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Chicken or the egg: Iso-Seq library preparation to analysis

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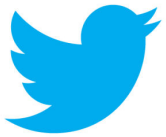
20 Years of Pioneering
Great British Bioscience

Outline

- Big picture
- Library prep
  - 5’ cap selection
  - normalization
- Analysis
  - Full pipeline
  - Different tools
- TAMA
  - Collapse
  - Merge
  - TAMA-GO



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#tamatools

Iso-Seq Webinar:
https://www.youtube.com/watch?v=Pwx_uEBuhZc&t=1071s

Big Picture

- What are you trying to find?
  - Whole transcriptome
  - Specific genes
  - Alternative splicing
  - Transcription start/termination sites
  - Rare genes/transcripts
  - Transcriptome without genome
- Need to design experiment according to your goals
  - Number of samples
  - Types of samples
  - Number of SMRT cells
  - Barcoding/multiplexing
  - 5’ cap selection
  - Normalization
  - Targeted sequencing
  - Depth

Chicken or the egg Iso-Seq planning:
None of the steps come first in planning.
They are all dependent on each other.

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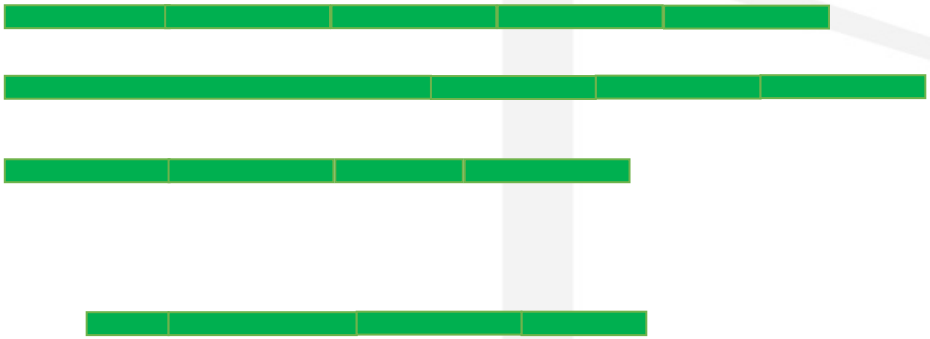
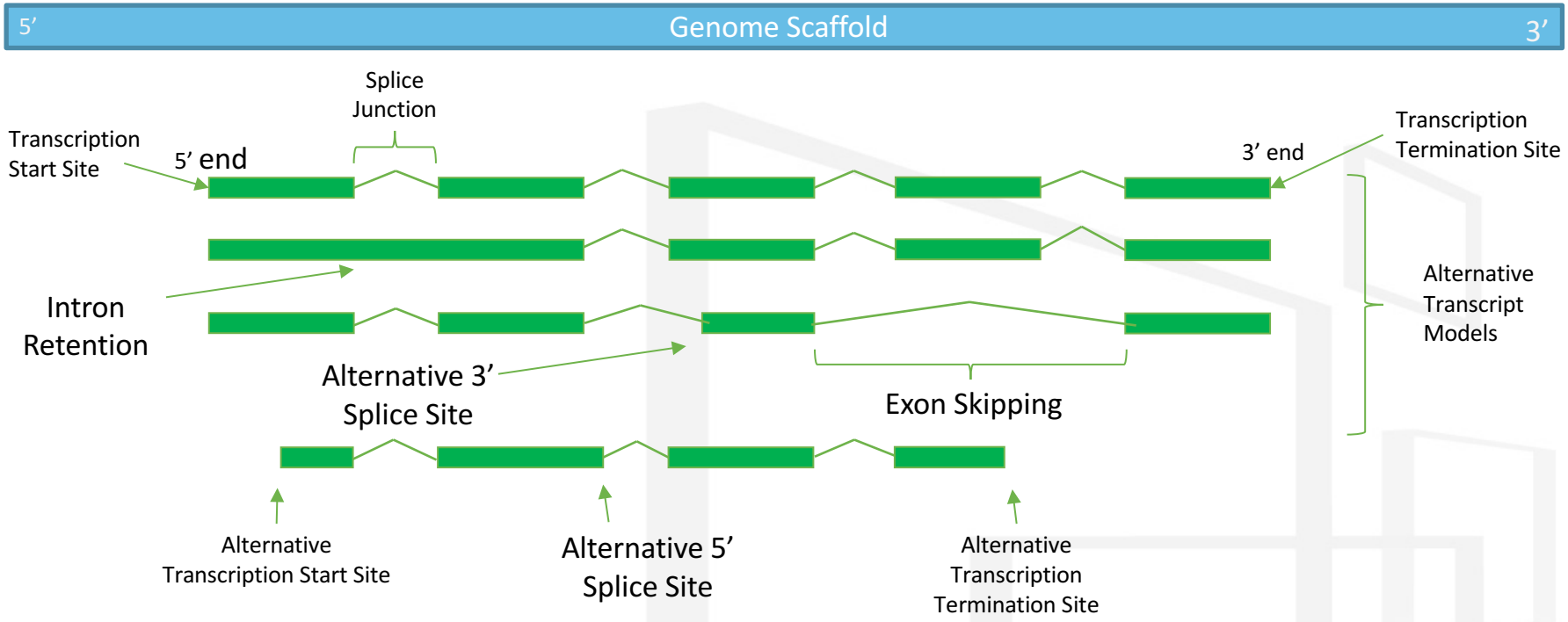
Transcripts without genome



Transcript Models



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Starting from nothing

- No prior information
  - No genome
  - No transcriptome
- With the least amount of starting information, you will need to do the most work to get good results
  - High depth/many SMRT cells
  - 5’ cap selection
  - Short read error correction
- Harder to identify
  - Splice junctions
  - Rare genes/transcripts
  - Gene groups
  - Paralogs

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With a genome

- Only a genome assembly available
- Can map to genome assembly
- Limited to assembly quality
- Transcript model focused/reference based transcriptomes
- Only need exon starts and ends to be accurate
- Want to make a transcriptome annotation
  - 5’ cap selection
  - Normalization
  - Many SMRT cells
  - Short read error correction

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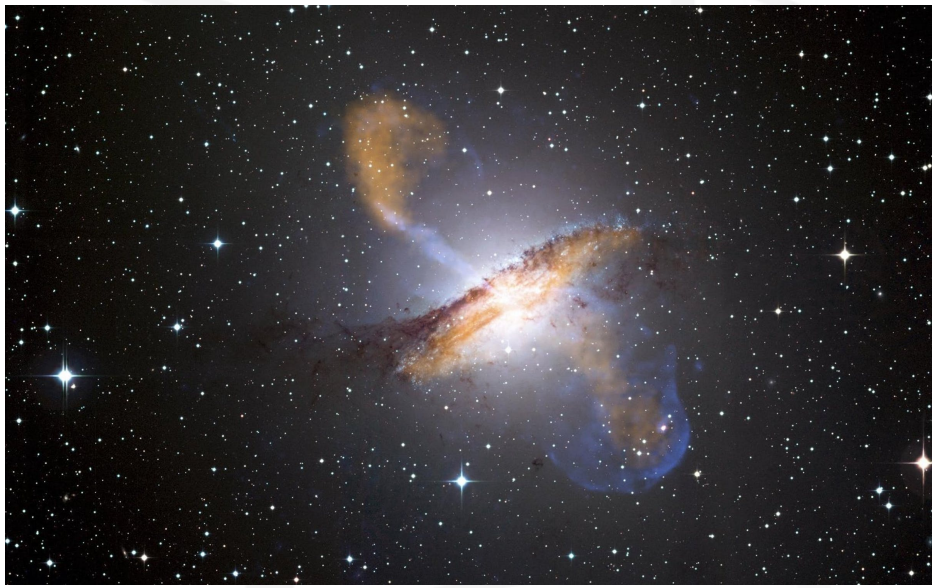
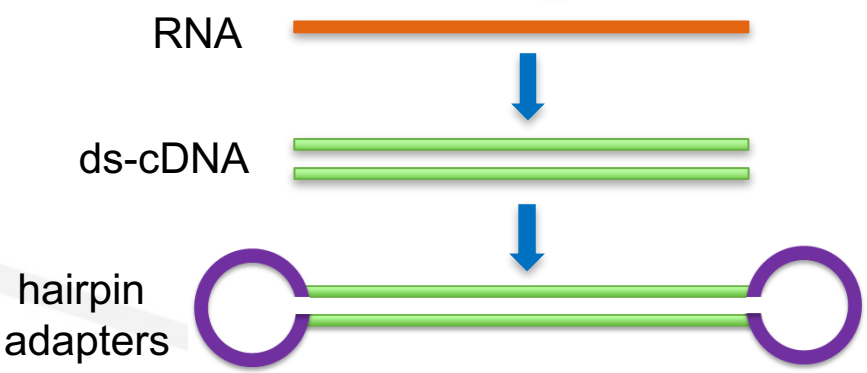
Genome and Transcriptome

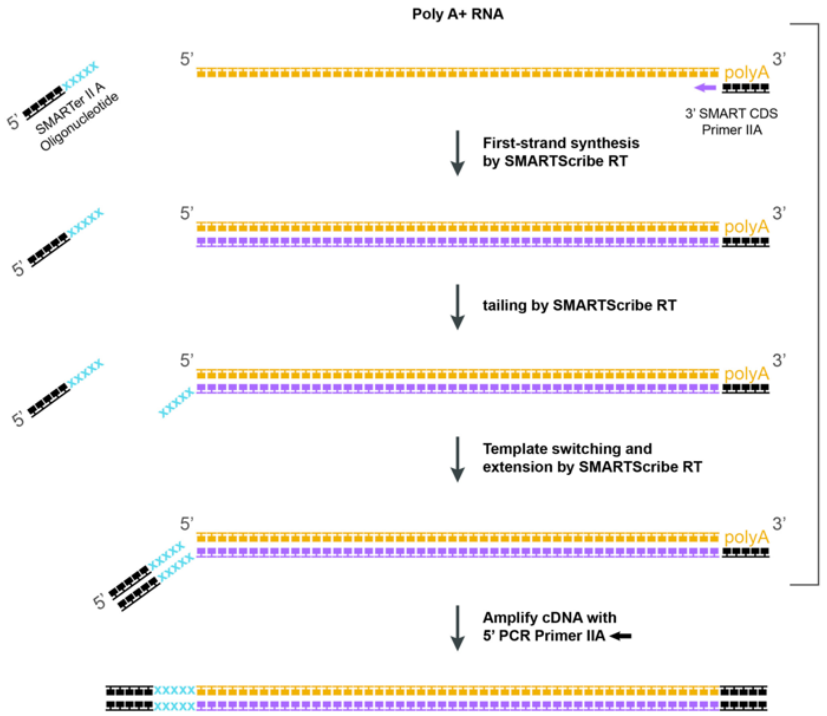
- Genome and Transcriptome
- Can map to assembly
- Limited to assembly quality
- Transcript model focused/reference based transcriptomes
- Only need exon starts and ends to be accurate
- Want to improve a transcriptome annotation
  - Depends on what you want



Standard Library Preparation

- Extract and purify RNA
- Create cDNA
  - Oligo-dT primer
  - 5’ end adapter ligation
- Attach hairpin adapters
- 5’ degradation?
- Overly abundant genes?

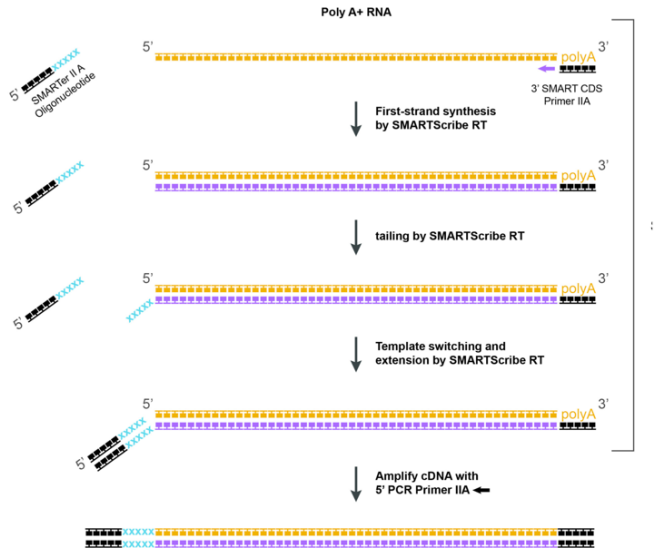




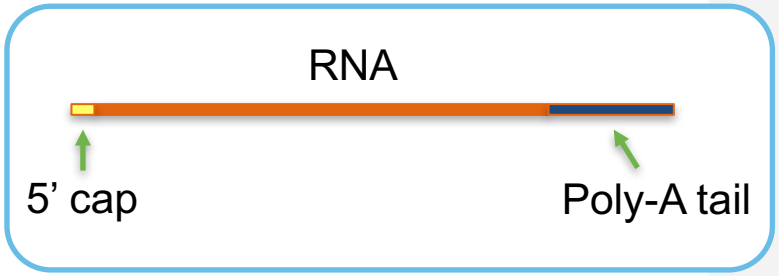
5’ Degradation

- 5’ degradation?

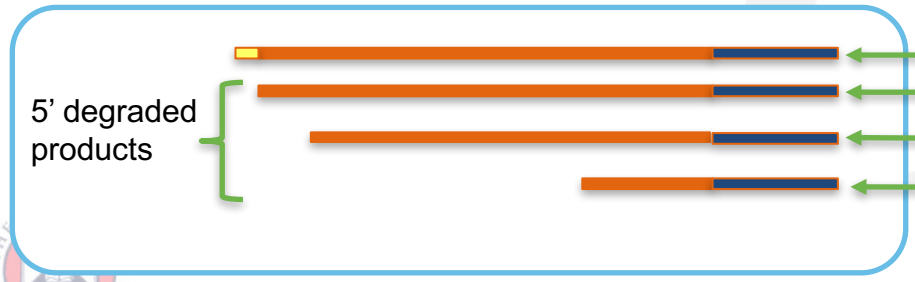
Standard Library Preparation



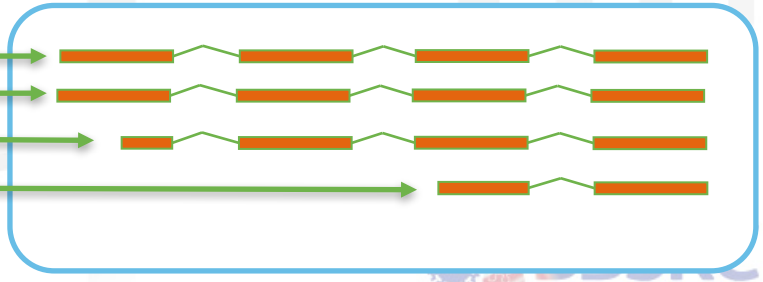
RNA structure



RNA from same transcript



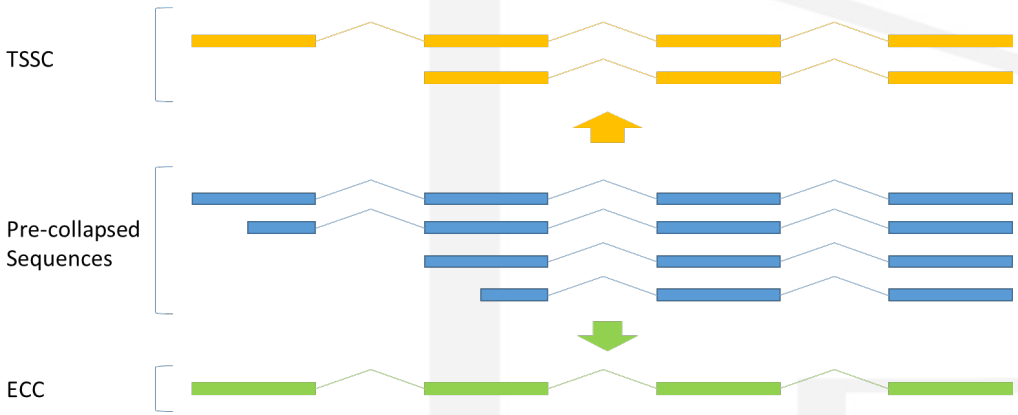
Resulting models



5’ Cap Selection

- Does 5’ cap selection make a difference?
  - Collapsed using Iso-Seq Tofu Collapse tool
  - Used both methods of collapsing to compare

TSSC – Transcription Start Site Collapse
ECC – Exon Cascade Collapse



	Pre-collapsed	TSSC	ECC	TSSC % decrease	ECC % decrease
No Cap	199,560	80,814	55,932	59.50%	72.00%
5’ Cap	11,881	9,368	8,468	21.20%	28.70%

Normalized long read RNA sequencing in chicken reveals transcriptome complexity similar to human
https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-017-3691-9

5’ Cap Comparison

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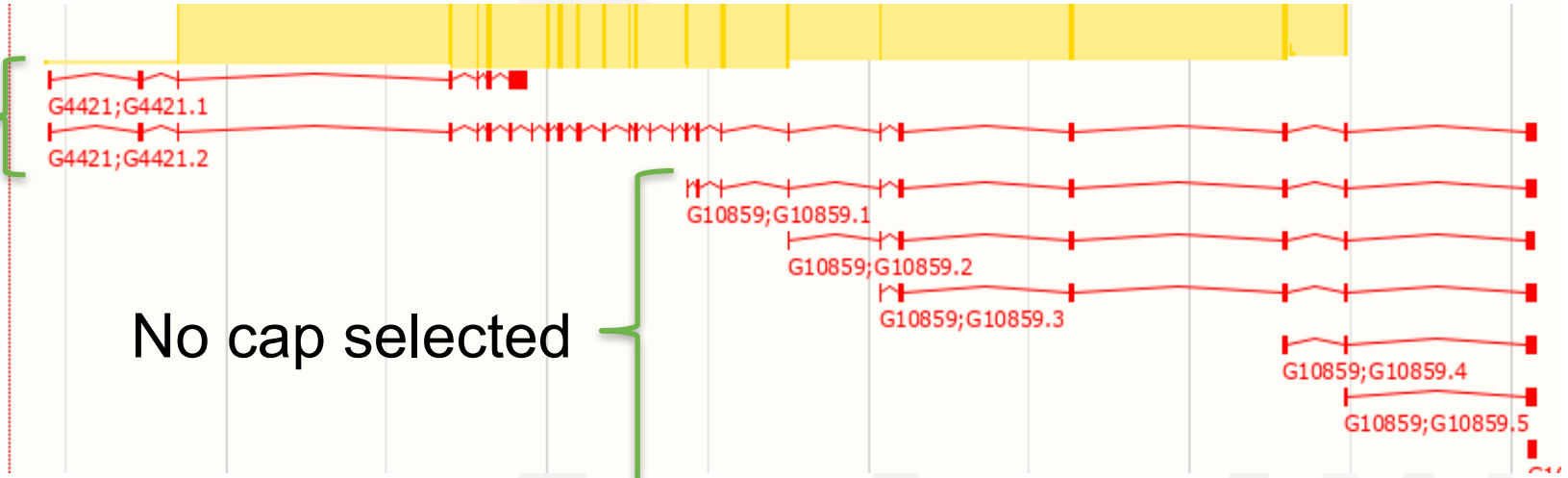
LEXOGEN
Enabling complete transcriptome sequencing

5’ cap selected

No cap selected



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5’ Cap Teloprime Kit

Step 2: Double-Strand Specific Ligation

Capped Poly(A) RNA

Degraded RNA

Enabling complete transcriptome sequencing

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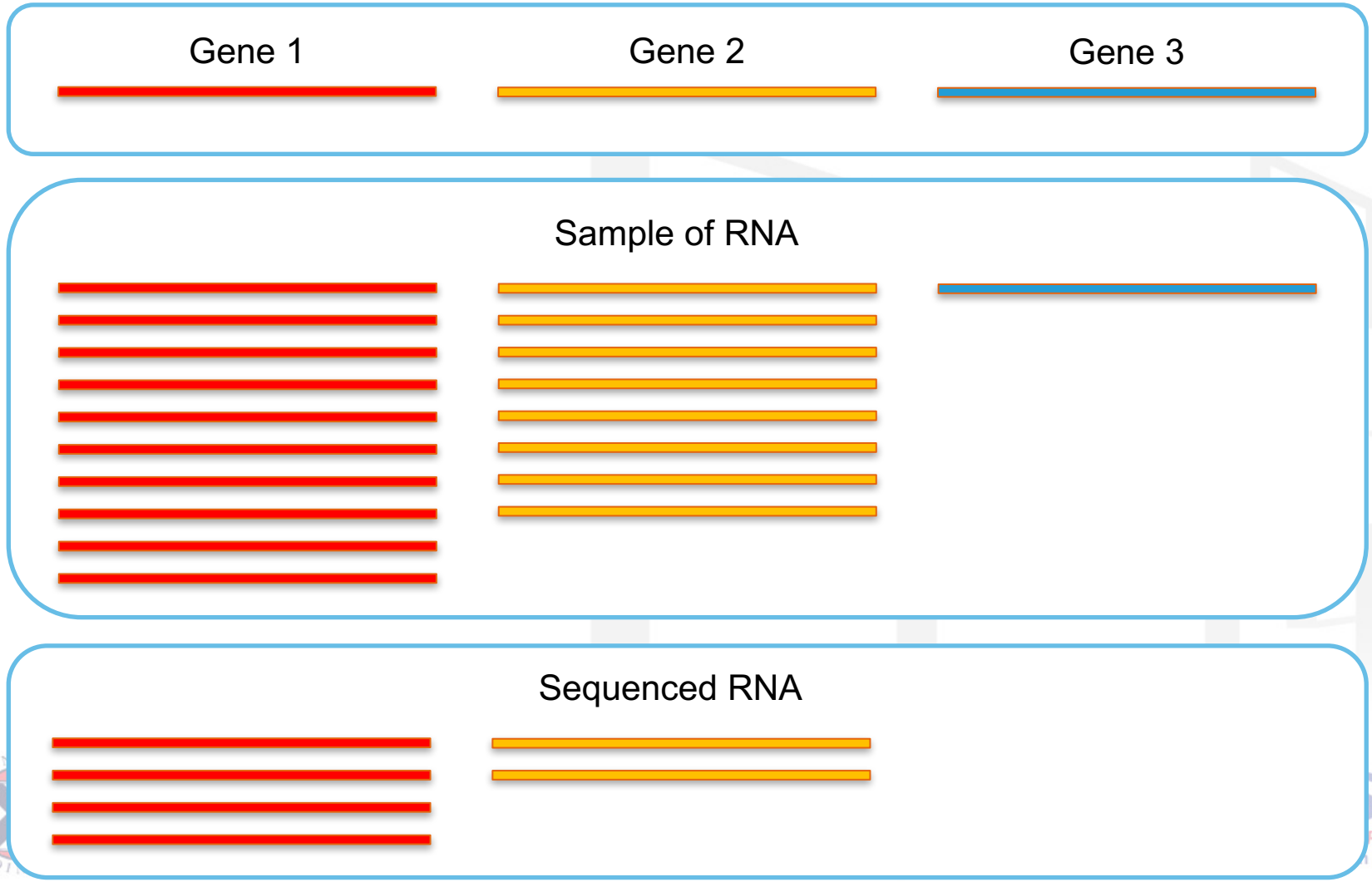
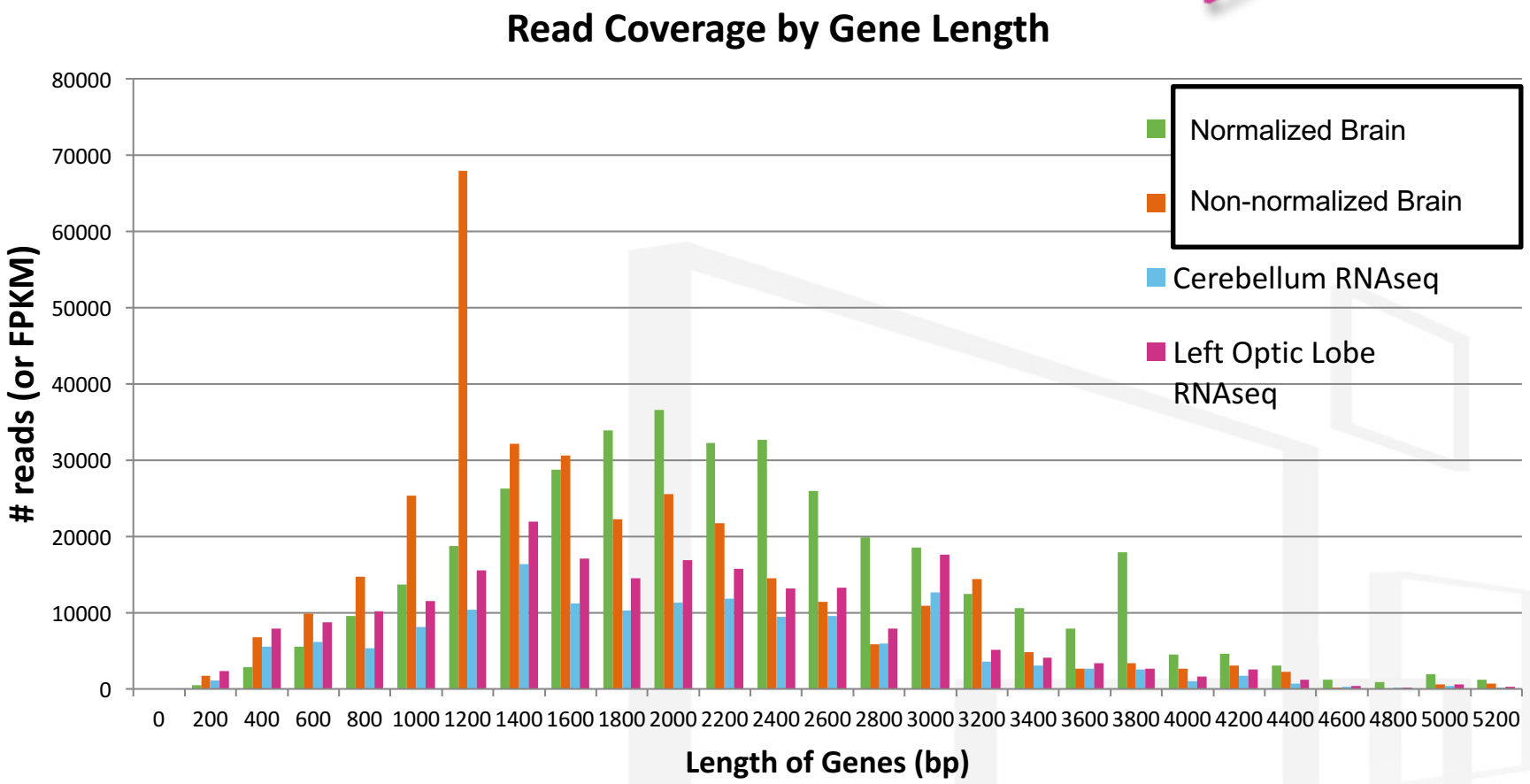
Over abundant genes/transcripts

- Overly abundant genes?



Normalization



- 2 genes in 1200bp bin for Sequel run associated with 37,679 reads
- Roughly 10% of sequencing spent on only 2 genes

Normalization results

	CCS	FLNC	Genes	Transcripts	Genes/FLNC	Trans/FLNC
Non-Norm.	566,307	197,544	11,934	39,909	0.06	0.20
Normalized	145,527	58,567	19,849	49,465	0.34	0.84

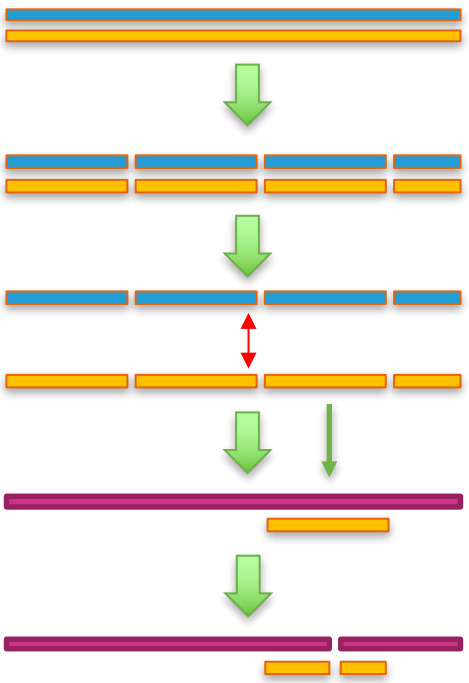
- >5x genes per FLNC with normalization
- >4x transcripts per FLNC with normalization
- Additional genes are mostly lncRNA

FLNC – Full length non-chimeric reads

Normalization Methods

- DSNase method
- Column based method

Cascade Effect Theory



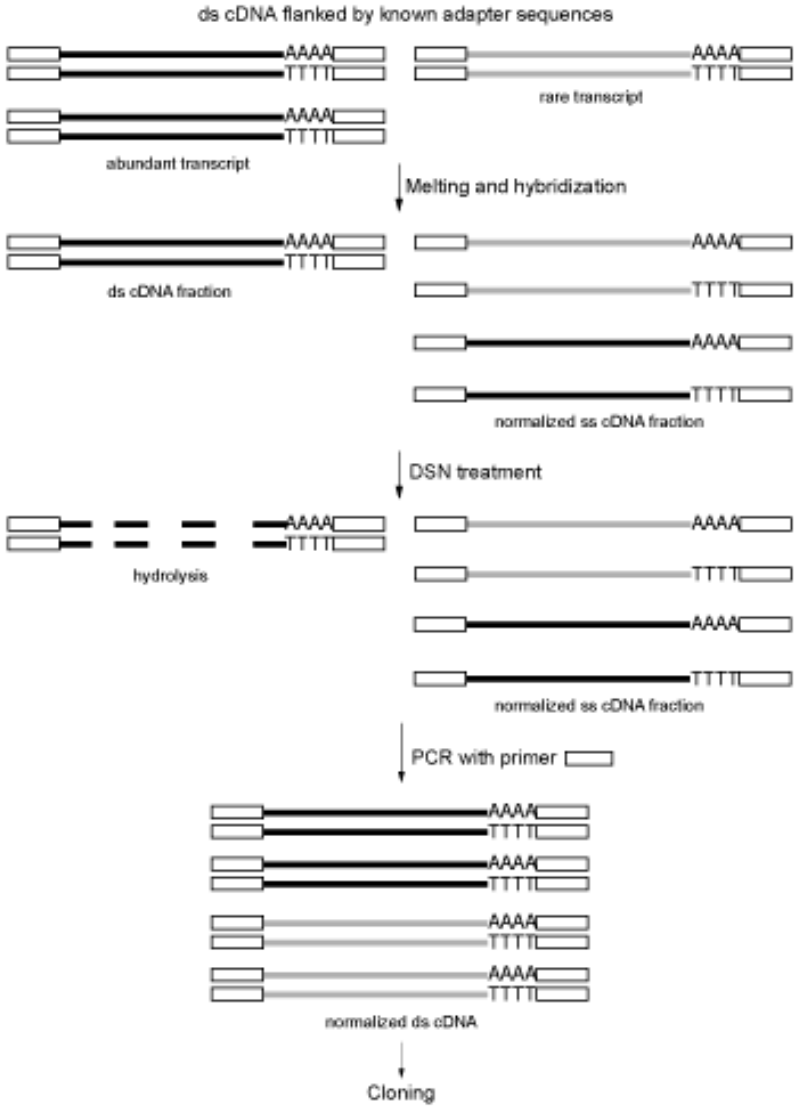
dsDNA

Cleaved dsDNA

Disassociation

Fragment Re-association

Fragment dsDNA Cleaving



From Evrogen website

Normalization Methods

- DSNase method
- Column based method

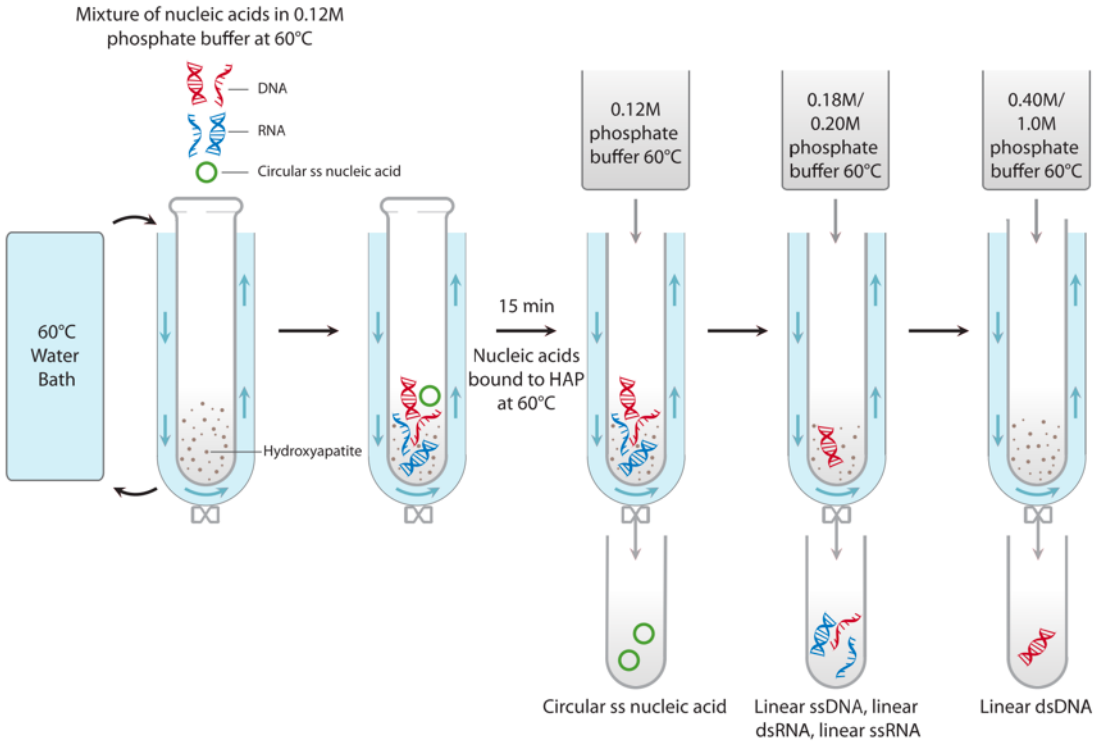
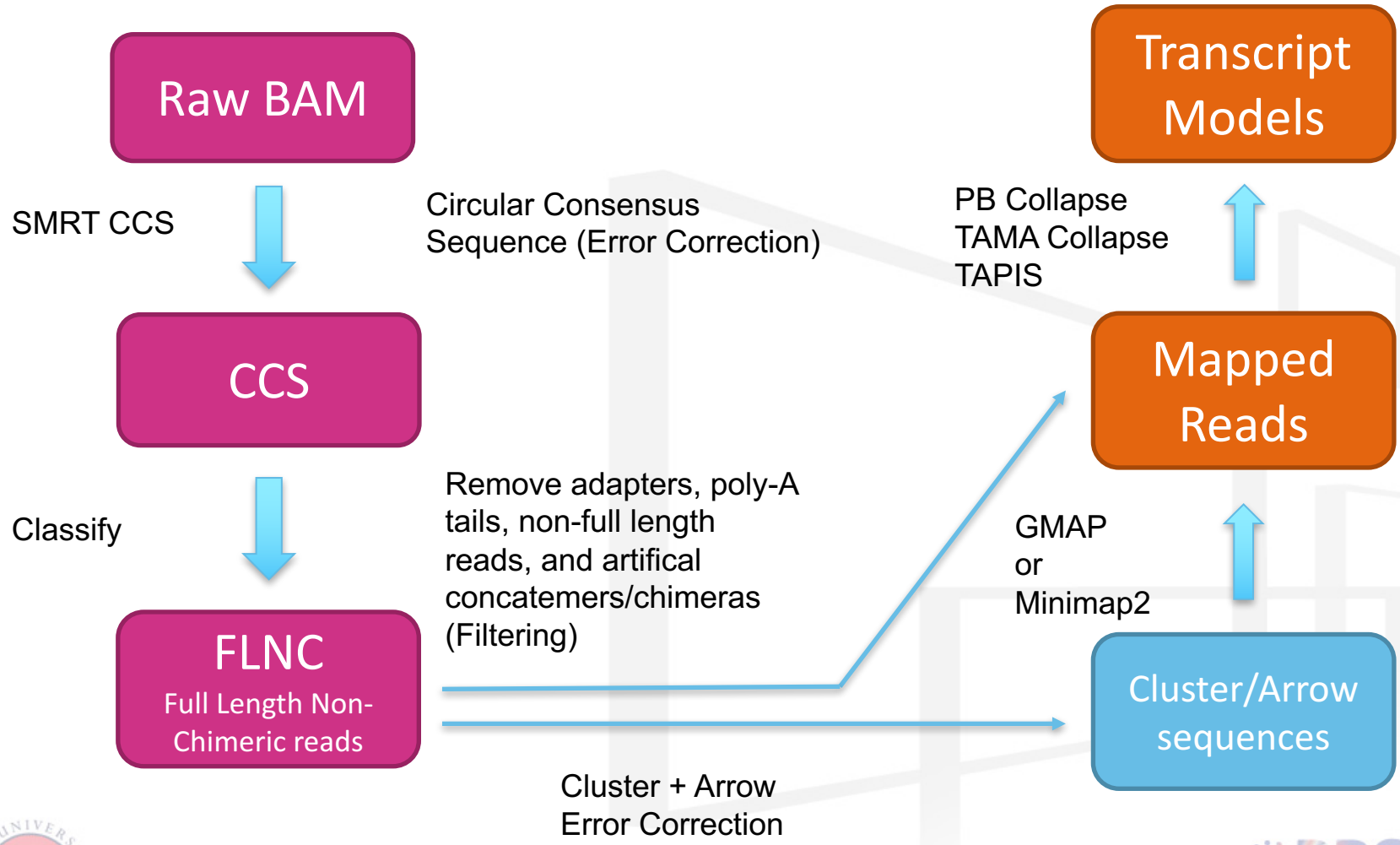


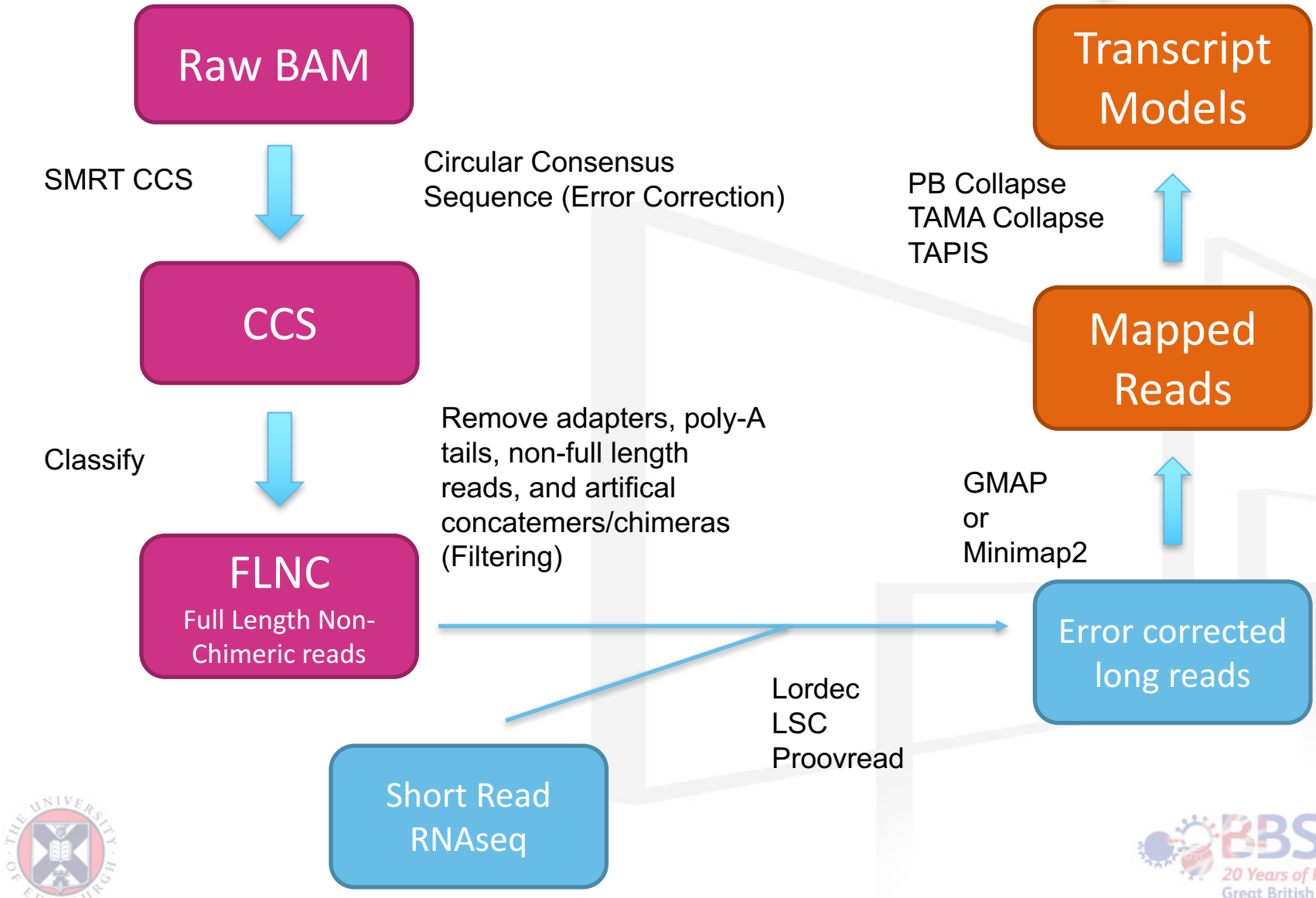
FIG. 1. Flow diagram depicting HAP-mediated separation of known and environmental viral nucleic acids.

From "Hydroxyapatite-Mediated Separation of Double-Stranded DNA, Single-Stranded DNA, and RNA Genomes from Natural Viral Assemblages"

Iso-Seq Analysis Pipeline

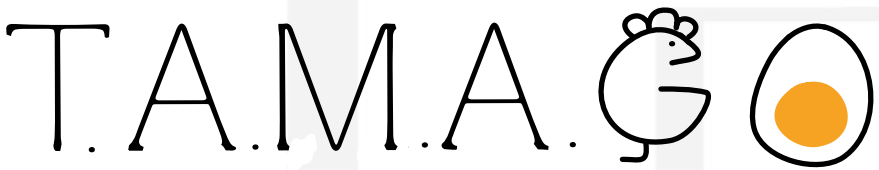


Iso-Seq pipeline w/ RNAseq



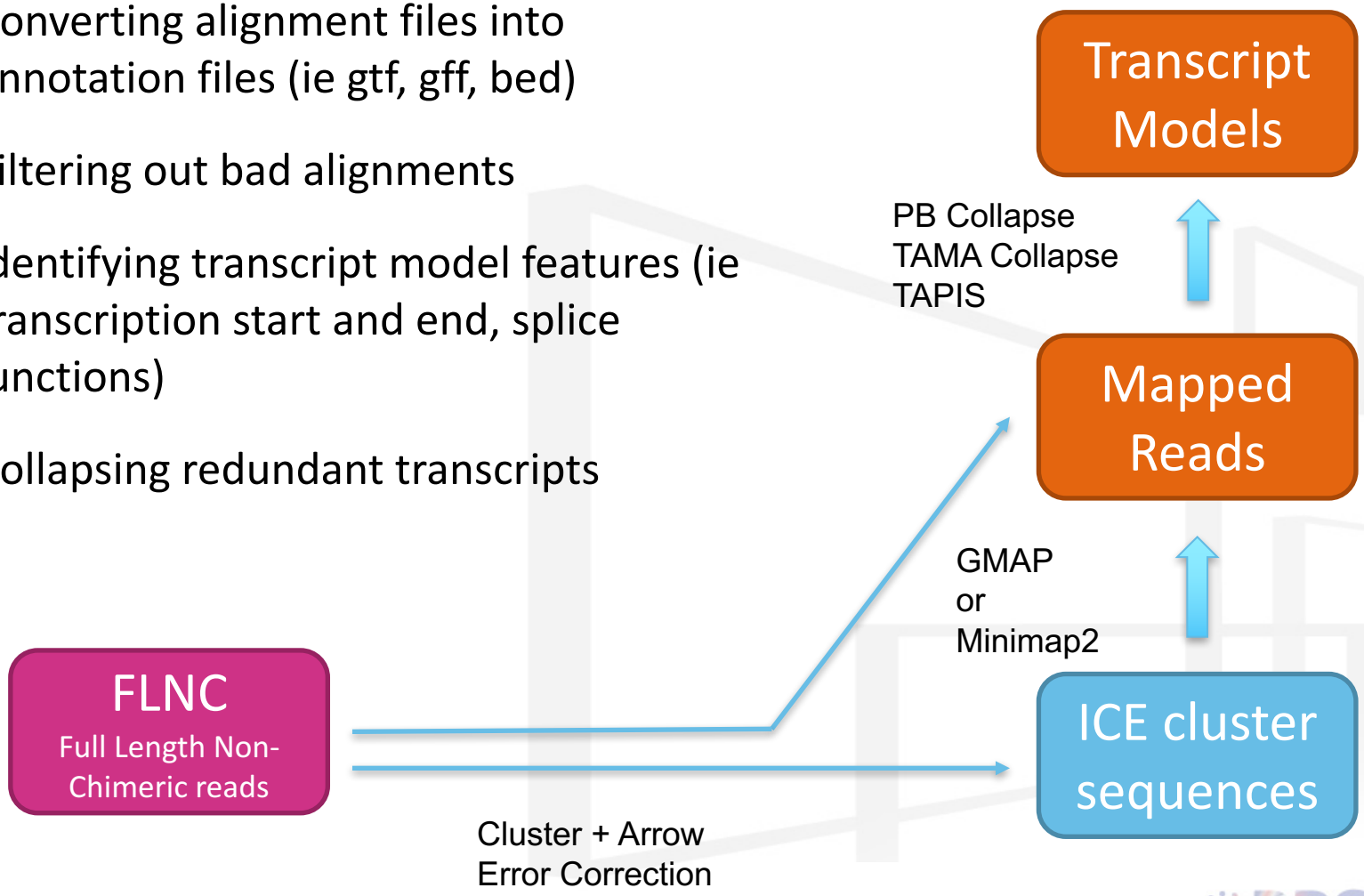
TAMA

- Transcriptome Annotation by Modular Algorithms
- TAMA Collapse
- TAMA Merge
- TAMA-GO



Collapse/Annotation

- Converting alignment files into annotation files (ie gtf, gff, bed)
- Filtering out bad alignments
- Identifying transcript model features (ie transcription start and end, splice junctions)
- Collapsing redundant transcripts



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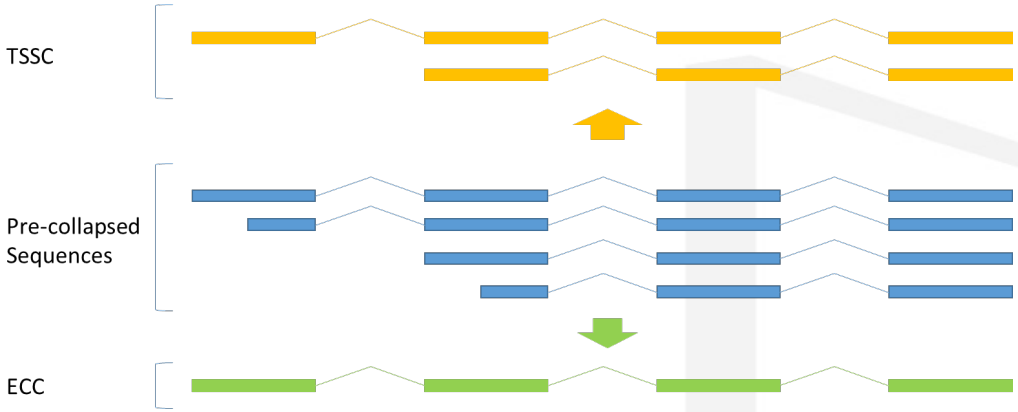
PacBio Collapse

TSSC – Transcription Start Site Collapse
ECC – Exon Cascade Collapse



Transcript Models

PB Collapse
TAMA Collapse
TAPIS

Mapped Reads

GMAP
or
Minimap2

ICE cluster sequences

FLNC
Full Length Non-Chimeric reads

Cluster + Arrow Error Correction

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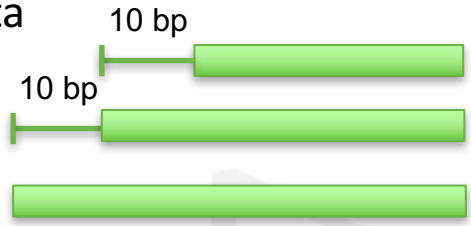
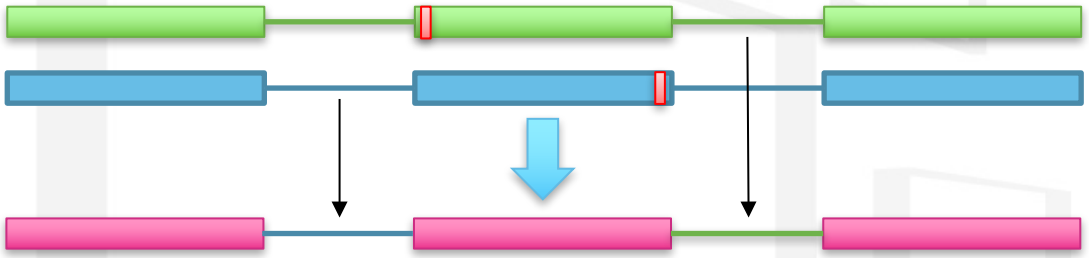
TAMA Collapse

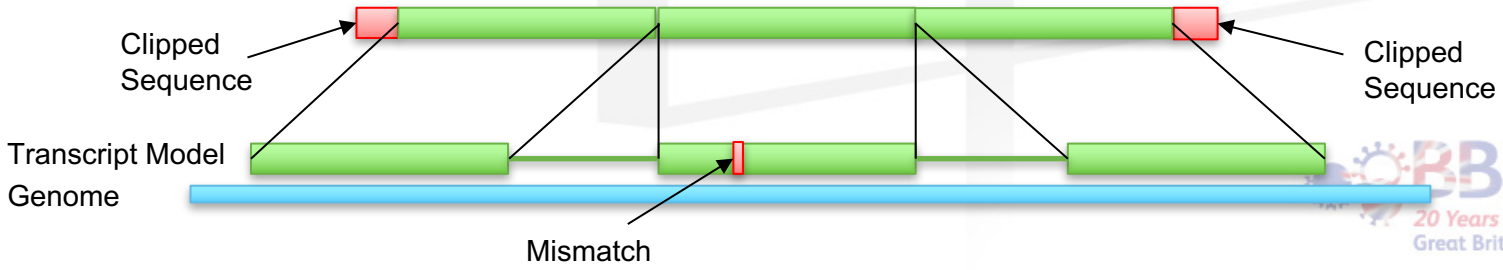
- Control over transcript collapsing
- Manages 5’ cap selected and non cap selected sequencing data
- Provides source information for all predicted events
  - Support for each final model
  - Support for each transcript feature (TSS/TTS, splice junctions)



- Flags uncertainties
  - Poly A truncation
  - Variation
  - Wobble



- Splice junction priority
  - Uses mapping mismatch information near splice junctions to choose best evidence



Using TAMA Collapse

TAMA Collapse trans_report

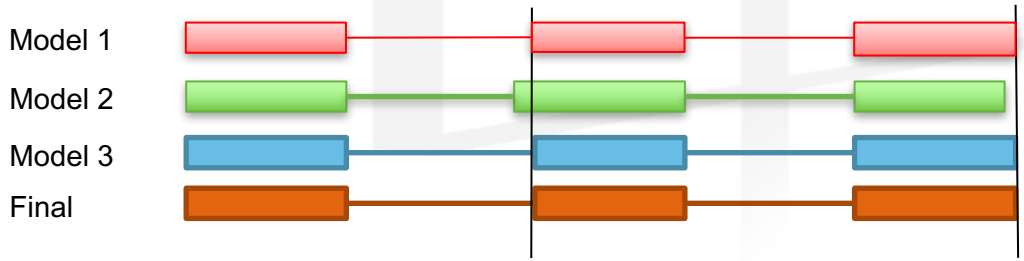
Line from trans_report.txt:

G1.6 47 100.0 99.3 99.62 93.33 52,0,0,0,0,0,0,4 0,0,0,0,0,0,0,20 0,0,0,0,0,0,0,1 0,0,0,0,0,0,2,0 0>0;0>0;0>0;0>0;0>0;0>0;10.G.A_1D_5M>0-10.G.A>0

Column/Field identities

transcript_id	G1.6
num_clusters	47
high_coverage	100
low_coverage	99.3
high_quality_percent	99.62
low_quality_percent	93.33
start_wobble_list	52,0,0,0,0,0,0,4
end_wobble_list	0,0,0,0,0,0,0,20
collapse_sj_start_err	0,0,0,0,0,0,0,1
collapse_sj_end_err	0,0,0,0,0,0,2,0
collapse_error_nuc	0>0;0>0;0>0;0>0;0>0;0>0;10.G.A_1D_5M>0-10.G.A>0

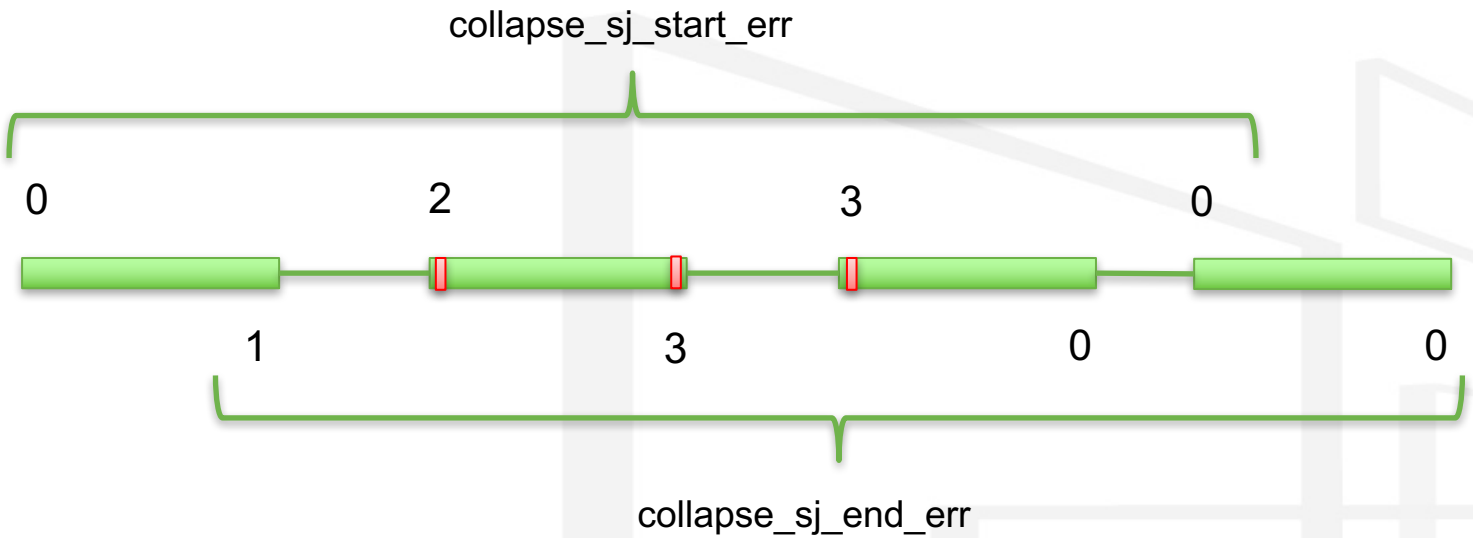
This is the interesting stuff!!



TAMA Collapse SJ Error

Column/Field identities

collapse_sj_start_err	0,2,3,0
collapse_sj_end_err	1,3,0,0

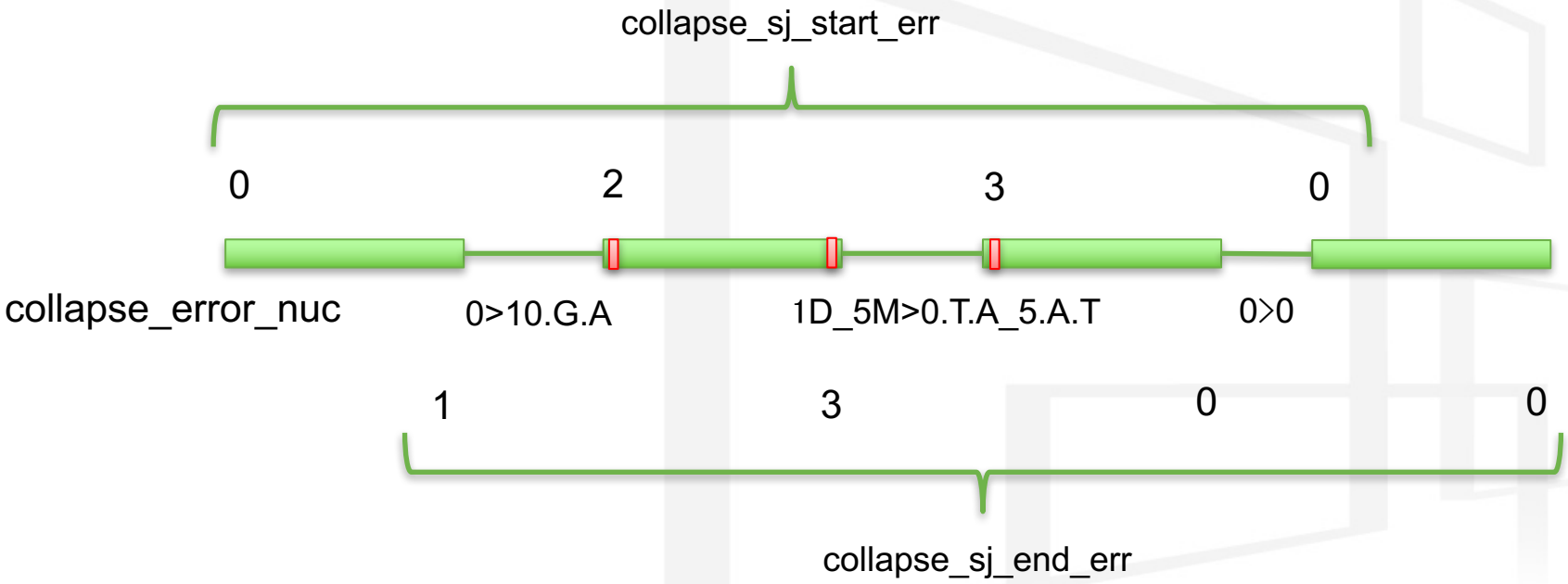


0	No mismatches on either side of the splice junction
1	One mismatch on the other side of the splice junction
2	One mismatch on the same side of the splice junction
3	There are mismatches on both sides of the splice junction

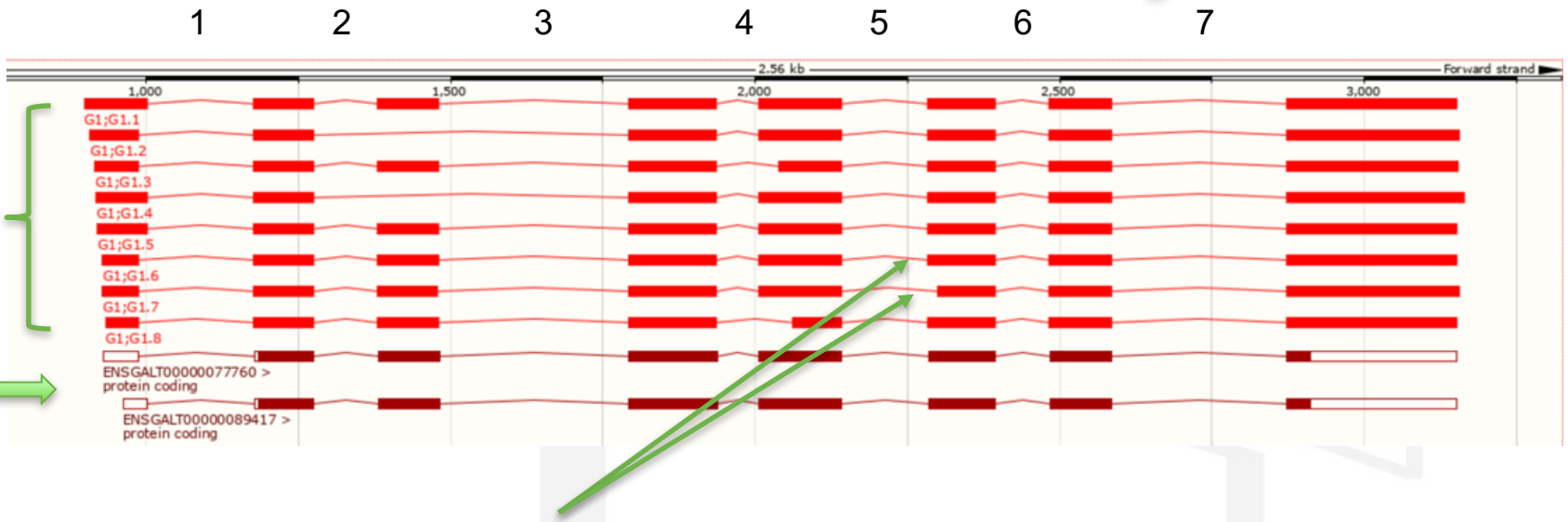
TAMA Collapse Error Nuc

Column/Field identities

collapse_sj_start_err	0,2,3,0
collapse_sj_end_err	1,3,0,0
collapse_error_nuc	0>10.G.A; 1D_5M>0.T.A_5.A.T;0>0



Local density error



transcript_id	clusters	high_cov	low_cov	high_quality_percent	low_quality_percent	start_wobble_list	end_wobble_list	collapse_sj_start_err	collapse_sj_end_err	collapse_error_nuc
G1.6	47	100.0	99.3	99.62	93.33	52,0,0,0,0,0,0,4	0,0,0,0,0,0,0,20	0,0,0,0,0,0,0,1	0,0,0,0,0,0,2,0	0>0;0>0;0>0;0>0;0>0;0>0;10.G.A_1D_5M>0-10.G.A>0
G1.7	1	100.0	100.0	93.98	93.98	0,0,0,0,0,0,0,0	0,0,0,0,0,0,0,0	0,3,0,2,3,3,1,3	3,0,1,3,3,2,3,0	3.G.C>9M_2I;0>0;0>0.T.A_5.A.T_6.T.A;1D_3M_1D_1M>0.T.C_2.C.T;7.A.C>9I_1M_2D;1D_6M>0;10.G.A>10M_1D

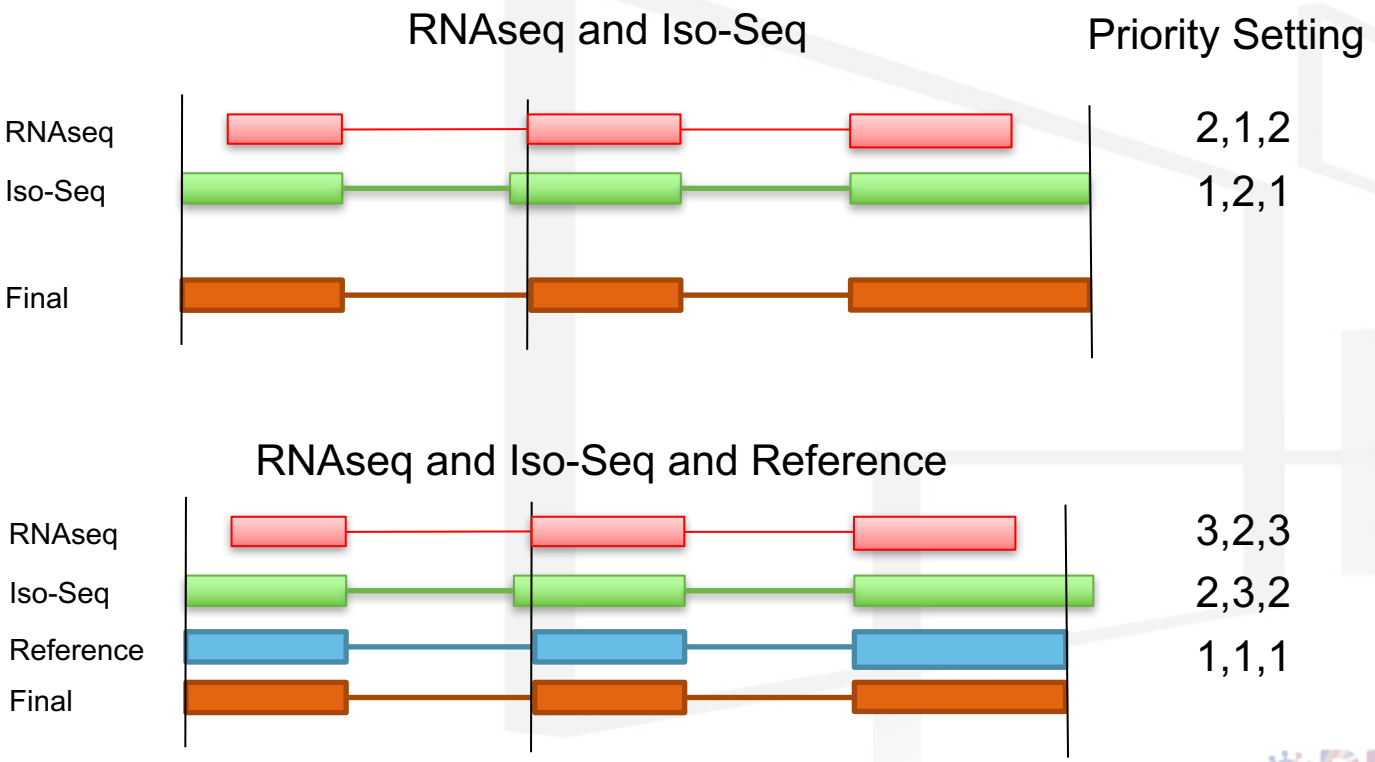
1	3.G.C>9M_2I
2	0>0
3	0>0.T.A_5.A.T_6.T.A
4	1D_3M_1D_1M>0.T.C_2.C.T
5	7.A.C>9I_1M_2D
6	1D_6M>0
7	10.G.A>10M_1D

TAMA Merge

- Allows merging of Iso-Seq, RNA-seq, and public annotations
- Provides control over merging thresholds
- Allows user defined priority of transcript features from different sources
  - Use transcription start and end sites from Iso-Seq and splice junctions from RNAseq
- Tracks all merging events and outputs it in report files
- https://github.com/GenomeRIK/tama

Using TAMA Merge

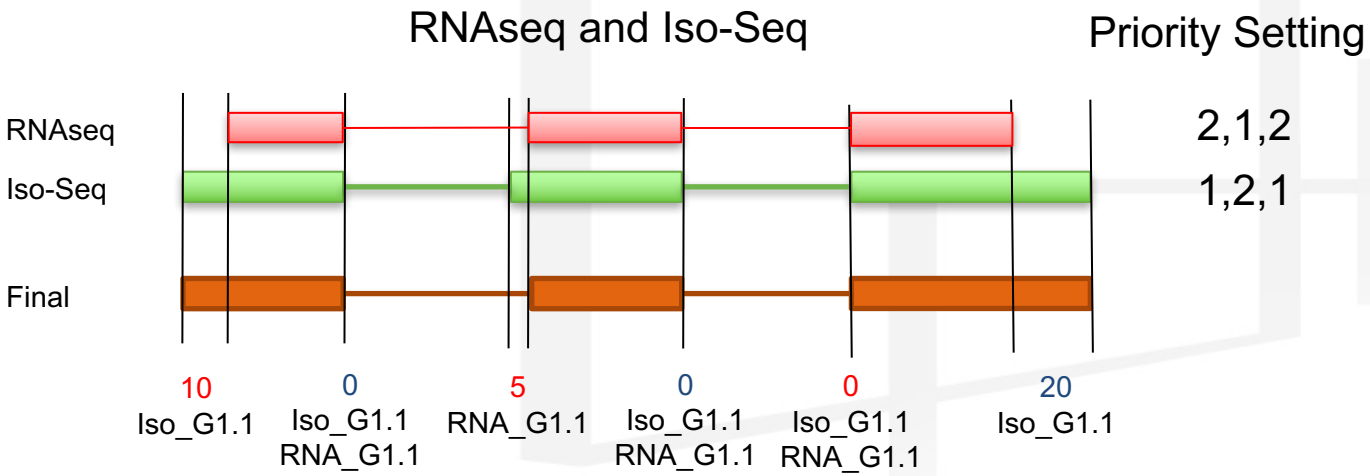
- Similar algorithm for merging transcripts as TAMA collapse
- Some nuanced (but important!) differences



TAMA Merge trans_report

G1.1 1 Iso,RNA 10,5,0 0,0,20 Iso_G1.1;RNA_G1.1; Iso_G1.1,RNA_G1.1 Iso_G1.1,RNA_G1.1; Iso_G1.1,RNA_G1.1; Iso_G1.1

start_wobble_list 10,5,0
end_wobble_list 0,0,20
exon_start_support Iso_G1.1;RNA_G1.1; Iso_G1.1,RNA_G1.1
exon_end_support Iso_G1.1,RNA_G1.1; Iso_G1.1,RNA_G1.1; Iso_G1.1



TAMA-GO ORF/NMD

1. Convert bed to fasta
2. Get open reading frames (ORF)
3. Blast amino acid sequences against the Uniprot/Uniref
4. Parse the Blastp output file for top hits
5. Create new bed file with CDS regions and NMD predictions

Example BED12 output line

```
1	481182	484817	G28;G28.23;none;5prime_degrade;no_hit;NMD1;F2	40	-	482403	484816	0,200,255	4	831,81,113,127	0,1198,2386,3508
```

TAMA-GO

- Suite of tools for various transcriptome annotation needs
- NMD/ORF predictions
- Format convertors
- More to come!

P.S. If you need a tool, please contact me.
I may have it but just haven't uploaded it yet.
If I don't have it, I may be able to make it for you.
Also if you want to contribute to the repo contact me!
GenomeRIK@gmail.com

Acknowledgement

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PACIFIC BIOSCIENCES®

Professor Dave Burt

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Jacqueline Smith

Katarzyna Miedzinska

Bob Paton

Lel Eory

Elizabeth Tseng

edinburgh genomics.

Karim Gharbi

Marian Thomson

EBRC Easter Bush Research Consortium

wellcome trust

BBSRC bioscience for the future

Contact

- You can reach me at GenomeRIK@gmail.com
- I also tweet updates for TAMA and Iso-Seq: @GenomeRIK
- TAMA tools: https://github.com/GenomeRIK/tama
- Normalized long read RNA sequencing in chicken reveals transcriptome complexity similar to human: https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-017-3691-9
- Iso-Seq Webinar: https://www.youtube.com/watch?v=Pwx_uEBuhZc&t=1071s