RNA analysis in the Guy's DNA Diagnostic Laboratory

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Introduction

- Why RNA?
- Alternative strategies
- Practical points
  - Starting material
  - RNA extraction → Sequencing
- Pitfalls/solutions
- Progress to date by disease

Why use cDNA sequencing?

- Screening strategy or further analysis

  1) DMD/X-linked Alports – patients with no genomic change

  2) COL6 (UCMD) – Mutation screening
- Will pick up the same point mutations as genomic sequencing but additionally:
  - Resolve some unclassified variants
  - Demonstrate splicing variants
  - Demonstrate inversions
  - Reduce sequencing load
- Better genotype/phenotype correlation

Materials and Methods

- Starting material:
  - Hair roots
  - Fibroblast cultures
  - Muscle/tissue samples
  - Blood (leukocytes)
- Which?
  - Expression patterns
  - Availability of samples

Extraction Methods

- Qiagen Rneasy Fibrous Tissue Kit
  - Tissue, HR, Fibroblast cultures
- PAXgene
  - Blood (leukocytes)

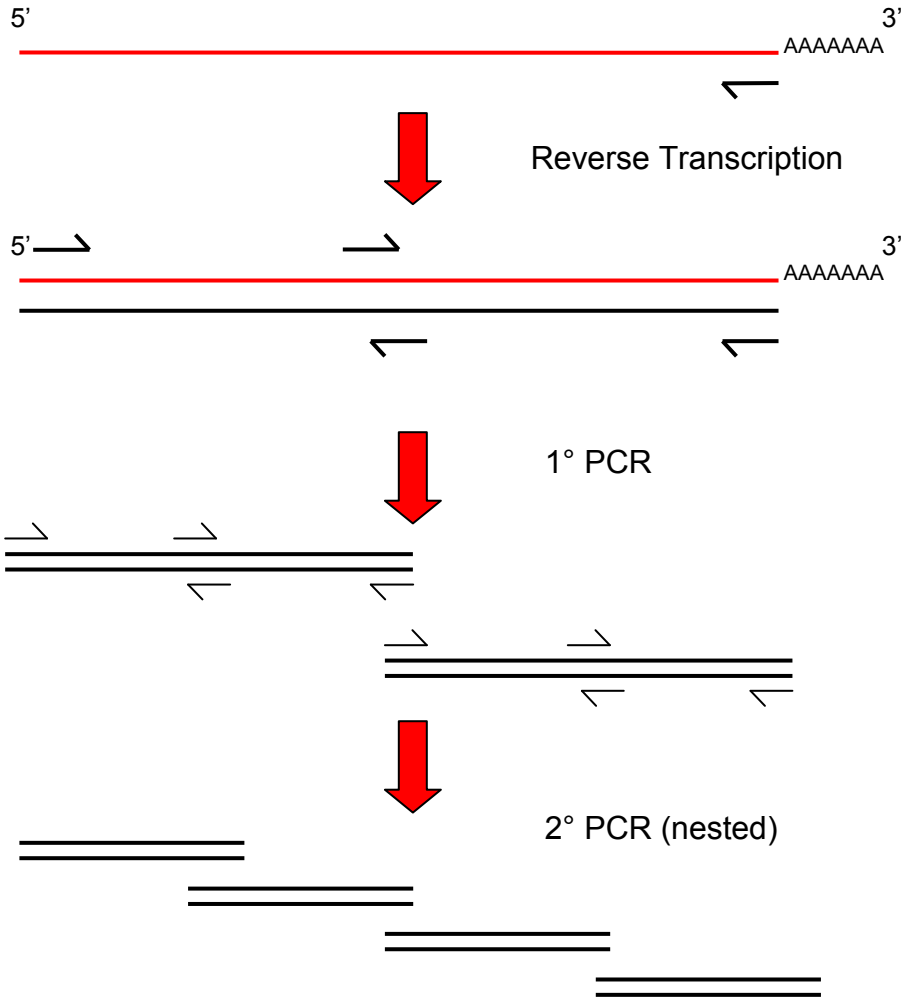
RNA yields from different tissues

Tissue Type	Average Yield	Sample 'Volume'	Proven efficacy		
			DMD	Alports	Col6
Hair roots	10ng/µl	10 hair roots	✓	✓	✓
Fibroblast culture	300ng/µl	0.5ml	UK	✓	✓
Muscle biopsy			✓	UK	✗
Blood	100ng/µl	3ml	**?**	**?**	✓

RT-PCR

- Reverse transcription reaction:
  - Superscript III (Invitrogen)
  - Up to 10kb transcript
- PCR
  - Split into 1° and 2° reactions
  - Why?
    - Increase specificity
    - Increase amount of product

RT-PCR


5’
3’
AAAAAAA
Reverse Transcription
5’
3’
AAAAAAA
1° PCR
2° PCR (nested)

Sequencing

- PCRs:
  - 10 µl reactions using Qiagen mpx mix
  - Cleaned with Ampure (Agencourt)
- Sequencing reaction:
  - 5ul reactions (0.25µl BigDye)
- Unidirectional/bidirectional
- Tagged primers
  - Ease of set up
  - Batching
- Seq. reactions cleaned using CleanSeq

Progress by disease

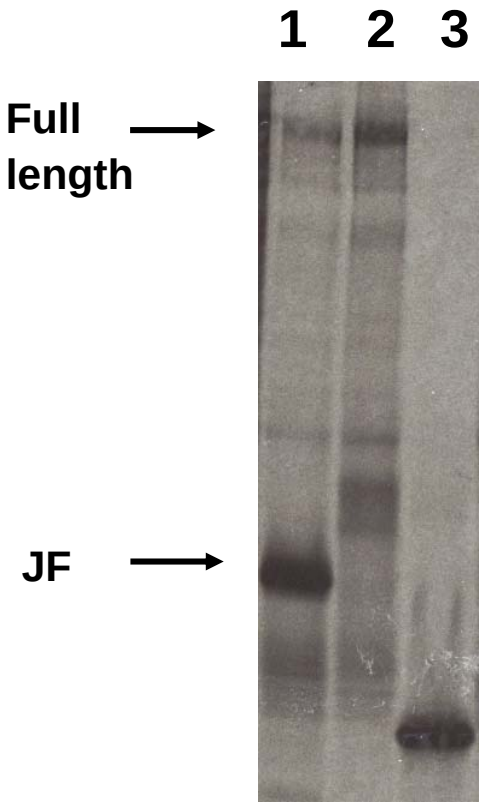
- Alports
  - Testing of potential splicing variants found on genomic screen
- DMD
  - 5 deep cryptic intronic splice site mutation
  - 4 inversions – 2 single exons
  - Splicing-out of exon 45 demonstrated in both hair roots and muscle RNA
- UCMD/BM
  - Main screening strategy
  - Mutations found in majority of patients screened
  - Of 11 patients screened, 10 have confirmed or putative mutations

BMD1-4 - deep cryptic intronic splice site mutations

Patient	*Exon*	*Transcripts detected*	*Mutation*
BMD1	25	a) wild-type b) 2 mutant	c.3432+2036 a>g
BMD2	32	a) wild-type b) 1 mutant	c. 4518+636 a>t
BMD3	44	a) wild-type b) 1 mutant	c.6439-9192 a>t
BMD4	44	a) wild-type b) 1 mutant	c.6439-55480_6439-55479insA

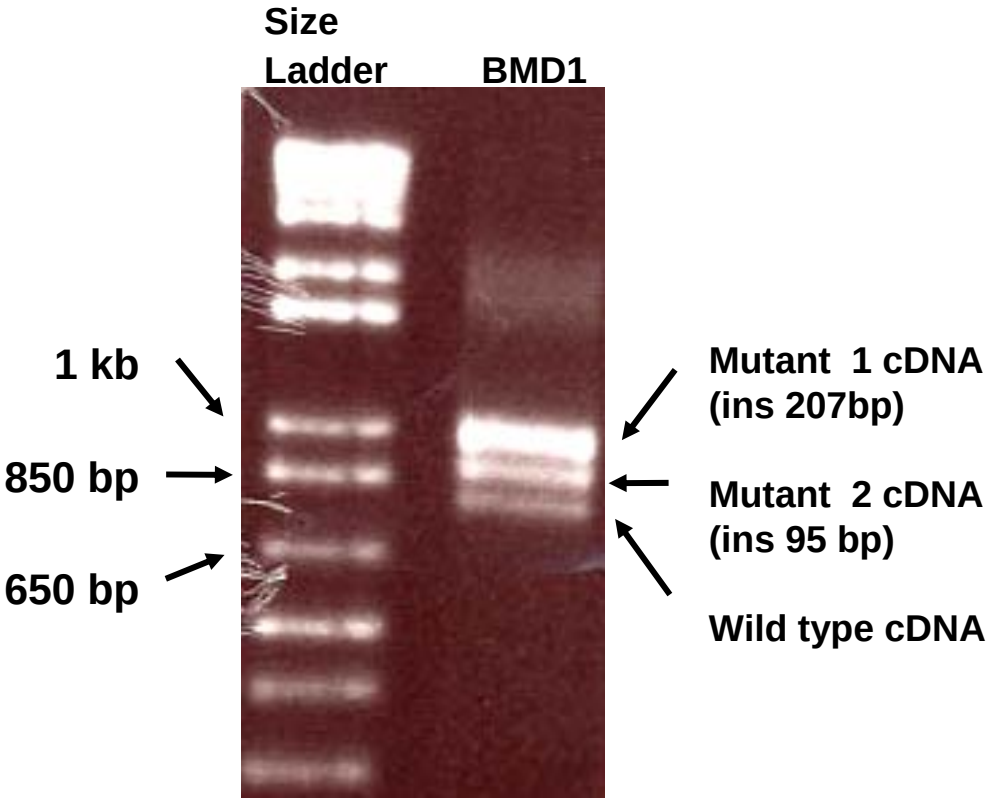
BMD1: RNA-based mutation analysis

PTT analysis

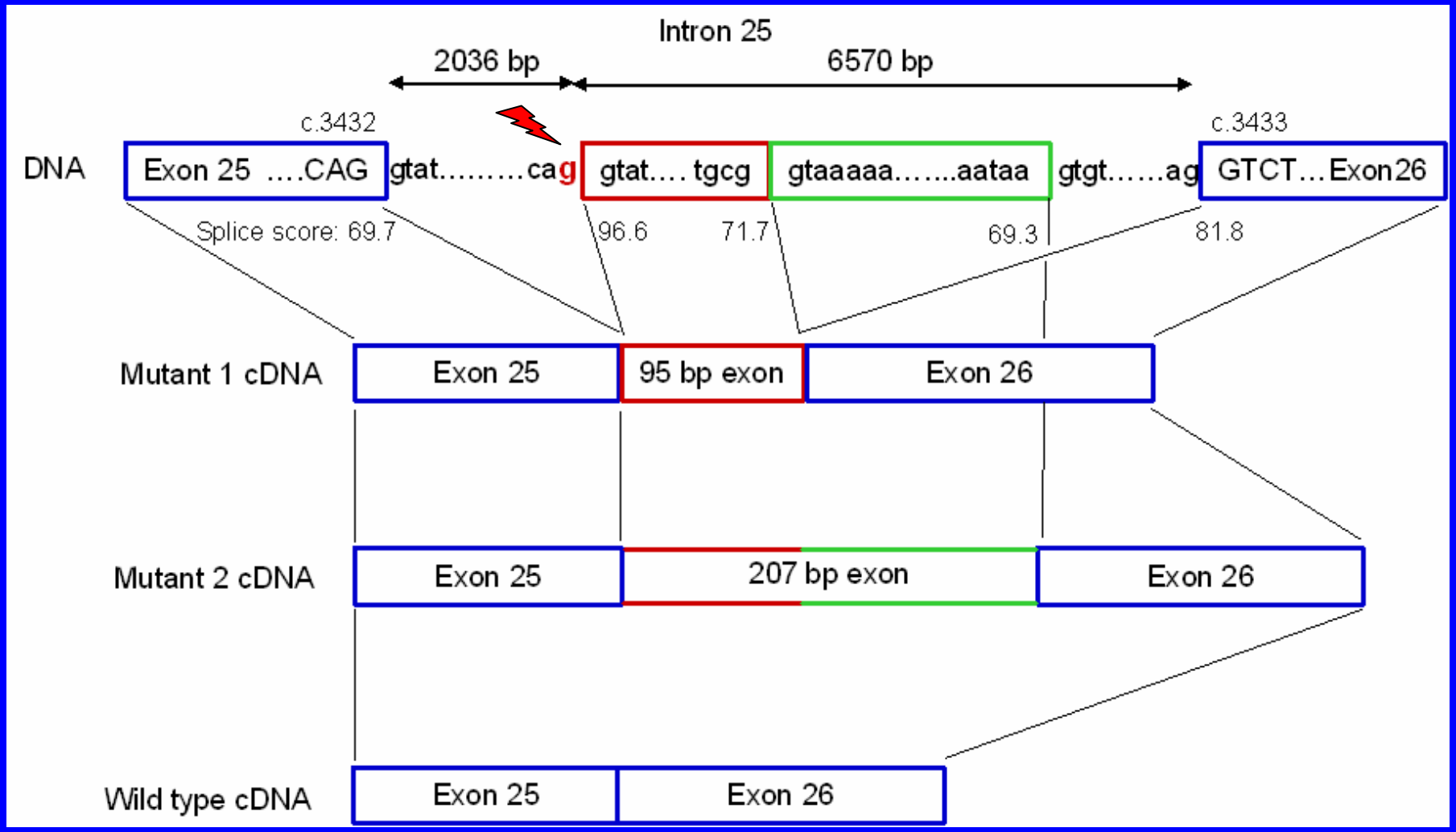


1 = BMD1
2 = Normal control
3 = DMD control

RT-PCR amplification

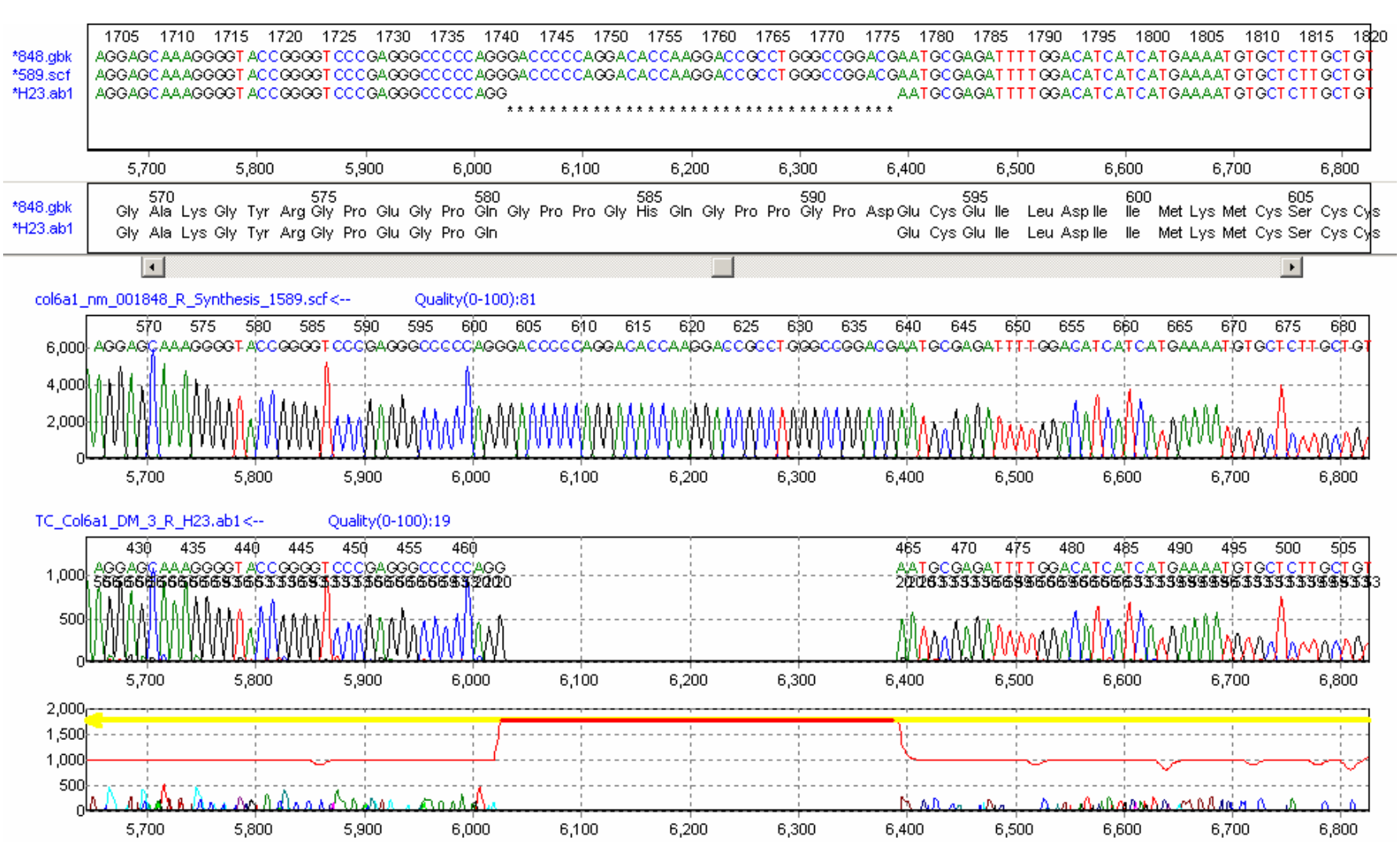


BMD1 - c.3432+2036 a>g



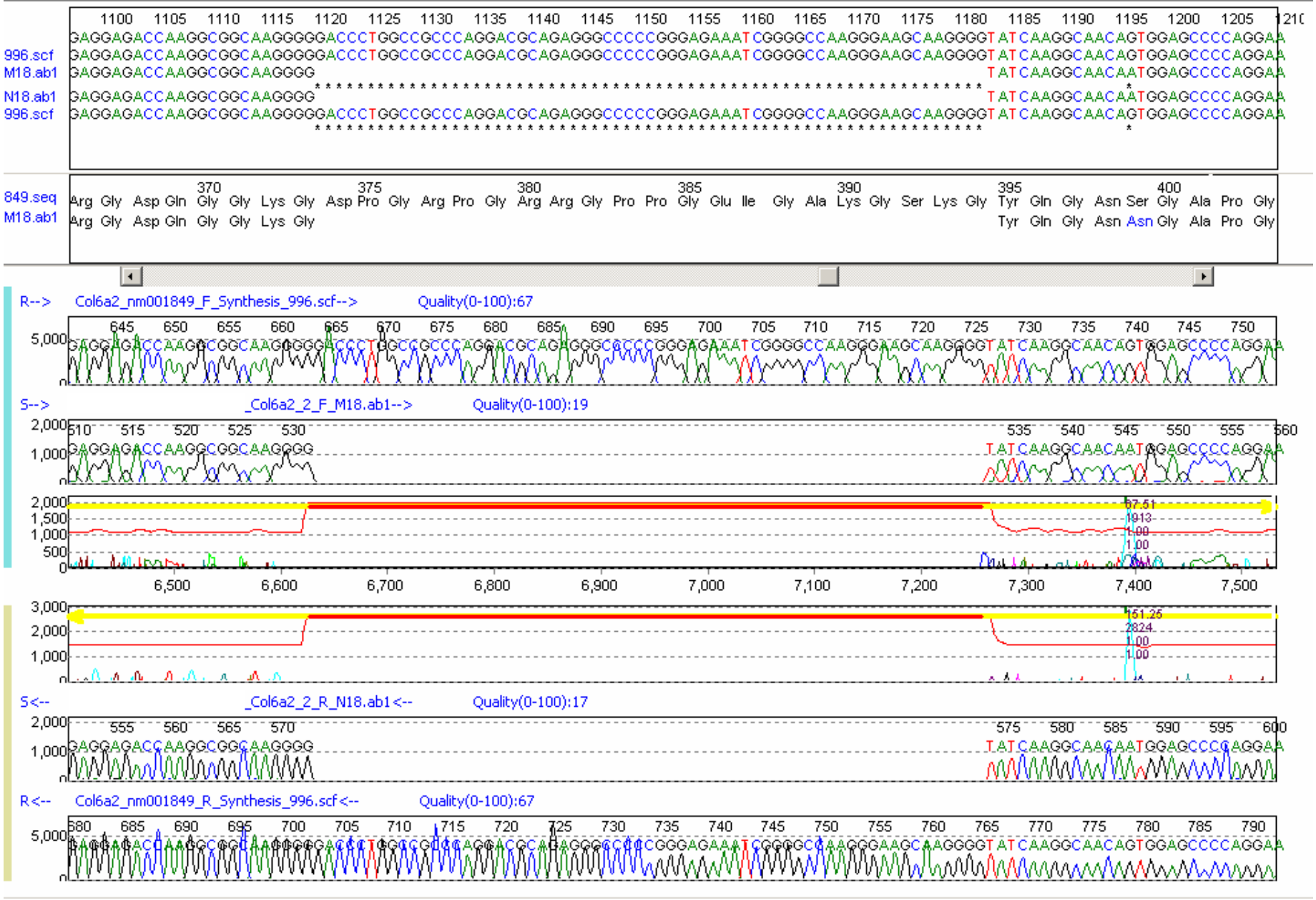
Col6a3: p.Gly2095_Lys2103del

Splicing-out/Deletion of exon 18



Col6a1: c.1776+1G>A; p.Gly581_Asp592del

Splicing-out of exon 27



Col6a2: c.1117_1179del; p.Gly373_Lys393del

Deletion/splicing-out of exon 13