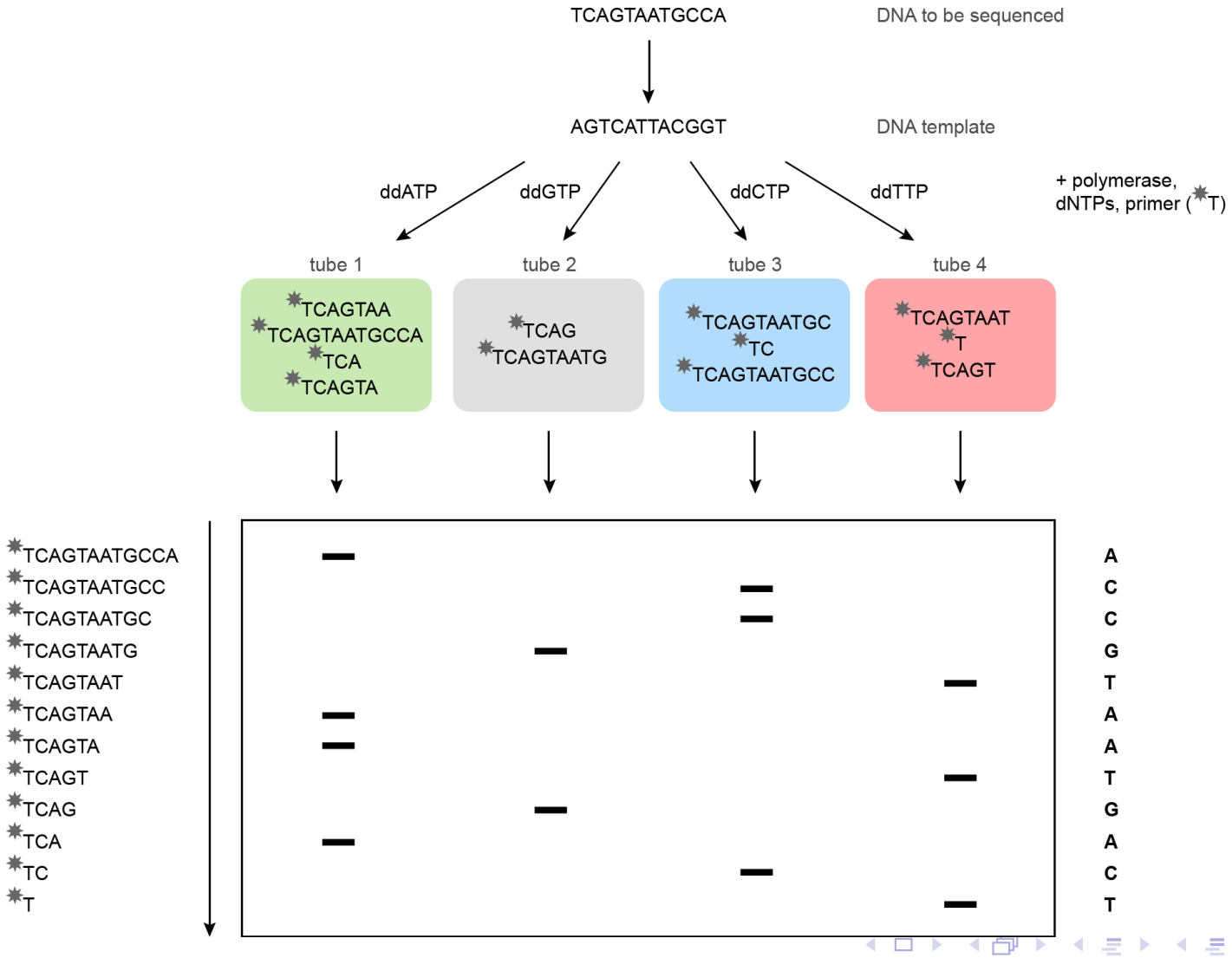
Sanger sequencing

Shortest fragments make the furthest progress. By considering the results of our race, we can reconstruct the nucleotide sequence.



Sanger sequencing

Each tube contains different length fragments.



The output Sanger sequencing machines are FASTA files

FASTA format is a text-based format for representing nucleotide sequences, in which nucleotides are represented using single-letter codes.

```
>gi|9626685|ref|NC_001477.1| Dengue virus type 1, complete genome
AGTTGTTAGTCTACGTGGACCGACAAGAACAGTTTCGAATCGGAAGCTTGCTTAACGTAGTTCTAACAGT
TTTTTATTAGAGAGCAGATCTCTGATGAACAACCAACGGAAAAAGACGGGTCGACCGTCTTTCAATATGC
TGAAACGCGCGAGAAACCGCGTGTCAACTGTTTCACAGTTGGCGAAGAGATTCTCAAAAGGATTGCTTTC
AGGCCAAGGACCCATGAAATTGGTGATGGCTTTTATAGCATTCCTAAGATTTCTAGCCATACCTCCAACA
GCAGGAATTTTGGCTAGATGGGGCTCATTCAAGAAGAATGGAGCGATCAAAGTGTTACGGGGTTTCAAGA
AAGAAATCTCAAACATGTTGAACATAATGAACAGGAGGAAAAGATCTGTGACCATGCTCCTCATGCTGCT
GCCCACAGCCCTGGCGTTCCATCTGACCACCCGAGGGGGAGAGCCGCACATGATAGTTAGCAAGCAGGAA
AGAGGAAAATCACTTTTGTTTAAGACCTCTGCAGGTGTCAACATGTGCACCCTTATTGCAATGGATTTGG
GAGAGTTATGTGAGGACACAATGACCTACAAATGCCCCCGGATCACTGAGACGGAACCAGATGACGTTGA
CTGTTGGTGCAATGCCACGGAGACATGGGTGACCTATGGAACATGTTCTCAAACTGGTGAACACCGACGA
GACAAACGTTCCGTCGCACTGGCACCACACGTAGGGCTTGGTCTAGAAACAAGAACCGAAACGTGGATGT
CCTCTGAAGGCGCTTGGAAACAAATACAAAAAGTGGAGACCTGGGCTCTGAGACACCCAGGATTCACGGT
GATAGCCCTTTTTCTAGCACATGCCATAGGAACATCCATCACCCAGAAAGGGATCATTTTTATTTTGCTG
ATGCTGGTAACTCCATCCATGGCCATGCGGTGCGTGGGAATAGGCAACAGAGACTTCGTGGAAGGACTGT
CAGGAGCTACGTGGGTGGATGTGGTACTGGAGCATGGAAGTTGCGTCACTACCATGGCAAAAGACAAACC
AACACTGGACATTGAACTCTTGAAGACGGAGGTCACAAACCCTGCCGTCCTGCGCAAACTGTGCATTGAA
GCTAAAATATCAAACACCACCACCGATTCGAGATGTCCAACACAAGGAGAAGCCACGCTGGTGGAAGAAC
AGGACACGAACTTTGTGTGTCGACGAACGTTCGTGGACAGAGGCTGGGGCAATGGTTGTGGGCTATTCGG
AAAAGGTAGCTTAATAACGTGTGCTAAGTTTAAGTGTGTGACAAAACTGGAAGGAAAGATAGTCCAATAT
GAAAACTTAAAATATTCAGTGATAGTCACCGTACACACTGGAGACCAGCACCAAGTTGGAAATGAGACCA
CAGAACATGGAACAACTGCAACCATAACACCTCAAGCTCCCACGTCGGAAATACAGCTGACAGACTACGG
AGCTCTAACATTGGATTGTTCACCTAGAACAGGGCTAGACTTTAATGAGATGGTGTTGTTGACAATGAAA
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TGAAACGCGCGAGAAACCGCGTGTCAACTGTTTCACAGTTGGCGAAGAGATTCTCAAAAGGATTGCTTTC
AGGCCAAGGACCCATGAAATTGGTGATGGCTTTTATAGCATTCCTAAGATTTCTAGCCATACCTCCAACA
GCAGGAATTTTGGCTAGATGGGGCTCATTCAAGAAGAATGGAGCGATCAAAGTGTTACGGGGTTTCAAGA
AAGAAATCTCAAACATGTTGAACATAATGAACAGGAGGAAAAGATCTGTGACCATGCTCCTCATGCTGCT
GCCCACAGCCCTGGCGTTCCATCTGACCACCCGAGGGGGAGAGCCGCACATGATAGTTAGCAAGCAGGAA
AGAGGAAAATCACTTTTGTTTAAGACCTCTGCAGGTGTCAACATGTGCACCCTTATTGCAATGGATTTGG
GAGAGTTATGTGAGGACACAATGACCTACAAATGCCCCCGGATCACTGAGACGGAACCAGATGACGTTGA
CTGTTGGTGCAATGCCACGGAGACATGGGTGACCTATGGAACATGTTCTCAAACTGGTGAACACCGACGA
GACAAACGTTCCGTCGCACTGGCACCACACGTAGGGCTTGGTCTAGAAACAAGAACCGAAACGTGGATGT
CCTCTGAAGGCGCTTGGAAACAAATACAAAAAGTGGAGACCTGGGCTCTGAGACACCCAGGATTCACGGT
GATAGCCCTTTTTCTAGCACATGCCATAGGAACATCCATCACCCAGAAAGGGATCATTTTTATTTTGCTG
ATGCTGGTAACTCCATCCATGGCCATGCGGTGCGTGGGAATAGGCAACAGAGACTTCGTGGAAGGACTGT
CAGGAGCTACGTGGGTGGATGTGGTACTGGAGCATGGAAGTTGCGTCACTACCATGGCAAAAGACAAACC
AACACTGGACATTGAACTCTTGAAGACGGAGGTCACAAACCCTGCCGTCCTGCGCAAACTGTGCATTGAA
GCTAAAATATCAAACACCACCACCGATTCGAGATGTCCAACACAAGGAGAAGCCACGCTGGTGGAAGAAC
AGGACACGAACTTTGTGTGTCGACGAACGTTCGTGGACAGAGGCTGGGGCAATGGTTGTGGGCTATTCGG
AAAAGGTAGCTTAATAACGTGTGCTAAGTTTAAGTGTGTGACAAAACTGGAAGGAAAGATAGTCCAATAT
GAAAACTTAAAATATTCAGTGATAGTCACCGTACACACTGGAGACCAGCACCAAGTTGGAAATGAGACCA
CAGAACATGGAACAACTGCAACCATAACACCTCAAGCTCCCACGTCGGAAATACAGCTGACAGACTACGG
AGCTCTAACATTGGATTGTTCACCTAGAACAGGGCTAGACTTTAATGAGATGGTGTTGTTGACAATGAAA
```

FASTA file are easy to manipulate by using scripting languages such as: Python, AWK, and Perl.

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- This is called *Large-Scale Sequencing*.
- A very good video about Sanger sequencing can be found here:

http://www.youtube.com/watch?v=bEFLBf5WEtc

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"Nowadays", thanks to NGS methodologies sequencers are capable to sequence the whole human genome in less that one week at a cost of less than 50.000 dollars.
Cost and time are decreasing at each new release, while speed, accuracy and resolution are improving dramatically.

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NGS sequencing methodologies are able to generate hundreds of millions of short *reads* (e.g. 30 bps - 100 bps) with their quality values.

Data produced after a typical NGS process

FASTQ format is a text-based format for storing both a biological sequence and its corresponding quality scores.

```
@HWI-1KL111:53:D176AACXX:1:1101:1221:1861 1:N:0:CGATGT
NTGCTTCCTCAAAAAATTACAAAAATGCCAGTGGAGTTGTGAACTCTTCACCTCGAAGTCATAGCGCCACAAATG
+
#4=DDFFFHHHGHJIIIJJIJJJJJHHIJJJFHIHGDGHIJJIJJJGJJJJJIJI<FHHHIGGIJHHFFFEDEEC
@HWI-1KL111:53:D176AACXX:1:1101:1197:1873 1:N:0:CGATGT
NTGATGCTTTCAACTGCATACTTAAAGCTTGCTCCAGTTTTGTCCATCTTGTTTAAAAAACAGATTCGAGGAAAA
+
#1:DBDDDFHBDFIEGHIIHHIDHGHGHAHII>C@GIIIIIEDHGGHEGF@BDH:BGHH6:==3@@##########
@HWI-1KL111:53:D176AACXX:1:1101:1228:1956 1:N:0:CGATGT
NTGAAGGGCTCTCCTTCCTGCACCAAGCTGGGAGGTTTCGGGCTCCTCTGGGGCATTCAATGCCTGCAAGCAAG
+
#1=DDFDDHHHHHJJJJJJIIJJJIJIGHHIGE@GHGHIIIIIJIJIIIFHEGIE<EDFFFFFFECEEECDD=CC
@HWI-1KL111:53:D176AACXX:1:1101:1371:1853 1:N:0:CGATGT
NGCCGGCGGACCGAAGAACGCAGGAAGGGGGCCGGGGGGACCCGCCCCCGGCCGGCCGCAGCCATGAACTCCAAC
+
#1=DDFFFHDFHHIGIIJIGIGHJHHHGB<>B3=BDDDD0<?B@BBBBD8B5<8B8BBBDDBB<CCACCADD:?>
@HWI-1KL111:53:D176AACXX:1:1101:1492:1889 1:N:0:CGATGT
NTATTTTTAGTAGAGACTGGATTTCTCCATGTTGGTCAGACAGGTCTCGAACTCCTGACCTCAGGTGATCTGCCT
+
#1=DDFFFFHHHFIIJIJJJGIJJJJJJBIIIEHIBGIIJJJIJBGIIJIDGGIJIJJIHJIEII7@GIIJJJIC
```

However...

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... as the quantities of sequence data increase exponentially thanks to NGS methodologies, ...

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... the analysis of such a large data (usually hundreds of GigaBytes) became the major "bottom-neck" of experimental investigations.

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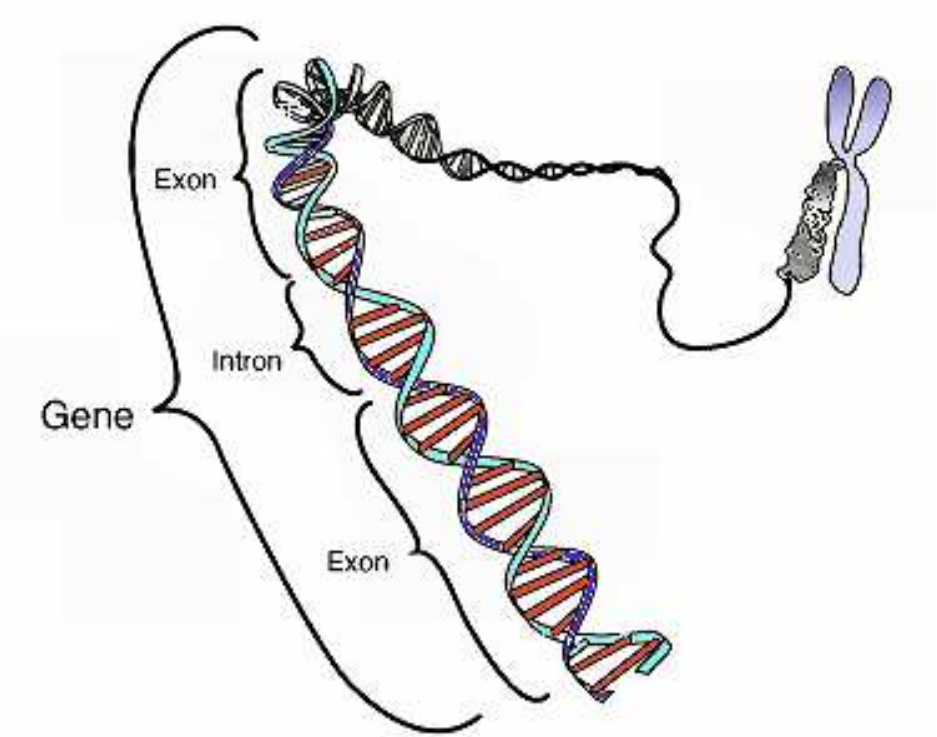

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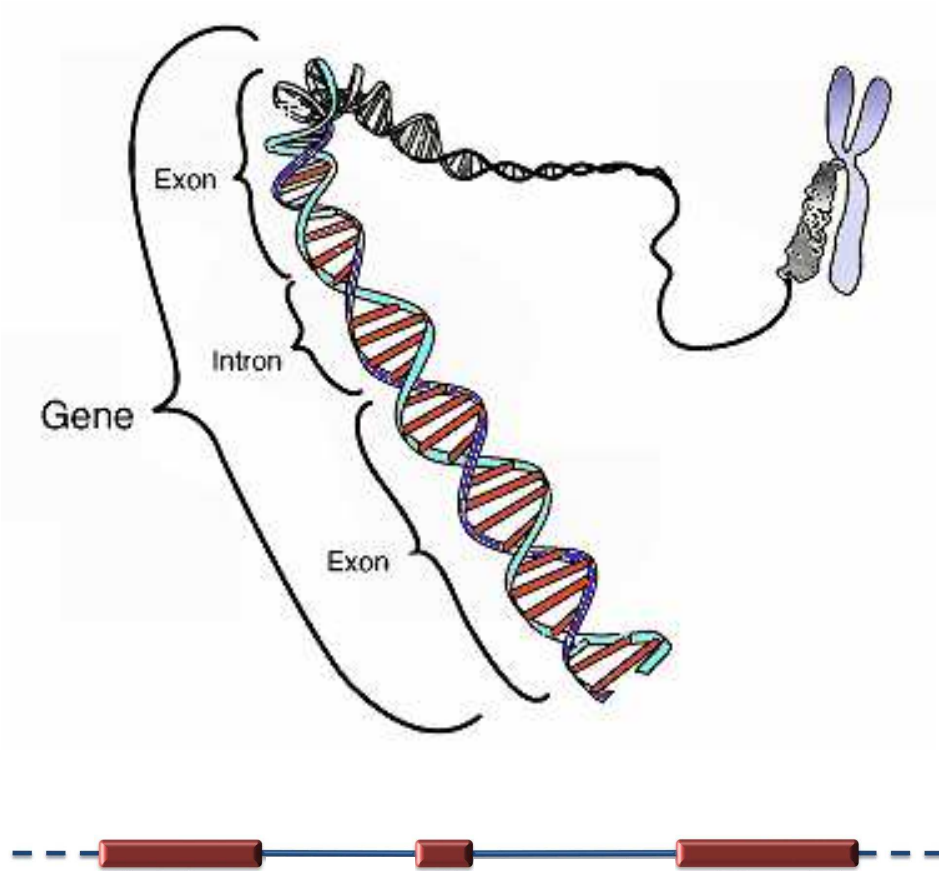

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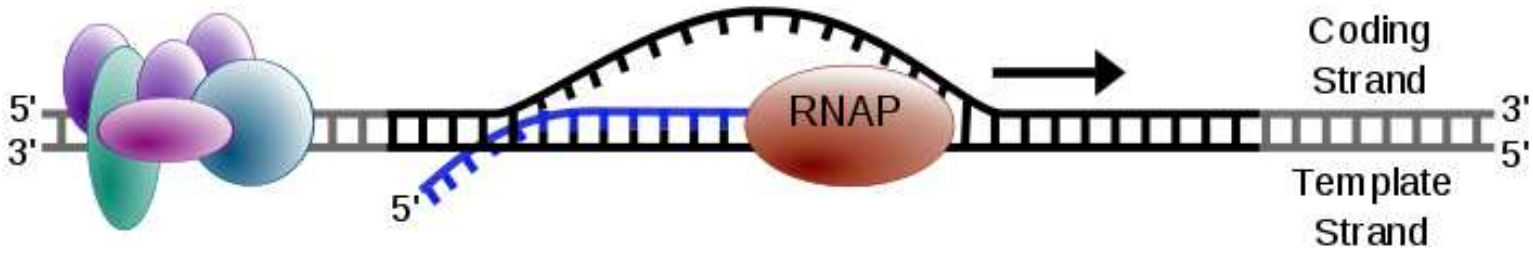

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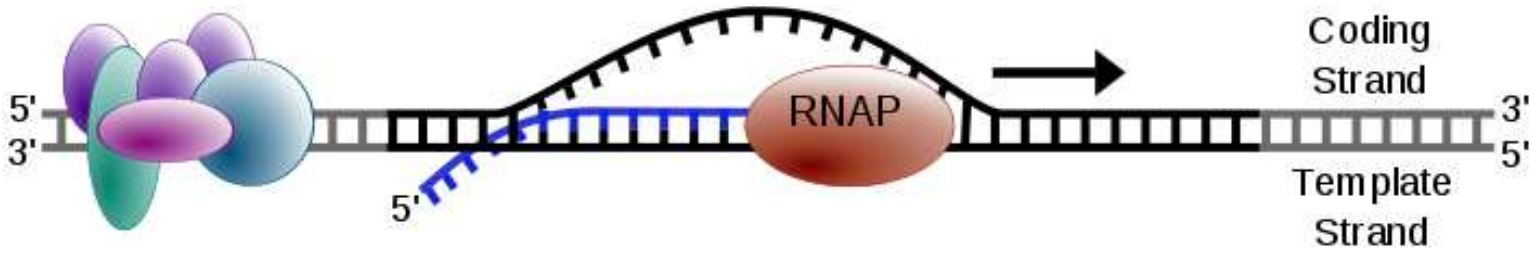

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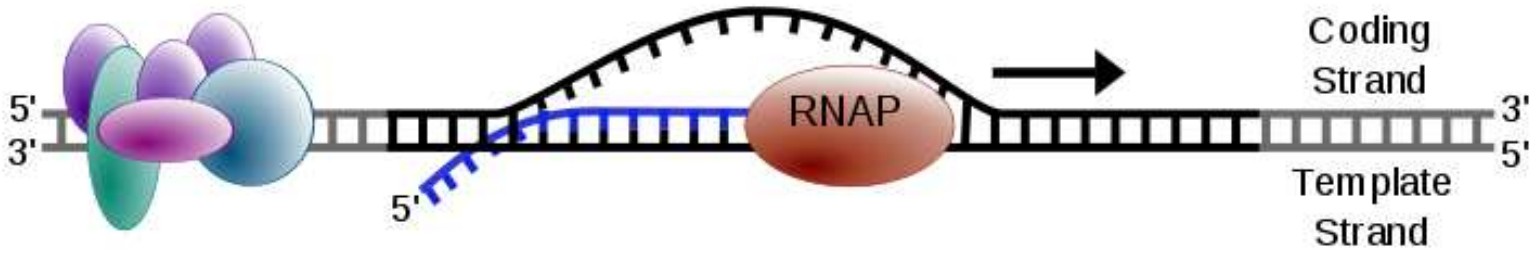

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Differently from DNA, RNA is very perishable.

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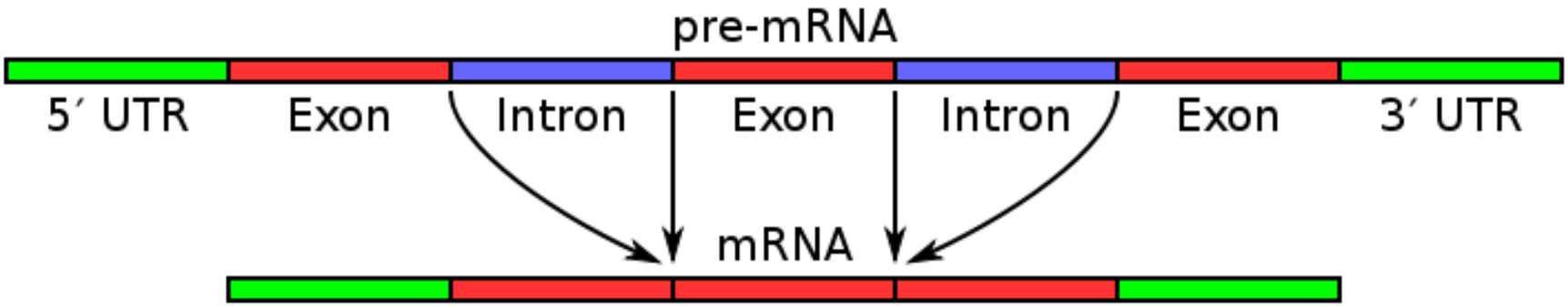
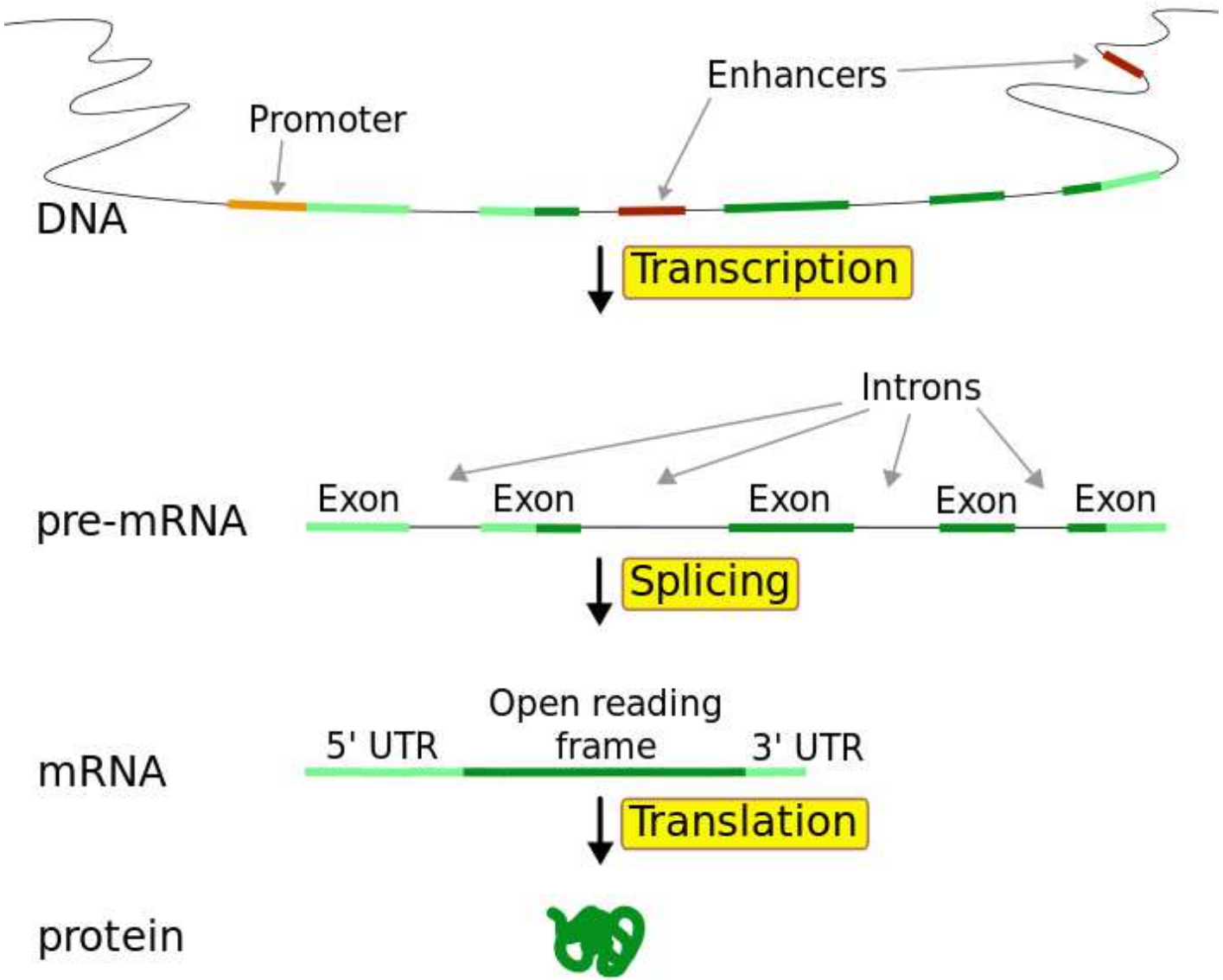


UTR stands for Untranslated Region.

Protein production



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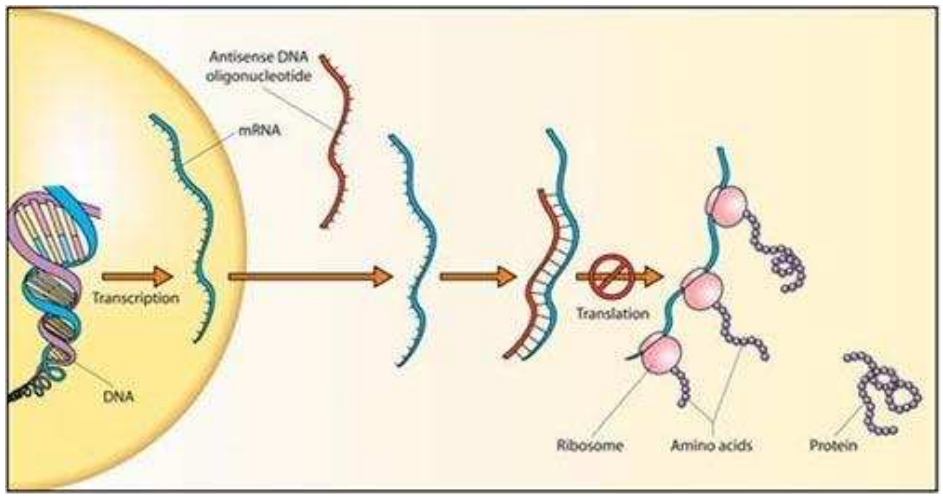

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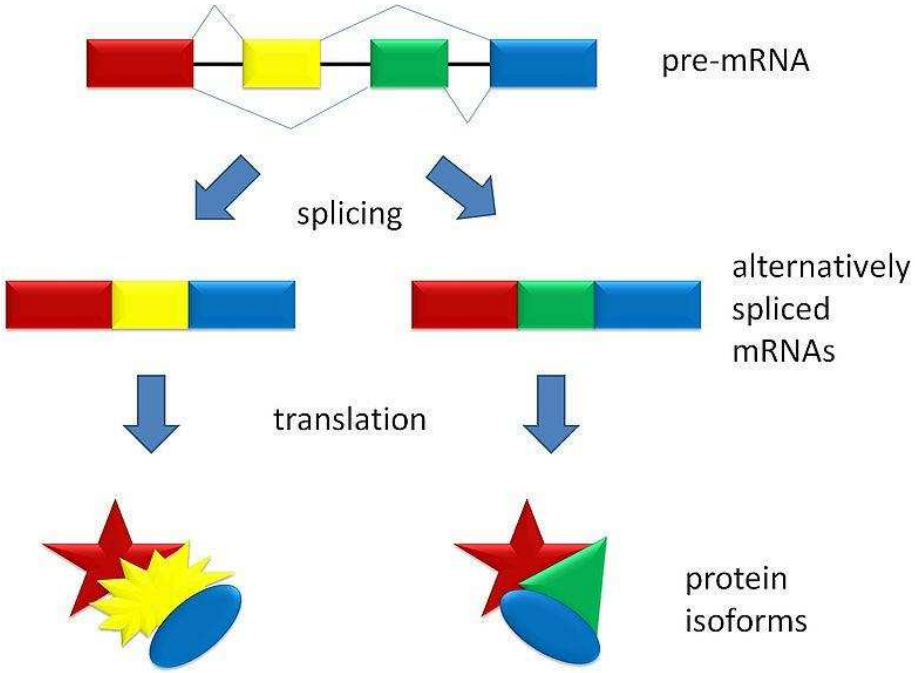

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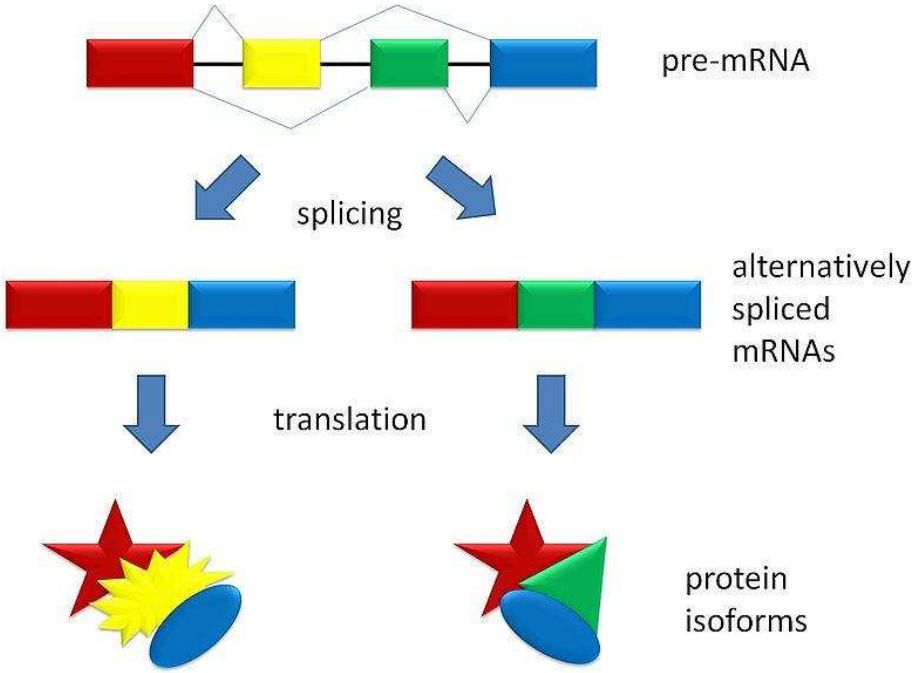

- Therefore, Alternative Splicing extends the complexity of gene expression.

Gene Expression

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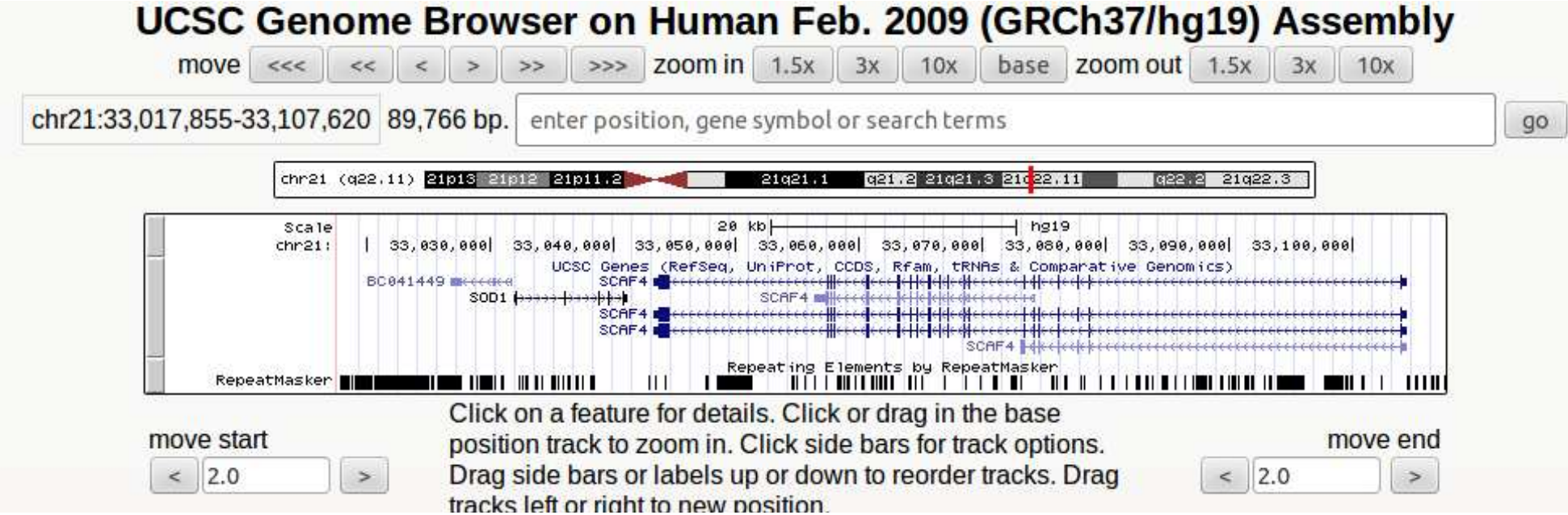
Genome Browser

A way to display and navigate across annotated genes is using a free software called: Genome Browser.



GTF

The Gene Transfer Format is a file format used to hold information about gene structure.

```
chr1    protein_coding  exon    34554   35174   .       -       .       gene_id "ENSG00000237613"; transcript_id "ENST00000417324";
chr1    protein_coding  stop_codon      35138   35140   .       -       0       gene_id "ENSG00000237613"; transcript_id "ENST00000417324";
chr1    protein_coding  CDS     35141   35174   .       -       1       gene_id "ENSG00000237613"; transcript_id "ENST00000417324";
chr1    processed_transcript    exon    35245   35481   .       -       .       gene_id "ENSG00000237613"; transcript_id "ENST00000461467";
chr1    protein_coding  CDS     35277   35481   .       -       2       gene_id "ENSG00000237613"; transcript_id "ENST00000417324";
chr1    protein_coding  exon    35277   35481   .       -       .       gene_id "ENSG00000237613"; transcript_id "ENST00000417324";
chr1    processed_transcript    exon    35721   36073   .       -       .       gene_id "ENSG00000237613"; transcript_id "ENST00000461467";
chr1    protein_coding  CDS     35721   35736   .       -       0       gene_id "ENSG00000237613"; transcript_id "ENST00000417324";
chr1    protein_coding  exon    35721   36081   .       -       .       gene_id "ENSG00000237613"; transcript_id "ENST00000417324";
chr1    protein_coding  start_codon     35734   35736   .       -       0       gene_id "ENSG00000237613"; transcript_id "ENST00000417324";
chr1    protein_coding  CDS     69091   70005   .       +       0       gene_id "ENSG00000186092"; transcript_id "ENST00000335137";
chr1    protein_coding  exon    69091   70008   .       +       .       gene_id "ENSG00000186092"; transcript_id "ENST00000335137";
chr1    protein_coding  start_codon     69091   69093   .       +       0       gene_id "ENSG00000186092"; transcript_id "ENST00000335137";
chr1    protein_coding  stop_codon      70006   70008   .       +       0       gene_id "ENSG00000186092"; transcript_id "ENST00000335137";
chr1    protein_coding  exon    367640  368634  .       +       .       gene_id "ENSG00000235249"; transcript_id "ENST00000426406";
chr1    protein_coding  CDS     367659  368594  .       +       0       gene_id "ENSG00000235249"; transcript_id "ENST00000426406";
chr1    protein_coding  start_codon     367659  367661  .       +       0       gene_id "ENSG00000235249"; transcript_id "ENST00000426406";
chr1    protein_coding  stop_codon      368595  368597  .       +       0       gene_id "ENSG00000235249"; transcript_id "ENST00000426406";
chr1    protein_coding  exon    621059  622053  .       -       .       gene_id "ENSG00000185097"; transcript_id "ENST00000332831";
chr1    protein_coding  stop_codon      621096  621098  .       -       0       gene_id "ENSG00000185097"; transcript_id "ENST00000332831";
chr1    protein_coding  CDS     621099  622034  .       -       0       gene_id "ENSG00000185097"; transcript_id "ENST00000332831";
chr1    protein_coding  start_codon     622032  622034  .       -       0       gene_id "ENSG00000185097"; transcript_id "ENST00000332831";
chr1    protein_coding  exon    860260  860328  .       +       .       gene_id "ENSG00000187634"; transcript_id "ENST00000420190";
chr1    protein_coding  exon    860530  860569  .       +       .       gene_id "ENSG00000187634"; transcript_id "ENST00000437963";
chr1    protein_coding  exon    861118  861180  .       +       .       gene_id "ENSG00000187634"; transcript_id "ENST00000342066";
chr1    protein_coding  exon    861302  861393  .       +       .       gene_id "ENSG00000187634"; transcript_id "ENST00000342066";
chr1    protein_coding  exon    861302  861393  .       +       .       gene_id "ENSG00000187634"; transcript_id "ENST00000420190";
chr1    protein_coding  exon    861302  861393  .       +       .       gene_id "ENSG00000187634"; transcript_id "ENST00000437963";
chr1    protein_coding  CDS     861322  861393  .       +       0       gene_id "ENSG00000187634"; transcript_id "ENST00000342066";
chr1    protein_coding  CDS     861322  861393  .       +       0       gene_id "ENSG00000187634"; transcript_id "ENST00000420190";
chr1    protein_coding  CDS     861322  861393  .       +       0       gene_id "ENSG00000187634"; transcript_id "ENST00000437963";
```

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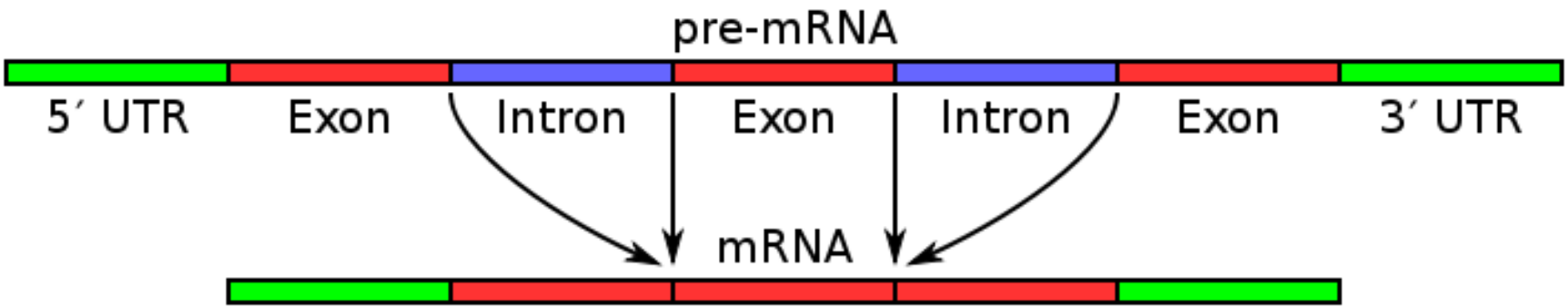

Introns are not sequenced.

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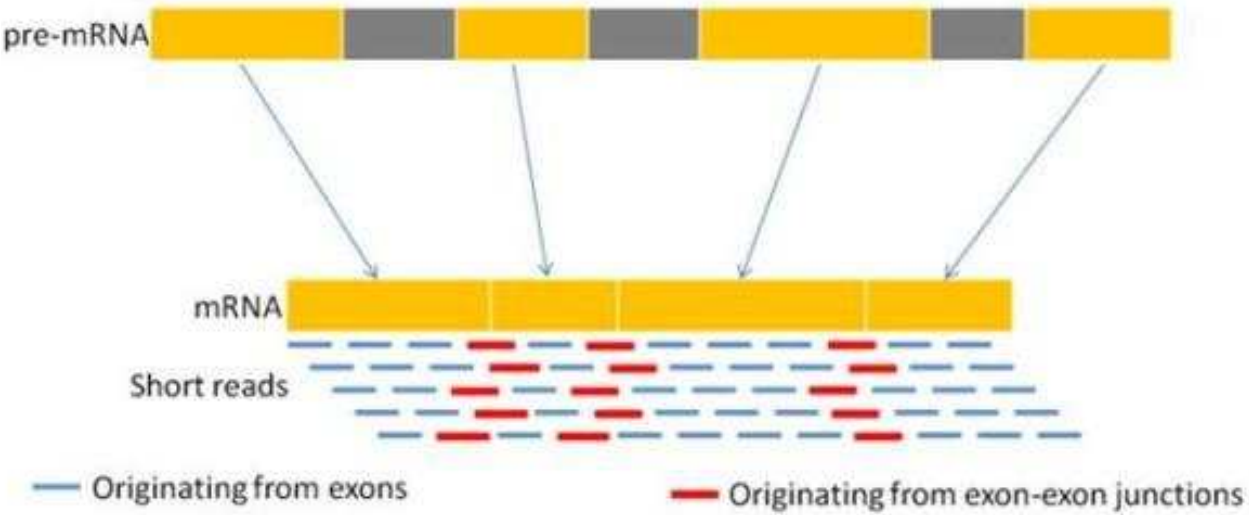
- RNA alignment, also called Transcript alignment, is particularly challenging due to splicing.



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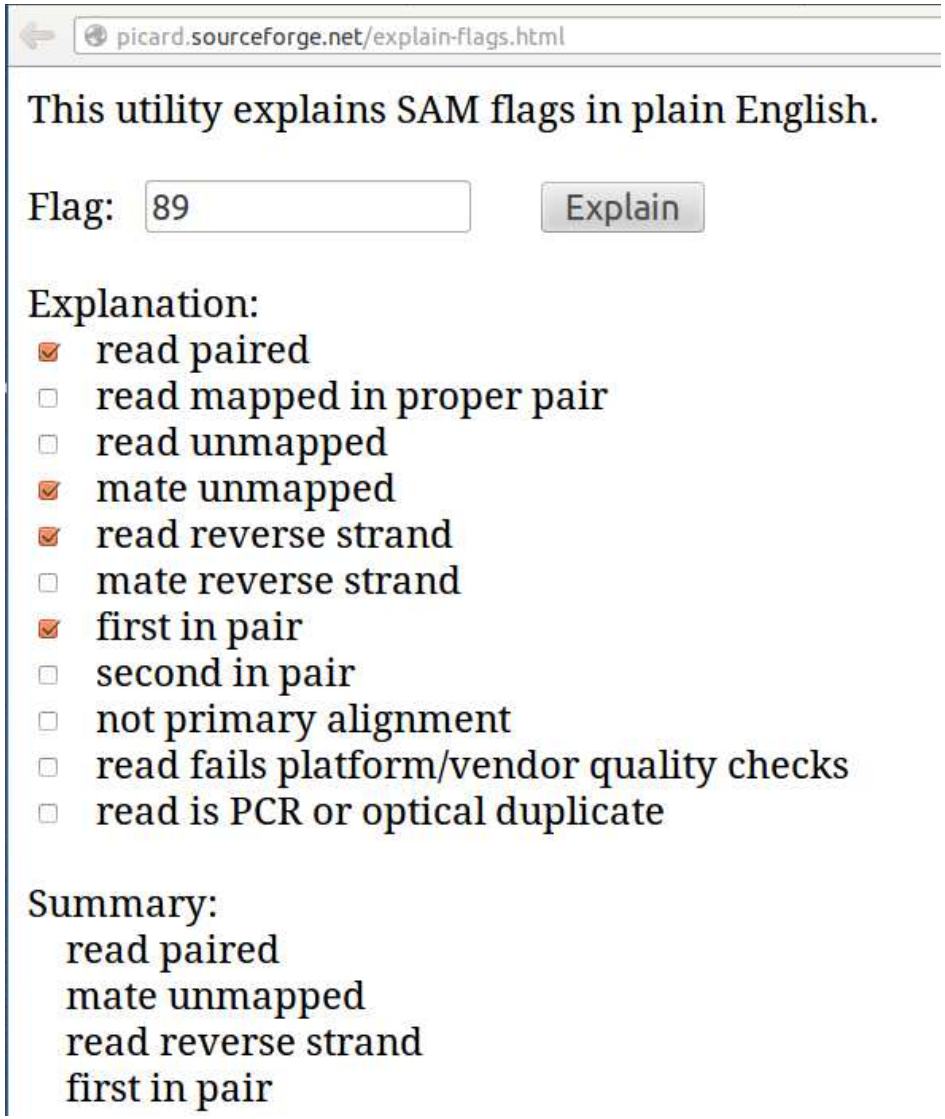
- Reads can be affected by sequencing errors.
- Therefore, the real task is to find the location where each short read best matches the reference, while allowing for errors and structural variations.

Alignment output: SAM format

```
HWI-1KL111:53:D176AACXX:1:1314:7404:89056       89      chr1    10535   3       75M     *       0       0
GTACCACCGAAATCTGTGCAGAGGAGAACGCAGCTCCGCCCTCGCGGTGCTCTCCGGGTCTGTGCTGAGGAGAAC     BAB@<2BBBBBABBBBABBBBABA@?
<7BBBBB=>@EFBIIFBFECCFGD?AEFIFIIFEIIFFDFFBDA=4+1=       AS:i:-5 XN:i:0  XM:i:1  XO:i:0  XG:i:0  NM:i:1
MD:Z:25C49      YT:Z:UU NH:i:2  CC:Z:chr15      CP:i:102520475  HI:i:0

HWI-1KL111:53:D176AACXX:1:2105:1895:21247       419     chr1    11650   3       75M     =       11718   143
TGGATTTTTGCCAGTCTAACAGGTGAAGCCCTGGAGATTCTTATTAGTGATTTGGGCTGGGGCCTGGCCATGTNN     ?=?BDDDAF@<DFGFDBGIHIJGEFH<<FE
F3:CGIA9??4?9B*?EGHGIGHIBA@@E;;EECA@?###        AS:i:-2 XN:i:0  XM:i:2  XO:i:0  XG:i:0  NM:i:2  MD:Z:73G0T0
YT:Z:UU NH:i:2  CC:Z:chr15      CP:i:102519446  HI:i:0

HWI-1KL111:53:D176AACXX:1:2210:10686:50410      329     chr1    11672   1       75M     *       0       0
GTGAAGCCCTGGAGATTCTTATTAGTGATTTGGGCTGGGGCCTGGCCATGTGTATTTTTTTAAATTTCCACTGAT
CC@FFFFFHHHHHJHJIIJJIJJJJHIIJJJJJJJJJJJJJJJIJJJHIJGIGGIJJJJJHHFFFFFFFEEEEEE     AS:i:0  XN:i:0  XM:i:0  XO:i:0
XG:i:0  NM:i:0  MD:Z:75 YT:Z:UU NH:i:3  CC:Z:chr15      CP:i:102519424  HI:i:0

HWI-1KL111:53:D176AACXX:1:1106:4169:92324       419     chr1    11685   3       75M     =       11790   180
GATTCTTATTAGTGATTTGGGCTGGGGCCTGGCCATGTGTATTTTTTTAAATTTCCACTGATGATTTTGCTGCAT     B?
@DBDDDHHBDAFGIIJJIEHJJJE<GGHGGBH@?D?DGHFGEGD@AHGIHGGGDGIIICEH:?EHFBDF@###      AS:i:0  XN:i:0  XM:i:0  XO:i:0
XG:i:0  NM:i:0  MD:Z:75 YT:Z:UU NH:i:2  CC:Z:chr15      CP:i:102519411  HI:i:0

HWI-1KL111:53:D176AACXX:1:1303:20062:7739       419     chr1    11708   3       75M     =       11771   138
GGGGCCTGGCCATGTGTATTTTTTTAAATTTCCACTGATGATTTTGCTGCATGGCCGGTGTTGAGAATGACTGCN
@<BBFFFDHFHB;ECGDGGHGIGGGEFBFHIGGGHII<FH@FEGHGBGGDFFHJICHGHHEFB@C;A@#######     AS:i:-1 XN:i:0  XM:i:1  XO:i:0
XG:i:0  NM:i:1  MD:Z:74G0       YT:Z:UU NH:i:2  CC:Z:chr15      CP:i:102519388  HI:i:0

HWI-1KL111:53:D176AACXX:1:2105:1895:21247       339     chr1    11718   3       75M     =       11650   -143
CATGTGTATTTTTTTAAATTTCCACTGATGATTTTGCTGCATGGCCGGTGTTGAGAATGACTGCGCAAATTTGCC     CCCCCCC?
9<@?;=EHHDHCEE@CIGIHCCDHFB2HDGAFDD<G@F?FCC@DHAFHBGGAC8:AFHFDDDDD@@<     AS:i:0  XN:i:0  XM:i:0  XO:i:0  XG:i:0
NM:i:0  MD:Z:75 YT:Z:UU NH:i:2  CC:Z:chr15      CP:i:102519378  HI:i:0
```

Flags


picard.sourceforge.net/explain-flags.html
This utility explains SAM flags in plain English.
Flag: 89
Explain
Explanation:
read paired
read mapped in proper pair
read unmapped
mate unmapped
read reverse strand
mate reverse strand
first in pair
second in pair
not primary alignment
read fails platform/vendor quality checks
read is PCR or optical duplicate
Summary:
read paired
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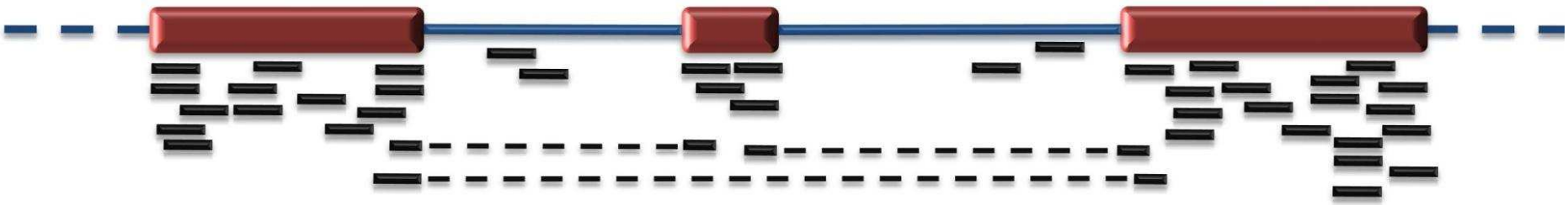
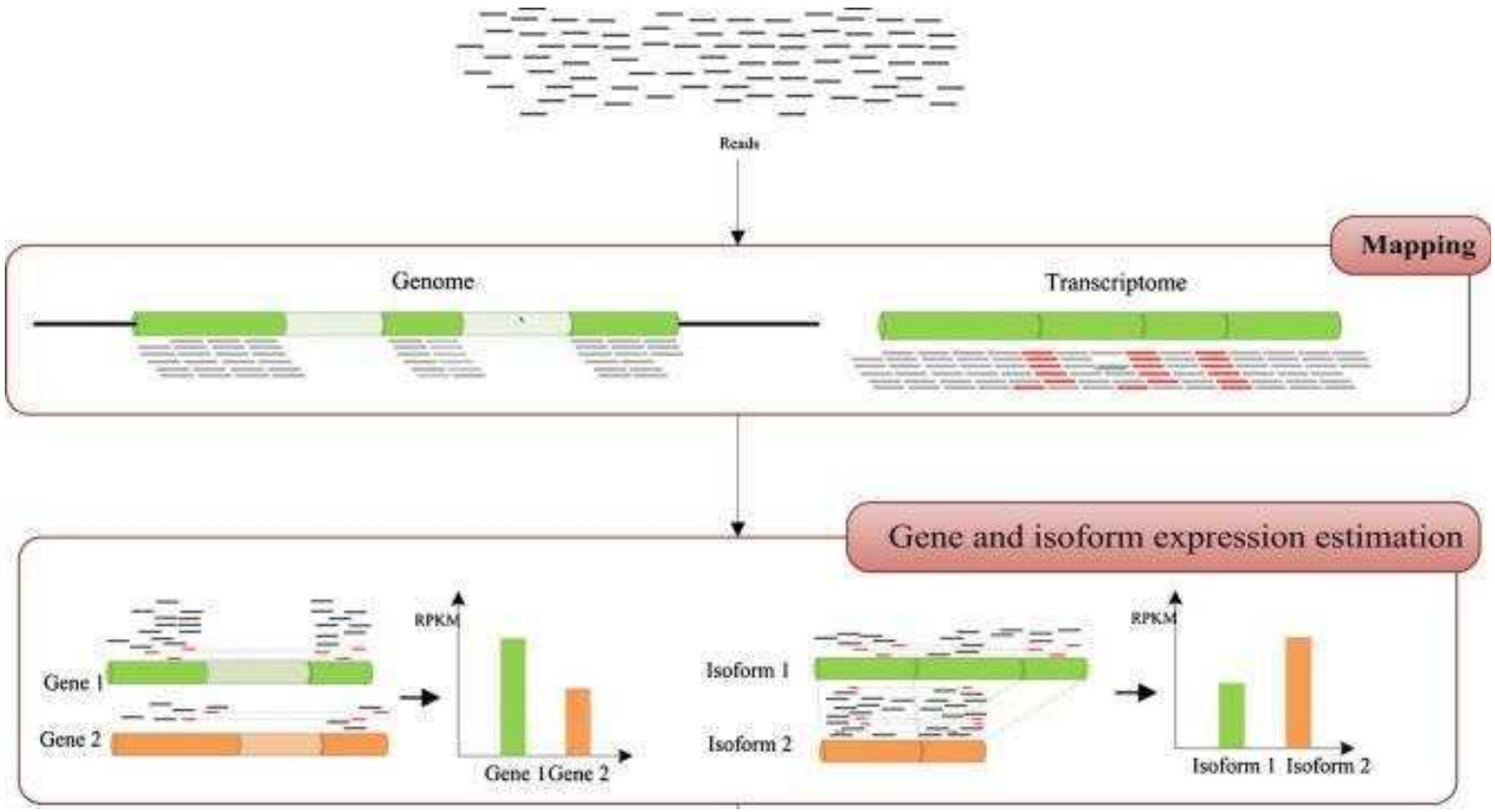


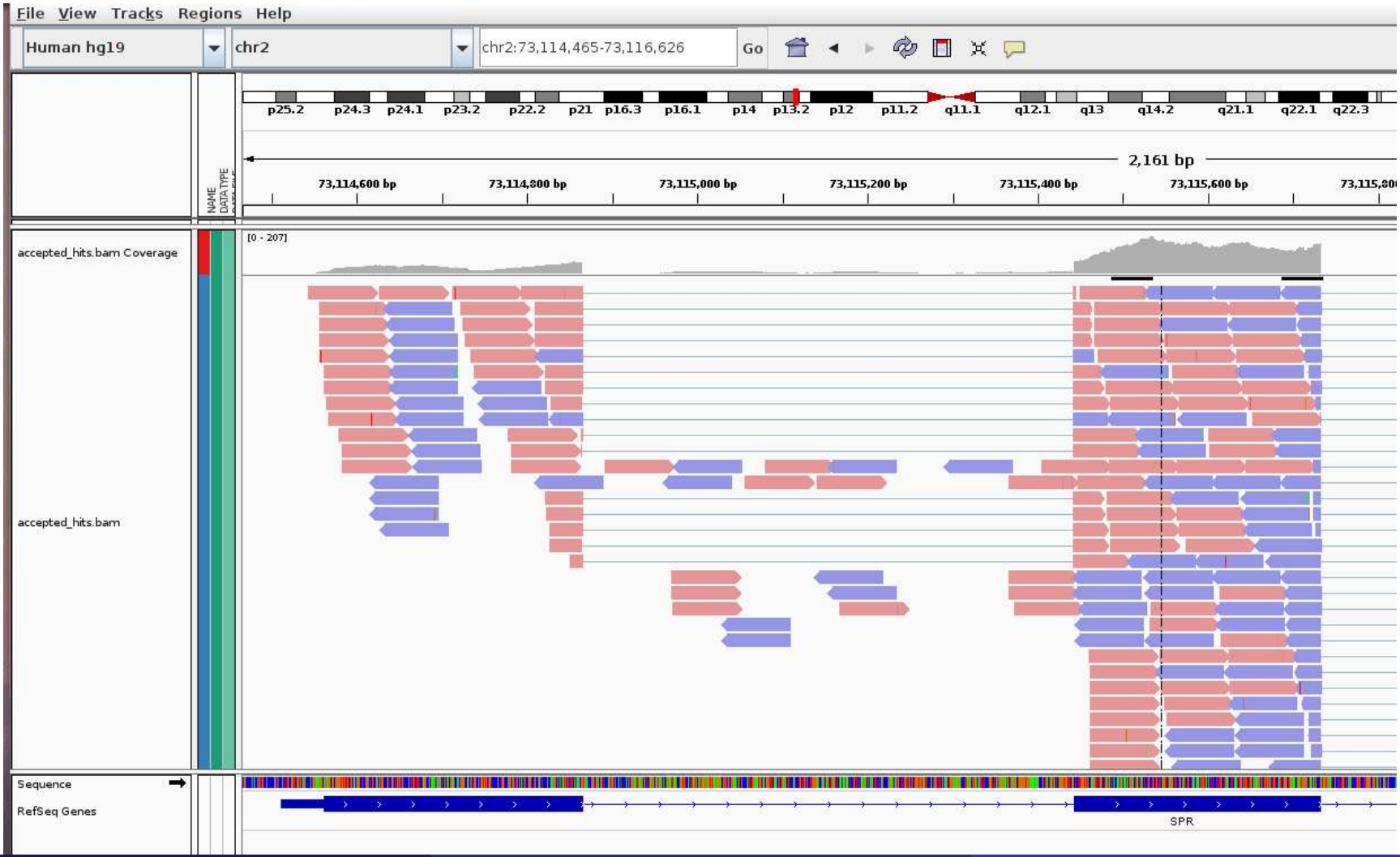
A pipeline to study gene expression and isoform expression

One of the most commonly used workflows is to map reads with a tool like *Tophat* and then use a tool like *HTSeq* to count the number of reads overlapping a gene.

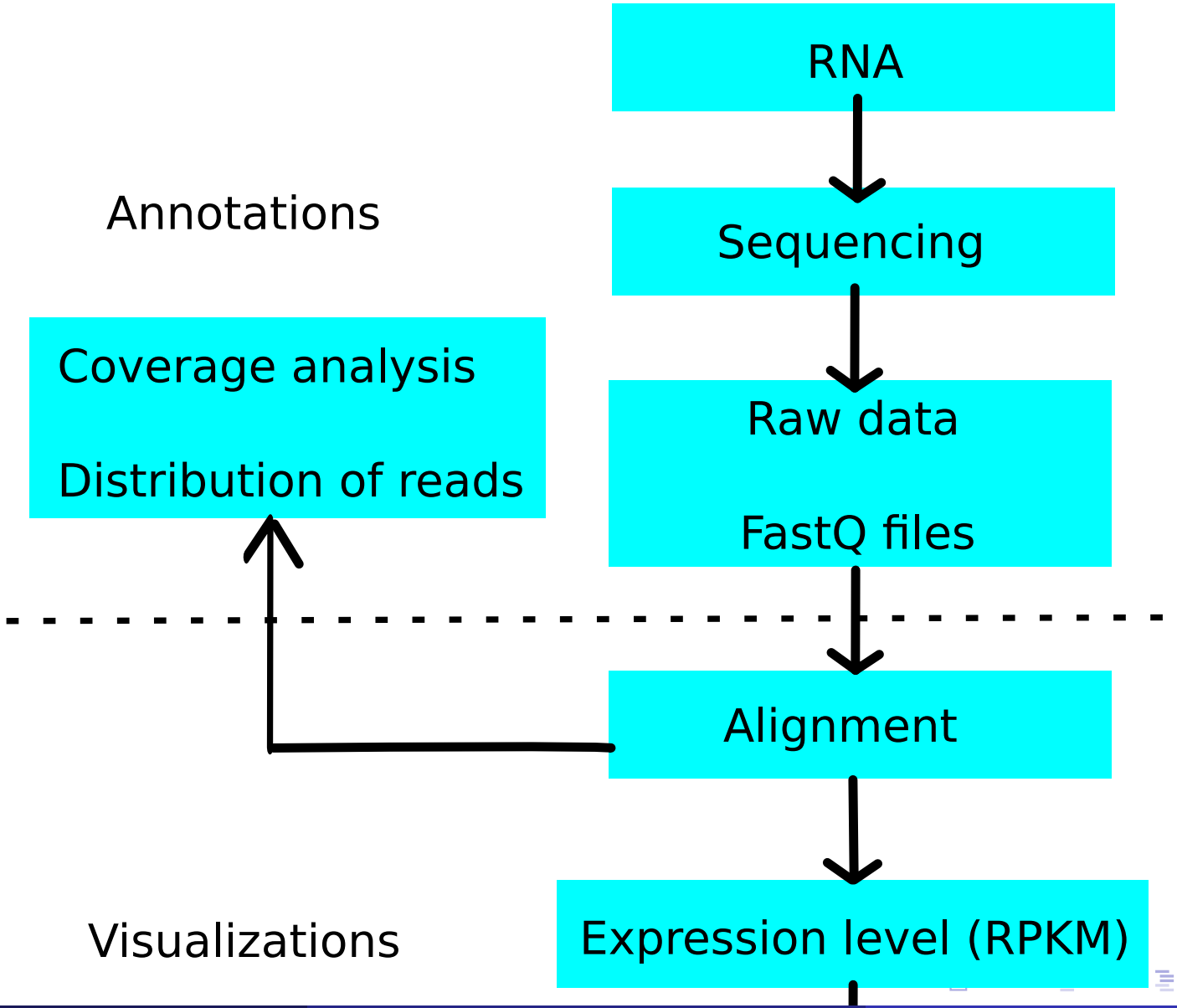


Integrated Genome Viewer

Some reads fall within junctions. First half falls on one exon and the second half on the other exon.



Overview of RNA-SEQ analysis workflow



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Novel Probabilistic models for RNA-SEQ are still needed.

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- *De novo transcriptome assembly* is the method of creating a transcriptome without the aid of a reference genome.

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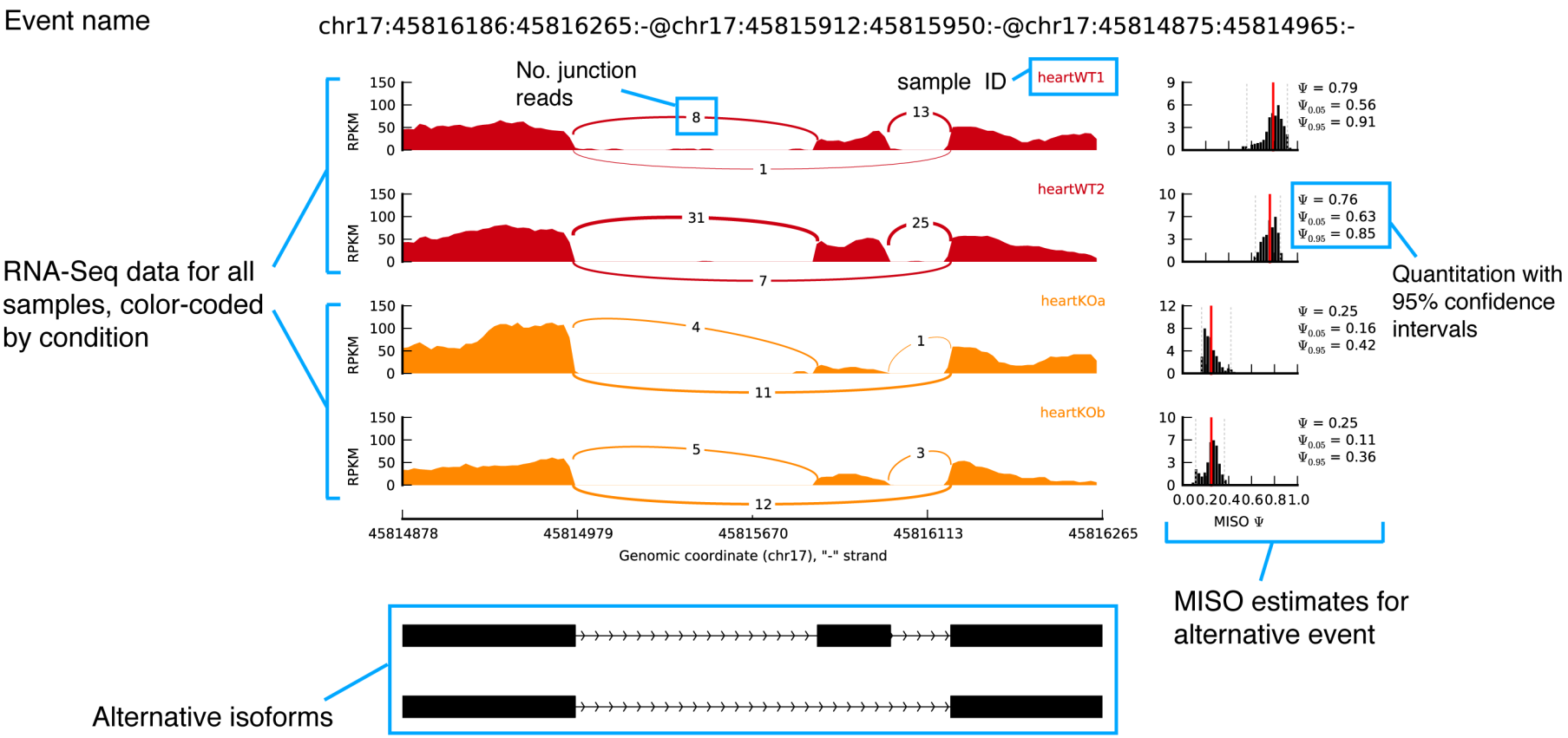
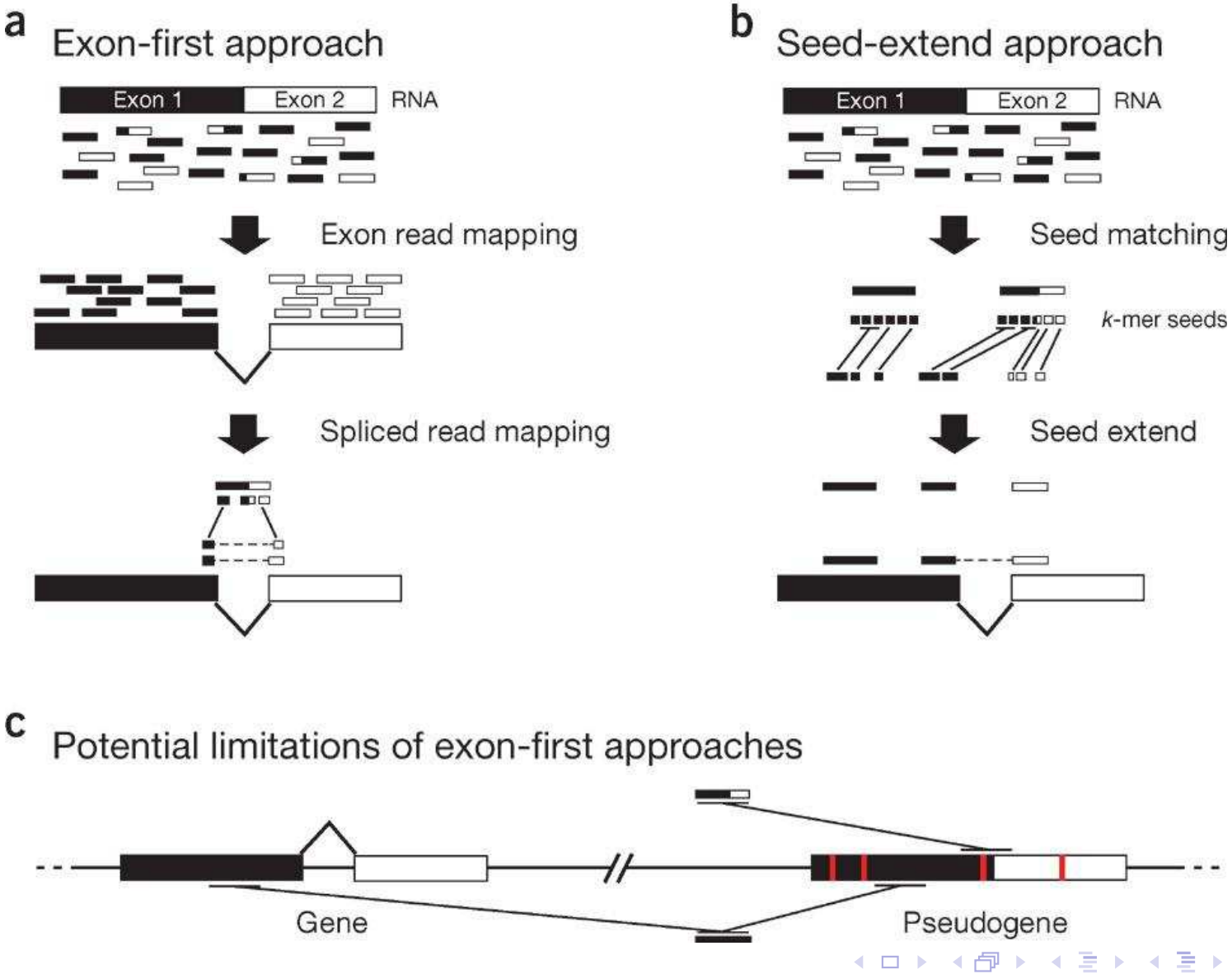
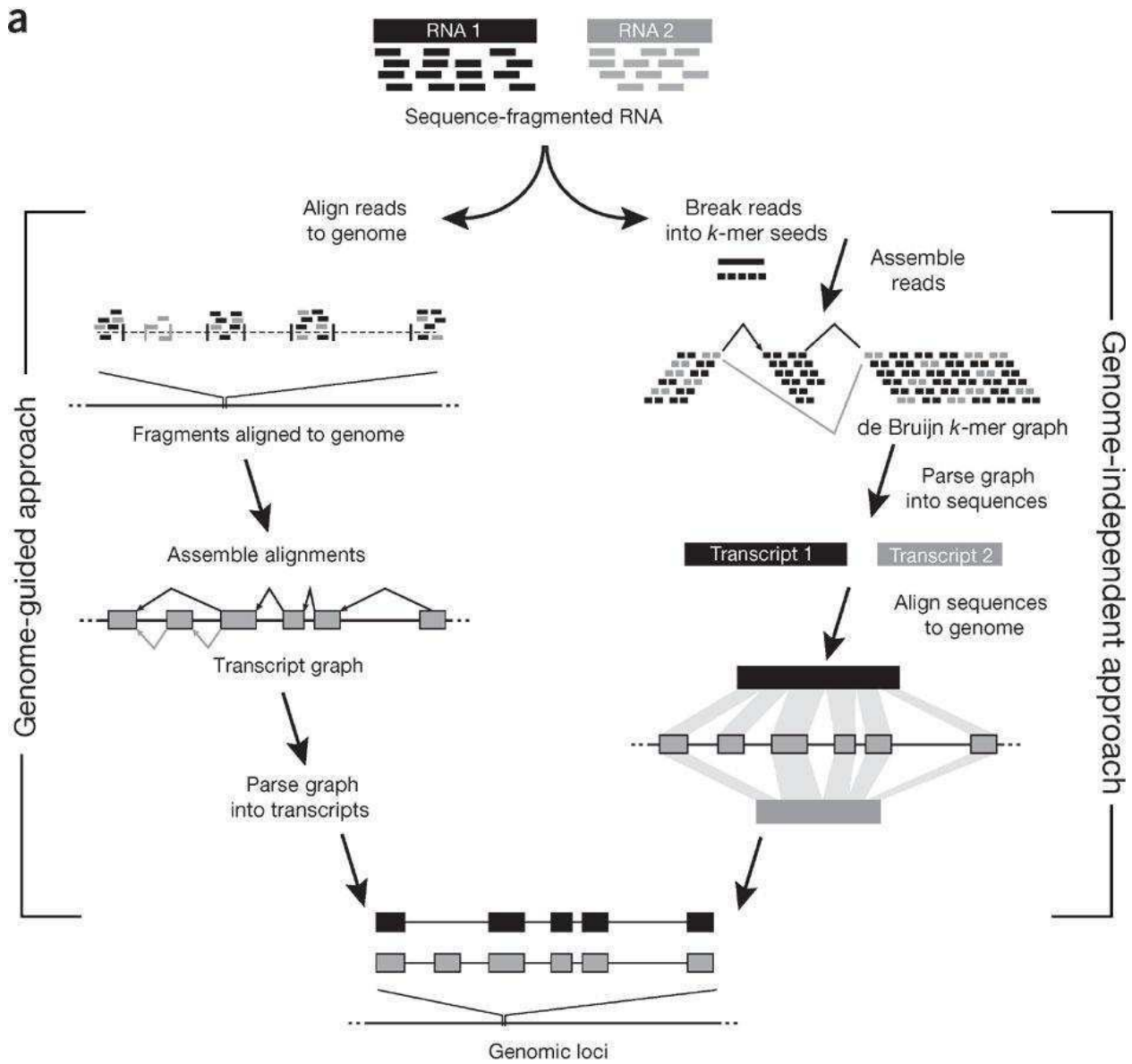


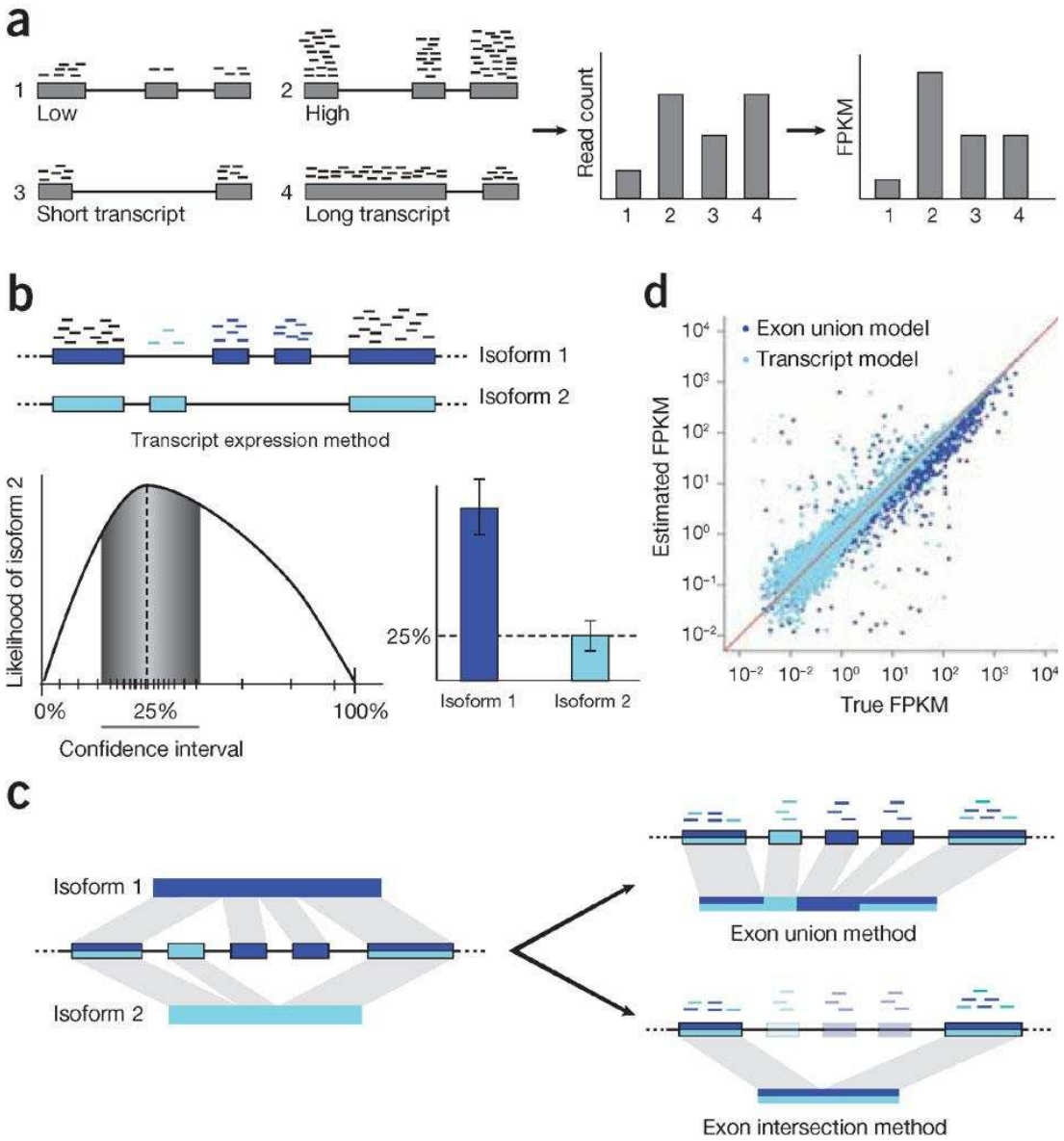
Alignment strategies of RNA-SEQ reads to the genome



Transcriptome reconstruction methods



Gene espression quantification with RNA-SEQ



Graph theory to locally assembly genes/transcripts

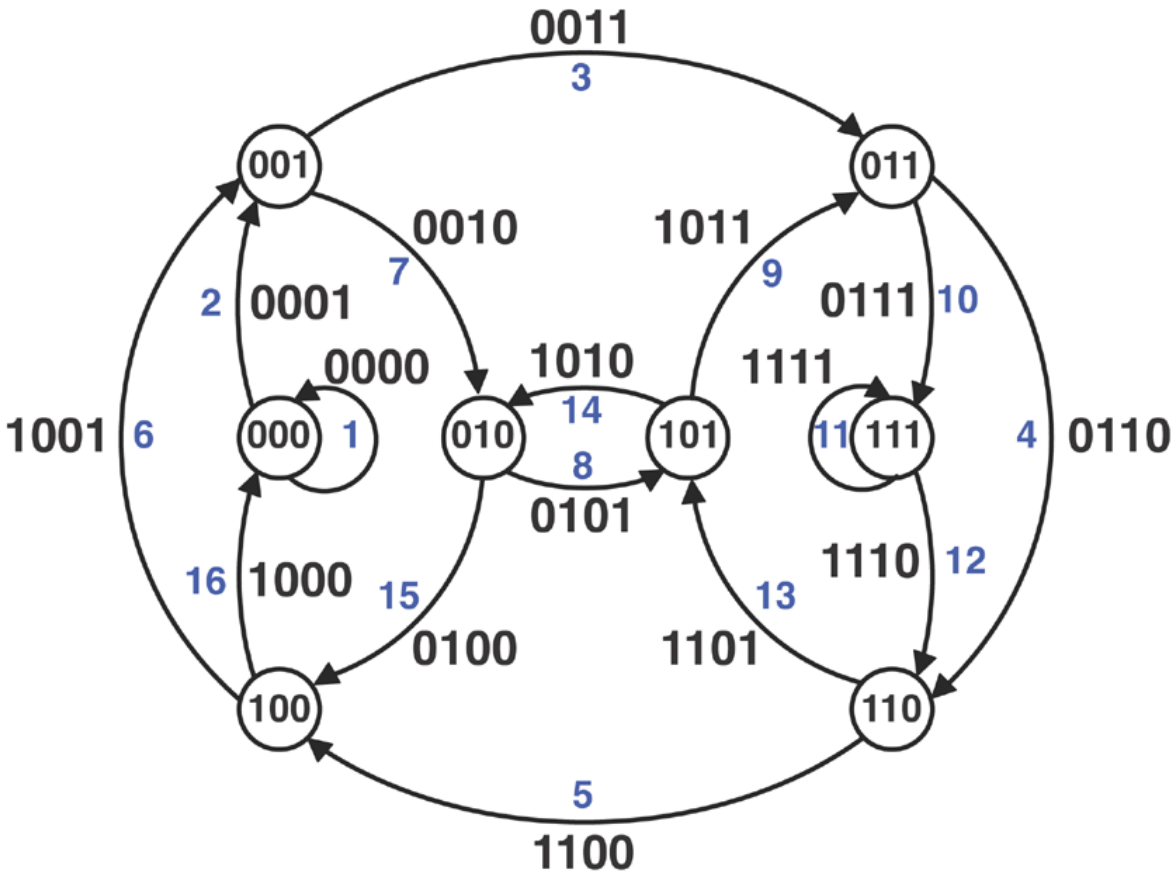
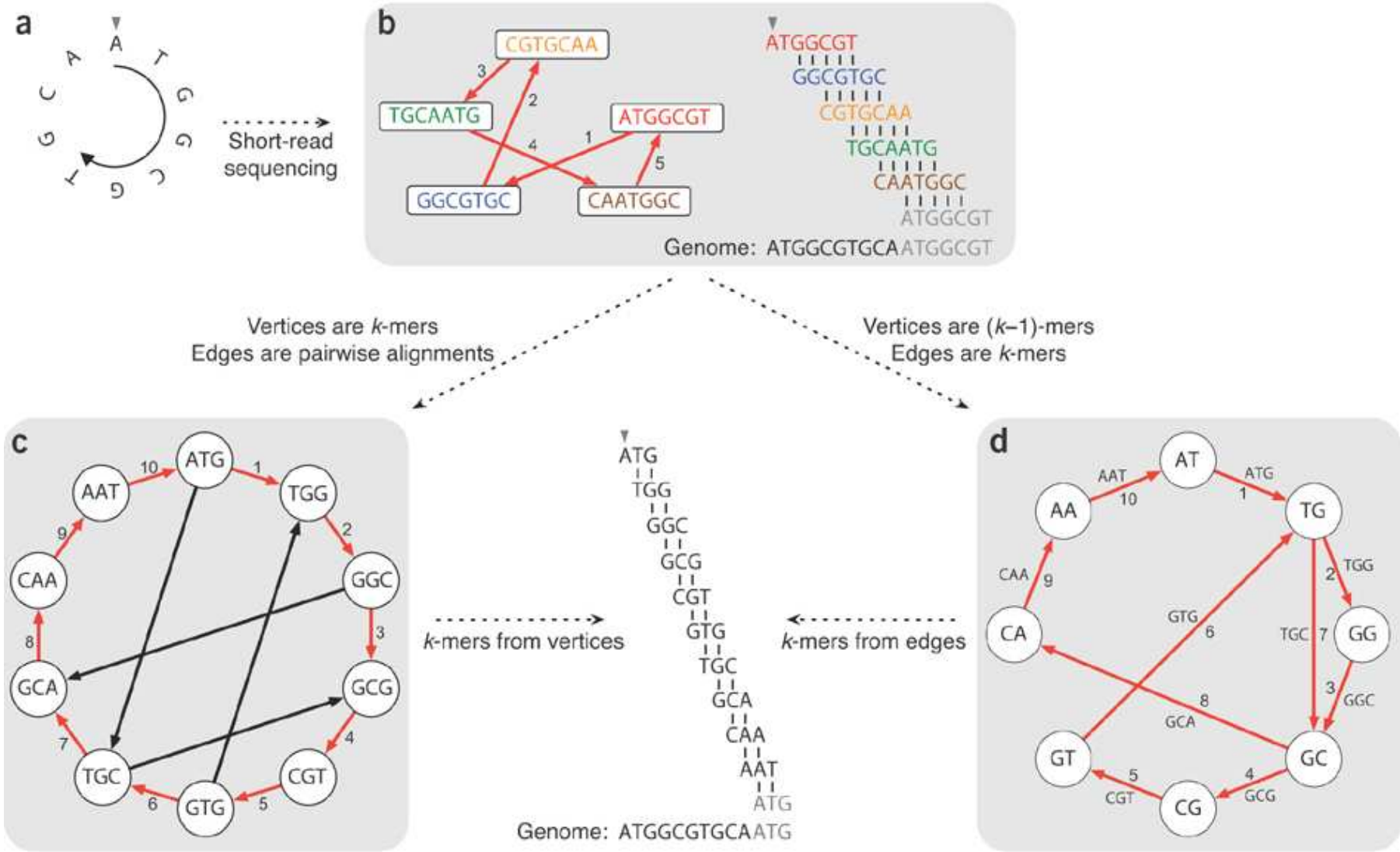
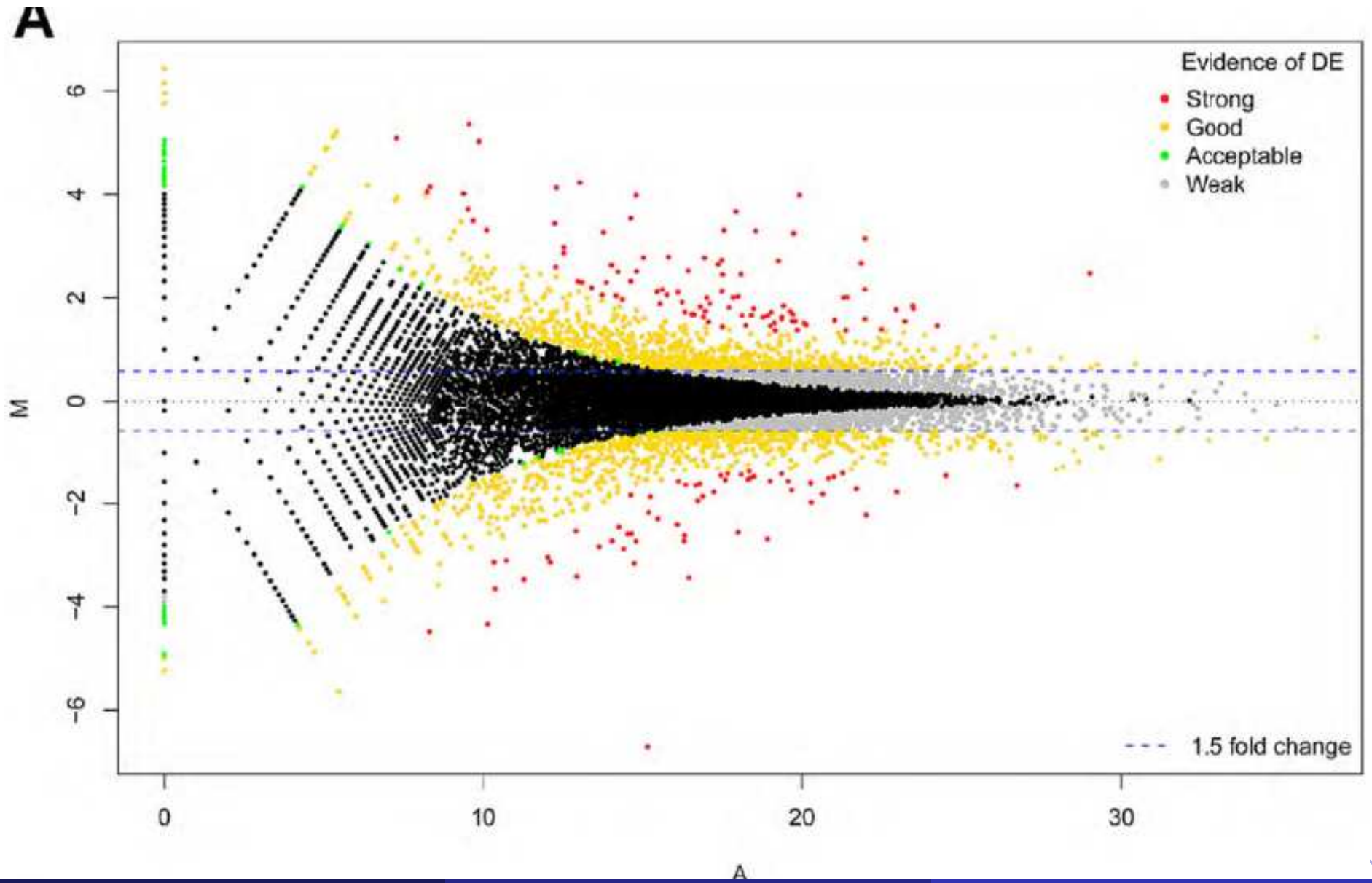


Figure:

Two strategies for genome assembly: from Hamiltonian cycles to Eulerian cycles



Differential Expression



THANK YOU !