Ensembl gene annotation project (*e!86*)
Macaca mulatta, Mmul_8.0.1

This document describes the annotation process of the high-coverage rhesus macaque Mmul_8.0.1 assembly, described in Figure 1. The first stage is Assembly Loading where databases are prepared and the assembly loaded into the database.

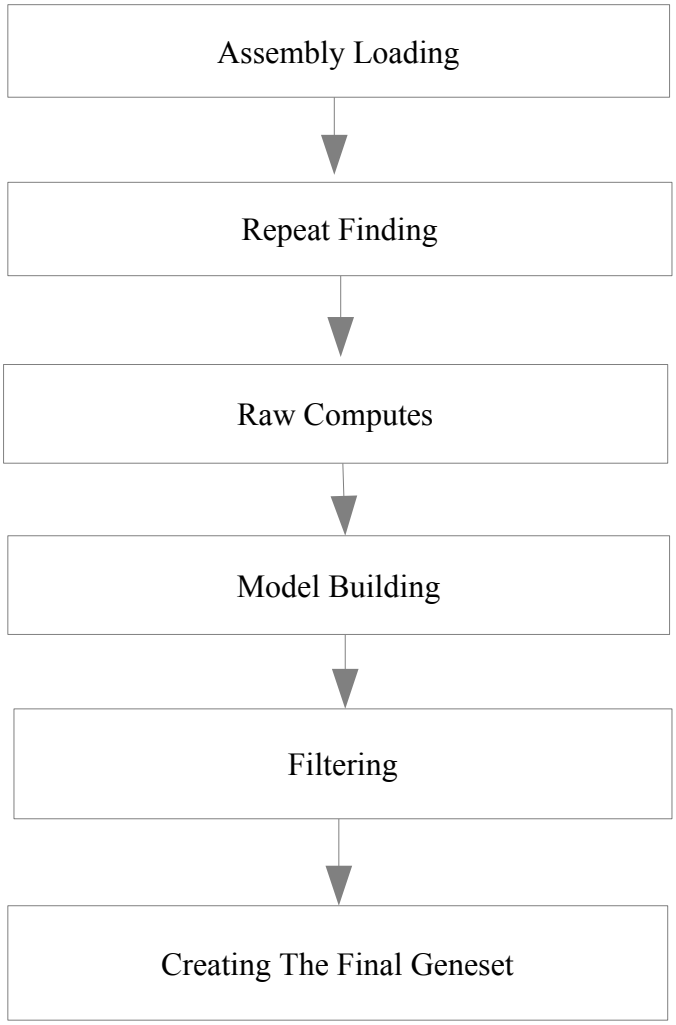

Figure 1: The Gene Annotation Pipeline

Repeat Finding

After loading into a database the genomic sequence was screened for sequence patterns including repeats using RepeatMasker [1] (version 4.0.5 with parameters '-nolow -species "primates"', using 'wublast' as the search engine), Dust [3] and TRF [4]. Both executions of RepeatMasker and Dust combined masked 54.39% of the assembly.

Raw computes

Transcription start sites were predicted using Eponine–scan [5] and FirstEF [6]. CpG islands [Micklem, G.] longer than 400 bases and tRNAs [7] were also predicted. The results of Eponine-scan, FirstEF, CpG, and tRNAscan are for display purposes only; they are not used in the gene annotation process.

Genscan [8] was run across repeat-masked sequence and the results were used as input for UniProt [9], UniGene [10] and Vertebrate RNA [11] alignments by WU-BLAST [12]. Passing only Genscan results to BLAST is an effective way of reducing the search space and therefore the computational resources required. This resulted in 7981783 UniProt, 10870513 UniGene and 10021427 Vertebrate RNA sequences aligning to the genome.

cDNA Alignments

Rhesus macaque cDNAs were downloaded from RefSeq and aligned to the genome using Exonerate. Only known or predicted mRNAs were used (NMs or XMs). A minimal sequence length of 60bp was and a cut-off of 95% identity and 50% coverage were required for an alignment to be kept. The cDNAs are mainly used for display purposes, but can be used to add UTR to the protein coding transcript models if they have an matching set of introns.

Species	Filename	Initial mRNA sequences	Sequences aligned
Rhesus macaque	GCF_000772875	55301	55092

Table 1: cDNA alignments

Model Generation

Various sources of transcript and protein data were investigated and used to generate gene models using a variety of techniques. The data and techniques employed to generate models are outlined here. The numbers of gene models generated are described in Table 2.

Pipeline	Source	Number of Models
RNA-seq	Nonhuman primate reference transcriptome resource	169437
Projection	GENCODE basic protein-coding from GRCh38 e83	67773
Protein-to-genome	Subset of UniProt vertebrate proteins	944789

Table 2: Gene Model Generation Overview

Protein-to-genome Pipeline: Generating coding models using UniProt proteins

Protein sequences were downloaded from UniProt and aligned to the genome in a splice aware manner using GenBlast [21]. The set of proteins aligned to the genome was a subset of UniProt proteins used to provide a broad, targeted coverage of the rhesus macaque genome. The set is known internally in Ensembl as the 'primates basic' set. The set consists of the following:

- Human PE level 1 & 2
- Other primates PE level 1, 2 & 3
- Mouse PE level 1 & 2
- Other mammals PE level 1 & 2
- Other vertebrates PE level 1 & 2

Note: PE level = protein existence level

A cut-off of 50 percent coverage and identity and an e-value of e-20 were used for GenBlast with the exon repair option turned on. The top 5 transcript models built by GenBlast for each protein passing the cut-offs were kept. This process produced 944789 transcript models in total.

RNA-seq Pipeline

RNA-seq data provided by the NHPRTR was used in the annotation. This consisted of paired end data from thirteen tissue samples: cerebellum, colon, frontal cortex, kidney, ovary, pituitary, temporal lobe, lung, liver, skeletal muscle, spleen, testes and thymus. A merged file contain reads from all tissues was also created. The merged was less likely to suffer from model fragmentation due to read depth. The available reads were aligned to the genome using BWA. The Ensembl RNA-seq pipeline was used to process the BWA alignments and create further split read alignments using Exonerate.

The split reads and the processed BWA alignments were combined to produce 169437 transcript models in total. The predicted open reading frames were compared to UniProt proteins using WU-BLAST. Models with poorly scoring or no BLAST alignments were split into a separate class and considered as potential lincRNAs (discussed further below).

Projection Pipeline

The Mmul_8.0.1 assembly was aligned to the human GRCh38 assembly using the LastZ whole genome alignment pipeline provided by the Ensembl Compara team. Once conserved alignment blocks were identified we used the WGA2GenesDirect module to project protein-coding transcript models from the e83 GENCODE Basic set from GRCh38 onto Mmul_8.0.1. If a projected transcript model had a structural issue (either a frameshift intron or a non-canonical splice site), we realigned the parent protein in the same region using GenBlast to see if we could produce a model that had a normal transcript structure. Using this method a total of 67773 were projected from GRCh38 to Mmul_8.0.1.

Filtering the Models

The filtering phase decided the subset of protein-coding transcript models, generated from the model-building pipelines, that comprise the final protein-coding gene set. Model are filtered based on information such as what pipeline they were generated using, how closely related the data are to the target species and how good the alignment coverage and precent identity to the original data are.

Models were filtered using the LayerAnnotation and GeneBuilder modules. The Apollo software [16] was used to visualise the results of filtering.

LayerAnnotation

The LayerAnnotation module was used to define a hierarchy of input data sets, from most preferred to least preferred. The output of this pipeline included all transcript models from the highest ranked input set. Models from lower ranked input sets are included only if their exons do not overlap a model from an input set higher in the hierarchy.

Note that models cannot exist in more than one layer. For UniProt proteins, models were also separate into clades, to help selection during the layering process. Each UniProt protein was in one clade only, for example mammal proteins were present in the mammal clade and were not present in the vertebrate clade to avoid aligning the proteins multiple times.

Layer 1:

- Projection models with >= 80 percent coverage and identity
- RNA-seq models with >= 80 percent coverage and identity
- Rhesus macaque UniProt proteins from PE levels 1 & 2 with >= 80 percent coverage and identity
- Other mammal UniProt proteins from PE levels 1 & 2 with >= 95 percent coverage and identity

Layer 2:

- Mammal UniProt proteins from PE levels 1 & 2 with >= 80 percent coverage and identity

Layer 3:

- Other vertebrate UniProt proteins from PE levels 1 & 2 with >= 95 percent coverage and identity
- Primate SwissProt proteins from PE level 3 with >= 95 percent coverage and identity

Layer 4:

- Primate SwissProt proteins from PE level 3 with >= 80 percent coverage and identity
- Other vertebrate UniProt proteins from PE levels 1 & 2 with >= 80 percent coverage and identity

Layer 5:

- Projection models with >= 50 percent coverage and identity

Addition of UTR to coding models

The set of coding models was extended into the untranslated regions (UTRs) using RNA-seq and cDNA and EST sequences. The source of the UTRs was prioritised with UTR coming from RNA-seq and known rhesus macaque cDNAs getting priority over UTR from predicted cDNAs.

Generating multi-transcript genes

The above steps generated a large set of potential transcript models, many of which overlapped one another. Redundant transcript models were collapsed and the remaining unique set of transcript models were clustered into multi-transcript genes where each transcript in a gene has at least one coding exon

that overlaps a coding exon from another transcript within the same gene.

At this stage the gene set comprised 22435 genes with 46702 transcripts.

Pseudogenes

The Pseudogene module was run to identify pseudogenes from within the set of gene models. A total of 286 genes were labelled as pseudogenes or processed pseudogenes.

Creating The Final Gene Set

Small ncRNAs

Small structured non-coding genes were added using annotations taken from RFAM [17] and miRBase [18]. WU-BLAST was run for these sequences and models built using the Infernal software suite [20].

lincRNAs

Long intergenic non-coding RNAs were identified from transcript models generated from the RNA-seq pipeline. As part of the RNA-seq pipeline the longest ORF of each transcript is translated and a BLAST is run against the UniProt vertebrate PE level 1 & 2 proteins. Models with either no hits or likely false positives were subjected to further analysis for evidence of Pfam domains. Models that had no evidence of protein domains were labelled as lincRNAs. The lincRNA pipeline identified 2938 lincRNA genes.

Cross-referencing

Before public release the transcripts and translations were given external references (cross-references to external databases). Translations were searched for signatures of interest and labelled where appropriate.

Stable Identifiers

Stable identifiers were assigned to each gene, transcript, exon and translation. When annotating a species for the first time, these identifiers are auto-generated. In all subsequent annotations for a species, the stable identifiers are propagated based on comparison of the new gene set to the previous gene set.

As rhesus macaque has been previously released in Ensembl a comparison was made to the previous gene set and as many stable identifiers as possible were mapped between the two annotations.

Final Gene Set Summary

The final gene set consists of 21099 protein coding genes, including 13 mitochondrial genes. These contain 45293 transcripts. A total of 286 pseudogenes were identified. 8039 small ncRNAs were added by the small ncRNA pipeline. 2938 lincRNAs were added via the lincRNA pipeline.

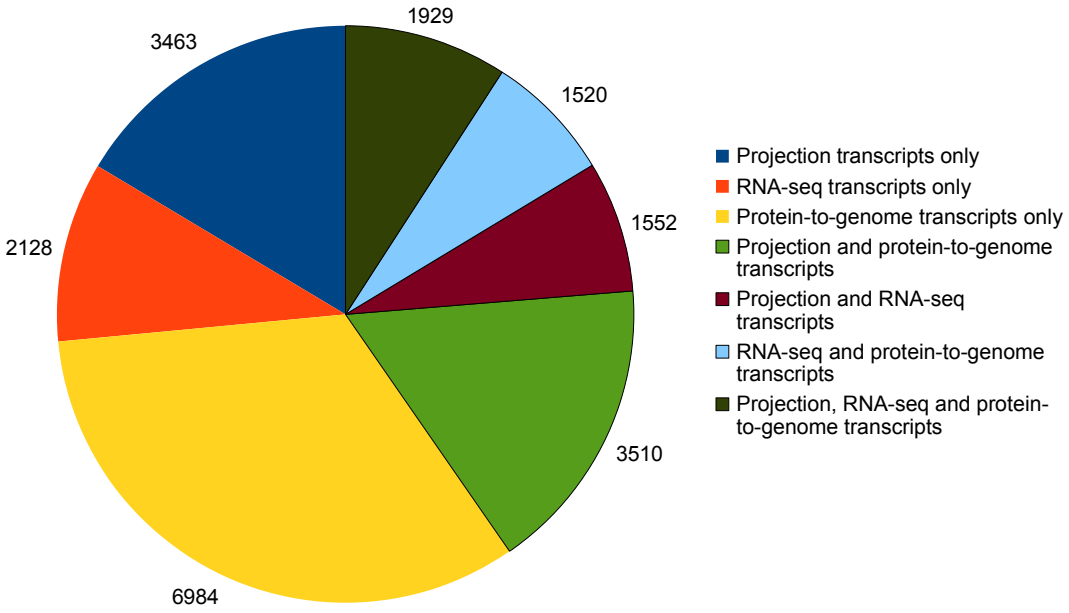

Figure 2: Transcript composition for protein-coding genes. The pie chart shows what pipelines the transcripts that make up the protein-coding gene set come from. Note that these values only correspond to the count of the original source of the final models. Often models are supported by evidence from more than one pipeline, in which case the longest model is usually kept.

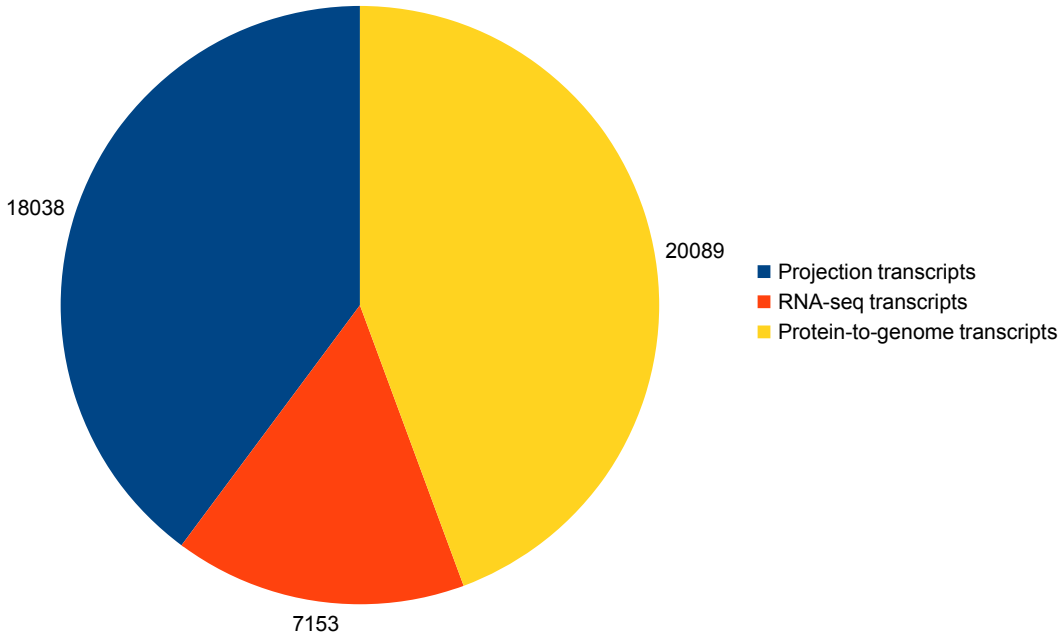

Figure 3: Pipeline source for the protein-coding transcript models. Note that these values only correspond to the count of the original source of the final models. Often models are supported by evidence from more than one pipeline, in which case the longest model is usually kept.

Further information

The Ensembl gene set is generated automatically, meaning that gene models are annotated using the Ensembl gene annotation pipeline. The main focus of this pipeline is to generate a conservative set of protein-coding gene models, although non-coding genes and pseudogenes may also annotated.

Every gene model produced by the Ensembl gene annotation pipeline is supported by biological sequence evidence (see the “Supporting evidence” link on the left-hand menu of a Gene page or Transcript page); *ab initio* models are not included in our gene set. *Ab initio* predictions and the full set of cDNA and EST alignments to the genome are available on our website.

The quality of a gene set is dependent on the quality of the genome assembly. Genome assembly can be assessed in a number of ways, including:

1. Coverage estimate
    - A higher coverage usually indicates a more complete assembly.
    - Using Sanger sequencing only, a coverage of at least 2x is preferred.
2. N50 of contigs and scaffolds
    - A longer N50 usually indicates a more complete genome assembly.
    - Bearing in mind that an average human gene may be 10-15 kb in length, contigs shorter than this length will be unlikely to hold full-length gene models.
3. Number of contigs and scaffolds
    - A lower number toplevel sequences usually indicates a more complete genome assembly.
4. Alignment of cDNAs and ESTs to the genome

- A higher number of alignments, using stringent thresholds, usually indicates a more complete genome assembly.

More information on the Ensembl automatic gene annotation process can be found at:

- Aken B et al.: **The Ensembl gene annotation system.** Database 2016. [PMCID: PMC4919035]
- Potter SC, Clarke L, Curwen V, Keenan S, Mongin E, Searle SM, Stabenau A, Storey R, Clamp M: **The Ensembl analysis pipeline.** *Genome Res.* 2004, **14(5):**934-41. [PMID: 15123589]
- http://www.ensembl.org/info/genome/genebuild/index.html
- https://github.com/Ensembl/ensembl-doc/blob/master/pipeline_docs/the_genebuild_process.txt

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