RT-PCR

? RT-priming

➩ Oligo dT:
It anneals to poyAmRNA:
No rRNA transcription
No for degraded RNA

➩Random examers
Reproducible and reliable results.
Dnase treatment,
Transcribe all RNAs

➩ Specific Primer
Only specific RNA,
The number of RT = targets to analyze
➩It is the most specific and sensible among RT, but time consuming

The three methods differ both in yield and in variety and specificity of the products

Reverse transcription

NB: RPA and oligo-dT Tm is lower than working T of reverse transcriptase

RPA→ they start RT in ≠ places producing more than one cDNA for every original target.

It is by definition a non specific method but with > cDNA yield. Useful for transcripts with strong secondary structure

cDNA from total RNA is mostly from rRNA ⇒ trouble for low expressed transcripts as they could not be reached by RPA ⇒amplification could not mirror the actual amount of target ⇒ non quantitative.

MONTE CARLO EFFECT

It is a statistical phenomenon affecting PCR for low copy number of target, causing deviation from the ideal model of PCR (different efficiencies of PCR for differently expressed cDNA).

Every sample has a certain P to be amplified or lost. The higher variance in the results from PCR reactions is linked to low starting template quantity (< 100 copies) and contributes to this statistical phenomenon, such that more qualitative information is produced, as compared to reactions with abundant targets.

MONTE CARLO EFFECT

Monte Carlo effects depends on [sample]: the less is the template the lower is the probability that its real amount could be related to the final quantity of the amplification product.

Less expressed transcripts can suffer from Montecarlo Effect. Many more replicate reactions are required to achieve statistical confidence in the results.

Possible methods

⇨2 steps- RT and then PCR

⇨ one step-in thermocycler with a RT pre-cycle of about1 h

NOTE

☞2 steps > yield than 1 step.

☞1 step – no contamination

☞∃ DNA polymerase with Rt activity with higher working T (even 60° C,) not for RPA and oligo dT.

RT-PCR

? Enzima

⇨ AMV: from Avian myeloblastosis virus. Suggested especially with specific primers, not so good with other types of priming.

⇨ MMLV: da Moloney murine leukemia virus. Good with oligo dT and random primers.

Combinations:

Specific Primer + AMV→ fresh tissues as well as FFPET

Oligo dT + Moloney → only fresh tissues

RPA +Moloney → fresh tissues as well as FFPET

⇨RNA from FFPE tissues- RPA

☞Maximizing the pairing with hexamers (short fragments do not tend to match)

☞Higher $[Mg^{2+}]$ (7.5 mM)

☞> [RPA] (0.1÷3.85 nmoli) and < enzyme (250 u)

☞RNA denaturation10' 65° C-4° C

☞Pre annealing @ 25° C

☞T= 37° C, t=45-60 min Vf =20 µl

☞Enzyme inactivation 15' @70° C.

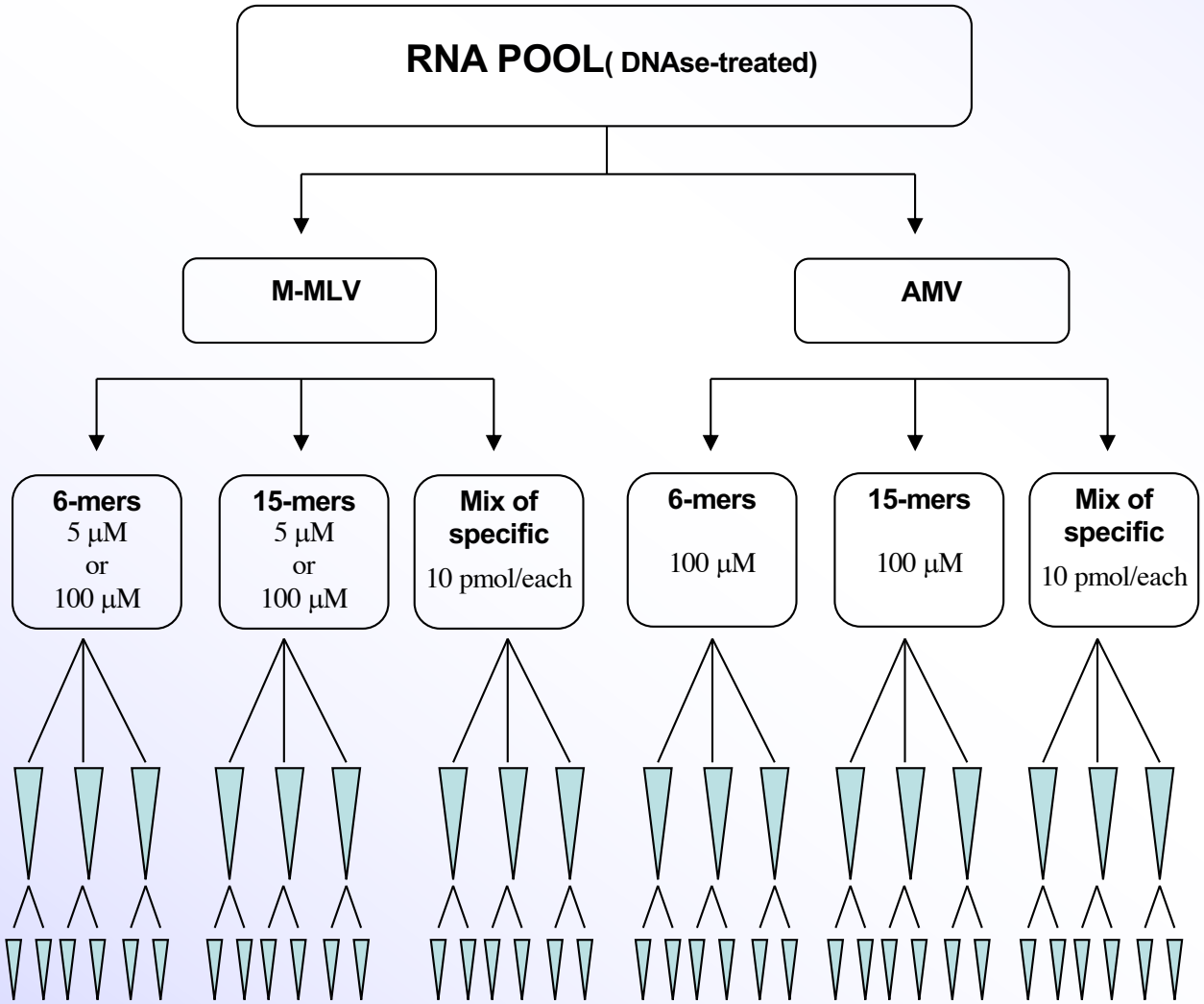
⇨ FFPET- AMV

☞Buffer AMV1x, dNTPs 1mM final, 15 pmoli antisense primer, 4 u Rnase inhibitor, AMV RT 2.5 u. Vf =10 µl, T= 42° C, t=45-60 min

☞For following PCR add 15 pmoli of sense primer, PCR buffer w/o Mg to have a final Mg conc of 1 mM or 1,5 mM.

☞Final [dNTPs] 0.2 mM in Vf=50µl

Design



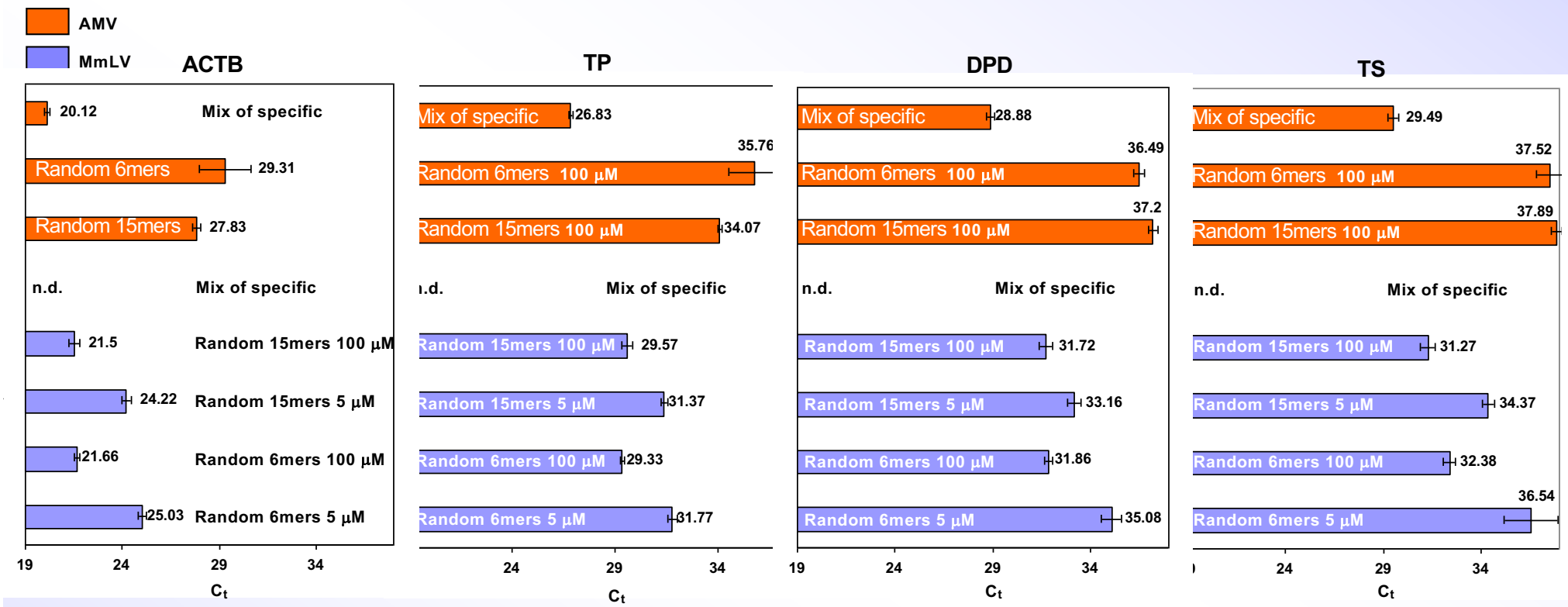
Targets:

Thymidylate synthase (TS)
Thymidine phosphorylase (TP)
Dihydropyrimidine dehydrogenase (DPD)
β-actin (ACTB)

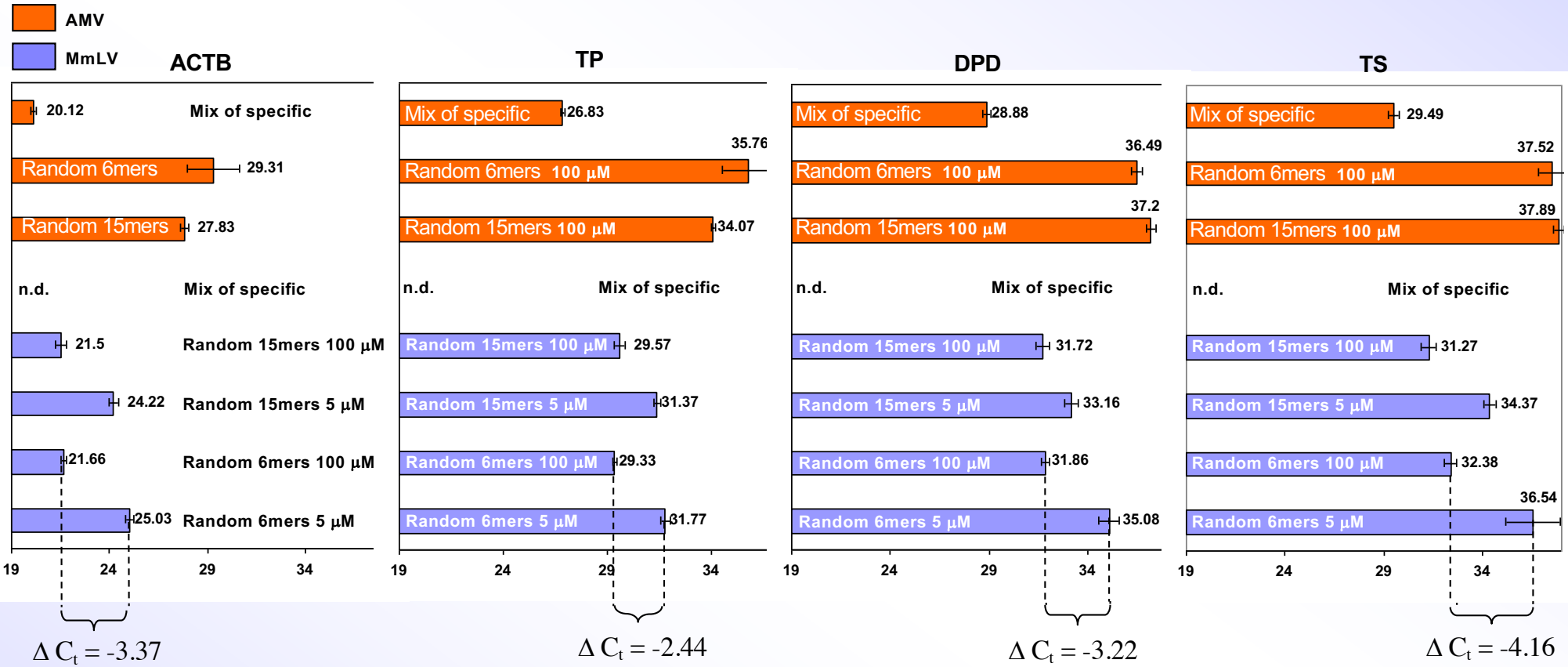
3 RT replicates

2 real time PCR replicates

Yield of the reverse transcription



Yield of the reverse transcription



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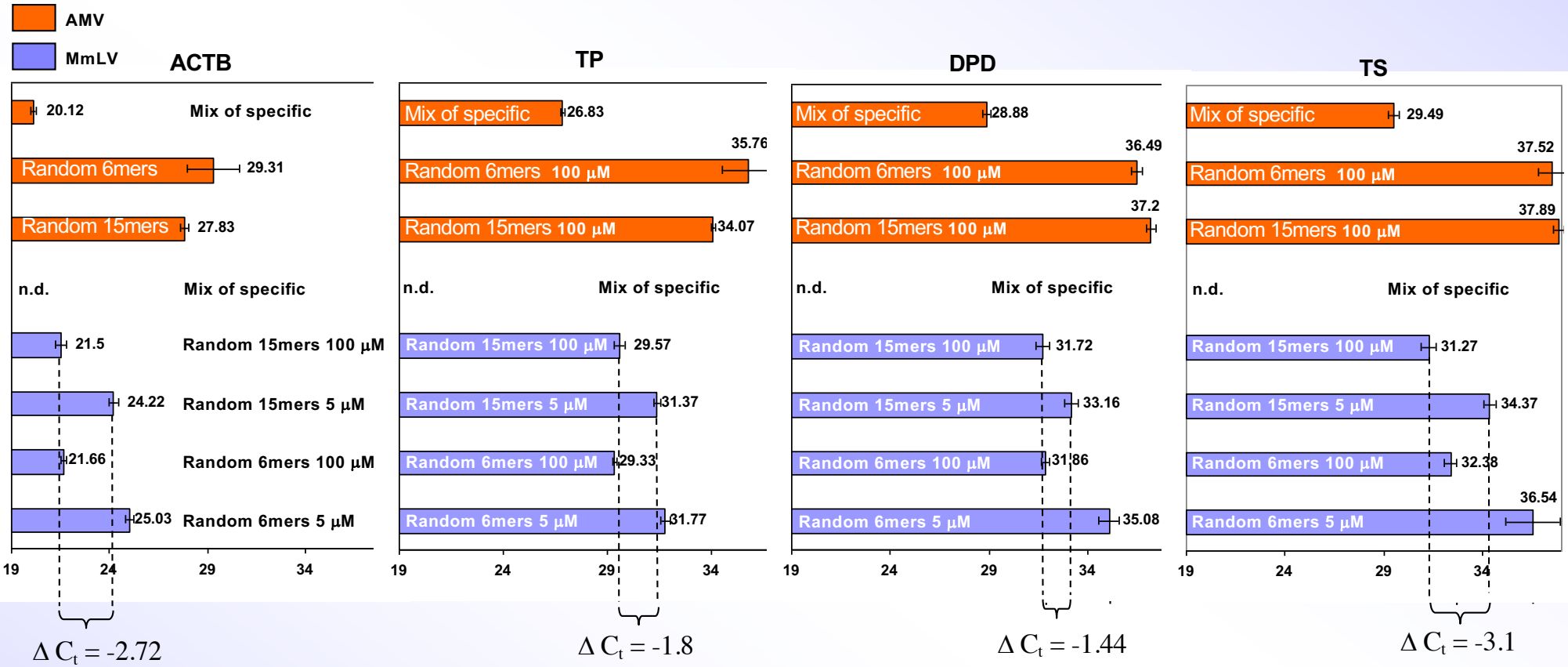
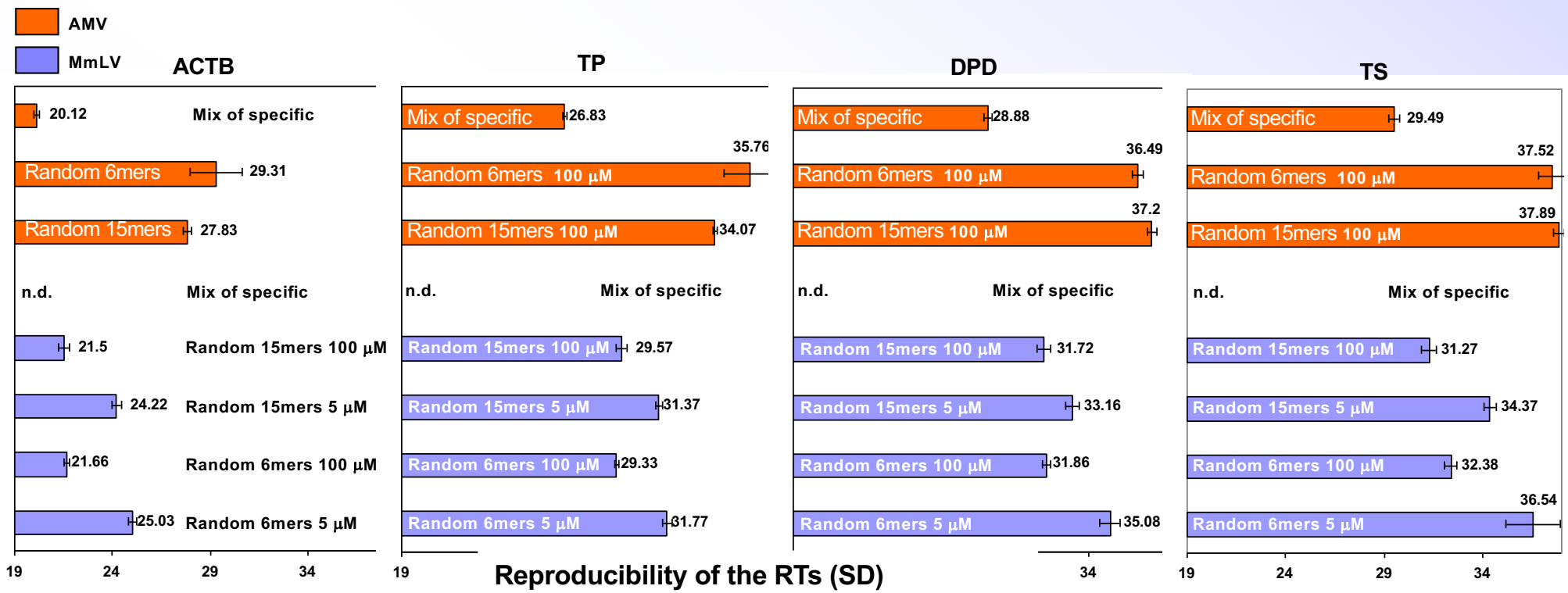


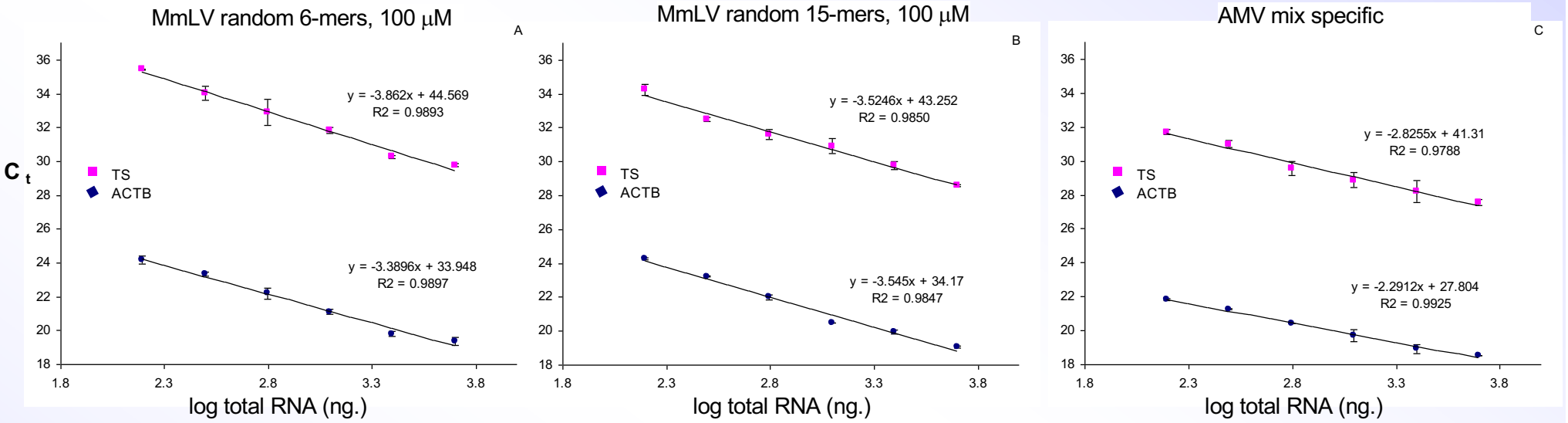
Yield of the reverse transcription



Reproducibility of the RTs (SD)

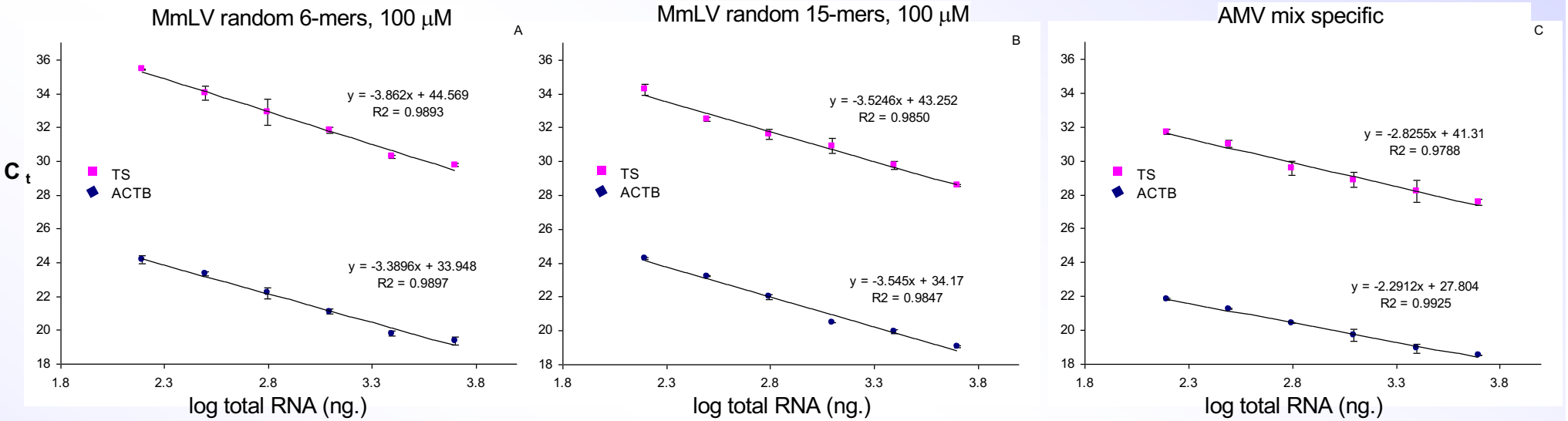
	ACTB	TP	DPD	TS
AMV + mix specific	**0.17**	**0.09**	**0.19**	**0.27**
AMV + 100 µM 6mers	1.36	1.24	0.26	0.68
AMV + 100 µM 15mers	0.48	0.20	0.25	0.28
MmLV + mix specific	n.a.	n.a.	n.a.	n.a.
MMLV + 100 µM 15mers	**0.22**	**0.18**	**0.33**	**0.32**
MmLV + 5 µM 15mers	0.29	0.28	0.35	0.38
MmLV + 100 µM 6mers	**0.17**	**0.11**	**0.21**	**0.30**
MmLV + 5 µM 6mers	0.24	0.21	0.50	1.40

Linearity of the reverse transcription



		Gene			
		TS	**TP**	**DPD**	**ACTB**
MMLV + 5 µM 6mers	Slope	-4.225	-2.587	-4.276	-3.032
	Efficiency (%)	72.45	143.54	71.34	113.71
	R^2	0.9827	0.9287	0.9834	0.9581
MMLV + 100 µM 6mers	Slope	-3.862	-3.568	-3.482	-3.390
	Efficiency (%)	81.52	90.67	93.72	97.25
	R^2	0.9893	0.9701	0.9662	0.9897
MMLV + 5 µM 15mers	Slope	-3.151	-2.513	-3.213	-3.152
	Efficiency (%)	107.68	149.97	104.77	107.61
	R^2	0.9899	0.9741	0.9849	0.9791
MMLV + 100 µM 15mers	Slope	-3.525	-3.073	-3.736	-3.545
	Efficiency (%)	92.19	111.51	85.21	91.47
	R^2	0.9850	0.9750	0.9902	0.9847
AMV + mix specific	Slope	-2.826	-2.746	-2.267	-2.291
	Efficiency (%)	125.90	131.27	176.19	173.18
	R^2	0.9788	0.9877	0.9767	0.9925

Linearity of the reverse transcription



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Efficiency of the real time-PCR

Comparison of C_t values and reaction efficiencies of cDNA amplification.
Criteria for inclusion: $C_t < 39$, SD < 0.5 and $R^2 > 0.98$

Method		cDNA dilution in PCR mix						
		1:10	1:40	1:160	1:640	1:2560	1:10240	**E%**
6-MERS M-MLV	**TS**	30.79 (0.01)	32.65 (0.04)	34.79 (0.33)	37.89 (0.42)	-	-	*84*
	TP	27.56 (0.04)	29.22 (0.04)	31.70 (0.05)	33.63 (0.15)	35.24 (0.35)	37.67 (0.40)	*98*
	DPD	28.97 (0.29)	30.82 (0.12)	32.71 (0.27)	34.99 (0.43)	-	-	*100*
	ACTB	20.43 (0.25)	21.30 (0.16)	23.20 (0.06)	25.43 (0.14)	27.76 (0.18)	30.09 (0.22)	*100*
15-MERS M-MLV	**TS**	29.37 (0.21)	30.61 (0.07)	33.34 (0.01)	35.98 (0.23)	-	-	*85*
	TP	28.30 (0.16)	29.54 (0.05)	31.64 (0.08)	33.38 (0.40)	35.96 (0.45)	38.02 (0.49)	*100*
	DPD	29.24 (0.14)	30.31 (0.14)	32.49 (0.31)	35.34 (0.48)	-	-	*97*
	ACTB	20.60 (0.13)	21.85 (0.17)	23.74 (0.21)	25.59 (0.11)	27.76 (0.19)	30.36 (0.20)	*100*
Mix of specific AMV	**TS**	-	29.88 (0.48)	31.66 (0.04)	34.17 (0.43)	35.80 (0.45)	38.60 (0.47)	*91*
	TP	-	26.76 (0.05)	28.86 (0.08)	31.01 (0.30)	33.45 (0.40)	36.26 (0.40)	*80*
	DPD	-	27.96 (0.08)	30.54 (0.18)	32.65 (0.28)	35.28 (0.35)	36.48 (0.48)	*86*
	ACTB	18.44 (0.04)	19.95 (0.14)	21.84 (0.05)	24.22 (0.23)	26.42 (0.02)	28.98 (0.11)	*92*

Quantitative analyses: RNA should be:

⇨Good quality

⇨ DNA free, especially for targets w/o intron/exon junction

⇨Inhibitors check and reduction

⇨Nuclease free for long storage periods

NOTES

☞There are several RT-PCR inhibitors in blood and tissues.

☞Inhibitors can be checked by serial dilutions of the template (Ct deviation from linearity)

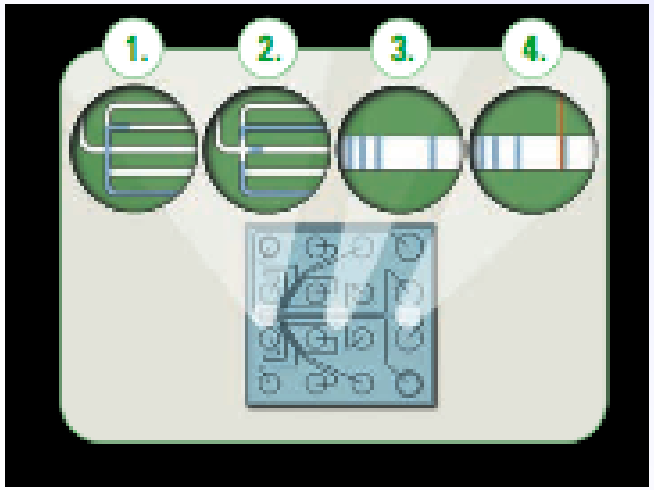
☞Exogenous target (es plants for human). Template + exogenous DNA are amplified togeter for exogenous targets. If Ct inctreases with the increment of huaman template ⇒ inhibitors

RNA quality control

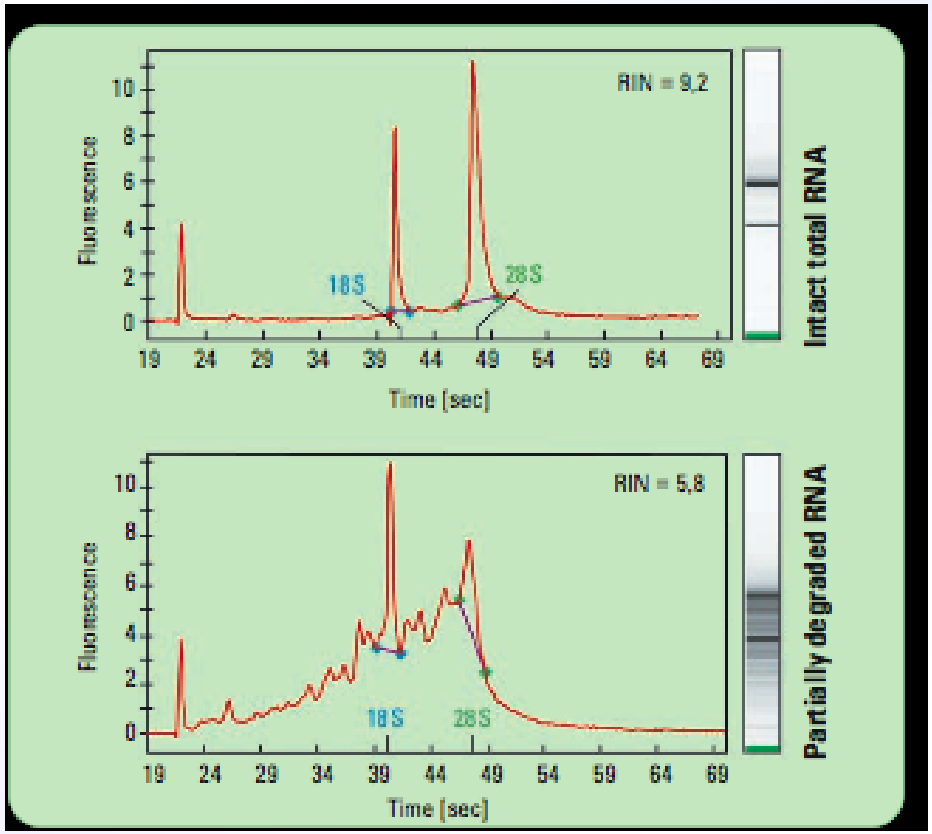
1. Evaluation of ribosomal bands of 18S and 28S with a capillary electrophoresis system (Agilent RNA LabChip). The system compares the peak area compared to 6 reference RNAs. RIN = 10 intact RNA, RIN = 1 completely degraded

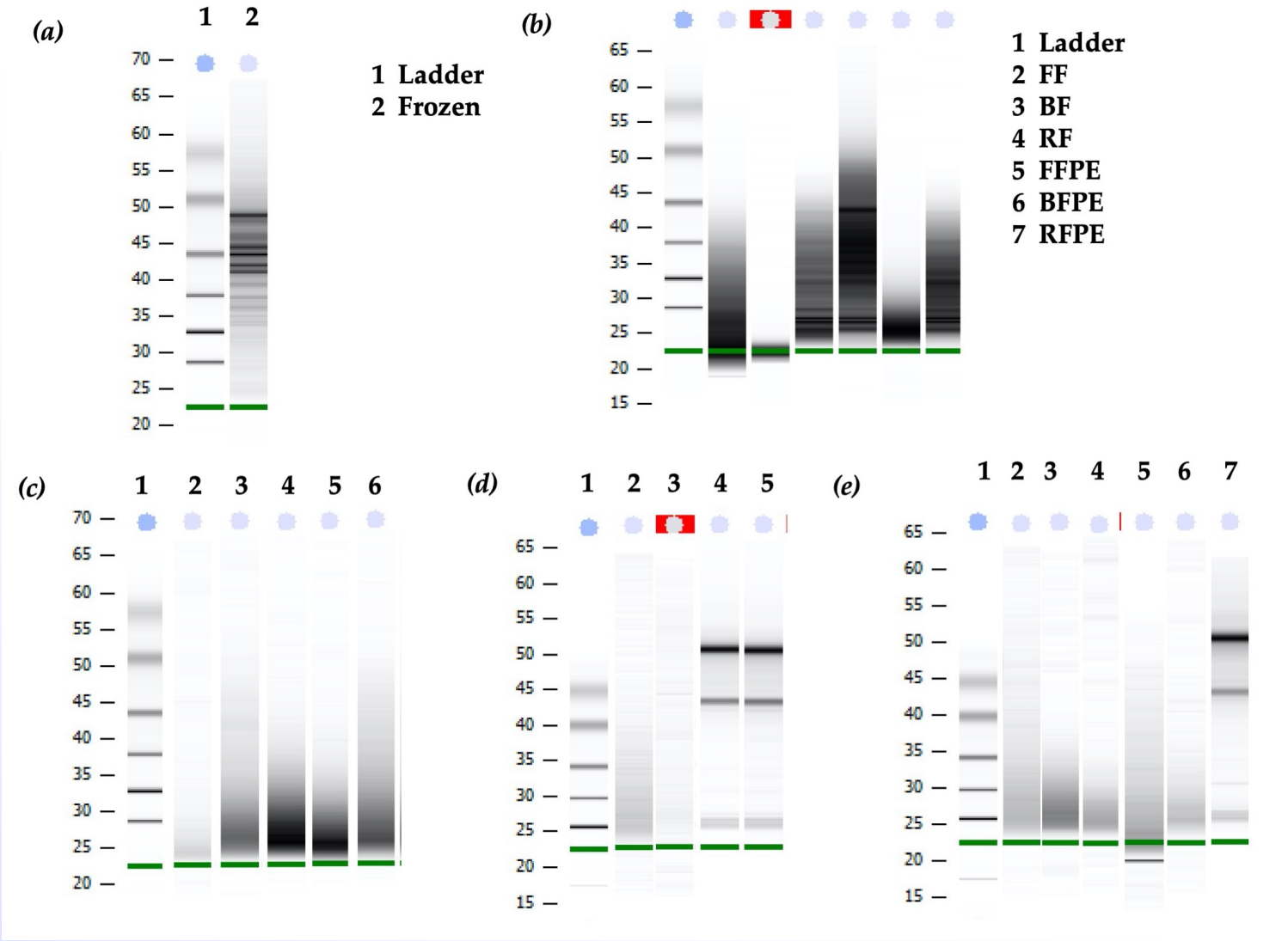
2. 3'-5 ’test with GAPDH: the reaction run using oligodT for RT. A multiplex PCR is done to quantify 3 amplicons (specific assays). The 3’ amplification fragments are all for the GAPDH, but one is close to 5 ', the second is positioned in the center and the third towards the terminal 3' of the RNA. The progression of RT towards the 5’ is related to the quality of RNA. A ratio of Ct 3 ’: 5’ = 1 indicates high integrity, while a ratio> 5 indicates degradation.

1. The sample moves through the microchannels from the sample well.
2. The sample is injected into the separation channel.
3. Sample components are electrophoretically separated.
4. Components are detected by their fluorescence and translated into gel-like images (bands) and electropherograms (peaks).
1.1. 1.2. 3. 4.



4
RNase degradation of RNA samples is a common reason for failed downstream experiments. The Agilent 2100 bioanalyzer provides RNA quality control results in both gel-like image as well as electrophoretic data making it easy to detect even small degradation effects. In addition an RNA Integrity Number (RIN) is provided for each total RNA sample allowing standardization.




(a)
1 2
70
65
60
55
50
45
40
35
30
25
20
1 Ladder
2 Frozen
(b)
65
60
55
50
45
40
35
30
25
20
15
1 Ladder
2 FF
3 BF
4 RF
5 FFPE
6 BFPE
7 RFPE
(c)
1 2 3 4 5 6
70
65
60
55
50
45
40
35
30
25
20
(d)
1 2 3 4 5
65
60
55
50
45
40
35
30
25
20
15
(e)
1 2 3 4 5 6 7
65
60
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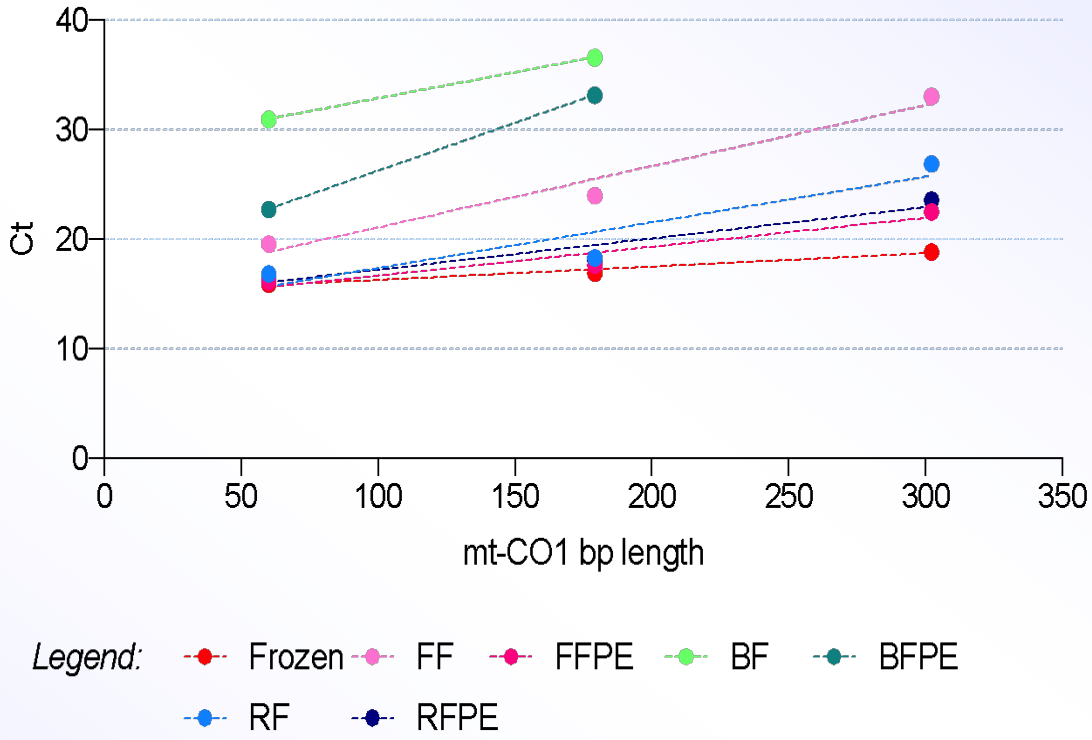
30
25
20
15


40
30
20
10
0
Ct
0
50
100
150
200
250
300
350
mt-CO1 bp length
Legend:
Frozen
FF
FFPE
BF
BFPE
RF
RFPE

RIN/RQI <5 are prone to substantial uncertainty and conclusions about quality are ambiguous.

Differential amplicons,

ΔAmp = CqAmplicon1 –CqAmplicon2 ,

and the amplicon ratio amplicon1/amplicon2 = $2^{\Delta Amp}$ (assuming 100% PCR efficiency and same fluorescence intensity per amplicon) as indicator of RNA integrity. A new RNA marker that is virtually resistant to RNases and found in most eukaryotic cells has been published: Mt-CO1- Mitochondrially encoded cytochrome C oxidase I.

Specimen	ΔAmp M-S (60-179)	ΔAmp L-S (302-60)
Frozen	1.00	2.93
FF	4.43	13.48
BF	5.63	N.A.
RF	1.48	10.05
FFPE	1.50	6.37
BFPE	10.41	N.A.
RFPE	1.44	6.87