V8 – Genomics data

Program for today:

- Read mapping
- SNP calling
- SNP frequencies in 1000 Genomes data
- Isoforms of genes (alternative splicing)
- Non-canonical translation
- Removing sequence redundancy

(1) Read mapping: range of usage

The accurate **alignment** of reads generated by NGS machines to a reference genome is a crucial part in many **application workflows**, such as

- genome resequencing (in contrast to de novo assembly),
- DNA methylation,
- RNA-Seq (transcriptomics),
- ChIP sequencing (e.g. histone marks, TFBS occupancies),
- SNP detection,
- detection of genomic structural variants, and
- metagenomics (sequencing mixtures of organisms).

Hatem et al.
BMC Bioinformatics (2013) 14:184

Read mapping tools

Numerous tools have been developed for this challenging task:

MAQ, RMAP, GSNAP,

Bowtie, Bowtie2,

BWA, SOAP2, Mosaik, FANGS, SHRIMP, BFAST,

MapReads, SOCS, PASS, mrFAST, mrsFAST, ZOOM,

Slider, SliderII, **RazerS**, RazerS3, Novoalign and

GPU-based tools such as SARUMAN and SOAP3.

Hatem et al.
BMC Bioinformatics (2013) 14:184

Read mapping techniques: (1) Hash tables

For most of the existing tools, the mapping process starts by building an **index** either for the reference genome or for the reads.

Then, the index is used to find the corresponding genomic positions for each read.

There are **two main types of techniques** for this: Hash tables + BWT

(1) The hash based methods either hash the reads or the genome.

The main idea for both types is to build a **hash table** for **subsequences** of the reads/genome.

The **key** of each entry is a subsequence

while the **value** is a list of positions

where the subsequence can be found.

Hatem et al.
BMC Bioinformatics (2013) 14:184

Key	Hashed index	Genomic location
"GCTAGC"	Key1	Chr1 123412 … …
"TTTAGC"	KeyN	Chr6 988472

Read mapping techniques: (2) Burrows Wheeler transform

The **BWT** of the string T = "abracadabra$" is "ard$rcaaaabb.
It is represented by the matrix M where each row is a rotation of the text, and the rows have been sorted lexicographically.
The transform corresponds to the last column labeled L.

Modern alignments
use an extension of BWT
named **FM index**
after Ferragina & Manzina

I	F	L
1	$ abracadabr	a
2	a $abracadab	r
3	a bra$abraca	d
4	a bracadabra	$
5	a cadabra$ab	r
6	a dabra$abra	c
7	b ra$abracad	a
8	b racadabra$	a
9	c adabra$abr	a
10	d abra$abrac	a
11	r a$abracada	b
12	r acadabra$a	b

www.wikipedia.org

Read mapping techniques: (2) Burrows Wheeler transform

C[c] is a table that, for each character c in the alphabet, contains the number of occurrences of lexically smaller characters in the text.

C[c] of "ard$rcaaaabb"						
c	$	a	b	c	d	r
C[c]	0	1	6	8	9	10

The function Occ(c, k) is the number of occurrences of character c in the prefix L[1..k].

Occ(c, k) of "ard$rcaaaabb"											
	a	r	d	$	r	c	a	a	a	a	b	b
	1	2	3	4	5	6	7	8	9	10	11	12
$	0	0	0	1	1	1	1	1	1	1	1	1
a	1	1	1	1	1	1	2	3	4	5	5	5
b	0	0	0	0	0	0	0	0	0	0	1	2
c	0	0	0	0	0	1	1	1	1	1	1	1
d	0	0	1	1	1	1	1	1	1	1	1	1
r	0	1	1	1	2	2	2	2	2	2	2	2

www.wikipedia.org

Read mapping techniques: (2) Burrows Wheeler transform

The FM-index itself is a compression of the string L together with C and Occ in some form, as well as information that maps a selection of indices in L to positions in the original string T.

FM index is used e.g. by the tools Bowtie and BWA

Soap uses a different variant of BWT.

www.wikipedia.org

Read alignment: features

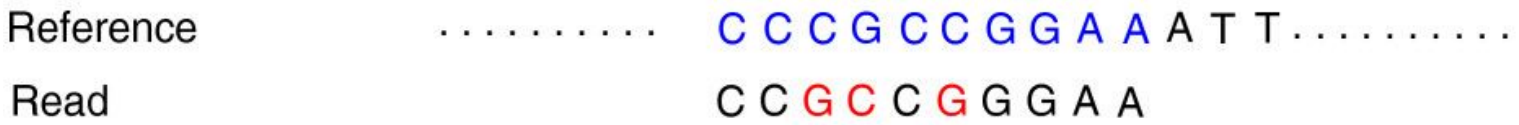
Crucial default options:

- Maximum number of mismatches in the **seed** (default 2). The seed are “the first few tens of base pairs of a read.” The seed part of a read is expected to contain less erroneous characters.
- Maximum number of **mismatches** in the read (2 to 10)
- **Seed length** (28 – 32).
- **Quality threshold**: It is equal to 70 for MAQ and Bowtie while it depends on the read length and the genome size for Novoalign.
- Splicing: This option is enabled for GSNAP.
- **Gapped alignment**: It is enabled for Bowtie2, GSNAP, BWA, Novoalign and MAQ while it is disabled for SOAP2.
- Minimum and maximum **insert sizes** for paired-end mapping: The insert size represents the distance between the two ends. (0 to 500)

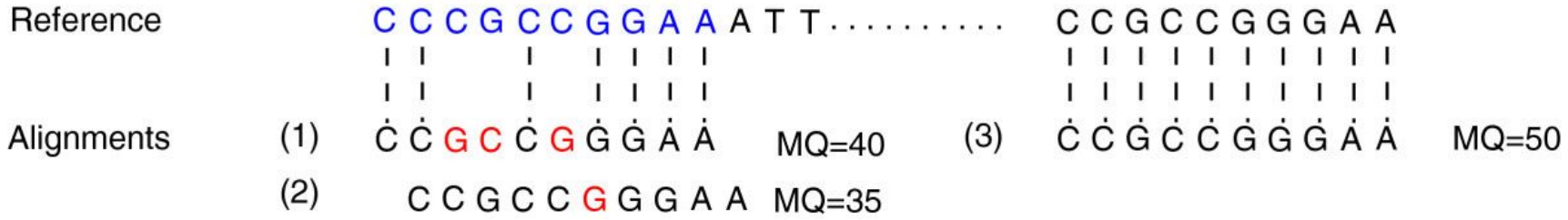
Hatem et al.
BMC Bioinformatics (2013) 14:184

Read alignment: evaluation criteria

The sequence in blue is the original genomic position where the simulated read was extracted from. After applying sequencing errors, the read does not exactly match to the original location (3 mismatches).



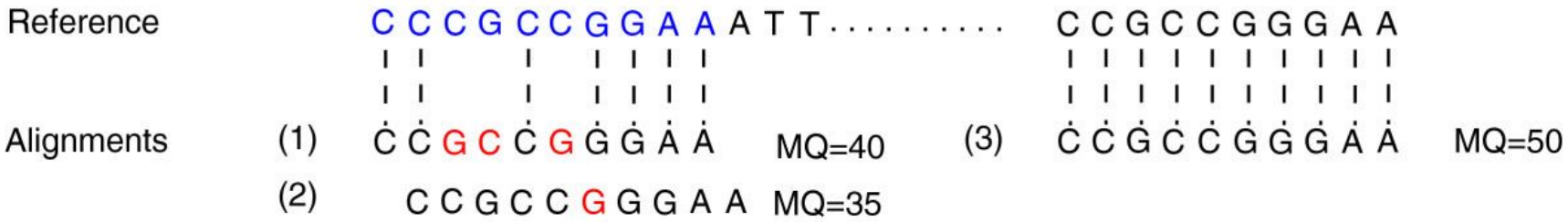
3 possible alignment locations for the read with their mapping quality score (MQ).



Naïve criterion: only consider the alignment (1) as the correct alignment.

Hatem et al.
BMC Bioinformatics (2013) 14:184

Read alignment: evaluation criteria



Ruffalo et al. criterion: consider also the mapping quality.

If the used threshold is 30, then (1) is *correctly mapped* while (2) and (3) are *incorrectly mapped-strict*.

If the threshold is 40, then (3) is considered as *incorrectly mapped relaxed* (no correct mapping available higher than the threshold).

Holtgrewe et al. criterion: considers all matches with distance *k*.

Here, it would detect (1) and (2) and consider them *correctly mapped* while (3) would be considered as *incorrectly mapped*.

Hatem *et al*: “We define a read to be correctly mapped if it is mapped while not violating the mapping criteria.“

Read alignment: throughput for simulated data

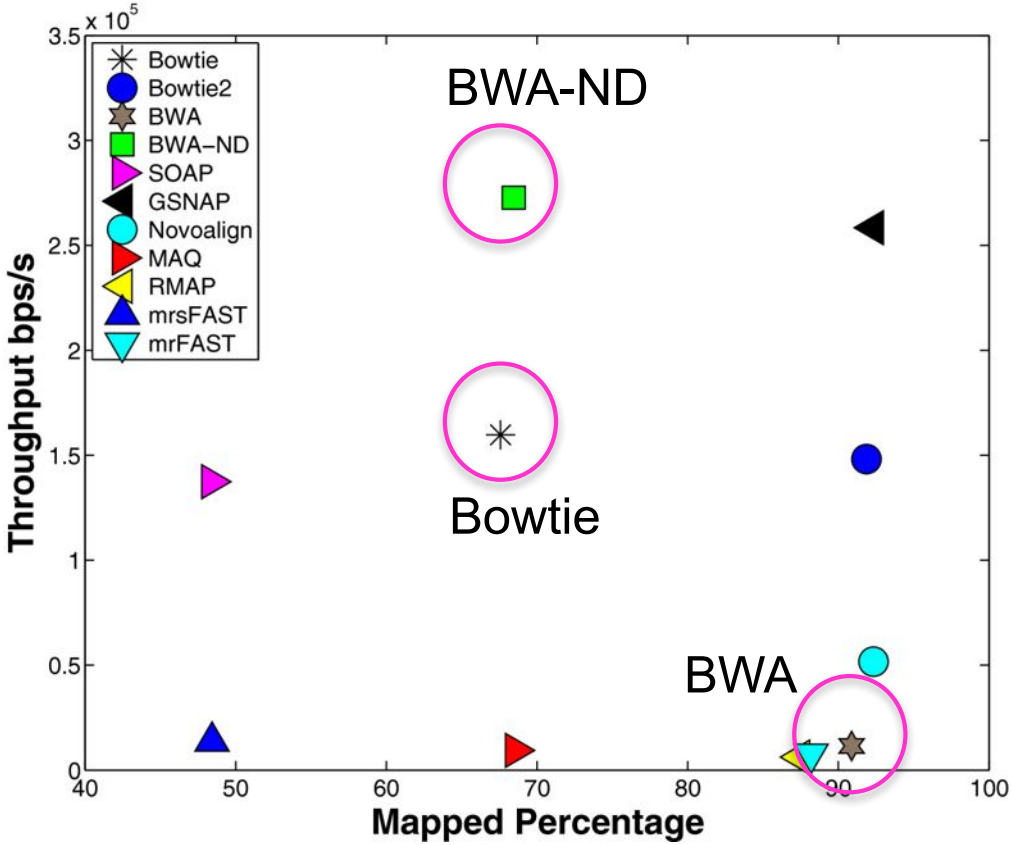
Task: map 1 million reads of length 125 extracted from the Human genome using `wgsim` with 0.09% SNP mutation rate, 0.01% indel mutation rate, and 2% uniform sequencing error rate.

Each tool was used with its own default options.

Bowtie only maps 68% of the reads, but achieves high throughput.

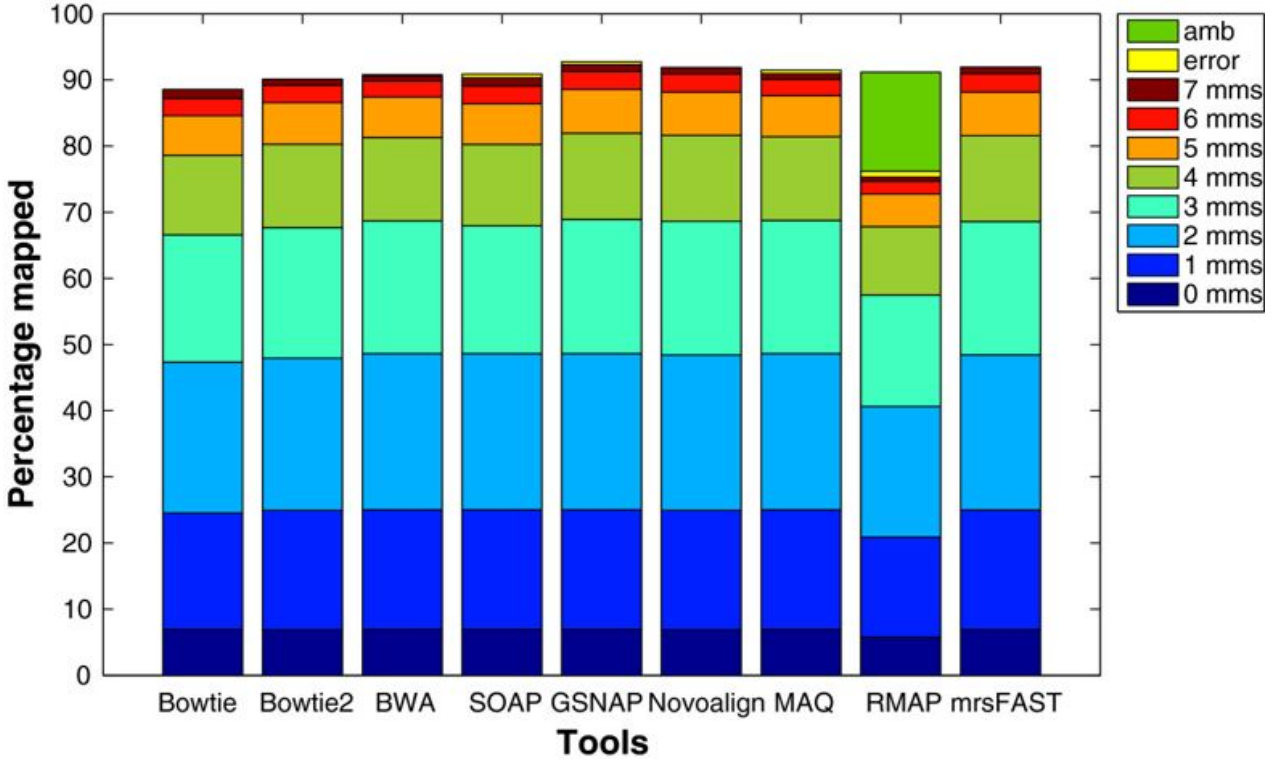
BWA maps 91% of the reads, but 15 x lower throughput.

However, when used with the same options as Bowtie, BWA achieves even a higher performance.



BWA-ND refers to BWA's results while using Bowtie's default options which are 2 mismatches in the seed, 3 mismatches in the whole read, and no gapped alignment.

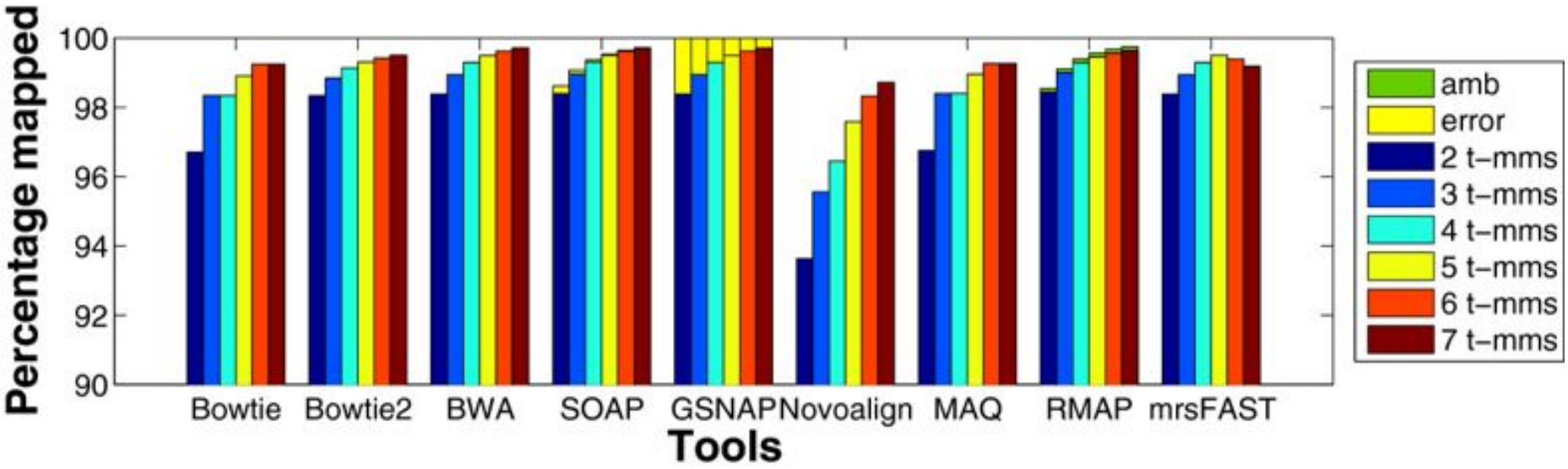
Read alignment: number of allowed mismatches



Mapping 1 million reads of length 125 extracted using `wgsim` from the Human genome while allowing up to 7 mismatches and a quality threshold of 140. The *error* is 0.6% for SOAP2 and MAQ and 0.45% for GSNAP.

Hatem et al.
BMC Bioinformatics (2013) 14:184

Read alignment: comparison on real data

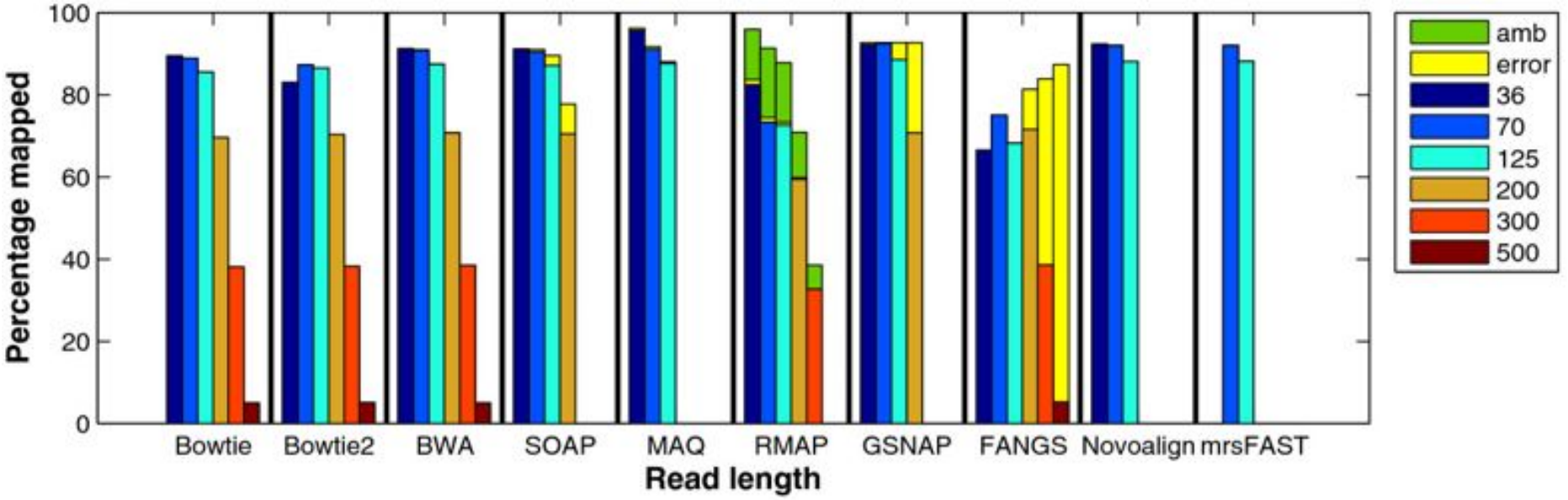


Comparing the different tools while changing the maximum allowed mismatches (T-mms) from 2 to 7.

A real mRNA data set of 1 million reads of length 51 bps extracted from the Spretus mouse strain and mapped against the mouse genome version mm9 was used in this experiment.

Hatem et al.
BMC Bioinformatics (2013) 14:184

Read alignment: effect of read length



The effect of changing the read length from 36 to 500. The reads were extracted from the Human genome.

Longer reads tend to have **more mismatches**. For a fixed number of mismatches, the read length decreases the percentage of mapped reads.

Hatem et al.
BMC Bioinformatics (2013) 14:184

Read alignment: SNP calling with different mappers

Tools	Log-odds ratio											
	5	100	200	300	400	500	600	700	800	900	1000	1000000
Bowtie	89479	24337	5082	2231	1076	648	426	281	0	0	0	1171
Bowtie2	200914	62178	10018	4200	2052	1156	767	537	0	0	0	2035
BWA	192050	52115	9028	4049	1894	1087	737	525	0	0	0	2067
SOAP2	174475	49302	8552	3824	1837	1030	704	508	0	0	0	1941
Novoalign	69798	17586	4061	1875	936	519	363	252	0	0	0	941
GSNAP	207920	69015	11416	4928	2482	1325	971	617	0	0	0	2602

The tools were used to map an mRNA dataset of 23 million reads extracted from the Spretus mouse strain. Then Partek is used to detect SNPs against mouse genome version mm9. A quality threshold of 70 was used for Bowtie and Novoalign while the remaining tools were allowed 5 mismatches.

Each column shows the number of SNPs detected with a log-odds ratio, which is a measure of the accuracy of the detected SNP, centered around the given values. The larger the log-odds ratio is, the more accurate the detected SNP becomes.

To understand the reason for the low number of SNPs detected by Bowtie and Novoalign, we carried out the same experiment while using a quality threshold of 100. The number of highly accurate SNPs increased to 1474 and 1100 for Bowtie and Novoalign, respectively.

Read alignment: conclusion

Mapping of short sequences is still subject of active development.

Genome indexing tools performed better than read indexing tools.

In general, there is no *the-best* tool among all of the tools; each tool was *the-best* in certain conditions.

Hatem et al.
BMC Bioinformatics (2013) 14:184

(2) Variant calling benchmark

-> Accurately detecting SNPs is critical e.g. for **medical diagnostics**.

Genome in a Bottle (GIAB) consortium:

public-private-academic consortium to develop the technical infrastructure (reference standards, reference methods, and reference data) to enable translation of whole human genome sequencing to clinical practice.

GIAB generated a set of highly confident variant calls for one individual in the 1000 Genome project:

they integrated 14 variant data sets from 5 NGS technologies, 7 read mappers and 3 variant calling methods, and manually cleaned up discordant data sets.

This highly accurate set of SNP and indel genotype calls can be used as **gold standard** variant genotype data set for systematic comparisons of variant callers.

Hwang et al., Scientific Reports
5, 17875 (2015)

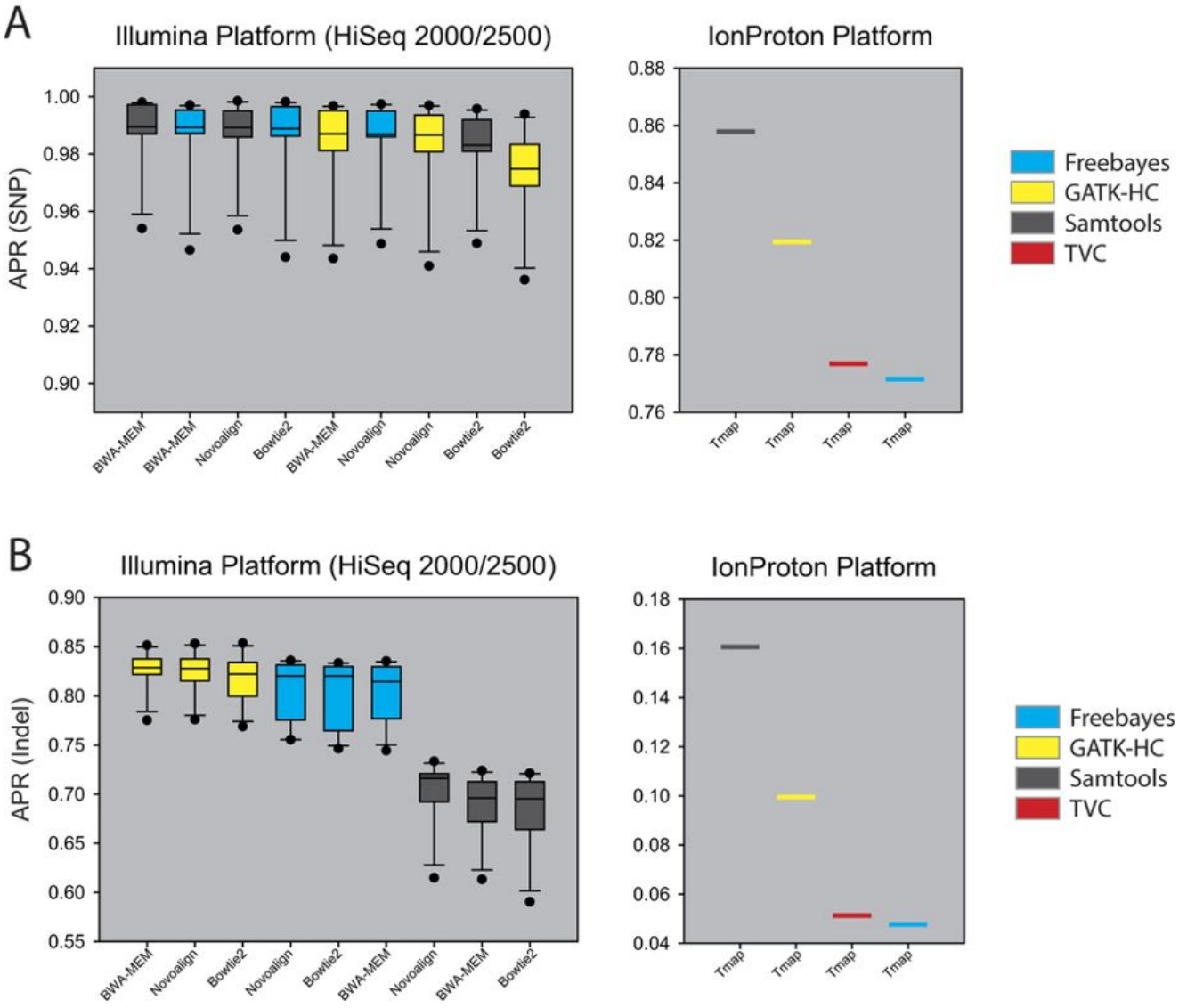
Variant calling: performance

Performance of variant calling pipelines measured by APR for

(A) SNP and

(**B**) indels.

APR: Area under a precision-recall curve.



Coverage 47 – 299 x

< 10 x

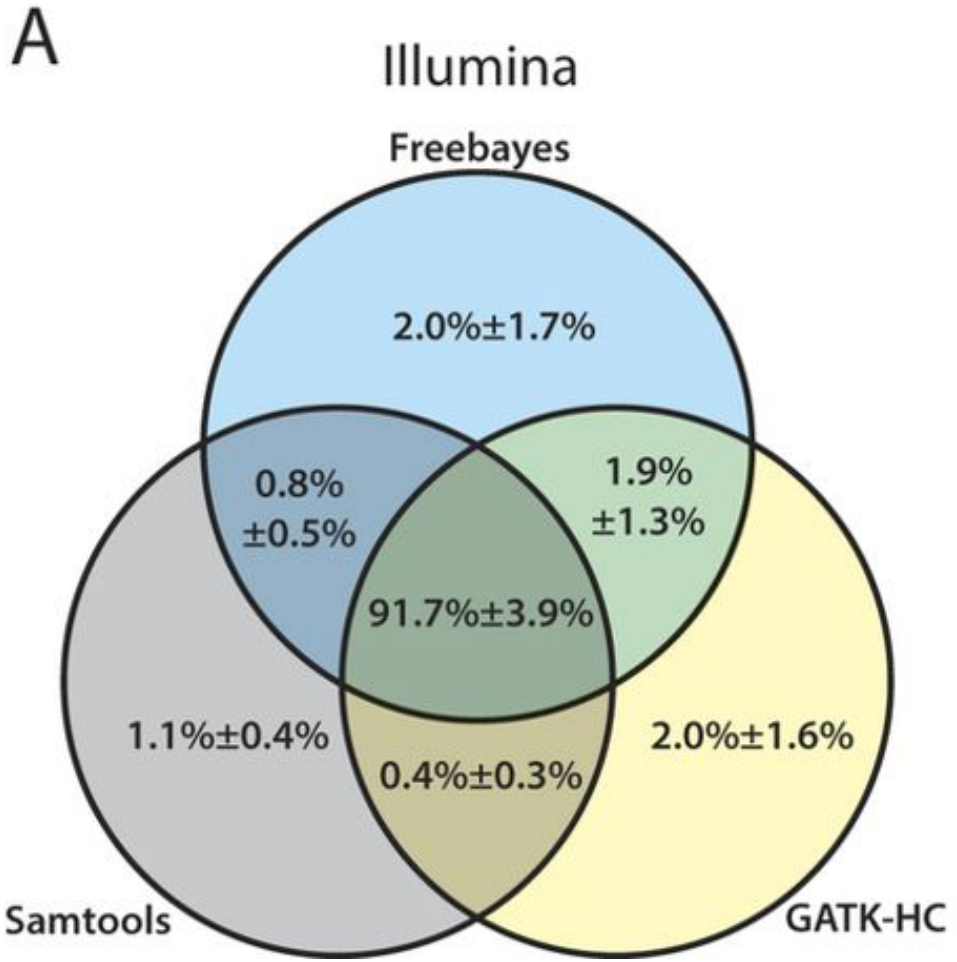
Hwang et al., Scientific Reports 5, 17875 (2015)

Variant calling: consistency

Mean percentage with standard deviation of confidence variant calls with quality ≥ 20 for Illumina data sets.

-> Generally good agreement (92% overlap of results).

For low coverage IonProton data, the overlap is only 15%.



Hwang et al., Scientific Reports 5, 17875 (2015)

Variant calling: recommendation

The authors recommend the use of BWA-MEM and Samtools pipeline for SNP calls and BWA-MEM and GATK-HC pipeline for indel calls.

Low coverage data is not suitable for reliable SNP calling.

Indels are detected at lower accuracy than SNPs.

Hwang et al., Scientific Reports
5, 17875 (2015)

(3) SNPs in 1000 Genomes project

The 1000 Genomes Project ran between 2008 and 2015 and created the largest public catalogue of human variation and genotype data up to date.

The goal of the 1000 Genomes Project was to find most genetic variants with frequencies of at least 1% in the populations studied.

http://www.internationalgenome.org/

Data set

We used only the European super-population with 503 individuals and focused on **autosomes** (chromosomes 1 – 22). Genes on sex chromosomes X and Y were ignored.

We kept autosomal SNPs with a minor allele frequency larger than zero → SNP exists

allele : variant form of a given gene

major allele : most common variant

minor allele: second-most common variant

We removed:

- genes starting with "SNO“ (small nuclear RNAs) or "MIR“ (microRNAs)
- genes with CDS start equal to the CDS end

Neininger & Helms, submitted

Problem: there exist many overlapping genes

Shown is overlap between 3 human genes: *MUTH, FLJ13949*, and *TESK2*.

Dark boxes : coding sequence.

Light boxes : untranslated regions.

Table 1. Frequency of Different Types of Overlaps Between Protein-Coding Genes in Human and Mouse Genomes

	Human		Mouse	
	Overlapping genes	Genes with overlapping exons	Overlapping genes	Genes with overlapping exons
Total	774	542	578	455
Embedded	126 (16.28%)	15 (2.77%)	53 (9.17%)	7 (1.54%)
Tail to tail	414 (53.49%)	360 (66.42%)	314 (54.32%)	280 (61.54%)
Head to head	234 (30.23%)	167 (30.81%)	211 (36.51%)	168 (36.92%)
Involving coding sequence		299 (55.17%)		232 (50.99%)
Coding–coding overlap		57 (10.52%)		31 (96.81%)

Veeramachaneni et al.
Genome Res. (2004) 14: 280-286

Overlapping genes

One could speculate that overlapping genes would be more conserved between species than non-overlapping genes because a mutation in the overlapping region would cause changes in both genes.

Then, one would expect that evolutionary selection against these mutations is stronger.

However, Veeramachaneni *et al*. found that this is not the case.

Overlapping human and mouse genes were similarly conserved as non-overlapping genes.

Veeramachaneni et al.
Genome Res. (2004) 14: 280-286

How to deal with overlapping genes

In the case of overlapping genes, it is problematic to define the **genomic regions** because they have a different meaning for the 2 overlapping genes.

Therefore, we distinguished 2 cases:

(1) Overlaps where one gene is located **inside another gene**.

Such genes inside other genes were excluded from the SNP analysis.

(2) **staggered overlaps** (genes overlap partially).

We collected all genes with staggered overlap. From each “bundle", only one gene was selected randomly to avoid overlapping genes.

In total, about 5% of all genes were removed due to overlaps.

Neininger & Helms, submitted

Refseq

The Reference Sequence (RefSeq) collection at NCBI provides a comprehensive, integrated, non-redundant, well-annotated set of sequences, including genomic DNA, transcripts, and proteins.

RefSeq transcript and protein records are generated in different ways:

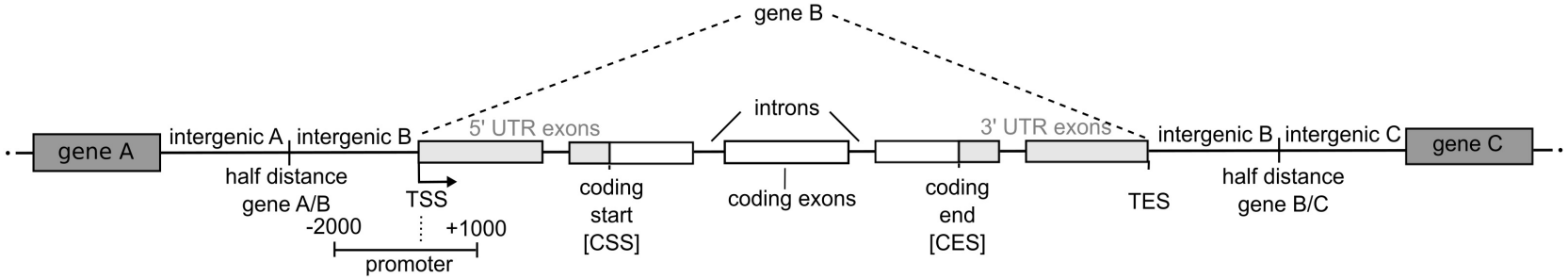
- Computation Eukaryotic Genome Annotation Pipeline
  Prokaryotic Genome Annotation Pipeline
- Manual curation
- Propagation from annotated genomes that are submitted to members of the International Nucleotide Sequence Database Collaboration (INSDC)

Research question:

Are the **Single Nucleotide Polymorphism (SNP) frequencies** in different genomic regions similar to eachother or not?

https://www.ncbi.nlm.nih.gov/refseq/about/

Definition of genomic regions



Every **gene** is located between two **intergenic regions**. Our definition for these is:

> First intergenic region : interval between the transcription start site (TSS) of the considered gene and the mid-upstream position between this TSS and the transcription end site (TES) of the closest upstream gene.
>
> Second intergenic region : defined analogously according to the TSS of the closest downstream gene.

Intragenic region of a gene : part between its TSS and its TES.

Gene **promoter** : region from 2000 bp upstream to 1000 bp downstream of the TSS.

Exons : intervals between the exon start positions and exon end positions (taken from UCSC genome browser).

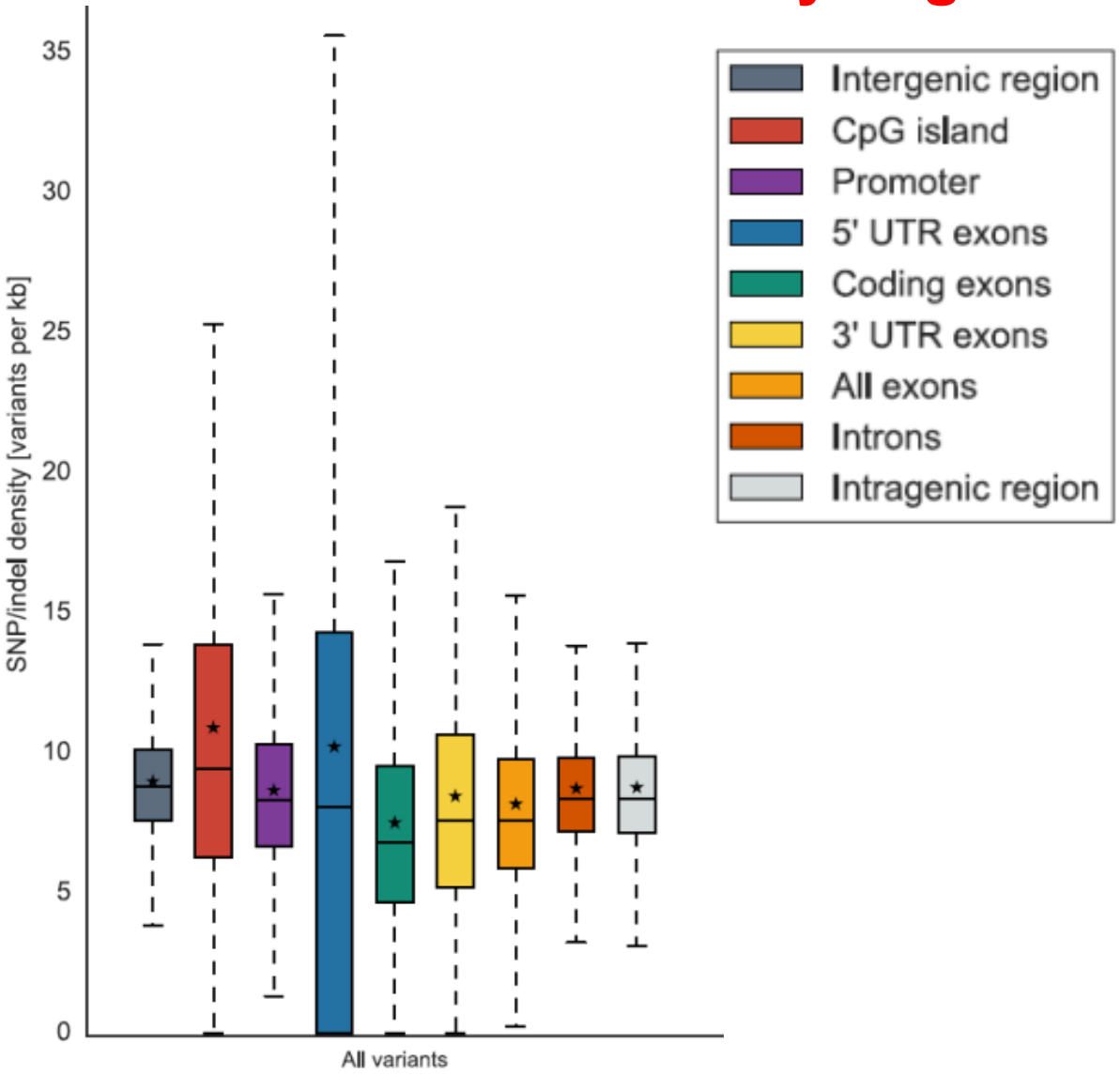
5' UTRs : exonic segments between the TSS and the CSS

3' UTRs : exonic regions between the CES and the TES.

Introns : regions between the exonic gene parts.

Neininger & Helms, submitted

SNP density in genomic regions



Neininger & Helms, submitted

Number of SNP variants per kb for different genomic regions.

→ lowest SNP density in coding exons (**green**)

→ highest SNP density in CpG islands (**red**, due to frequent deamination of methylated cytosines into thymines)

Second-highest SNP density in intergenic regions (**grey**, low evolutionary pressure)

(4) Isoforms of genes

Gene isoforms are mRNAs that are produced from the same locus but are different in their

- transcription start sites (TSSs),
- protein coding DNA sequences (CDSs) and/or
- untranslated regions (UTRs),

All these processes may potentially alter gene function.

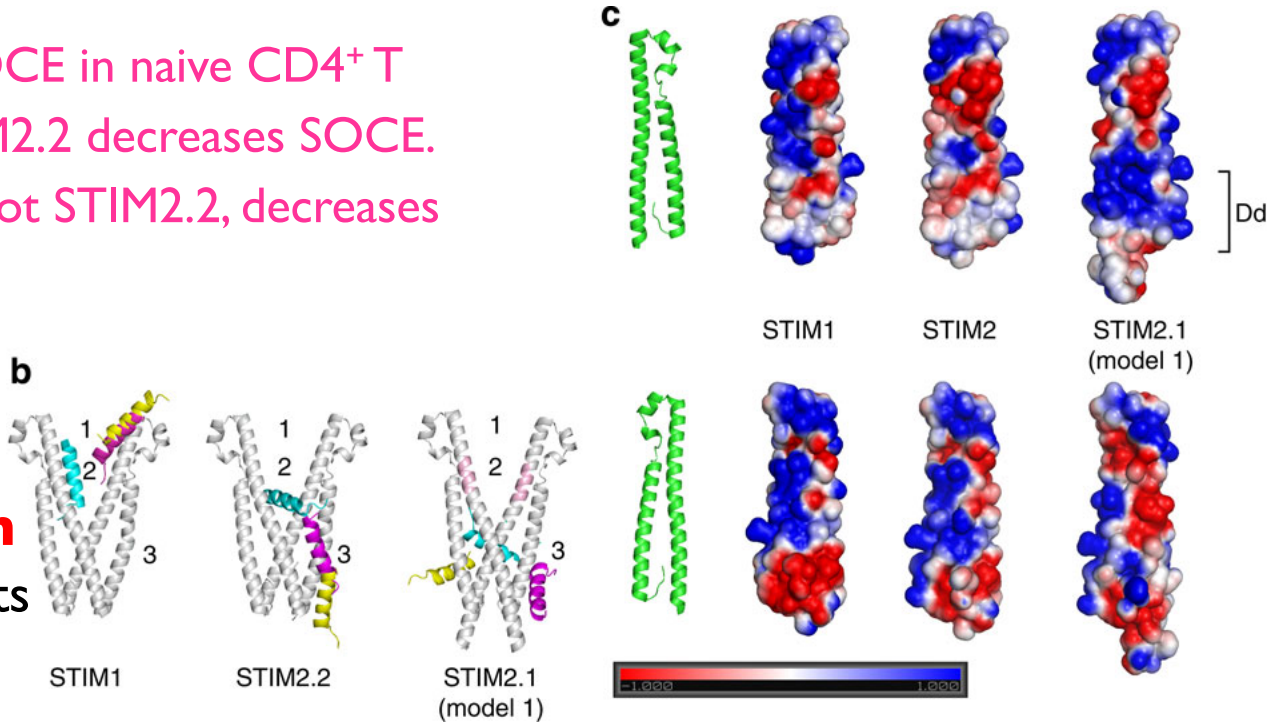
www.wikipedia.org

Alternative splicing may affect PP interactions: STIM2 splice variant

STIM proteins regulate store-operated calcium entry (SOCE) by sensing Ca^{2+} concentration in the ER and forming oligomers to trigger Ca^{2+} entry through plasma membrane-localized Orai1 channels.

Niemeyer and co-workers characterized a *STIM2* splice variant which retains an additional 8-AA exon within the region encoding the channel-activating domain.

STIM2.1 knockdown increases SOCE in naive $CD4^+$ T cells, whereas knockdown of STIM2.2 decreases SOCE. Overexpression of STIM2.1, but not STIM2.2, decreases SOCE.



STIM2.1 interaction with Orai1 is impaired and prevents Orai1 activation.

Miederer, ..., Lee, ..., Helms, Niemeyer
Nature Commun 6, 6899 (2015)

Alternative splicing

Alternative splicing (AS) of mRNA can generate a wide range of mature RNA transcripts.

It is estimated that AS of pre-mRNA occurs in 95% of multi-exon human genes.

There is abundant evidence for the expression of **multiple transcripts** in cells.

However, it is less clear whether these transcripts are expressed more or less equally across tissues or whether it would be biologically relevant to designate one transcript per gene as **dominant** and the rest as **alternative**.

Ezkurdia et al J Proteome Res. (2015) 14: 1880–1887.

Evidence from mRNA expression

3 contrasting large-scale expression studies came to different conclusions.

(1) An EST-based study with 13 different tissues predicted that primary tissues generally had a **single dominant transcript** per gene.

(2) In contrast, a large-scale study using RNAseq found that > 75% of protein-coding genes had **cell-line-specific dominant transcripts**.

Those genes with the most splice variants had more dominant transcripts.

(3) A second RNAseq study (Illumina Human BodyMap project) found that ca. 50% of the genes expressed in the 16 tissues studied had the same major transcript in all tissues, whereas another third of the genes had major transcripts that were tissue-dependent.

One curious result in this study was that the major transcript was noncoding in close to 20% of the protein-coding genes.

Ezkurdia et al J Proteome Res. (2015) 14: 1880–1887.

Detect isoforms in proteomic data

Ezkurdia *et al*. performed a re-analysis of 8 HT proteomics MS data sets.

At least 2 peptides were detected for 12 716 (63.9%) of the protein-coding genes but alternative protein isoforms only for 246 genes (1.2%).

→ the vast majority of genes had peptide evidence for just **one protein isoform**.

The isoform with the highest number of peptides was the **main proteomics isoform**.

A unique main proteomics isoform was identified for 5011 genes.

Ezkurdia et al J Proteome Res. (2015) 14: 1880–1887.

Comparison proteomics - RNAseq

CCDS variants are based on genomic evidence and are variants that are mutually agreed on by teams of manual annotators from NCBI, the Sanger Institute, EBI and UC Santa Cruz.

A total of 13 297 genes were annotated with a single CCDS variant. This unique manually curated variant agreed with the main proteomics isoform for 98.6% of the 3331 genes that were compared.

APPRIS annotates principal isoforms on the basis of conservation of structure and function and selected a **main isoform** for 15 172 of the coding genes.

Ezkurdia *et al.* were able to compare the APPRIS principal isoforms and the main proteomics isoforms over 4186 genes. The main proteomics isoform agreed with the isoform with the most conserved protein features for 97.8% of these genes.

In contrast, the **longest isoform** coincided with the main proteomics isoform only for 89.6% of the genes.

Ezkurdia et al J Proteome Res. (2015) 14: 1880–1887.

(5) Alternative translation: example TrpV6 channel protein

MUSCLE multiple sequence alignment of the translated 5′-UTR of TRPV6

Identical aa residues (compared with the human sequence) are *shaded*;

annotated N termini with the first Met^{+1} are in ***red***;

* : stop codon in frame

– : gap

Fecher-Trost et al. J. Biol. Chem. (2013) 288: 16629

The mammalian sequences upstream of the first AUG codon are conserved, but the one from rabbit contains an in-frame stop codon. In contrast, sequences from the other organisms contain several stop codons upstream of the annotated AUG and are not conserved. Sequence identity is highest among the 40 amino acids upstream of the first Met residue (position +1). This suggests that translation in mammals may start at a non-AUG

Alternative translation of human TRPV6

Nucleotide alignment of 5′-UTR TRPV6 sequences including the AUG triplet encoding the first methionine (*red*, +1) of the human protein.

Red, putative initiation sites;

underlined, STOP-codon in frame.

Experiments in the Flockerzi group (Medical department, Homburg) showed that translation starts at Thr^{-40} .

Fecher-Trost et al. J. Biol. Chem. (2013) 288: 16629

HT discovery of alternative translation: ribosome profiling

Protocol resembles ChIP-Seq.

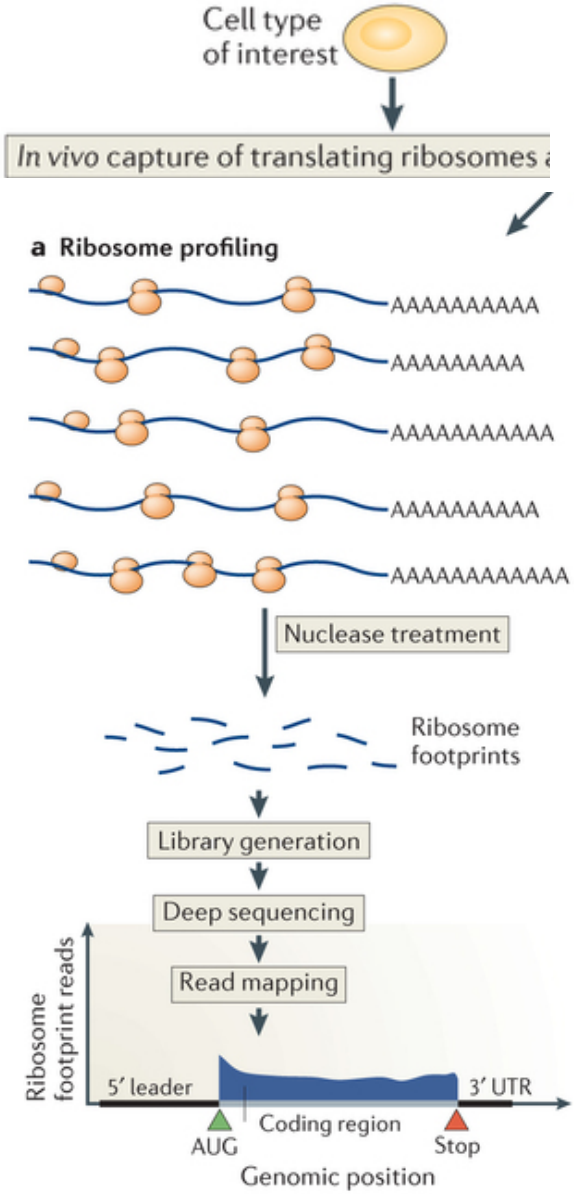
Halt translation by applying ribosome inhibitors.

Isolate ribosome-bound mRNAs by size.

Then treat sample with a nonspecific nuclease.

This results in protected mRNA fragments termed 'footprints'.

These ribosome footprints are isolated and converted to a library for deep sequencing.



Brar, Weissman, Nature Rev Mol Cell Biol 16, 651–664 (2015)

PreTIS: predict alternative translation initiation sites

```
  1 CGGUGAGGGU UCUCGGGCGG GGCCUGGGAC AGGCAGCUCC GGGGUCCGCG GUUUCACAUC
 61 GGAAACAAAA CAGCGGCUGG UCUGGAAGGA ACCUGAGCUA CGAGCCGCGG CGGCAGCGGG
121 GCGGCGGGGA AGCGUAUACC UAAUCUGGGA GCCUGCAAGU GACAACAGCC UUUGCGGUCC
181 UUAGACAGCU UGGCCUGGAG GAGAACACAU GAAAGAAAGA ACCUCAAGAG GCUUUGUUUU
241 CUGUGAAACA GUAUUUCUAU ACAGUUGCUC CAAUGACAGA GUUACCUGCA CCGUUGUCCU
301 ACUUCCAGAA UGCACAGAUG UCUGAGGACA ACCACCUGAG CAAUACUGUA CGUAGCCAGA
361 AUGACAAUAG AGAACGGCAG GAGCACAACG ACAGACGGAG CCUUGGCCAC CCUGAGCCAU
421 ...
```

Suppose that a ribosome profiling experiment detected 2 start sites for this mRNA sequence: CUG at position -78 and CUG at position -120 (**blue colored codons**). These start sites are then considered TP start sites. All near-cognate start sites not listed in the ribosome profiling dataset and upstream of the most downstream reported true start site are then considered TN (**red colored codons**).

Light red colored codons : start sites not considered as false starts in the analyses since they are located downstream of the most downstream reported true start site.

Grey colored downstream part : annotated CDS sequence

Italic (purple) upstream part : -99 upstream window needed to calculate some features.

All marked start sites (TP and TN) exhibit a surrounding window of ±99 nucleotides as well as a downstream in–frame stop codon. In total, this mRNA sequence would provide 2 **true start sites** and 9 **false start sites** out of 23 putative starts.

Data sets used for ML classifier

Cell line	Description	Genes	Start codons	TPs	TNs	Used for
HEK293	Human embryonic kidney cells	3,566	AUG and near-cognate	4,482	49,520	Human prediction model
HEK293	Human embryonic kidney cells	391	AUG	332	447	Validation set
Mouse ES	Mouse embryonic stem cells	1,632	AUG and near-cognate	3,009	19,864	Mouse prediction model

Three different datasets were used in this study to establish a human and mouse prediction model and to cross-validate the regression models. numbers indicate the filtered start sites used in the prediction approach.

doi:10.1371/journal.pcbi.1005170.t001

We only included curated mRNA sequences with available mRNA RefSeq identifier (starting with NM_).

Raw data is very unbalanced (number of TPs and TNs very different)

→ need to balance data sets (select random TN data points)

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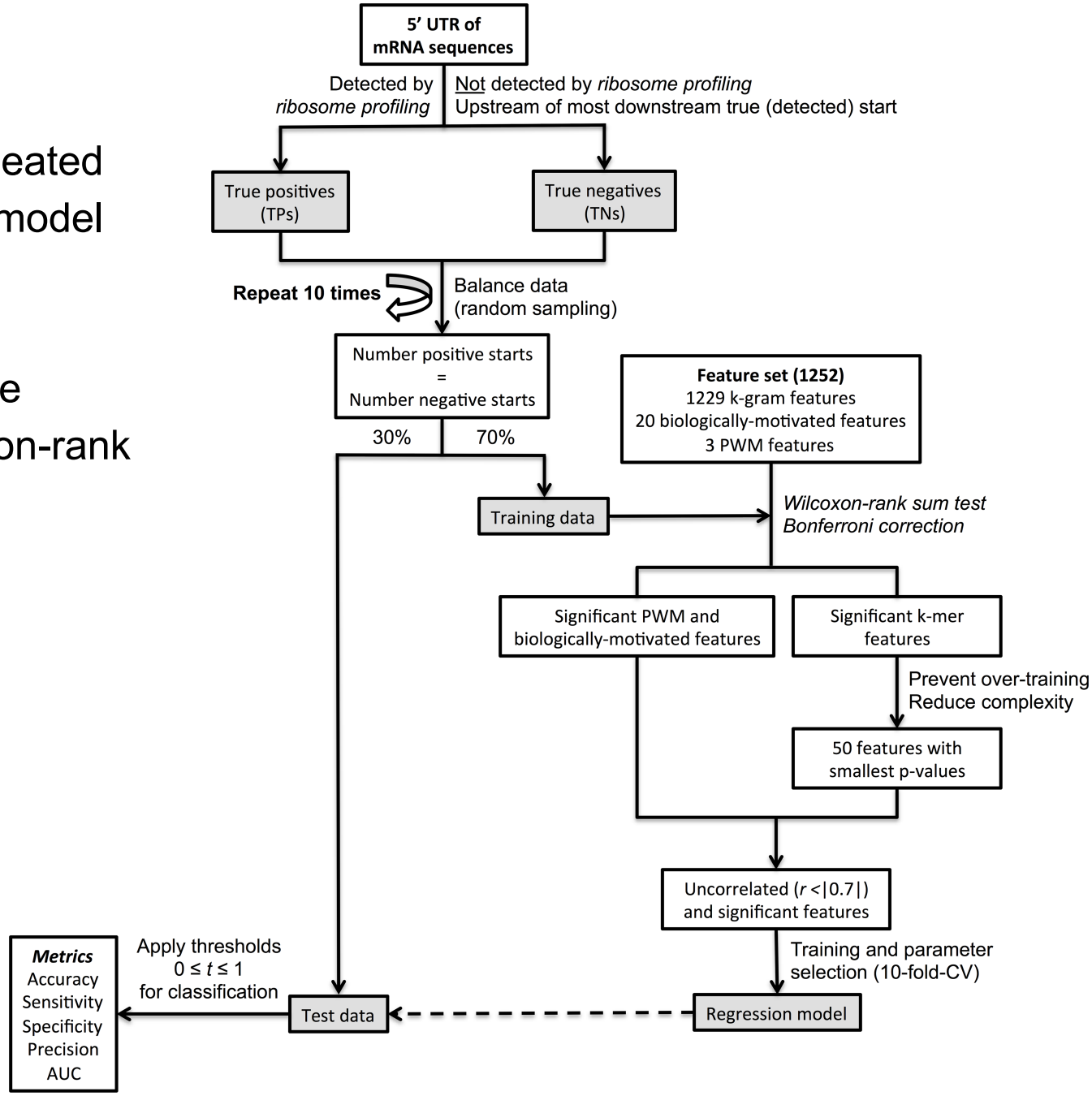
Flow-chart

Data balancing was repeated 10 times to investigate model robustness.

Significant features were identified by the Wilcoxon-rank sum test.



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Features used

Mean value and standard deviation of the 44 features that were used in the best human model.

PWM : probability weight matrix

$$\mathrm{PWM}_{(nt,i)} = log\left(\frac{PFM_{(nt,i)}}{bg_{nt}}\right)$$

Entries of position–frequency–matrix (PFM) : sum of occurrences of a nucleotide at position *i* divided by the total number of sequences contained in *S*.

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	Feature	True starts	False starts	P–value
1.	**5' UTR length**	414.41±270.48	675.41±545.35	$< 10^{-310}$
2.	**5' UTR conservation**	0.4±0.16	0.33±0.16	8.2×10^{-190}
3.	**PWM positive**	2.75±1.5	-0.14±2.82	5.5×10^{-173}
4.	K-mer: upstream AUG	0.22±0.57	0.59±0.9	5.1×10^{-144}
5.	**5' UTR: percentage A**	0.18±0.05	0.2±0.05	9.6×10^{-100}
6.	**Kozak sequence context**	2.67±1.07	2.3±1.11	9.2×10^{-95}
7.	**Translational efficiency of flanking sequence**	83.75±20.11	77.12±21.4	1.1×10^{-83}
8.	K-mer: position -12 is C	0.13±0.34	0.3±0.46	2.7×10^{-77}
9.	K-mer: upstream Asparagine	1.25±1.37	1.61±1.61	4.0×10^{-43}
10.	K-mer: downstream AUG	1.14±1.15	0.92±1.1	9.2×10^{-41}
11.	K-mer: upstream A	17.24±7.43	18.81±7.89	4.0×10^{-40}
12.	K-mer: in-frame upstream Alanine	3.69±2.6	3.16±2.29	4.0×10^{-37}
13.	K-mer: upstream Alanine	10.27±4.5	9.38±4.6	6.2×10^{-37}
14.	**5' UTR: percentage G**	0.32±0.06	0.31±0.05	7.1×10^{-37}
15.	**Codon conservation**	0.23±0.42	0.12±0.32	3.2×10^{-36}
16.	K-mer: position -3 is A	0.31±0.46	0.2±0.4	3.4×10^{-35}
17.	K-mer: upstream CCG	2.98±2.43	2.56±2.31	7.1×10^{-34}
18.	K-mer: downstream CCA	2.04±1.54	1.75±1.45	1.1×10^{-32}
19.	K-mer: position -12 is A	0.3±0.46	0.19±0.4	4.0×10^{-32}
20.	K-mer: in-frame upstream Methionine	0.07±0.29	0.2±0.48	3.3×10^{-31}
21.	K-mer: upstream Arginine	12.15±4.34	11.33±4.64	1.5×10^{-29}
22.	K-mer: upstream Histidine	1.7±1.52	1.97±1.65	2.2×10^{-27}
23.	K-mer: GCC	6.4±3.87	5.77±3.75	1.1×10^{-25}
24.	K-mer: position 4 is G	0.37±0.48	0.28±0.45	2.3×10^{-25}
25.	K-mer: upstream Threonine	3.56±2.08	3.91±2.19	4.9×10^{-25}
26.	K-mer: upstream CGG	3.14±2.51	2.77±2.41	3.2×10^{-24}
27.	K-mer: upstream C	30.4±8.98	28.96±9.04	1.0×10^{-23}
28.	K-mer: position -2 is G	0.23±0.42	0.32±0.47	1.2×10^{-23}
29.	K-mer: upstream Stop	2.3±1.71	2.66±2.0	1.4×10^{-23}
30.	K-mer: UAG	1.34±1.2	1.57±1.35	5.6×10^{-23}
31.	K-mer: upstream CAU	0.58±0.85	0.73±0.95	3.4×10^{-22}
32.	K-mer: upstream Serine	9.44±3.29	8.93±3.14	5.7×10^{-22}
33.	K-mer: downstream Glutamine	3.57±2.01	3.26±1.88	2.4×10^{-21}
34.	K-mer: AGG	4.29±2.51	4.7±2.69	2.1×10^{-20}
35.	K-mer: AGC	4.4±2.43	4.02±2.19	2.1×10^{-20}
36.	K-mer: downstream ACC	1.45±1.26	1.27±1.17	2.0×10^{-19}
37.	K-mer: UAA	1.22±1.42	1.51±1.76	6.2×10^{-19}
38.	K-mer: downstream Proline	9.3±5.63	8.56±5.47	3.5×10^{-18}
39.	K-mer: upstream CAA	0.75±0.92	0.91±1.06	1.3×10^{-17}
40.	K-mer: in-frame upstream Histidine	0.54±0.77	0.67±0.87	1.7×10^{-17}
41.	K-mer: upstream GAU	0.63±0.85	0.77±0.96	2.1×10^{-16}
42.	K-mer: in-frame upstream GCC	1.21±1.4	1.02±1.22	6.7×10^{-16}
43.	K-mer: in-frame upstream GCG	1.14±1.42	0.97±1.27	6.2×10^{-14}
44.	**PWM negative**	1.94±1.34	1.59±1.09	1.6×10^{-08}

Mean value and standard deviation of the 44 features that were used in the best human model (biologically-motivated and PWM features are shown in bold). All 4,482 true and 49,520 false start sites were considered for this analysis. All listed features showed significant differences between true and false start sites (P–values < 1.6×10^{-8}). Note that due to numerical reasons, very small p–values (< 10^{-310}) are represented as 0.0 in python programming language (*scipy version 0.17.0*). The PWM–scores are based on the test data (compare to Fig 4).

doi:10.1371/journal.pcbi.1005170.t003

Evaluation

	Accuracy	Specificity	Sensitivity	Precision	AUC	Threshold
HEK293						
Linear SVR	0.80±0.01	0.80±0.01	0.81±0.01	0.80±0.01	0.80±0.01	0.62±0.01
RBF SVR	0.82±0.01	0.81±0.01	0.83±0.02	0.82±0.01	0.82±0.01	0.55±0.02
Polynomial SVR	0.80±0.01	0.80±0.01	0.81±0.02	0.80±0.01	0.80±0.01	0.59±0.02
Linear Regression	0.80±0.01	0.80±0.01	0.81±0.01	0.80±0.01	0.80±0.01	0.55±0.01
Mouse ES						
Linear SVR	0.75±0.01	0.75±0.01	0.76±0.01	0.75±0.01	0.76±0.01	0.65±0.03
RBF SVR	0.76±0.01	0.76±0.01	0.76±0.02	0.76±0.01	0.76±0.01	0.58±0.03
Polynomial SVR	0.75±0.02	0.75±0.01	0.76±0.02	0.75±0.02	0.75±0.02	0.62±0.03
Linear Regression	0.76±0.01	0.75±0.01	0.76±0.01	0.75±0.01	0.76±0.01	0.55±0.01

The prediction was repeated 10 times to evaluate the model robustness. Shown are the average performance measures.

doi:10.1371/journal.pcbi.1005170.t002

All human models perform very similarly with accuracies of about 80%

while the average performance of the mouse model is lower with average accuracies of about 76%,

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Is model transferable to other species?

Performance of the best human HEK293 model applied to the mouse ES dataset

→ model is reasonably transferable,

suggests universal translation code

	Unbalanced datasets			
	Mouse ES		**Mouse ES**	
Threshold	$t = 0.54$		$t = 0.52$	
	TP	TN	TP	TN
Predicted positive	2,161	4,569	2,273	5,072
Predicted negative	848	15,295	736	14,792
Total	3,009	19,864	3,009	19,864
Accuracy	0.76		0.75	
Sensitivity	0.72		0.76	
Specificity	0.77		0.74	
Precision	0.32		0.31	
	Balanced datasets			
	Mouse ES		**Mouse ES**	
Threshold	$t = 0.54$		$t = 0.52$	
	TP	TN	TP	TN
Predicted positive	2,161	689	2,273	763
Predicted negative	848	2,320	736	2,246
Total	3,009	3,009	3,009	3,009
Accuracy	0.74		0.75	
Sensitivity	0.72		0.76	
Specificity	0.77		0.75	
Precision	0.76		0.75	

doi:10.1371/journal.pcbi.1005170.t004

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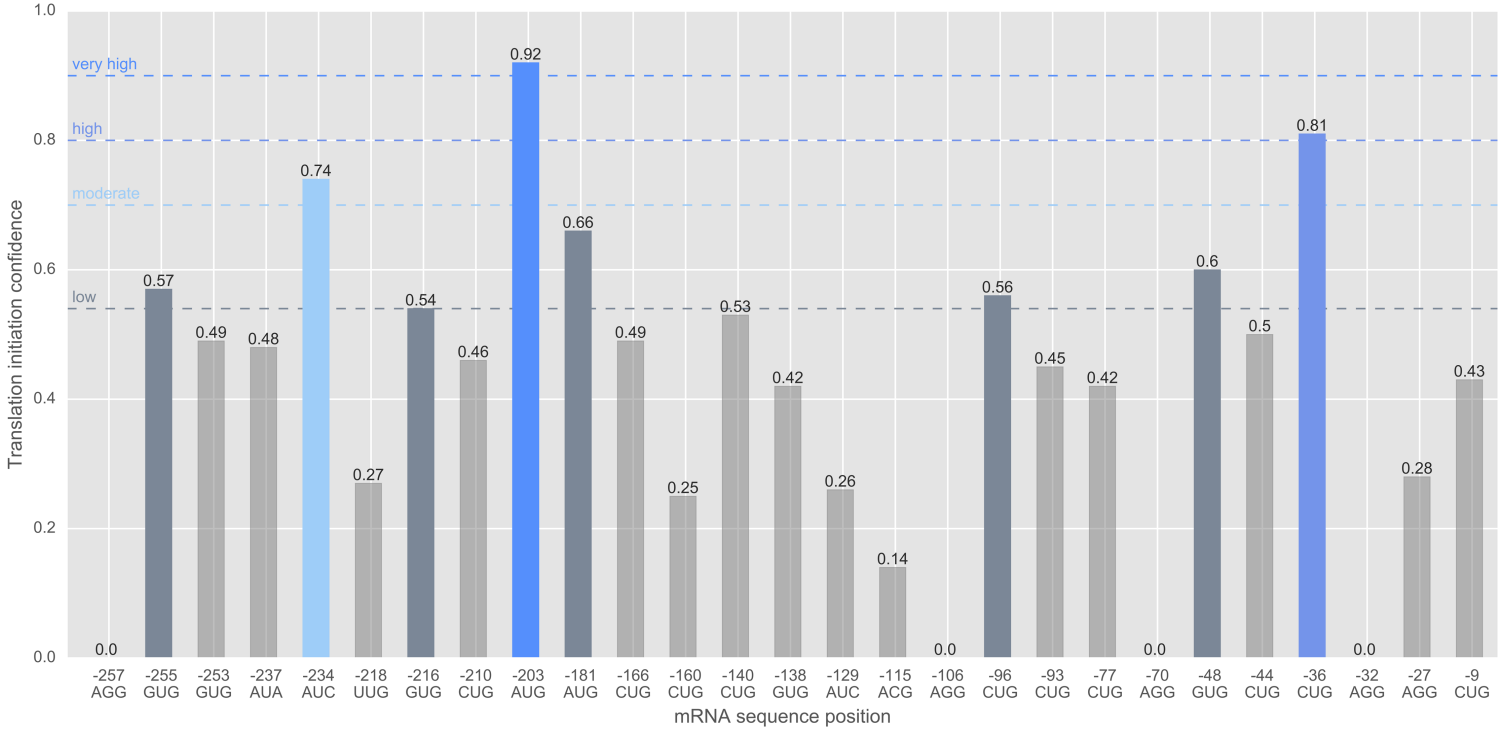
Alternative start codons of human gene GIMAP5



AUG at position -203 is a hot candidate with a very high confidence value of 0.92 of being a true start site.

Predicted start sites were subdivided into 4 confidence groups and highlighted by different colors and dashed lines:

- very high (best candidates with $c \geq 0.9$),
- high ($0.8 \leq c < 0.9$),
- moderate ($0.7 \leq c < 0.8$) and
- low ($t = 0.54 \leq c < 0.7$) initiation confidence c.

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Virtual SNP analysis of gene GIMAP5

Mutation matrix showing the impact of the flanking sequence context of 4 putative start sites of gene *GIMAP5* on the predicted initiation confidence.

In each case, only one nucleotide is mutated with respect to the reference sequence (top line). Grey : start was predicted as true translational start (predicted initiation confidence > 0.54). white : start was classified as false start.

Mutations at the start sites itself were not considered. The numbers reflect the predicted initiation confidence values

(A) *CUG at position -36*

SNPs	-15	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	**1**	**2**	**3**	4	5	6	7	8	9	10	11	12	13
	U	C	A	G	U	G	A	C	U	G	C	C	A	C	C	**C**	**U**	**G**	G	A	G	G	A	C	A	G	G	G
A	**0.80**	**0.80**		**0.80**	**0.83**	**0.82**		**0.73**	**0.84**	**0.82**	**0.81**	**0.84**		**0.85**	**0.83**				**0.80**		**0.82**	**0.86**		**0.83**		**0.86**	**0.89**	**0.85**
C	**0.81**		**0.80**	**0.64**	**0.83**	**0.81**	**0.75**		**0.80**	**0.82**			**0.67**			■			**0.78**	**0.81**	**0.82**	**0.86**	**0.79**		**0.80**	**0.82**	**0.81**	**0.83**
G	**0.80**	**0.79**	**0.79**		**0.77**		**0.78**	**0.78**	**0.78**		**0.76**	**0.80**	**0.74**	**0.80**	**0.80**			■		**0.73**			**0.77**	**0.79**	**0.73**			
U		**0.76**	**0.78**	**0.83**		**0.81**	**0.82**	**0.80**		**0.84**	**0.83**	**0.81**	**0.70**	**0.83**	**0.80**		■		**0.74**	**0.77**	**0.86**	**0.86**	**0.81**	**0.80**	**0.77**	**0.85**	**0.83**	**0.84**

(B) *CUG at position -44*

SNPs	-15	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	**1**	**2**	**3**	4	5	6	7	8	9	10	11	12	13
	C	C	A	G	A	G	C	C	U	C	A	G	U	G	A	**C**	**U**	**G**	C	C	A	C	C	C	U	G	G	A
A	0.49	0.49		**0.57**		0.49	**0.55**	0.49	0.49	0.51		0.46	**0.66**	**0.61**					**0.54**	0.52		0.52	0.50	**0.54**	0.51	**0.54**	**0.56**	
C			0.50	0.34	0.49	0.47			0.48		0.50	0.52	0.50	**0.58**	0.48	■					0.48				0.48	**0.54**	0.52	0.47
G	0.49	0.48	0.49		0.42		0.51	0.46	0.45	0.51	0.45		**0.57**		0.46			■	**0.60**	0.44	0.49	0.47	0.45	0.47	0.45			0.48
U	0.51	0.49	0.48	0.52	0.47	0.48	**0.56**	0.49		0.49	0.51	0.49		**0.55**	0.45		■		0.50	0.46	0.51	0.50	0.50	0.50		**0.56**	0.53	0.50

(C) *AUA at position -237*

SNPs	-15	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	**1**	**2**	**3**	4	5	6	7	8	9	10	11	12	13
	U	G	G	G	G	G	A	C	A	C	A	C	U	C	C	**A**	**U**	**A**	A	U	C	U	C	U	A	C	U	U
A	0.48	0.49	0.50	**0.56**	**0.54**	0.49		0.48		0.50		0.50	**0.63**	0.51	0.50	■		■		0.53	0.48	0.48	0.49	0.50		0.48	0.50	0.48
C	0.48	0.51	0.50	0.33	0.52	0.46	0.45		0.46		0.50		0.46						0.44	**0.54**		0.47		0.48	0.45		0.47	0.46
G	0.46						0.44	0.44	0.44	0.52	0.45	0.46	**0.55**	0.40	0.46				0.50	0.47	0.49	0.43	0.44	0.45	0.43	0.42	0.43	0.45
U		0.50	0.48	0.52	0.52	0.48	0.48	0.47	0.49	0.50	0.52	0.45		0.46	0.45		■		0.45		0.51		0.49		0.47	0.49		

(D) *CUG at position -160*

SNPs	-15	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	**1**	**2**	**3**	4	5	6	7	8	9	10	11	12	13
	C	C	U	C	C	U	U	A	A	C	U	G	C	G	U	**C**	**U**	**G**	C	U	C	A	A	C	C	U	C	C
A	0.23	0.24	0.25	0.47	0.26	0.26	0.25			0.20	0.25	0.31	0.46	0.33	0.30				0.29	0.31	0.24			0.25	0.26	0.26	0.30	0.28
C			0.25			0.24	0.20	0.26	0.24		0.24	0.30		0.31	0.28	■				0.29		0.24	0.24			0.23		
G	0.23	0.23	0.24	0.40	0.21	0.25	0.22	0.21	0.22	0.28	0.20		0.34		0.26			■	0.32	0.23	0.25	0.20	0.20	0.22	0.21	0.20	0.24	0.24
U	0.25	0.25		0.44	0.25			0.25	0.27	0.26		0.27	0.25	0.30			■		0.25		0.27	0.24	0.23	0.24	0.25		0.27	0.27

(6) Removing sequence redundancy

Let's assume we want to know whether the **amino acid composition** of certain protein sequences differs in one genomic region from the other regions.

For example, we want to know whether **transmembrane (TM) segments** of membrane proteins are more hydrophobic than the rest of the protein sequence

To check this, we could simply analyze all protein sequences from NCBI, predict the TM segments in them and compare the amino acid compositions.

However, this search would likely be **biased** by

- what proteins have been sequenced and which ones not, and
- by duplicated sequencing experiments.

→ It is very important to **remove sequence redundancy** before such analyses!

This can be done by software tools such as CDhit or BlastClust

BlastClust

blastclust -i infile -o outfile -p F -L .9 -b T -S 95

The sequences in "infile" will be clustered and the results will be written to "outfile".

The input sequences are identified as nucleotide (-p F); "-p T", or protein.

To register a pairwise match two sequences will need to be 95% identical (-S 95) over an area covering 90% of the length (-L .9) of each sequence (-b T) .

https://www.ncbi.nlm.nih.gov/Web/Newsltr/Spring04/blastlab.html

Take home messages

- Usually one removes sequence redundancy when correlating sequence features with properties of proteins etc.

- Check for overlapping genes

- Which isoform is relevant?

  There are substantial differences between what is expressed at the transcript level and what is expressed at the protein level.

  CCDS and APPRIS appear good resources.

- Which translated variant is relevant? May want to try PreTIS

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