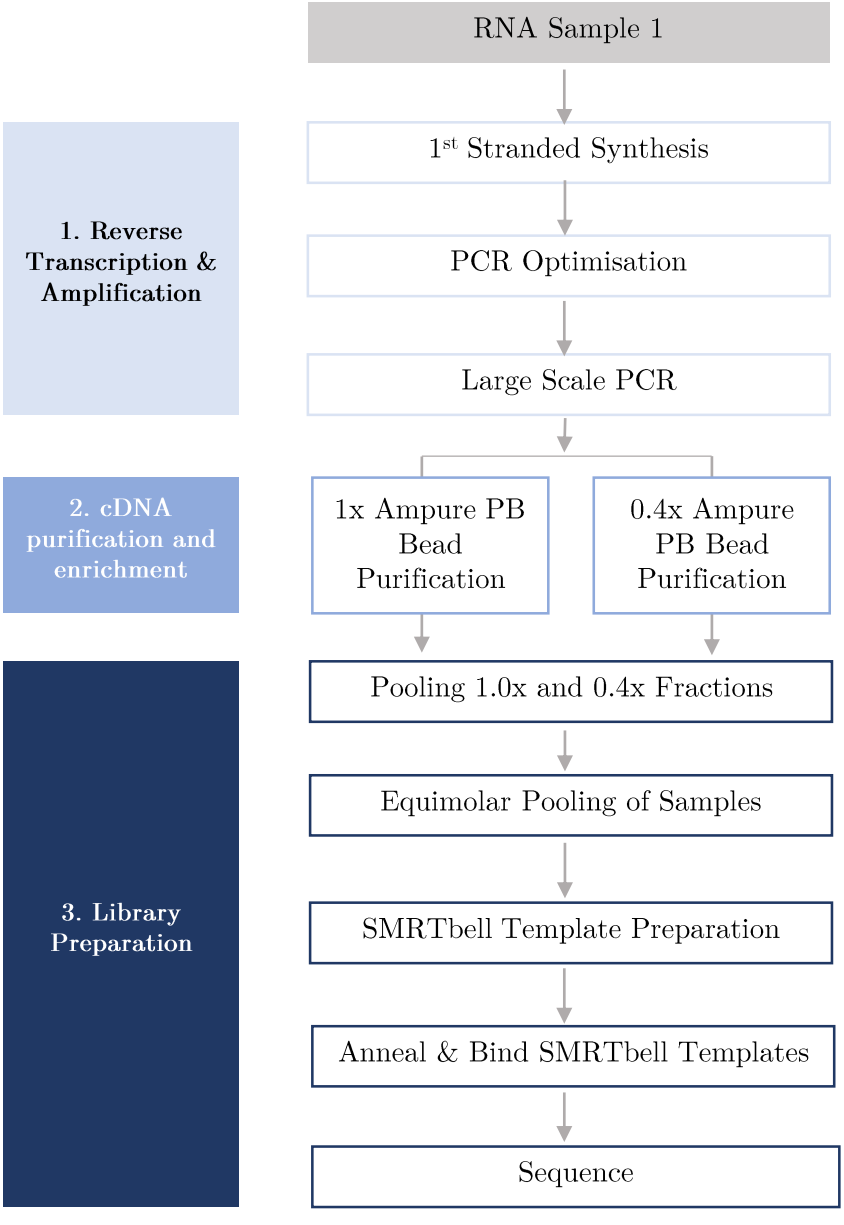
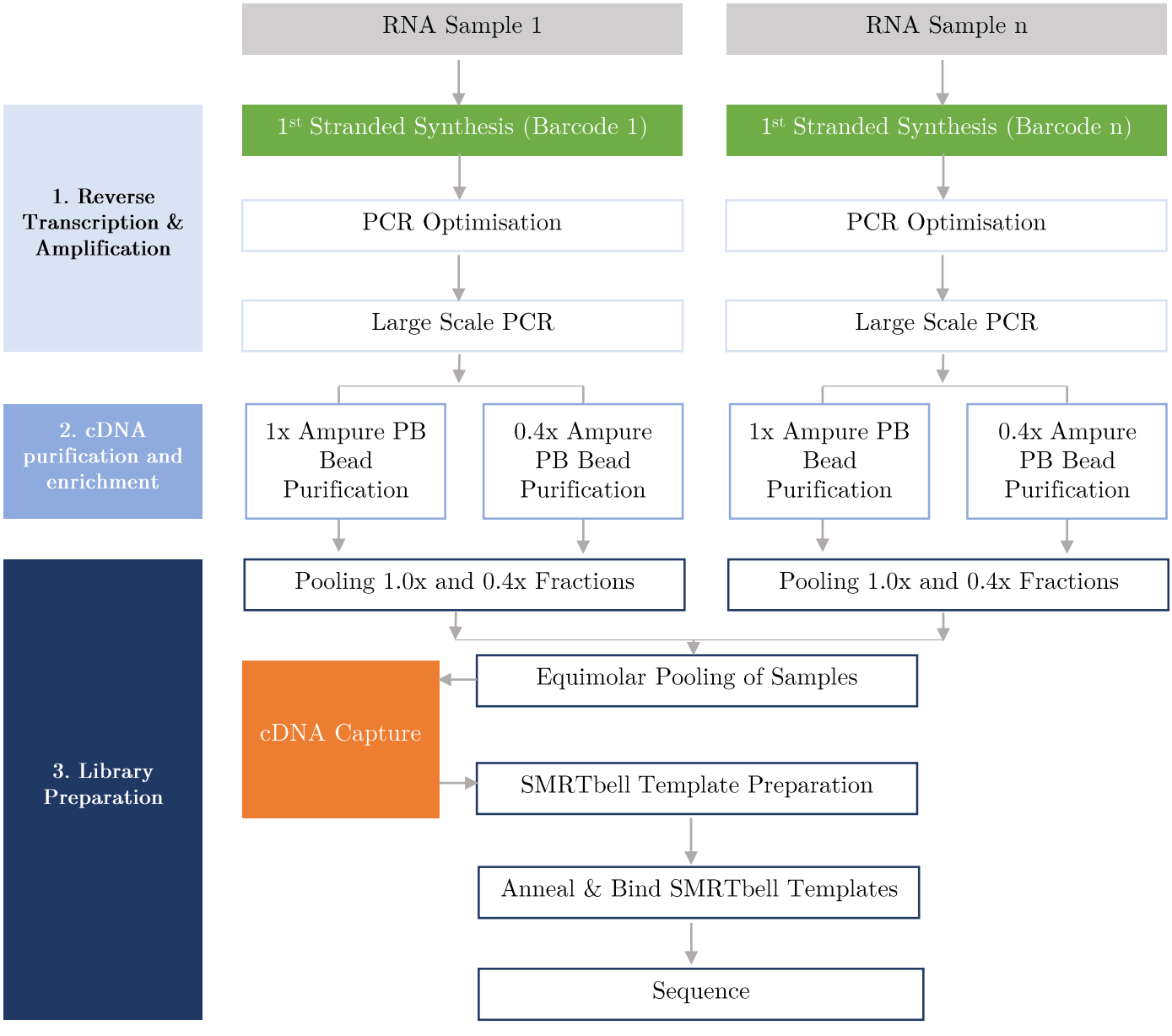
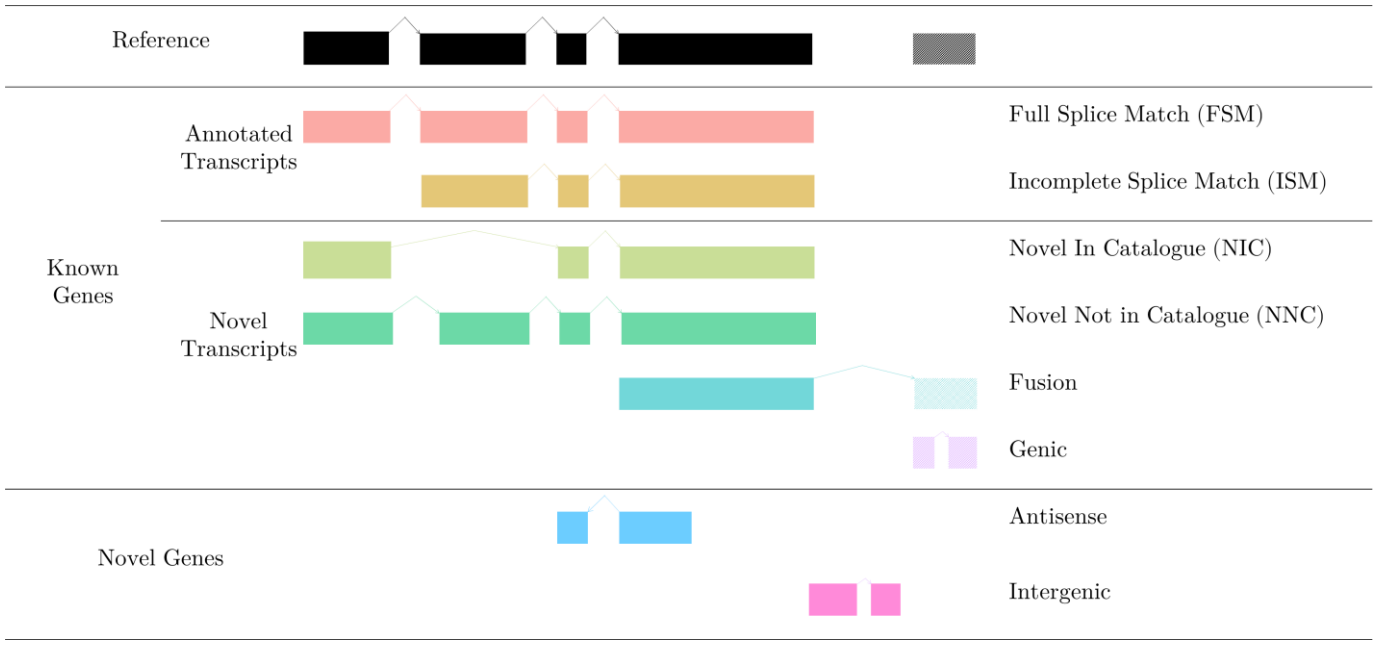
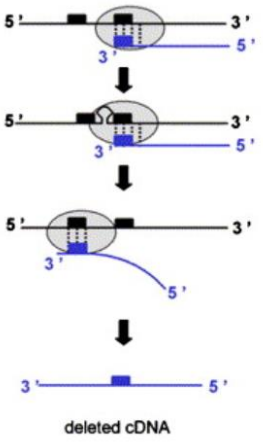

RNA Sample 1
1st Stranded Synthesis
PCR Optimisation
Large Scale PCR
1. Reverse Transcription & Amplification
1x Ampure PB Bead Purification
0.4x Ampure PB Bead Purification
2. cDNA purification and enrichment
Pooling 1.0x and 0.4x Fractions
Equimolar Pooling of Samples
SMRTbell Template Preparation
Anneal & Bind SMRTbell Templates
Sequence
3. Library Preparation

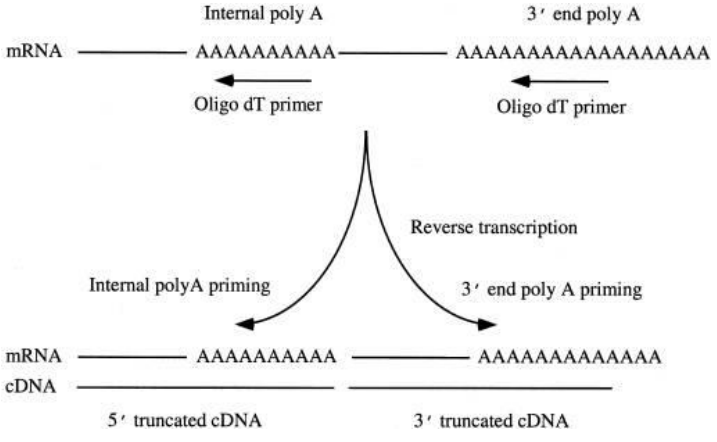

1. Reverse Transcription & Amplification
2. cDNA purification and enrichment
3. Library Preparation
RNA Sample 1
RNA Sample n
1st Stranded Synthesis (Barcode 1)
1st Stranded Synthesis (Barcode n)
PCR Optimisation
PCR Optimisation
Large Scale PCR
Large Scale PCR
1x Ampure PB Bead Purification
0.4x Ampure PB Bead Purification
1x Ampure PB Bead Purification
0.4x Ampure PB Bead Purification
Pooling 1.0x and 0.4x Fractions
Pooling 1.0x and 0.4x Fractions
Equimolar Pooling of Samples
cDNA Capture
SMRTbell Template Preparation
Anneal & Bind SMRTbell Templates
Sequence


Reference
Annotated Transcripts
Known Genes
Novel Transcripts
Novel Genes
Full Splice Match (FSM)
Incomplete Splice Match (ISM)
Novel In Catalogue (NIC)
Novel Not in Catalogue (NNC)
Fusion
Genic
Antisense
Intergenic

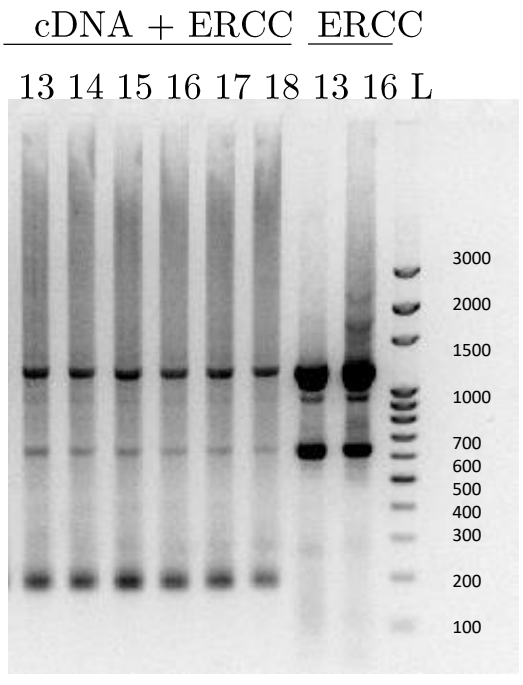
a


5'
3'
3'
5'
deleted cDNA

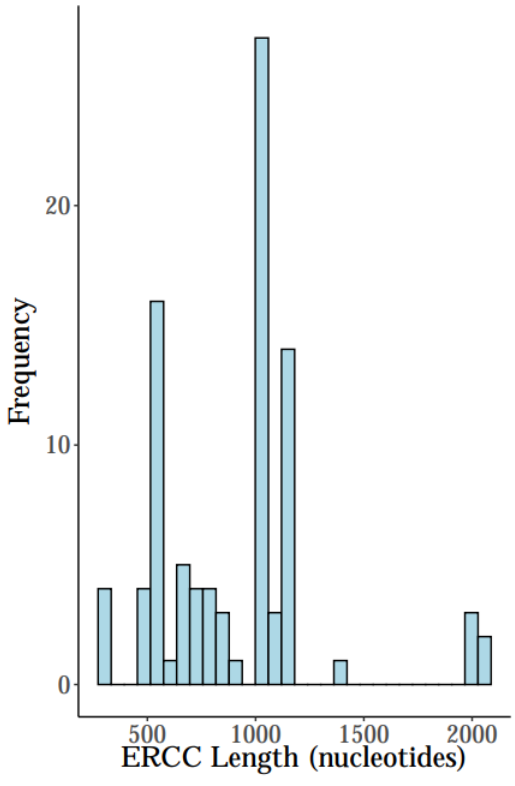
b


Internal poly A
3′ end poly A
mRNA
AAAAAAAAAA
AAAAAAAAAAAAAAAAAA
Oligo dT primer
Oligo dT primer
Reverse transcription
Internal polyA priming
3′ end poly A priming
mRNA
AAAAAAAAAA
AAAAAAAAAAAAA
cDNA
5′ truncated cDNA
3′ truncated cDNA

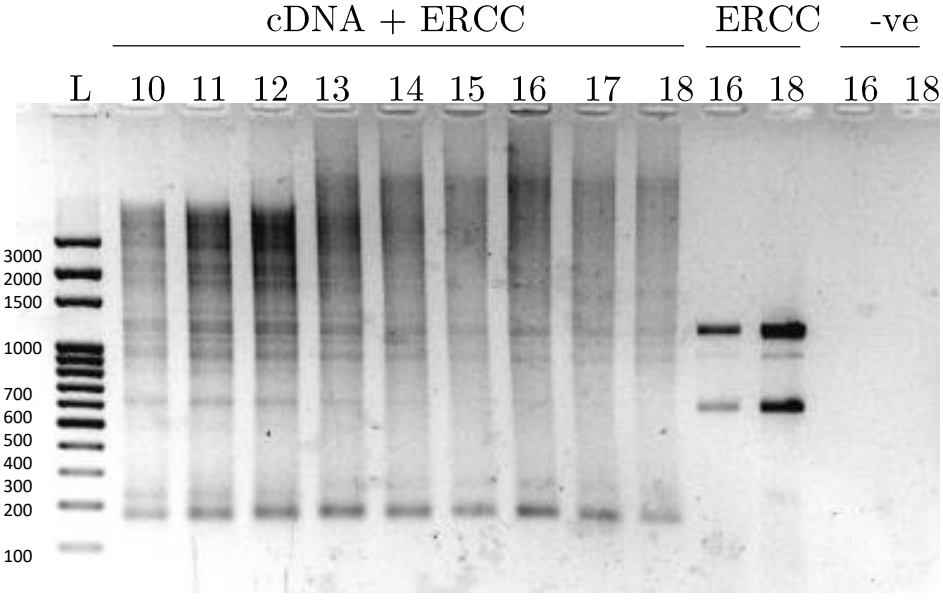
a

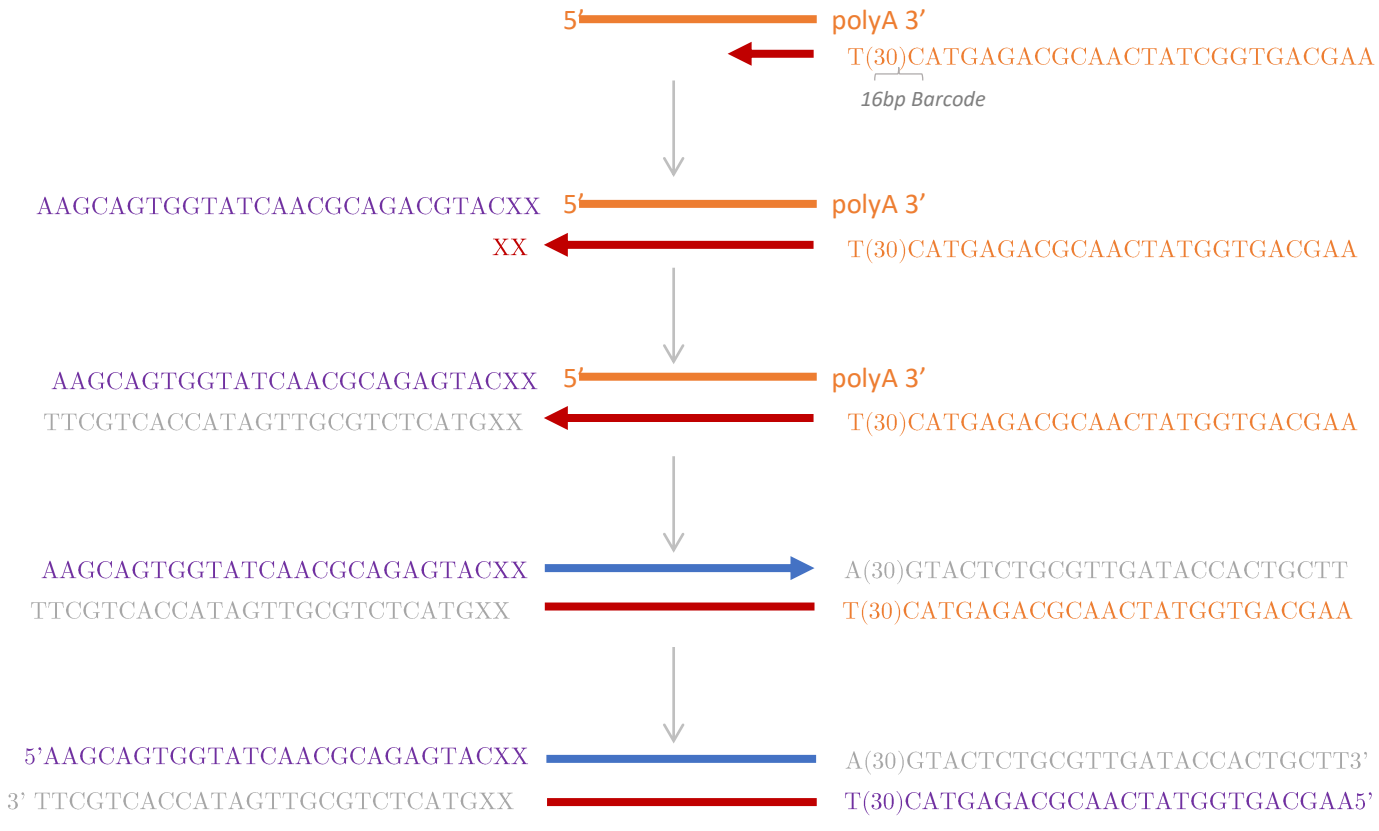

cDNA + ERCC
ERCC
13 14 15 16 17 18 13 16 L
3000
2000
1500
1000
700
600
500
400
300
200
100

b


Frequency
20
10
0
500
1000
1500
2000
ERCC Length (nucleotides)

c


cDNA + ERCC
ERCC
-ve
L 10 11 12 13 14 15 16 17 18 16 18 16 18
3000
2000
1500
1000
700
600
500
400
300
200
100


5'
polyA 3'
T(30)CATGAGACGCAACTATCGGTGACGAA
16bp Barcode
AAGCAGTGGTATCAACGCAGACGTACXX
5'
polyA 3'
XX
T(30)CATGAGACGCAACTATGGTGACGAA
AAGCAGTGGTATCAACGCAGAGTACXX
5'
polyA 3'
TTCGTCACCATAGTTGCGTCTCATGXX
T(30)CATGAGACGCAACTATGGTGACGAA
AAGCAGTGGTATCAACGCAGAGTACXX
A(30)GTACTCTGCGTTGATACCACTGCTT
TTCGTCACCATAGTTGCGTCTCATGXX
T(30)CATGAGACGCAACTATGGTGACGAA
5'AAGCAGTGGTATCAACGCAGAGTACXX
A(30)GTACTCTGCGTTGATACCACTGCTT3'
3' TTCGTCACCATAGTTGCGTCTCATGXX
T(30)CATGAGACGCAACTATGGTGACGAA5'

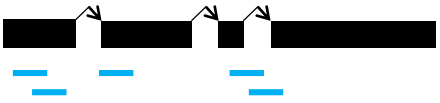
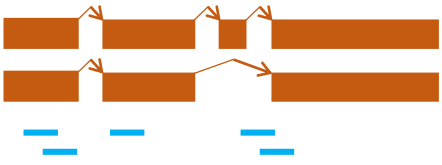
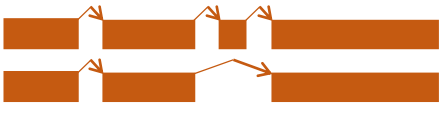
a)

b) Without Multiplexing

Strand	Sequence
Plus strand start	AATGTACTTCGTTCAGTTACGTATTGCTAAGCAGTGGTATCAACGCAGAGTACATGGG
Plus strand end	AAAAAAAAGTACTCTGCGTTGATACCACTGCTT
Minus strand start	AATGTACTTCGTTCAGTTACGTATTGCTAAGCAGTGGTATCAACGCAGAGTACTTTTTTTTT
Minus strand end	CCCATGTACTCTGCGTTGATACCACTGCTT

c) With Multiplexing

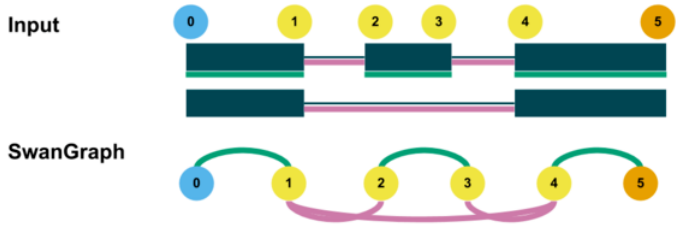
Strand	Sequence
Plus strand start	AATGTACTTCGTTCAGTTACGTATTGCTAAGCAGTGGTATCAACGCAGAGTACATGGG
Plus strand end	AAAAAAAA<Barcode>GTACTCTGCGTTGATACCACTGCTT
Minus strand start	AATGTACTTCGTTCAGTTACGTATTGCT<Barcode>AAGCAGTGGTATCAACGCAGAGTACTTTTTTTT
Minus strand end	CCCATGTACTCTGCGTTGATACCACTGCTT

	Analysis	Annotation	Expression
1.		Reference genome	RNA-Seq reads
2.		Iso-Seq defined transcriptome	RNA-Seq reads
3.		Iso-Seq defined transcriptome	Iso-Seq reads

Isoform	Associated Gene	Structural Category	Associated Transcript	Length	FL Sample 1	FL Sample 2
PB.1.1	Gene 1	FSM	ENMUST1	3000	10	10
PB.1.2	Gene 1	ISM	ENMUST1	1500	100	90
PB.1.3	Gene 1	ISM	ENMUST2	2400	20	20
PB.1.4	Gene 1	ISM	ENMUST3	2900	5	4
PB.1.5	Gene 1	ISM	ENMUST3	2190	5	2
PB.1.6	Gene 1	FSM	ENMUST4	2420	1000	1250
PB.1.7	Gene 1	FSM	ENMUST4	2560	1	4

Isoform	Associated Gene	Structural Category	Associated Transcript	Length	FL Sample 1	FL Sample 2
PB.1.1	Gene 1	FSM	ENMUST1	3000	110	100
PB.1.3	Gene 1	ISM	ENMUST2	2400	20	20
PB.1.4	Gene 1	ISM	ENMUST3	2900	10	6
PB.1.7	Gene 1	FSM	ENMUST4	2560	1001	1254

a)



Blue Nodes: Transcription Start Sites
Orange Nodes: Transcription End Sites
Yellow Nodes: Internal Spice Sites

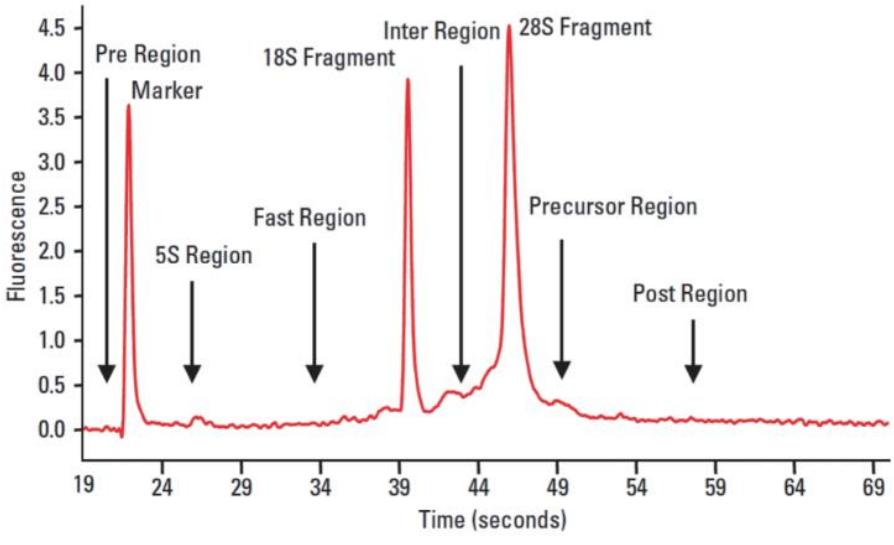
Green Edges: splice junction pair spanning exon
Pink Edges: splice junction pair spanning intron

b)

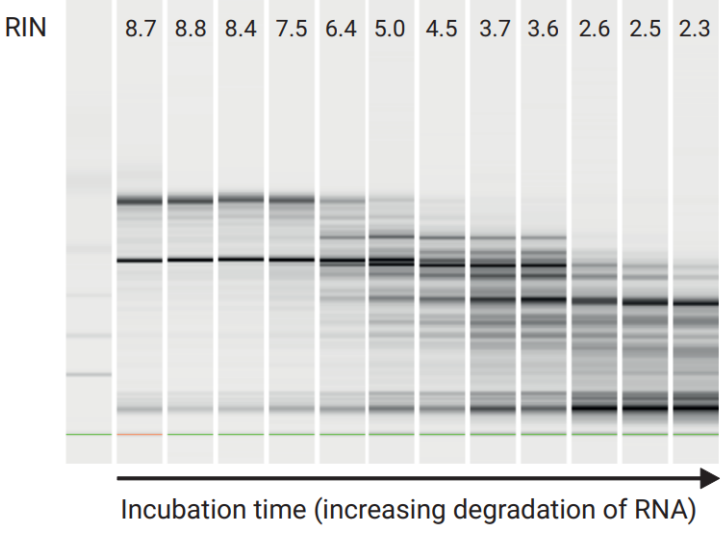
ENMUST00000014268.4

Associated Transcript	Mean Expression, Length	Structure
PB.6948.1 (FSM)	0.08FL, 3939bp	
PB.6948.7 (FSM)	645FL, 1600bp	
PB.6948.160 (ISM)	0.083FL, 1079bp	
PB.6948.35 (ISM)	46.7FL, 1000bp	
PB.6948.81 (ISM)	69.2FL, 812bp	
PB.6948.115 (ISM)	32.0FL, 663bp	
PB.6948.15 (ISM)	0.083FL, 847bp	

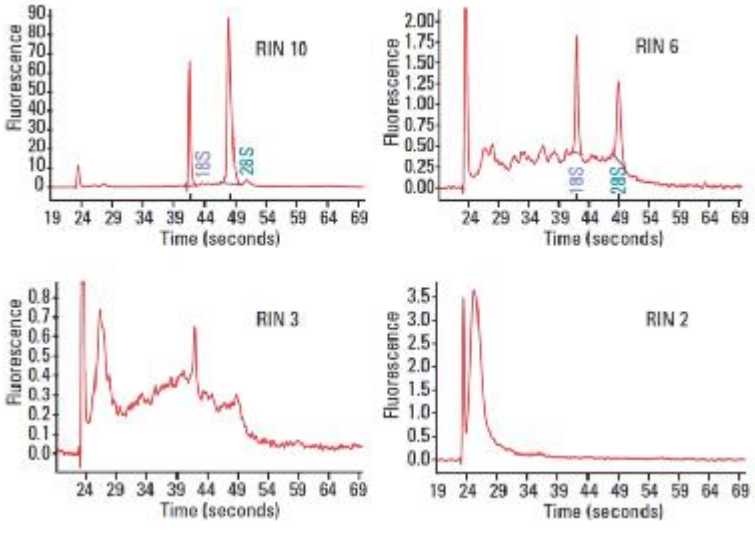
a)


Pre Region
Marker
5S Region
18S Fragment
Fast Region
Inter Region
28S Fragment
Precursor Region
Post Region
Fluorescence
Time (seconds)

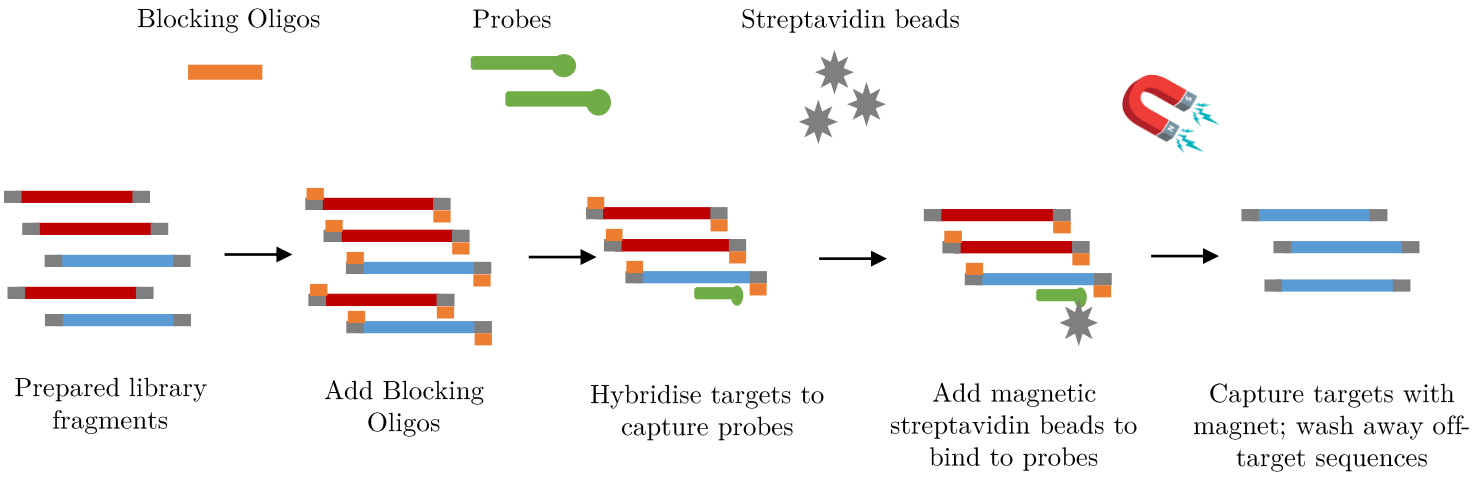
b)


RIN
8.7 8.8 8.4 7.5 6.4 5.0 4.5 3.7 3.6 2.6 2.5 2.3
Incubation time (increasing degradation of RNA)

c)

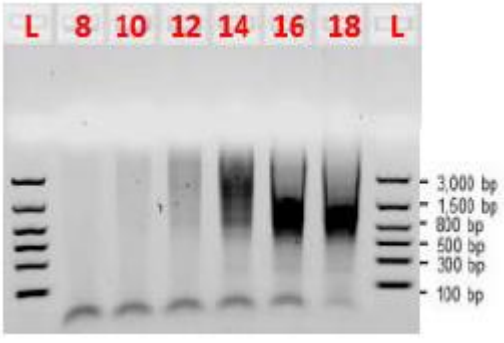

RIN 10
RIN 6
RIN 3
RIN 2
18S
28S
Fluorescence
Time (seconds)

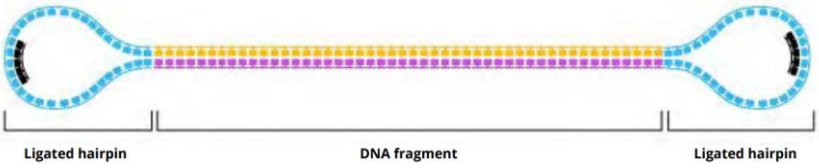
a)

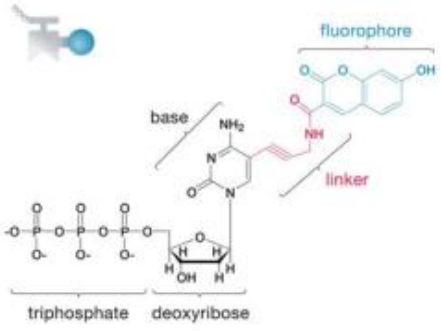

Blocking Oligos
Probes
Streptavidin beads
Prepared library fragments
Add Blocking Oligos
Hybridise targets to capture probes
Add magnetic streptavidin beads to bind to probes
Capture targets with magnet; wash away off-target sequences

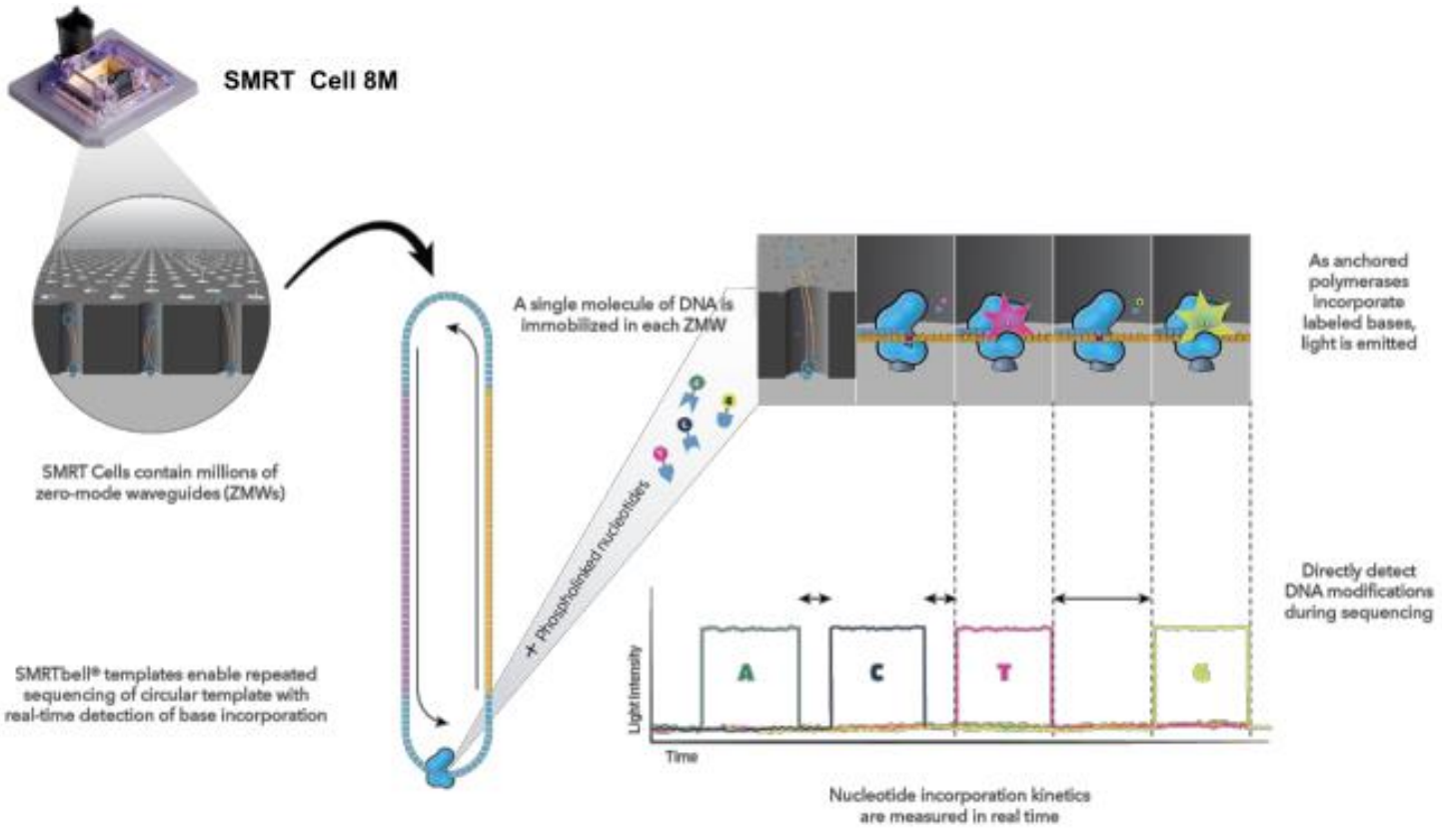
b)

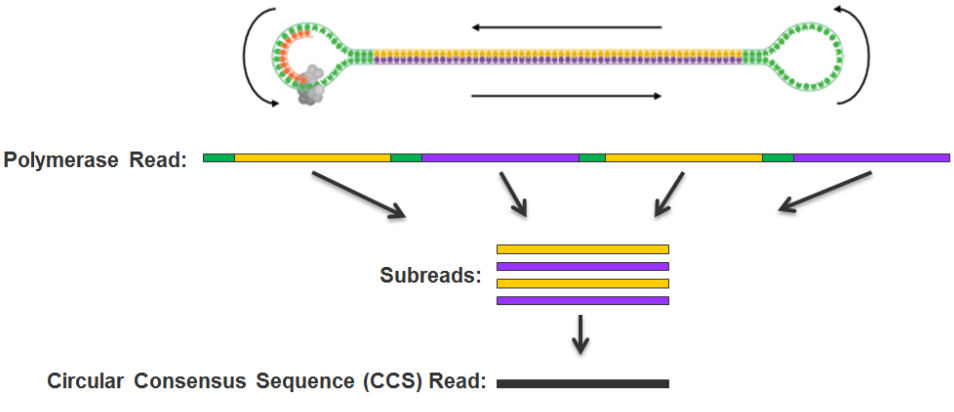
1
Combine DNA with blockers
Dry down DNA
Perform hybridization reaction
Total time: 15 minutes
Total time: Variable
Total time: 4–16 hours
2
Prepare buffers
Total time: 15 minutes*
3
Wash streptavidin beads
Total time: 15 minutes*
4
Perform bead capture
Total time: 45 minutes
5
Perform washes
Total time: 30 minutes
6
Perform post-capture PCR
Total time: 30 minutes
7
Purify post-capture PCR fragments
Total time: 30 minutes


L
8
10
12
14
16
18
L
3,000 bp
1,500 bp
800 bp
500 bp
300 bp
100 bp


Sequencing Primer
Ligated hairpin
DNA fragment
Ligated hairpin


fluorophore
base
linker
triphosphate
deoxyribose


SMRT Cell 8M
SMRT Cells contain millions of zero-mode waveguides (ZMWs)
A single molecule of DNA is immobilized in each ZMW
+ Phospholinked nucleotides
As anchored polymerases incorporate labeled bases, light is emitted
Directly detect DNA modifications during sequencing
SMRTbell® templates enable repeated sequencing of circular template with real-time detection of base incorporation
Light intensity
Time
A
C
T
G
Nucleotide incorporation kinetics are measured in real time


Polymerase Read:
Subreads:
Circular Consensus Sequence (CCS) Read: