EST clustering

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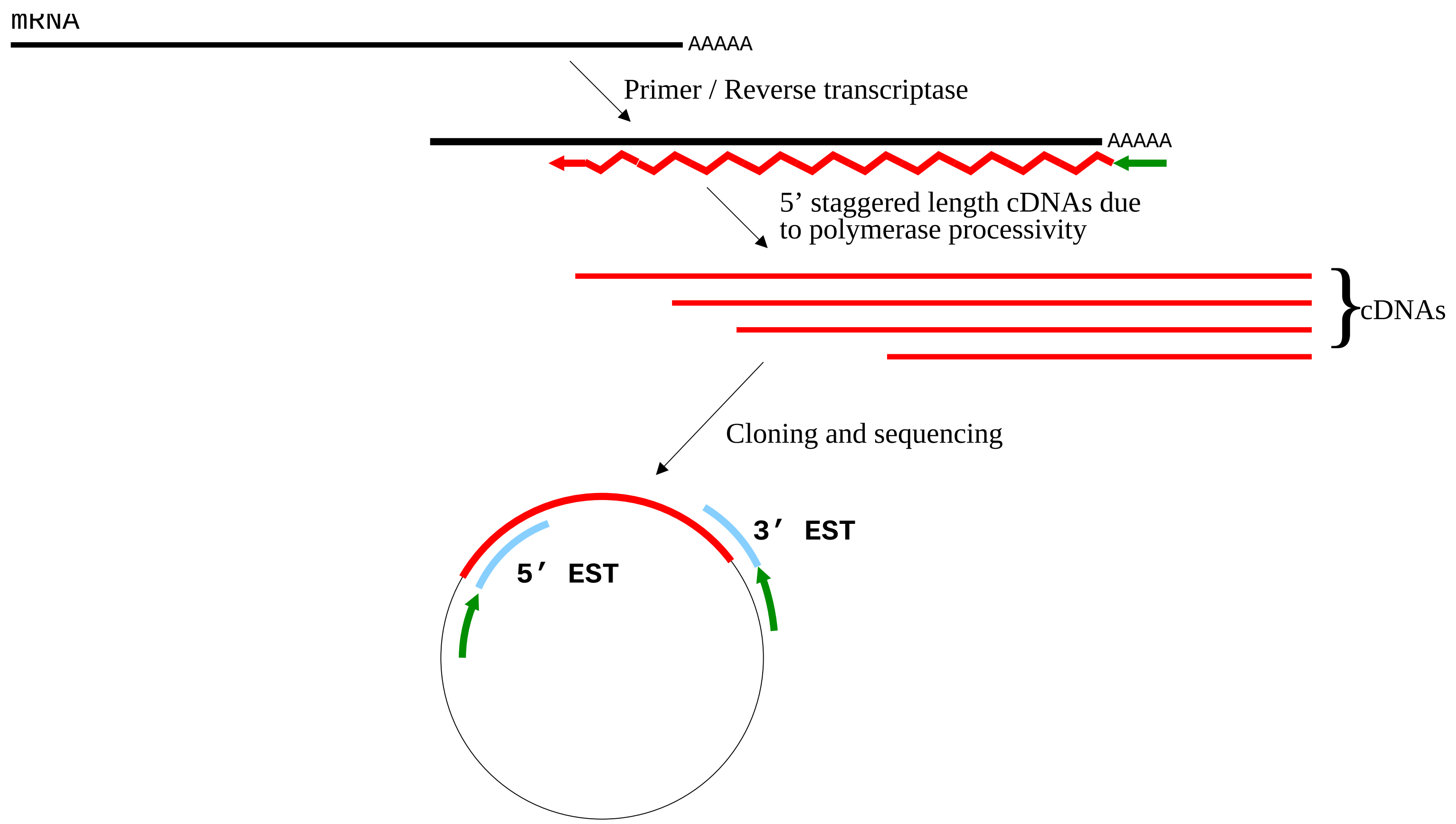
Expressed sequence tags (ESTs)

ESTs represent partial sequences of cDNA clones (average ~ 360 bp).

Single-pass reads from the 5’ and/or 3’ ends of cDNA clones.


mRNA
AAAAA
Primer / Reverse transcriptase
AAAAA
5’ staggered length cDNAs due
to polymerase processivity
cDNAs
Cloning and sequencing
3’ EST
5’ EST

Interest for ESTs

ESTs represent the most extensive available survey of the transcribed portion of genomes.

ESTs are indispensable for gene structure prediction, gene discovery and genomic mapping.

Characterization of splice variants and alternative polyadenilation.

In silico differential display and gene expression studies (specific tissue expression, normal/disease states).

SNP data mining.

High-volume and high-throughput data production at low cost.

There are 12,323,094 of EST entries in GenBank (dbEST) (August 16, 2002):

- 4,550,451 entries of human ESTs;
- 2,633,209 entries of mouse ESTs;
- ...

Low data quality of ESTs

High error rates ($\sim 1/100$) because of the sequence reading single-pass.

Sequence compression and frame-shift errors due to the sequence reading single-pass.

A single EST represents only a partial gene sequence.

Not a defined gene/protein product.

Not curated in a highly annotated form.

High redundancy in the data $\Rightarrow$ huge number of sequences to analyze.

Improving ESTs: Clustering, Assembling and Gene indices

The value of ESTs is greatly enhanced by clustering and assembling.

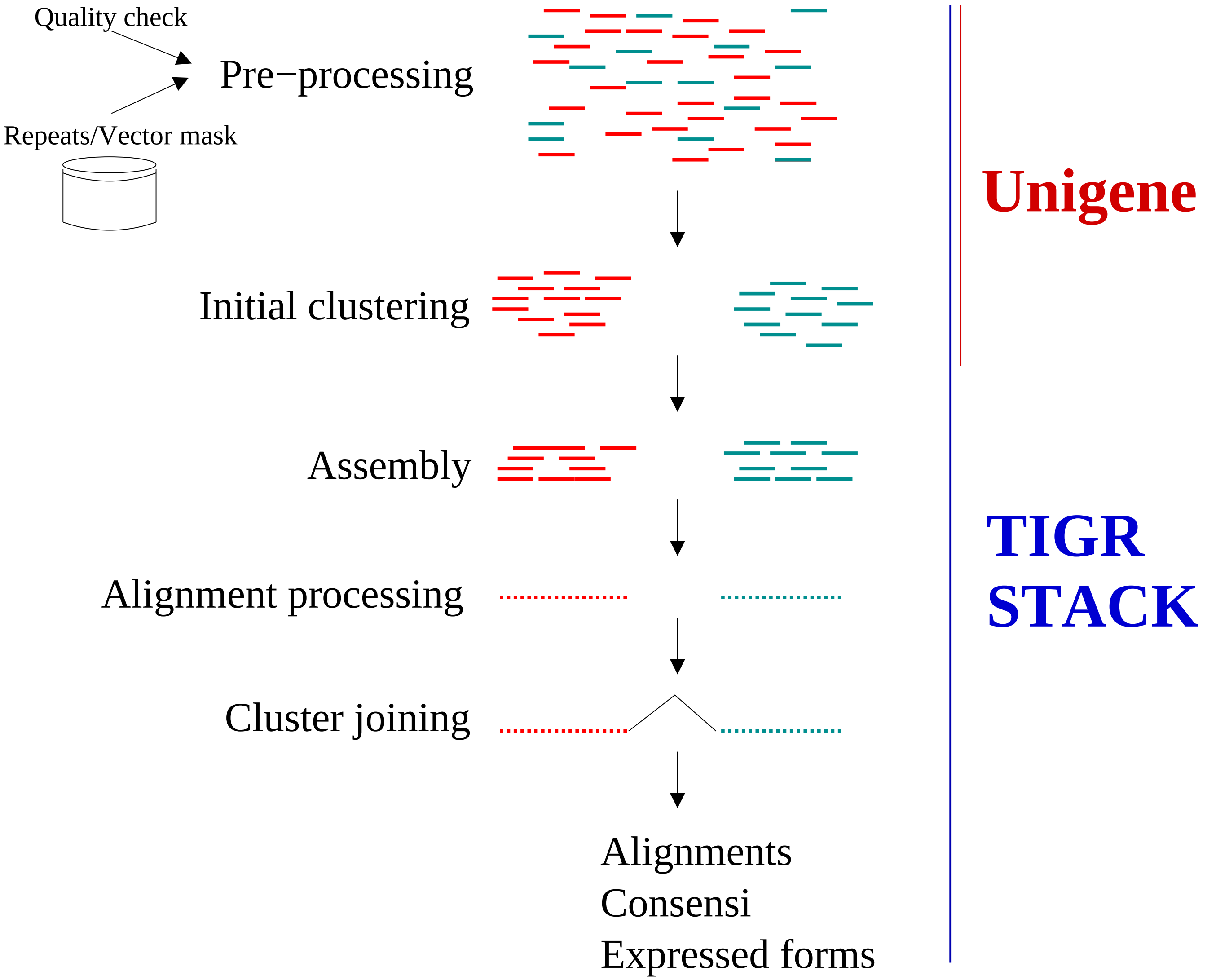
- solving redundancy can help to correct errors;
- longer and better annotated sequences;
- easier association to mRNAs and proteins;
- detection of splice variants;
- fewer sequences to analyze.

Gene indices: All expressed sequences (as ESTs) concerning a single gene are grouped in a single index class, and each index class contains the information for only one gene.

Different clustering/assembly procedures have been proposed with associated resulting databases (gene indices):

- UniGene (http://www.ncbi.nlm.nih.gov/UniGene)
- TIGR Gene Indices (http://www.tigr.org/tdb/tgi.shtml)
- STACK (http://www.sambi.ac.za/Dbases.html)

EST clustering pipeline



Quality check

Pre−processing

Repeats/Vector mask

Initial clustering

Assembly

Alignment processing

Cluster joining

Alignments
Consensi
Expressed forms

Unigene

TIGR STACK

Pre-processing

Data source

The data sources for clustering can be in-house, proprietary, public database or a hybrid of this (chromatograms and/or sequence files).

Each EST must have the following information:

- A sequence ID (ex. sequence-run ID);
- Location in respect of the poly A (3’ or 5’);
- The CLONE ID from which the EST has been generated;
- Organism;
- Tissue and/or conditions;
- The sequence.

The EST can be stored in FASTA format:

```
>T27784 EST16067 Human Endothelial cells Homo sapiens cDNA 5’
CCCCCGTCTCTTTAAAAATATATATATTTTAAATATACTTAAATATATATTTCTAATATC
TTTAAATATATATATATATTTNAAAGACCAATTTATGGGAGANTTGCACACAGATGTGAA
ATGAATGTAATCTAATAGANGCCTAATCAGCCCACCATGTTCTCCACTGAAAAATCCTCT
TTCTTTGGGGTTTTTCTTTCTTTCTTTTTTGATTTTGCACTGGACGGTGACGTCAGCCAT
GTACAGGATCCACAGGGGTGGTGTCAAATGCTATTGAAATTNTGTTGAATTGTATACTTT
TTCACTTTTTGATAATTAACCATGTAAAAAATGAACGCTACTACTATAGTAGAATTGAT
```

Pre-processing

EST pre-processing consists in a number of essential steps to minimize the chance to cluster unrelated sequences.

- Screening out low quality regions:
  - Low quality sequence readings are error prone.
  - Programs as Phred (*Ewig et al., 98*) read chromatograms and assesses a quality value to each nucleotide.
- Screening out contaminations.
- Screening out vector sequences (vector clipping).
- Screening out repeat sequences (repeats masking).
- Screening out low complexity sequences.

Dedicated software are available for these tasks:

- RepeatMasker (*Smit and Green*, http://ftp.genome.washington.edu/RM/RepeatMasker.html);
- VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen);
- Lucy (*Chou and Holmes*, 01);
- ...

Vector-clipping and contaminations

Vector-clipping

- Vector sequences can skew clustering even if a small vector fragment remains in each read.
- Delete 5’ and 3’ regions corresponding to the vector used for cloning.
- Detection of vector sequences is not a trivial task, because they normally lies in the low quality region of the sequence.
- UniVec is a non-redundant vector database available from NCBI: http://www.ncbi.nlm.nih.gov/VecScreen/UniVec.html

Contaminations

- Find and delete:
  - ▷ bacterial DNA, yeast DNA, and other contaminations;
  - ▷ ...

Standard pairwise alignment programs are used for the detection of vector and other contaminants (for example cross-match, BLASTN, FASTA). They are reasonably fast and accurate.

Repeats masking

Some repetitive elements found in the human genome:

	Length	Copy number	Fraction of the genome
LINEs (long interspersed elements)	6-8 kb	850,000	21%
SINEs (short interspersed elements)	100-300 bp	1,500,000	13%
LTR (autonomous)	6-11 kb	450,000	8%
LTR (non-autonomous)	1.5-3 kb		
DNA transposons (autonomous)	2-3 kb	300,000	3%
DNA transposons (non-autonomous)	80-3000 bp		
SSRs (simple sequence repeats or microsatellite and minisatellites)			3%

Repeats masking

Repeated elements:

- They represent a big part of the mammalian genome.
- They are found in a number of genomes (plants, ...)
- They induce errors in clustering and assembling.
- They should be masked, not deleted, to avoid false sequence assembling.
- ... but also interesting elements for evolutionary studies.
- SSRs important for mapping of diseases.

Tools to find repeats:

- RepeatMasker has been developed to find repetitive elements and low-complexity sequences. RepeatMasker uses the cross-match program for the pairwise alignments (http://repeatmasker.genome.washington.edu/cgi-bin/RepeatMasker).
- MaskerAid improves the speed of RepeatMasker by ~ 30 folds using WU-BLAST instead of cross-match (http://sapiens.wustl.edu/maskeraid)
- RepBase is a database of prototypic sequences representing repetitive DNA from different eukaryotic species.: http://www.girinst.org/Repbase_Update.html.

Low complexity masking

Low complexity sequences contains an important bias in their nucleotide compositions (poly A tracts, AT repeats, etc.).

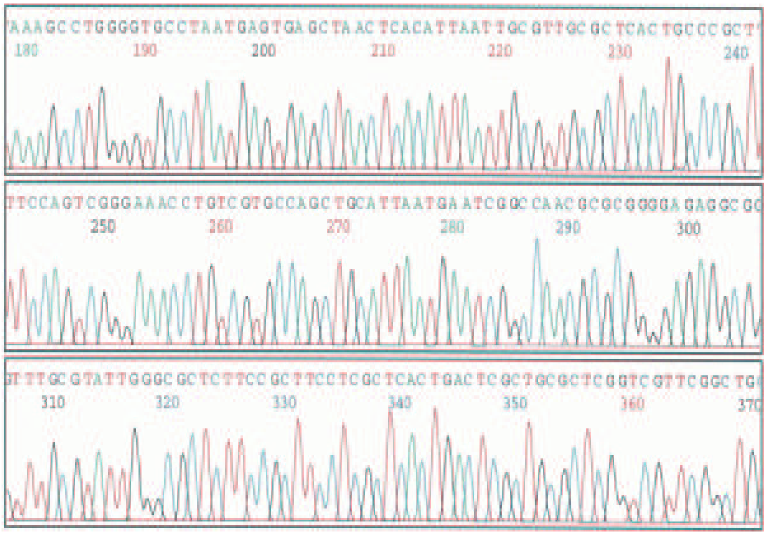
Low complexity regions can provide an artifactual basis for cluster membership.

Clustering strategies employing alignable similarity in their first pass are very sensitive to low complexity sequences.

Some clustering strategies are insensitive to low complexity sequences, because they weight sequences in respect to their information content (ex. d2-cluster).

Programs as DUST (NCBI) can be used to mask low complexity regions.

Pre-processing



Base calling
Select high quality reads

```
CCCCCGTCTCTTTAAAAATATATATATTTTAAATATACTTAAATATATATTTCTAATATC
TTTAAATATATATATATATATTTNAAAGACCAATTTATGGGAGANTTGCACACAGATGTGAA
ATGAATGTAATCTAATAGANGCCTAATCAGCCCACCATGTTCTCCACTGAAAAATCCTCT
TTCTTTGGGGTTTTTCTTTCTTTCTTTTTTGATTTTGCACTGGACGGTGACGTCAGCCAT
GTACAGGATCCACAGGGGTGGTGTCAAATGCTATTGAAATTNTGTTGAATTGTATACTTT
TTCACTTTTTGATAATTAACCATGTAAAAAATGAACGCTACTACTATAGTAGAATTGAT
```

Vector clipping

```
CCCCCGTCTCTTTAAAAATATATATATTTTAAATATACTTAAATATATATTTCTAATATC
TTTAAATATATATATATATATTTNAAAGACCAATTTATGGGAGANTTGCACACAGATGTGAA
ATGAATGTAATCTAATAGANGCCTAATCAGCCCACCATGTTCTCCACTGAAAAATCCTCT
TTCTTTGGGGTTTTTCTTTCTTTCTTTTTTGATTTTGCACTGGACGGTGACGTCAGCCAT
GTACAGGATCCACAGGGGTGGTGTCAAATGCTATTGAAATTNTGTTGAATTGTATACTTT
TTCACTTTTTGATAATTAACCATGTAAAAAATGXXXXXXXXXXXXXXXXXXXXXXXXXX
```

Repeat/Low complexity masking

```
CCCCCGTCTCTTTAAAANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNTTNAAAGACCAATTTATGGGAGANTTGCACACAGATGTGAA
ATGAATGTAATCTAATAGANGCCTAATCAGCCCACCATGTTCTCCACTGAAAAATCCTCT
TTCTTTGGGGTTTTTCTTTCTTTCTTTTTTGATTTTGCACTGGACGGTGACGTCAGCCAT
GTACAGGATCCACAGGGGTGGTGTCAAATGCTATTGAAATTNTGTTGAATTGTATACTTT
TTCACTTTTTGATAATTAACCATGTAAAAAATGXXXXXXXXXXXXXXXXXXXXXXXXXX
```

Sequence ready for clustering

```
CCCCCGTCTCTTTAAAANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNTTNAAAGACCAATTTATGGGAGANTTGCACACAGATGTGAA
ATGAATGTAATCTAATAGANGCCTAATCAGCCCACCATGTTCTCCACTGAAAAATCCTCT
TTCTTTGGGGTTTTTCTTTCTTTCTTTTTTGATTTTGCACTGGACGGTGACGTCAGCCAT
GTACAGGATCCACAGGGGTGGTGTCAAATGCTATTGAAATTNTGTTGAATTGTATACTTT
TTCACTTTTTGATAATTAACCATGTAAAAAATG
```

Clustering

EST clustering

The goal of the clustering process is to incorporate overlapping ESTs which tag the same transcript of the same gene in a single cluster.

For clustering, we measure the similarity (distance) between any 2 sequences. The distance is then reduced to a simple binary value: accept or reject two sequences in the same cluster.

Similarity can be measured using different algorithms:

- Pairwise alignment algorithms:
  - ▷ Smith-Waterman is the most sensitive, but time consuming (ex. cross-match);
  - ▷ Heuristic algorithms, as BLAST and FASTA, trade some sensitivity for speed
- Non-alignment based scoring methods:
  - ▷ d2_cluster algorithm: based on word comparison and composition (word identity and multiplicity) (*Burke et al.*, 99). No alignments are performed ⇒ fast.
- Pre-indexing methods.
- Purpose-built alignments based clustering methods.

Loose and stringent clustering

Stringent clustering:

- Greater initial fidelity;
- One pass;
- Lower coverage of expressed gene data;
- Lower cluster inclusion of expressed gene forms;
- Shorter consensi.

Loose clustering:

- Lower initial fidelity;
- Multi-pass;
- Greater coverage of expressed gene data;
- Greater cluster inclusion of alternate expressed forms.
- Longer consensi;
- Risk to include paralogs in the same gene index.

Supervised and unsupervised EST clustering

Supervised clustering

- ESTs are classified with respect to known reference sequences or ”seeds” (full length mRNAs, exon constructs from genomic sequences, previously assembled EST cluster consensus).

Unsupervised clustering

- ESTs are classified without any prior knowledge.

The three major gene indices use different EST clustering methods:

- TIGR Gene Index uses a stringent and supervised clustering method, which generate shorter consensus sequences and separate splice variants.
- STACK uses a loose and unsupervised clustering method, producing longer consensus sequences and including splice variants in the same index.
- A combination of supervised and unsupervised methods with variable levels of stringency are used in UniGene. No consensus sequences are produced.

Assembling, processing, and cluster joining

Assembly and processing

A multiple alignment for each cluster can be generated (assembly) and consensus sequences generated (processing).

A number of program are available for assembly and processing:

- PHRAP (http://www.genome.washington.edu/UWGC/analysistools/Phrap.cfm);
- TIGR_ASSEMBLER (*Sutton et al.*, 95);
- CRAW (*Burke et al.*, 98);
- ...

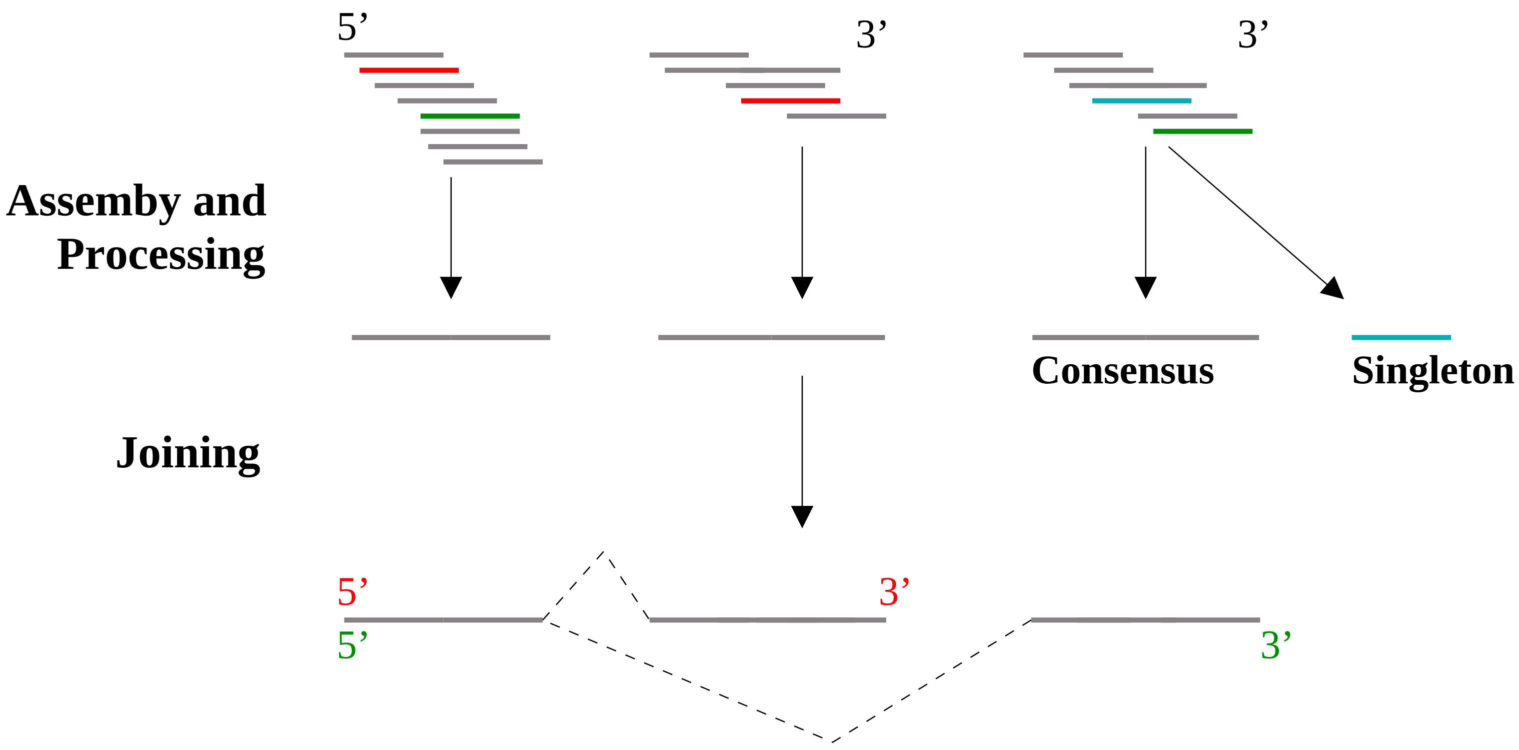
Assembly and processing result in the production of consensus sequences and singletons (helpful to visualize splice variants).

Cluster joining

All ESTs generated from the same cDNA clone correspond to a single gene.

Generally the original cDNA clone information is available ($\sim 90\%$).

Using the cDNA clone information and the 5' and 3' reads information, clusters can be joined.



UniGene

UniGene Gene Indices available for a number of organisms.

UniGene clusters are produced with a supervised procedure: ESTs are clustered using GenBank CDSs and mRNAs data as ”seed” sequences.

No attempts to produce contigs or consensus sequences.

UniGene uses pairwise sequence comparison at various levels of stringency to group related sequences, placing closely related and alternatively spliced transcripts into one cluster.

UniGene web site: http://www.ncbi.nlm.nih.gov/UniGene.

UniGene procedure

Screen for contaminants, repeats, and low-complexity regions in GenBank.

- Low-complexity are detected using Dust.
- Contaminants (vector, linker, bacterial, mitochondrial, ribosomal sequences) are detected using pairwise alignment programs.
- Repeat masking of repeated regions (RepeatMasker).
- Only sequences with at least 100 informative bases are accepted.

Clustering procedure.

- Build clusters of genes and mRNAs (GenBank).
- Add ESTs to previous clusters (megablast).
- ESTs that join two clusters of genes/mRNAs are discarded.
- Any resulting cluster without a polyadenilation signal or at least two 3' ESTs is discarded.
- The resulting clusters are called anchored clusters since their 3' end is supposed known.

UniGene procedure

Ensures 5’ and 3’ ESTs from the same cDNA clone belongs to the same cluster.

ESTs that have not been clustered, are reprocessed with lower level of stringency. ESTs added during this step are called guest members.

Clusters of size 1 (containing a single sequence) are compared against the rest of the clusters with a lower level of stringency and merged with the cluster containing the most similar sequence.

For each build of the database, clusters IDs change if clusters are split or merged.

TIGR Gene Indices

TIGR produces Gene Indices for a number of organisms (http://www.tigr.org/tdb/tgi).

TIGR Gene Indices are produced using strict supervised clustering methods.

Clusters are assembled in consensus sequences, called tentative consensus (TC) sequences, that represent the underlying mRNA transcripts.

The TIGR Gene Indices building method tightly groups highly related sequences and discard under-represented, divergent, or noisy sequences.

TIGR Gene Indices characteristics:

- separate closely related genes into distinct consensus sequences;
- separate splice variants into separate clusters;
- low level of contamination.

TC sequences can be used for genome annotation, genome mapping, and identification of orthologs/paralogs genes.

TIGR Gene Indices procedure

EST sequences recovered form dbEST (http://www.ncbi.nlm.nih.gov/dbEST);

Sequences are trimmed to remove:

- ▷ vectors
- ▷ polyA/T tails
- ▷ adaptor sequences
- ▷ bacterial sequences

Get expressed transcripts (ETs) from EGAD (http://www.tigr.org/tdb/egad/egad.shtml):

- ▷ EGAD (Expressed Gene Anatomy Database) is based on mRNA and CDS (coding sequences) from GenBank.

Get Tentative consensus and singletons from previous database build.

TIGR Gene Indices procedure

Supervised and strict clustering:

- Use ETs, TCs, and CDSs as template;
- Compare cleaned ESTs to the template using FLAST (a rapid pairwise comparison program).
- Sequences are grouped in the same cluster if both conditions are true:
  - ▷ they share $\geq 95\%$ identity over 40 bases or longer regions
  - ▷ < 20 bases of mismatch at either end

Each cluster is assembled using CAP3 assembling program to produce tentative consensus (TC) sequences.

- CAP3 can generate multiple consensus sequences for each cluster
- CAP3 rejects chimeric, low-quality and non-overlapping sequences.
- New TCs resulting from the joining or splitting of previous TCs, get a new TC ID.

TIGR Gene Indices procedure

Builded TCs are loaded in the TIGR Gene Indices database and annotated using information from GenBank and/or protein homology.

Track of the old TC IDs is maintained through a relational database.

References:

- Quackenbush *et al.* (2000) *Nucleic Acid Research*,**28**, 141-145.
- Quackenbush *et al.* (2001) *Nucleic Acid Research*,**29**, 159-164.

STACK

STACK concentrates on human data.

Based on ”loose” unsupervised clustering, followed by strict assembly procedure and analysis to identify and characterize sequence divergence (alternative splicing, etc).

The ”loose” clustering approach, d2_cluster, is not based on alignments, but performs comparisons via non-contextual assessment of the composition and multiplicity of words within each sequence.

Because of the ”loose” clustering, STACK produces longer consensus sequences than TIGR Gene Indices.

STACK also integrates $\sim 30\%$ more sequences than UniGene, due to the ”loose” clustering approach

STACK procedure

Sub-partitioning.

- Select human ESTs from GenBank;
- Sequences are grouped in tissue-based categories (”bins”). This will allow further specific tissue transcription exploration.
- A ”bin” is also created for sequences derived from disease-related tissues.

Masking.

- Sequences are masked for repeats and contaminants using cross-match:
  - ▷ Human repeat sequences (RepBase);
  - ▷ Vector sequences;
  - ▷ Ribosomal and mitochondrial DNA, other contaminants.

STACK procedure

”Loose” clustering using d2_cluster.

- The algorithm looks for the co-occurrence of n-length words ($n = 6$) in a window of size 150 bases having at least 96% identity.
- Sequences shorter than 50 bases are excluded from the clustering process.
- Clusters highly related sequences.
- Clusters also sequences related by rearrangements or alternative splicing.
- Because d2_cluster weights sequences according to their information content, masking of low complexity regions is not required.

Assembly.

- The assembly step is performed using Phrap.
- STACK don’t use quality information available from chromatograms.
- The lack of trace information is largely compensated by the redundancy of the ESTs data.
- Sequences that cannot be aligned with Phrap are extracted from the clusters (singletons) and processed later.

STACK procedure

Alignment analysis.

- The CRAW program is used in the first part of the alignment analysis.
- CRAW generates consensus sequence with maximized length.
- CRAW partitions a cluster in sub-ensembles if $\geq 50\%$ of a 100 bases window differ from the rest of the sequences of the cluster.
- Rank the sub-ensembles according to the number of assigned sequences and number of called bases for each sub-ensemble (CONTIGPROC).
- Annotate polymorphic regions and alternative splicing.

Linking.

- Joins clusters containing ESTs with shared clone ID.
- Add singletons produced by Phrap in respect to their clone ID.

STACK procedure

STACK update.

- New ESTs are searched against existing consensus and singletons using cross-match.
- Matching sequences are added to extend existing clusters and consensus.
- Non-matching sequences are processed using d2_cluster against the entire database and the new produces clusters are renamed ⇒ Gene Index ID change.

STACK outputs.

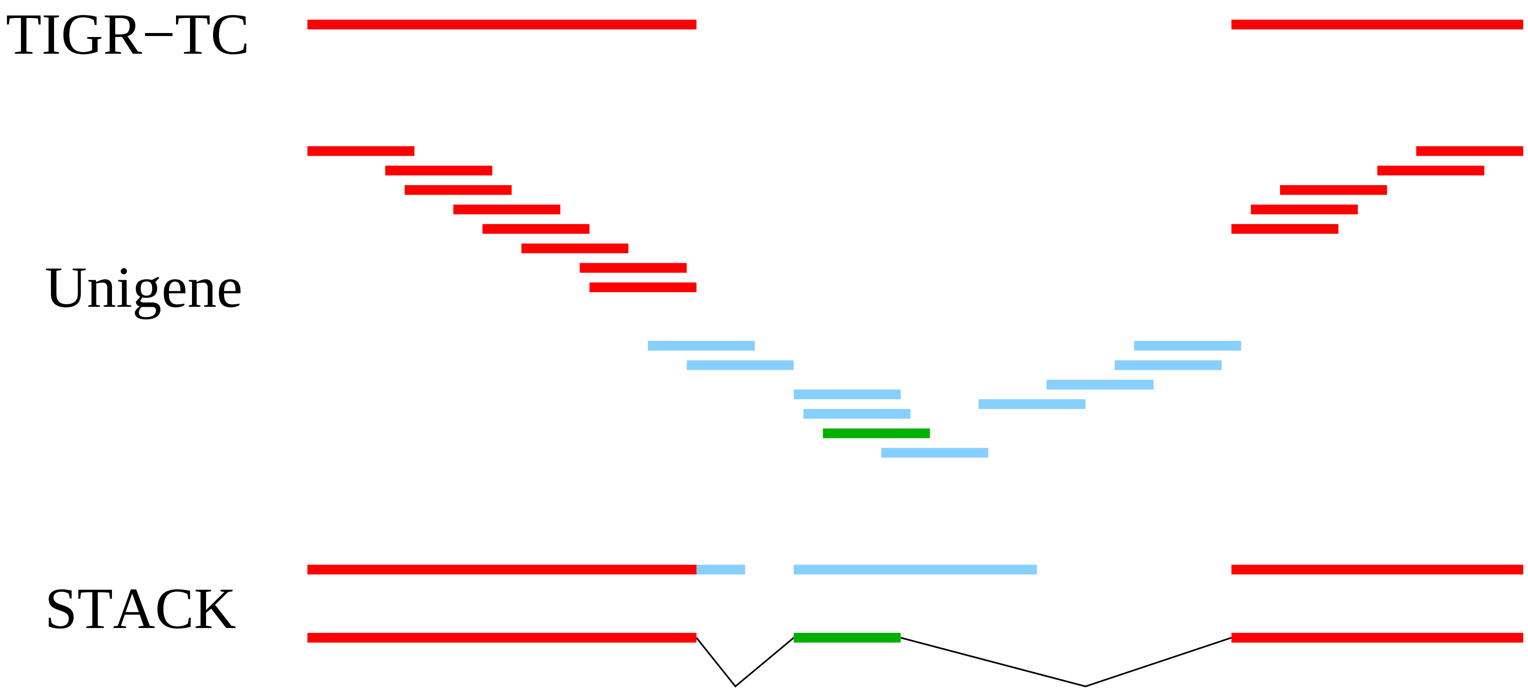
- Primary consensus for each cluster in FASTA format.
- Alignments from Phrap in GDE (Genetic Data Environment) format.
- Sequence variations and sub-consensus (from CRAW processing).

References.

- Miller *et al.* (1999) *Genome Research*,**9**, 1143-1155.
- Christoffels *et al.* (2001) *Nucleic Acid Research*,**29**, 234-238.

EST clustering procedures

Clean, short, and tight



TIGR−TC

Unigene

STACK

Long and loose

trEST

trEST is an attempt to produce contigs from clusters of ESTs and to translate them into proteins.

trEST uses UniGene clusters and clusters produced from in-house software.

To assemble clusters trEST uses Phrap and CAP3 algorithms.

Contigs produced by the assembling step are translated into protein sequences using the ESTscan program, which corrects most of the frame-shift errors and predicts transcripts with a position error of few amino acids.

You can access trEST via the HITS database (http://hits.isb-sib.ch).

The end