CGT Applications: PCR assays to support ATMP development

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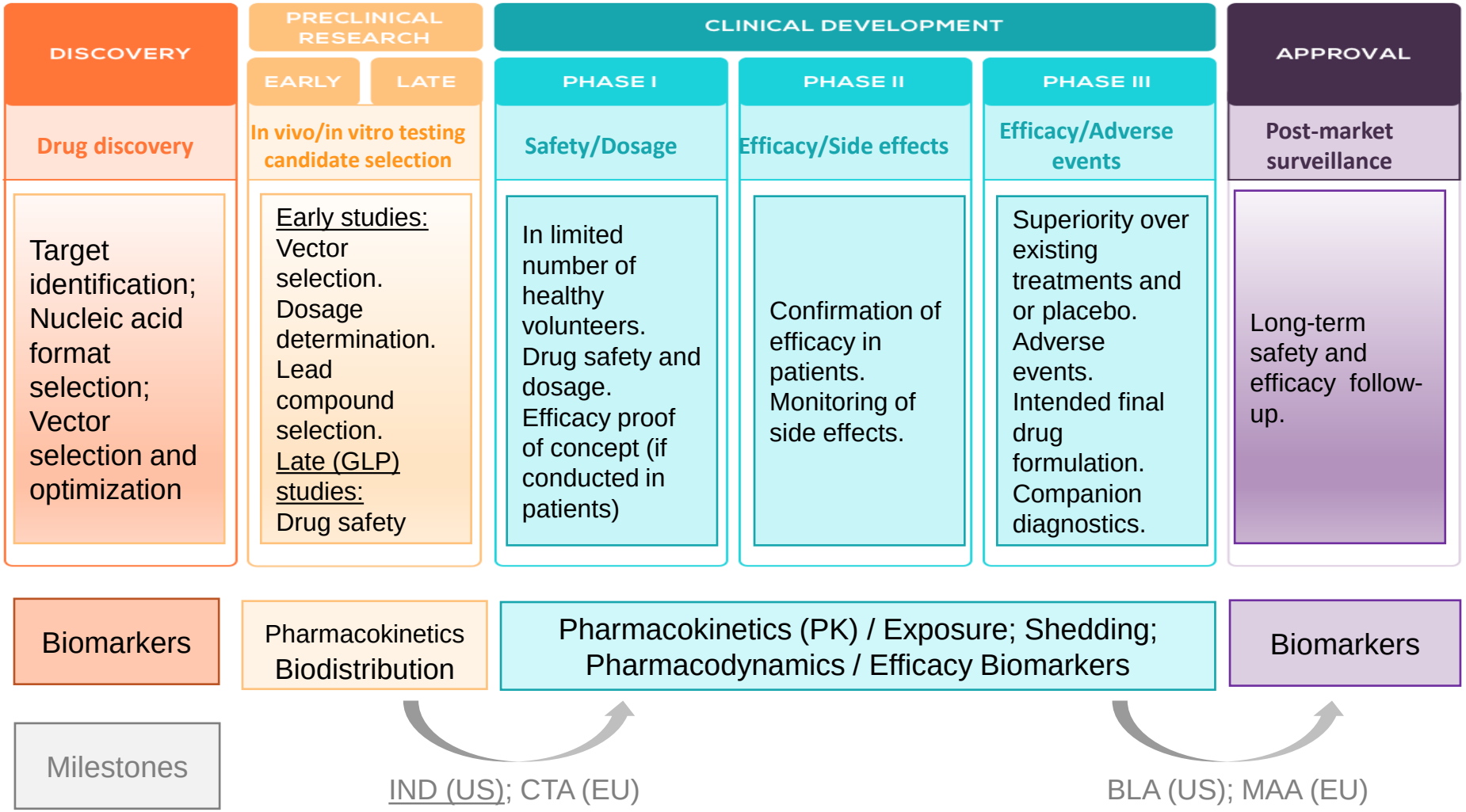
15TH EBF OS – Barcelona - November 17th, 2022

Bioanalysis during ATMP drug development process



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Therapeutic modalities evolution: complexity and main BA platforms

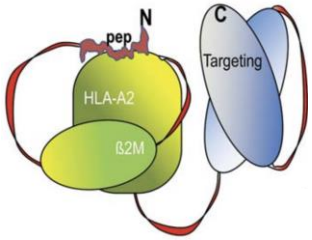
Liquid Chromatography / Mass-Spectrometry (LC-MS)

LMW Compounds
siRNA
Oligos

Peptides

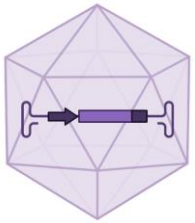
Replacement proteins

mAbs, ADCs

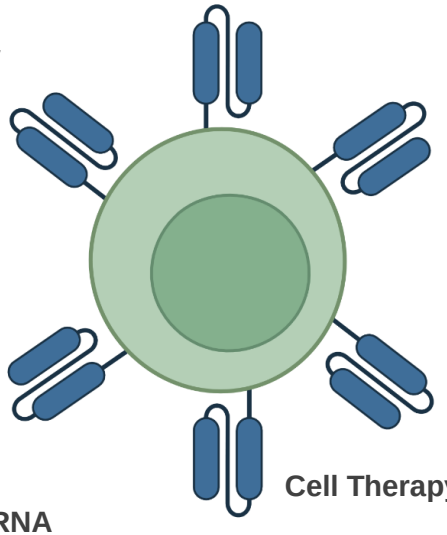


Multidomain fusion proteins

Viral-based gene therapy



Encapsulated RNA



Cell Therapy

Ligand binding assays (LBA); Cell-based assays (CBA); Flow cytometry

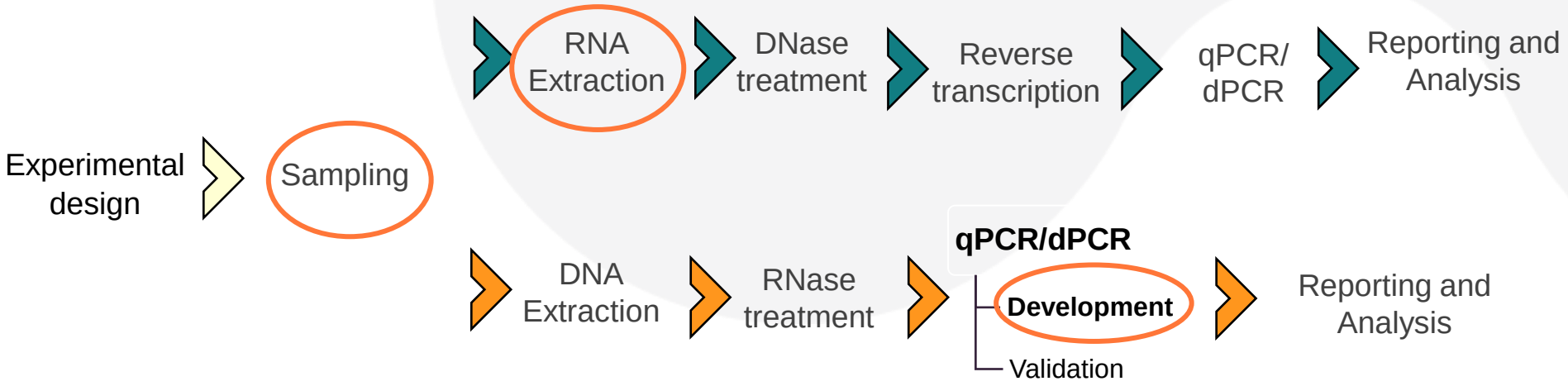
PCR

Guidelines and Guidances for assay validation
-> FDA, EMA, ICHM10…

MIQE quidelines, ISO 20395, white papers, webinars, discussion groups

Adapted from Revers and Furczon (2010); Weidle et al (2012); BioRender

It’s not only about PCR

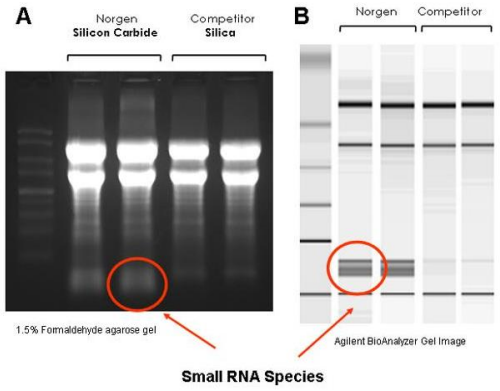


Nucleic Acid extraction and quality control

Experimental design → Sampling → **RNA Extraction** → DNase treatment → Reverse transcription → qPCR/ dPCR → Reporting and Analysis

Sampling → **DNA Extraction** → RNase treatment → qPCR/ dPCR → Reporting and Analysis

- Kit (Brand/Column/Magnetic Beads etc.)
- Lysis (TissueLyzer, Liquid Nitrogen, TRIzol, CTAB)
- Robot/Manual
- Carrier DNA/RNA
- Extraction efficiency (validated DNA/RNA/miRNA Spikes)
- RNase/DNAse Treatment
- Extraction to concentrate target
- Representativeness of extracted material



Quality of extracted material

- Spectroscopy (case study 1)
- Electrophoresis
- Validated PCR assay (case study 2)

Case study 1

- RNA tissue biodistribution study
- Formulated RNAi therapeutics
- Question: does the formulation allow penetration of the vector in the disease-relevant tissue(s)?
- Stage: Go / No Go criteria for the formulation
- Two-tailed PCR assay validation (**Poster A7 -** Focus 2, November 17th, 10:20-11:00)

RNA extraction

Specimen	Extraction Kit	Input (mg)	Avg. A260/A230	Avg. A260/A280	Mean yield* (ng/uL)	%CV of Yield
Brain	A	20 mg	2,0	2,0	161	nd
Kidney	B	10 mg	2,2	2,0	345	20,0
Liver	B	10 mg	2,2	2,1	684	19,7
Muscle	B	5 mg	0,4	1,9	14	13,6
Blood	B	100 uL	0,1	2,8	2	10,8
Blood	B + tissue lyser	100 uL	0,4	3,2	1	17,0
Blood	C	2 mL	0,3	2,1	58	10,5
Urine	D	10 mL	0,6	2,0	9	2,1

*n=15 / specimen, manual

Inhibition

Internally validated Two-Tailed RT-PCR assay (A7)

Delta Cq between:

- control reaction

- reaction containing 6uL of extracted nucleic acid at various dilutions

	Dilution (Times)					
Summary	**0x**	**2x**	**5x**	**10x**	**20x**	**50x**
Brain	1.07	0.81	ND	0.36	ND	ND
Liver	6.89	6.08	ND	2.73	ND	ND
Kidney	0.89	0.22	ND	0.9	ND	ND
Blood	0.23	ND	0.7	ND	0.6	ND
Muscle	0.7	0.39	ND	0.83	ND	ND
CSF	0.05	0.42	0.74	0.45	ND	ND
Plasma	2.11	ND	1.21	0.45	0.35	0.04

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Case study 2

- Pharmacodynamic assay development and optimization
- Aim: circulating extracellular target RNA detection assay as a surrogate for biopsies
- Hepatocytes secrete high levels of mRNAs in exosomes that can be collected and purified from serum or urine which reflect levels of mRNA expression from liver
  - *Taylor (2013)* *The origin, function, and diagnostic potential of RNA within extracellular vesicles present in human biological fluids*
  - *Sehgal et al (2013)* Tissue-specific gene silencing monitored in circulating RNA
- Extraction to concentrate target
- Assessing the quality of minute amounts of starting nucleic acid

Amount and purity of extracted RNA by spectrophotometry

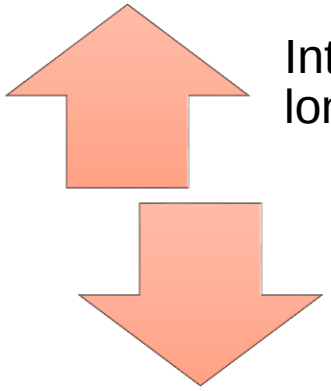
Sample name	RNA (ng/ul)	A260	A260/A280	A260/A230
A1	N/A	-0.02	-0.51	-0.02
A2	N/A	-0.01	-0.18	-0.01
A3	N/A	0.04	0.37	0.02
R1	N/A	0	-0.12	0
R2	N/A	0.02	0.2	0
R3	N/A	0.04	0.34	0
R4	N/A	0	0	0
R5	N/A	-0.01	-0.19	-0.01
R6	N/A	0.01	0.11	0
R7	N/A	0	0.03	0
R8	N/A	0	-0.09	-0.01
R9	N/A	-0.01	-0.19	-0.01
P1	**123.4**	**3.11**	**2.02**	**1.6**
P2	**109.2**	**2.77**	**2**	**1.1**
P3	**57.5**	**2.45**	**2**	**0.46**
NTC1	N/A	-0.01	1.64	-0.01
NTC2	N/A	-0.02	-1.33	-0.02
NTC3	N/A	-0.03	1.76	-0.02

Only control spiked samples (P) exhibit detectable levels of RNA
=> Adequateness of the chosen RNA extraction procedure for small amount of plasma.

Use qPCR to detect nucleic acids and assess their quality

PCR Assay to evaluate RNA quality

Assay	Copies/Genome	Amplicon length (bp)
18S Short	Multiple	74
18S Medium	Multiple	200
18S Long	Multiple	617



Intact mRNA: Cq's of the short and long amplicons are virtually the same

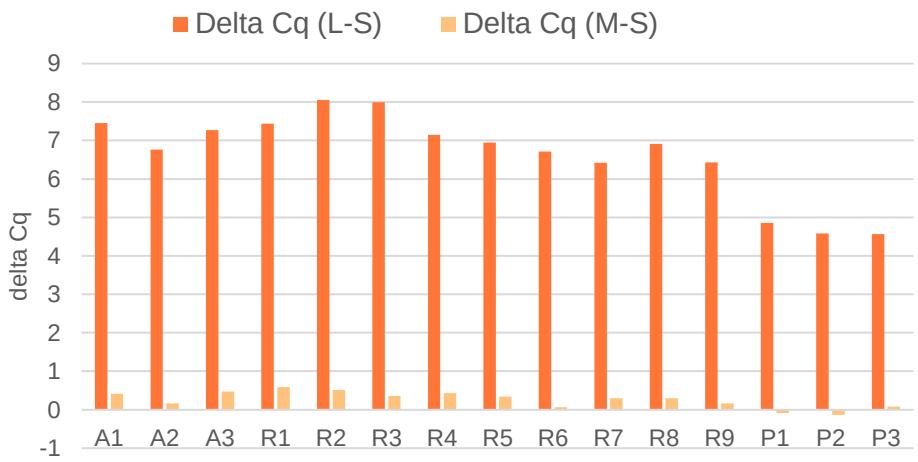
≠ Fragmented RNA: Cq of the longer amplicon is increased relatively to the shorter amplicon

Reference: Bjorkman et al (2016)

Assess RNA integrity by PCR

Difference in quantification cycles (Delta Cq) between long and short and Medium and Short amplicons for the 18S mRNA.

Sample	18S-S	18S-M	18S-L	*Delta Cq (L-S)*	*Delta Cq (M-S)*
A1	22.18	22.60	29.63	7.45	0.41
A2	18.78	18.95	25.54	6.76	0.17
A3	17.81	18.28	25.08	7.27	0.47
R1	19.83	20.41	27.27	7.44	0.58
R2	19.74	20.25	27.79	8.05	0.51
R3	20.38	20.74	28.37	7.99	0.36
R4	18.75	19.17	25.89	7.14	0.43
R5	18.81	19.15	25.76	6.95	0.34
R6	18.72	18.79	25.44	6.72	0.06
R7	17.43	17.72	23.85	6.42	0.29
R8	17.76	18.06	24.67	6.91	0.30
R9	17.78	17.94	24.20	6.43	0.16
P1	10.02	9.94	14.88	4.86	-0.09
P2	10.19	10.06	14.77	4.58	-0.14
P3	10.40	10.48	14.96	4.56	0.08



- Human cell-free mRNA can be extracted from limited volumes of plasma.
- RNAs are 70-200 bp long: RNAs longer than 200 bp are not detected (expected for cf RNA)
  => Proceed with assay development

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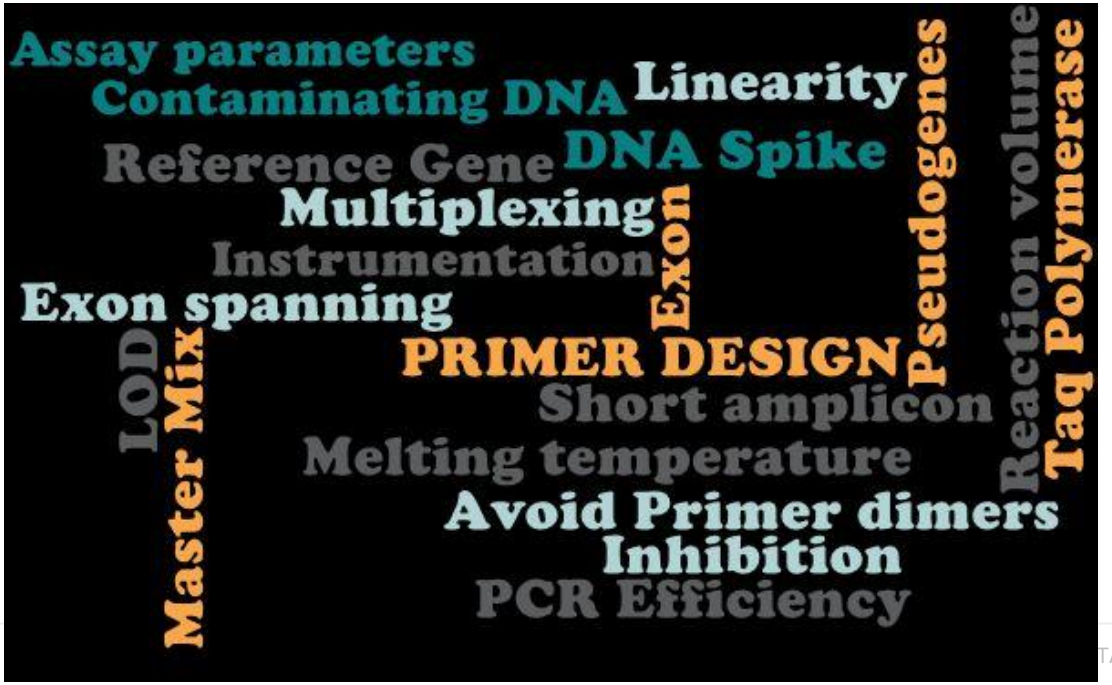
qPCR/dPCR development

Experimental design → Sampling

→ RNA Extraction → DNase treatment → Reverse transcription → qPCR/dPCR → Reporting; Data Analysis

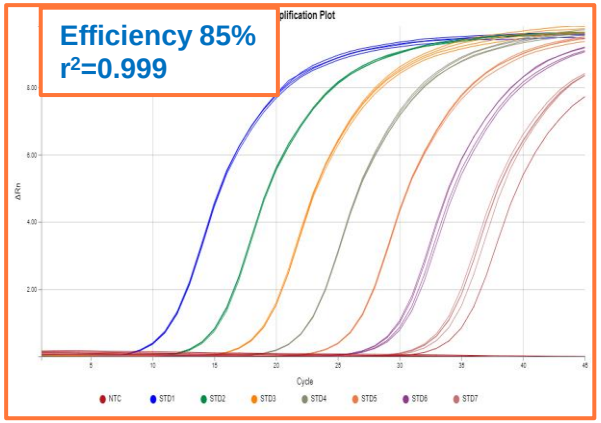
→ DNA Extraction → RNase treatment → **qPCR/dPCR**
- **Development**
- Validation

→ Reporting: Data Analysis



Example A

PRIMERS ONLY

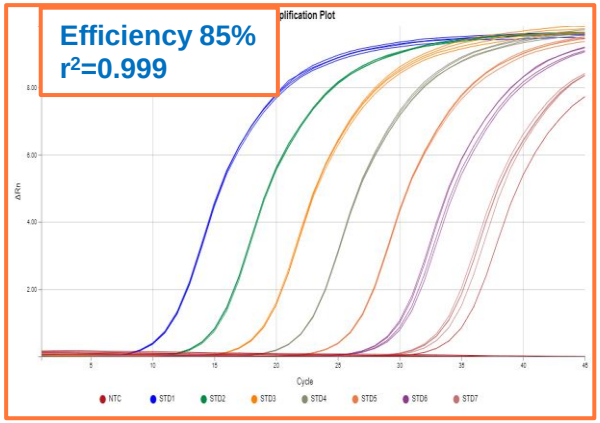


Standards	Cq-primers	Copies per reaction
STD1	10.8	2.00E+07
STD2	14.8	2.00E+06
STD3	18.6	2.00E+05
STD4	22.0	2.00E+04
STD5	26.0	2.00E+03
STD6	29.3	2.00E+02
STD7	33.1	2.00E+01

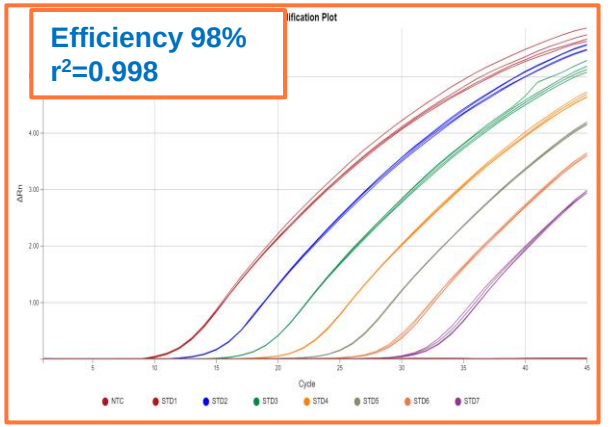
Example A

PRIMERS ONLY

PRIMERS AND PROBE

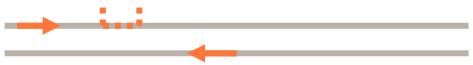


Standards	Cq-primers	Copies per reaction	Cq-primer+probe
STD1	10.8	2.00E+07	12.2
STD2	14.8	2.00E+06	16.1
STD3	18.6	2.00E+05	19.4
STD4	22.0	2.00E+04	22.8
STD5	26.0	2.00E+03	26.0
STD6	29.3	2.00E+02	29.4
STD7	33.1	2.00E+01	32.9



Example B

DESIGN 1



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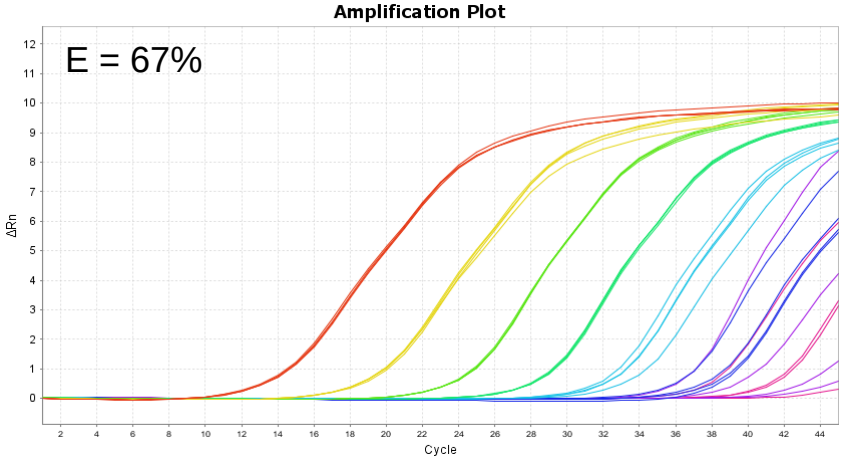
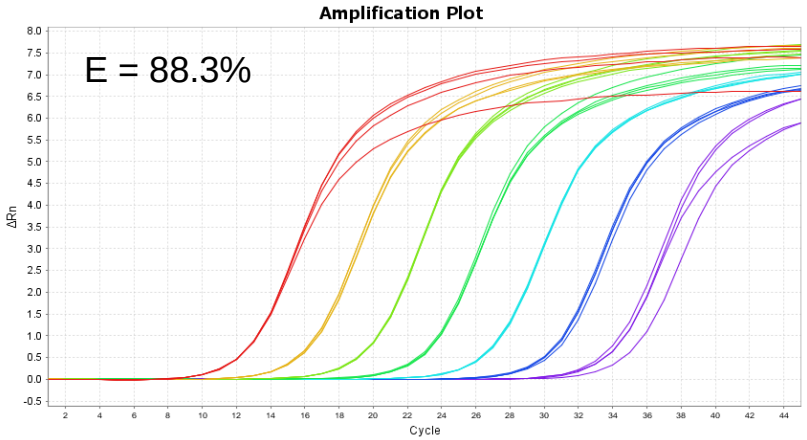
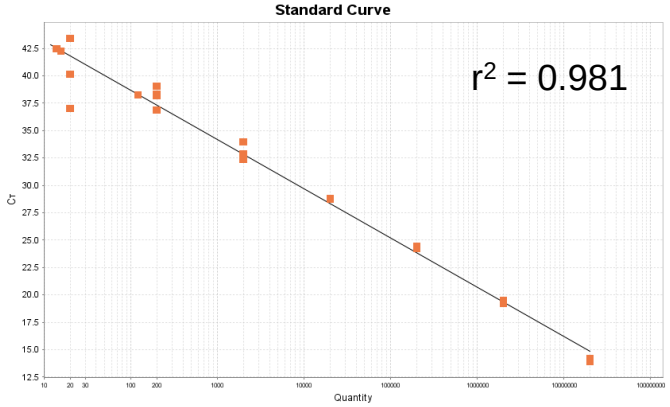
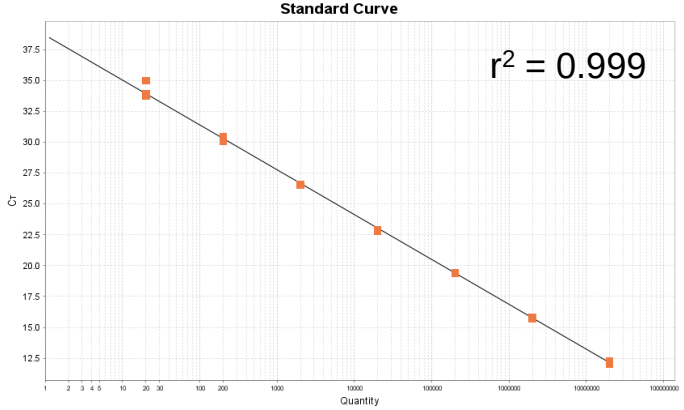






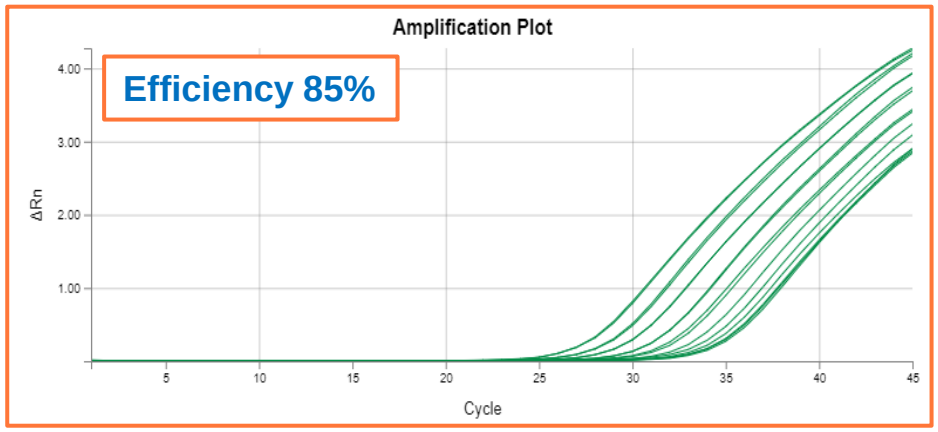


Case study 3

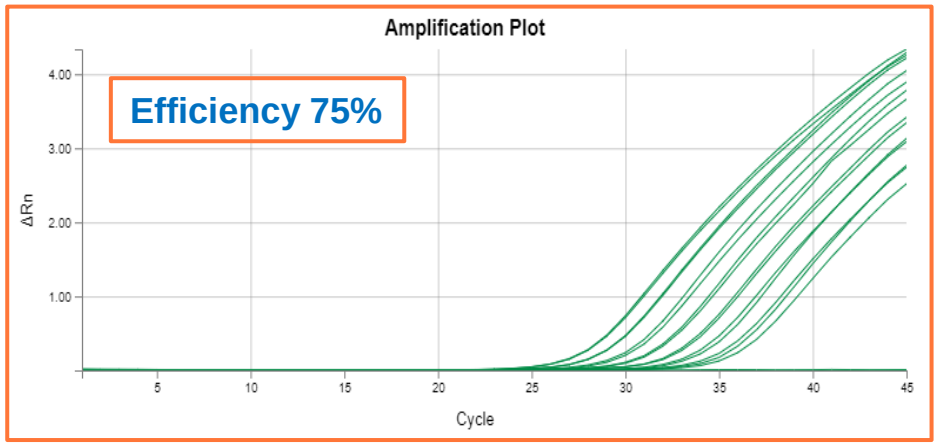
- Case study: Development of a highly sensitive proximal biomarker assay
- Matrix: genomic DNA
- Efficiency value: an absolute roadblock?

  In BA assays based on calibration curves and QCs in matrix

Assay Development



Sample	Cq	Copies per reaction	Cq-CV%	Quantity-CV%	%RE
STD 1 Matrix	27.8	1920	0.2	3.5	3.8
STD 2 Matrix	29.0	960	0.3	5.7	0.5
STD 3 Matrix	29.9	480	0.0	0.2	10.2
STD 4 Matrix	31.4	240	0.0	0.7	-12.4
STD 5 Matrix	32.4	120	1.0	20.7	1.5
STD 6 Matrix	33.7	60	1.0	21.6	-12.0
STD 7 Matrix	34.7	30	0.8	17.2	-3.1
STD 8 Matrix	35.0	20	0.3	6.1	18.5
Efficiency		85.00			
Acceptance criteria			**≤2**	**≤20-25**	**≤20-25**



Sample	Cq	Copies per reaction	Cq-CV%	Quantity-CV%	RE%
STD 1 TE-LPA	28.2	1920	0.2	2.4	-4.6
STD 2 TE-LPA	29.2	960	0.1	1.2	4.7
STD 3 TE-LPA	30.5	480	0.3	5.3	1.3
STD 4 TE-LPA	31.8	240	0.2	3.8	0.6
STD 5 TE-LPA	33.0	120	0.4	7.3	0.4
STD 6 TE-LPA	34.3	60	1.2	22.0	0.4
STD 7 TE.LPA	35.6	30	1.0	20.5	-2.0
STD 8 TE-LPA	-	20	-	-	-
Efficiency		75.00			
Acceptance criteria			**≤2**	**≤20-25**	**≤20-25**

The MAE

- Highly sensitive assay
- Minimal Accepted Efficiency value
- Target value for validation
- Note: absence of inhibition was demonstrated

Run #	E
1	93
2	87
3	90
4	98
5	89
6	86
7	93
8	87
n	*8*
mean	*90,4*
SD	*4,1*
%CV	*4,5*
min	*86,0*
max	*98*
median	*89,5*

Case study 4: sampling and design

- Challenge: studies in non-classical organisms or samples
- Sourcing of representative tissues for assay development and validation?
- Nature of the blank matrix?

Concluding remarks

- Context of Use
- Quality of PCR assay results relies on the quality of the upstream steps
- Reporting?

Thank you!

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