

Validação de métodos em diagnóstico molecular

Gustavo Barra



Essa é mais uma iniciativa educativa da

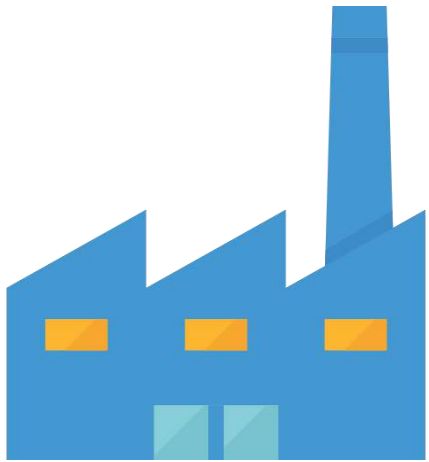
Homenagem e agradecimento

Dr. Marcio Mendes Biasolli
Família Controllab



Ilustração livro Paí, Paí vini-çá
Autor: Marcio Mendes Biasolli

Conceitos e definições

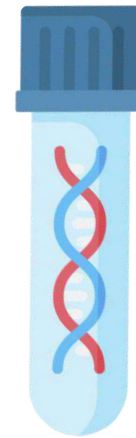


Verificação
E, P, ~~LOD~~, IR,



Validação

- E, P, LOD, LOQ, IR
- Comparação
- Recuperação
- Clínica
- Em andamento
- Baseada no método



**Diagnóstico
Molecular**

- Detectar
- Tipar
- Quantificar
- DNA/RNA

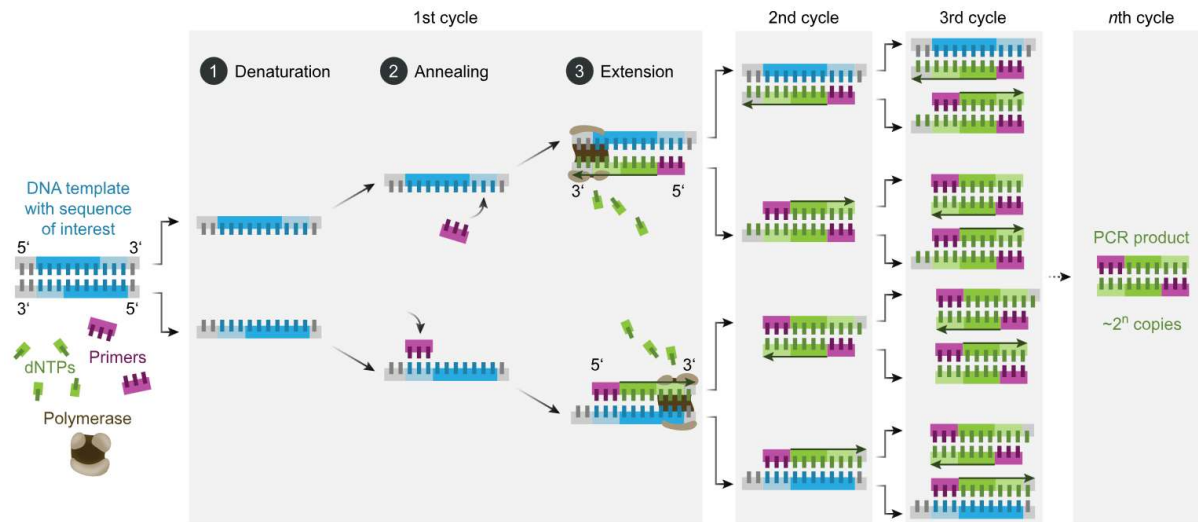


**Teste in-house
(LDT)**

- Novo/modificado
- Exclusivos
- Validade clínica
- PCR, NGS, HPLC, MALDI-TOF

Diagnóstico molecular

qPCR



Cq

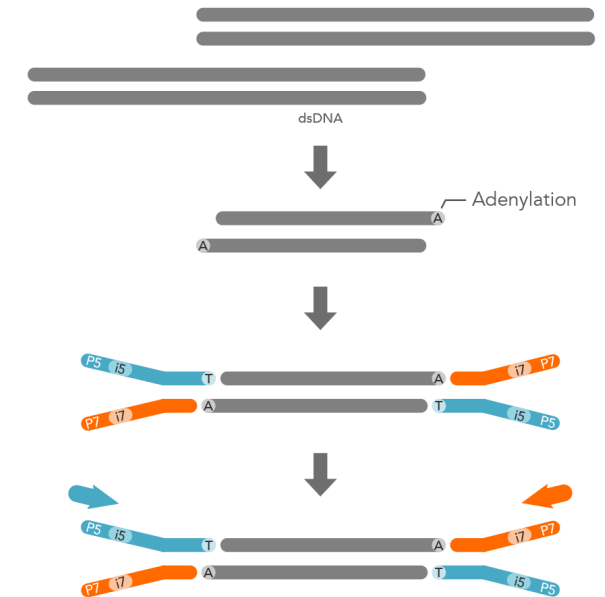
NGS

Fragmentation

End repair and A-tailing

Ligation

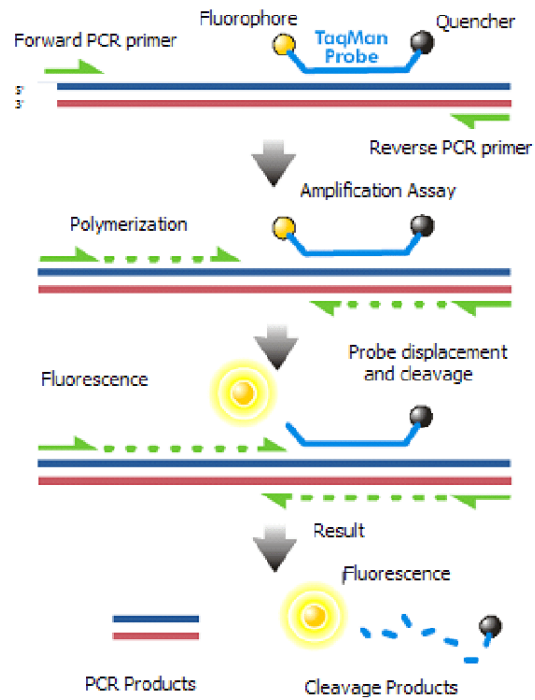
PCR amplification



ACTGCTGA

Diagnóstico molecular

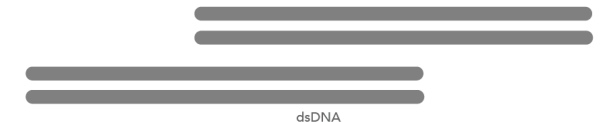
qPCR



Cq

NGS

Fragmentation



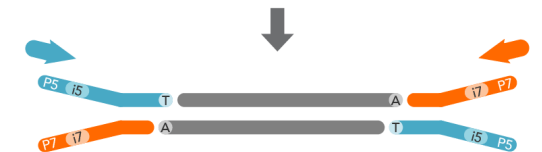
End repair and A-tailing



Ligation



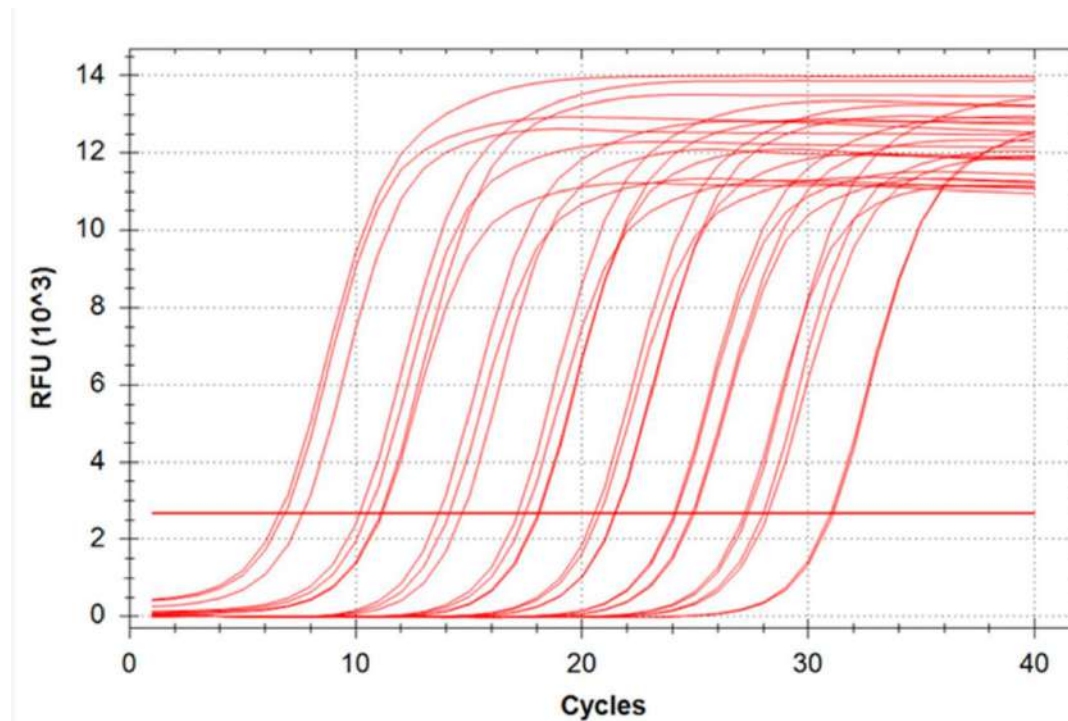
PCR amplification



ACTGCTGA

Diagnóstico molecular

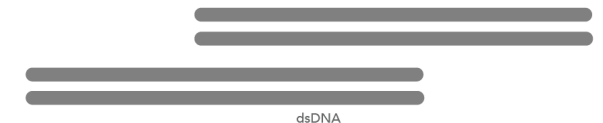
qPCR



Cq

NGS

Fragmentation



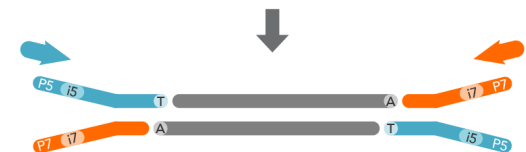
End repair and A-tailing



Ligation



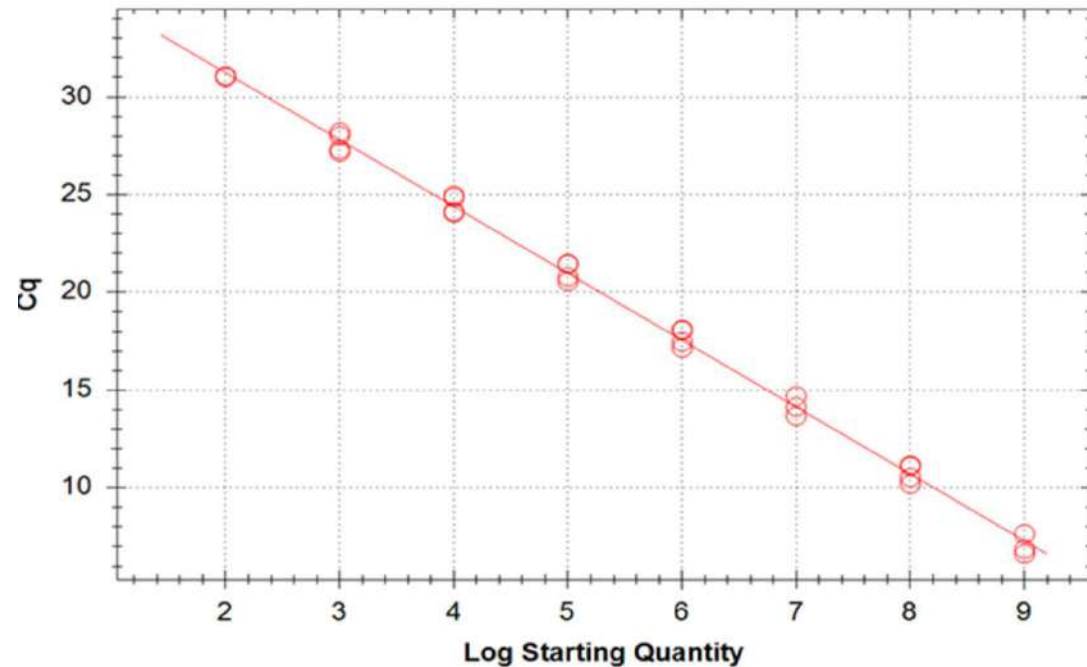
PCR amplification



ACTGCTGA

Diagnóstico molecular

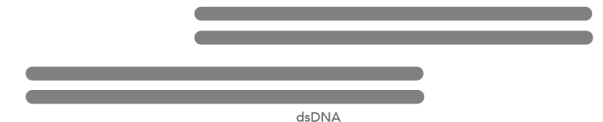
qPCR



Cq

NGS

Fragmentation



End repair and A-tailing



Ligation



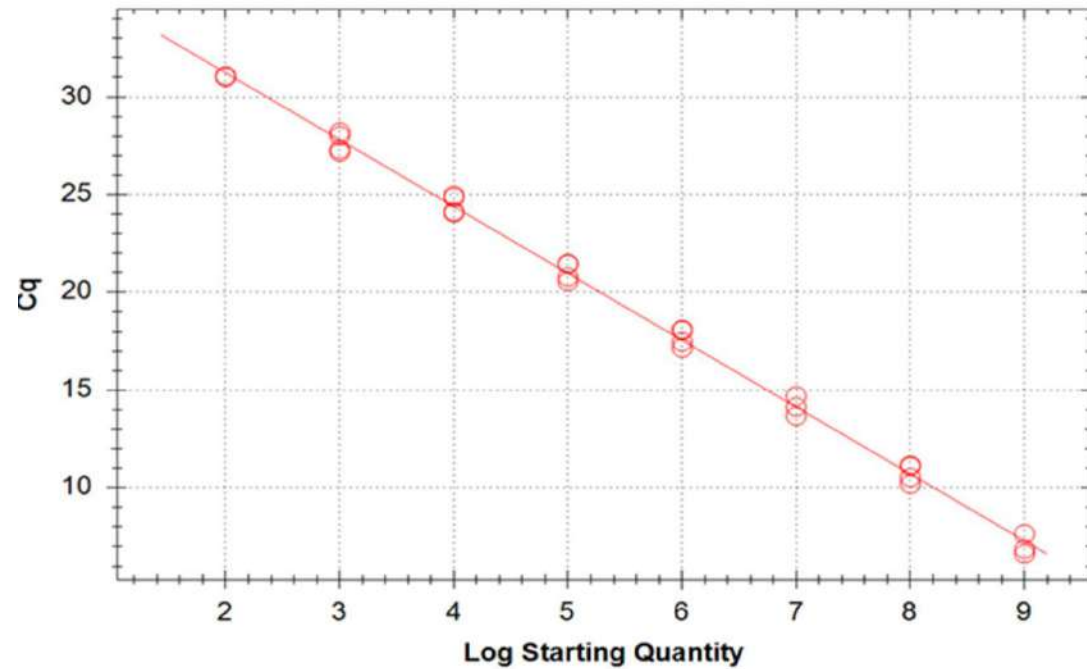
PCR amplification



ACTGCTGA

Diagnóstico molecular

qPCR



Cq

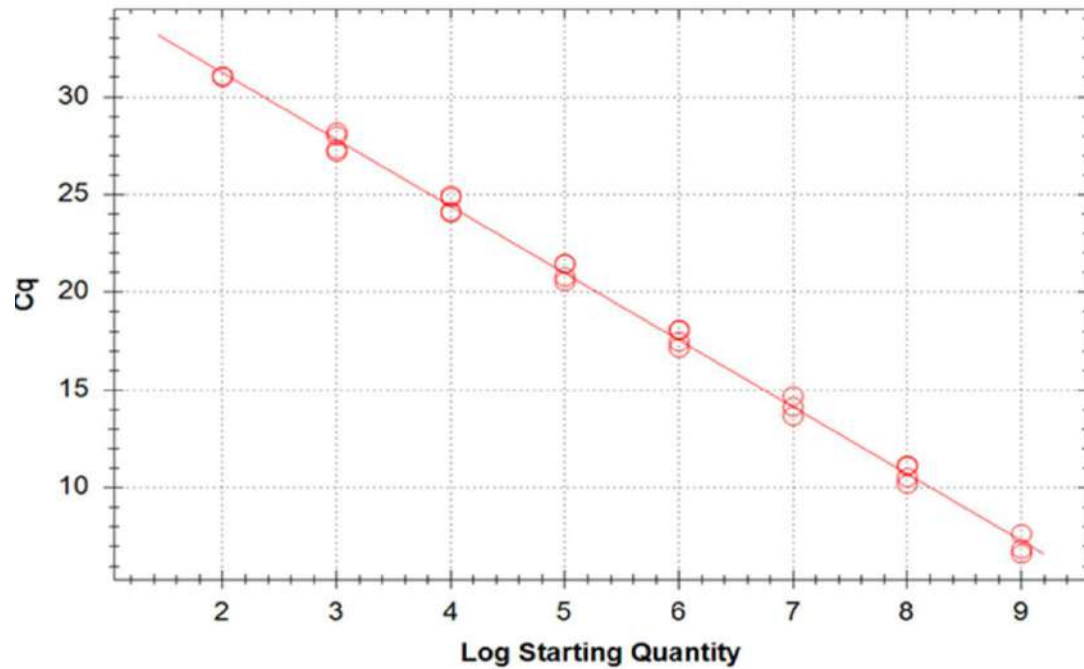
NGS



ACTGCTGA

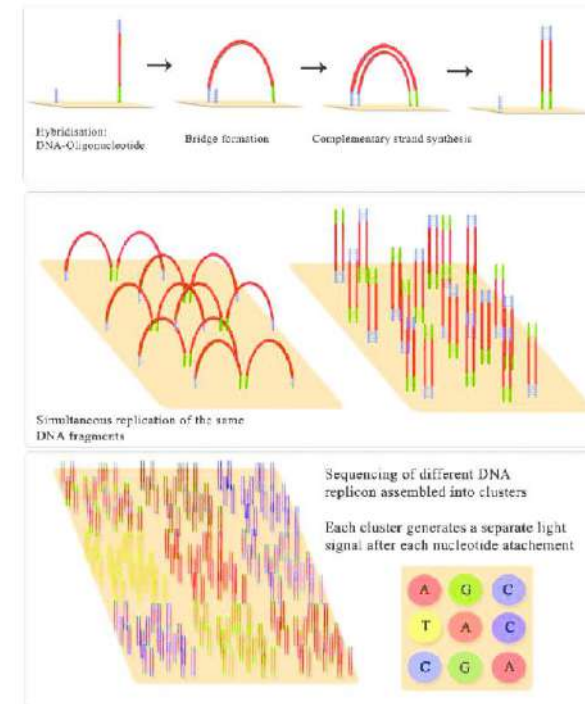
Diagnóstico molecular

qPCR



Cq

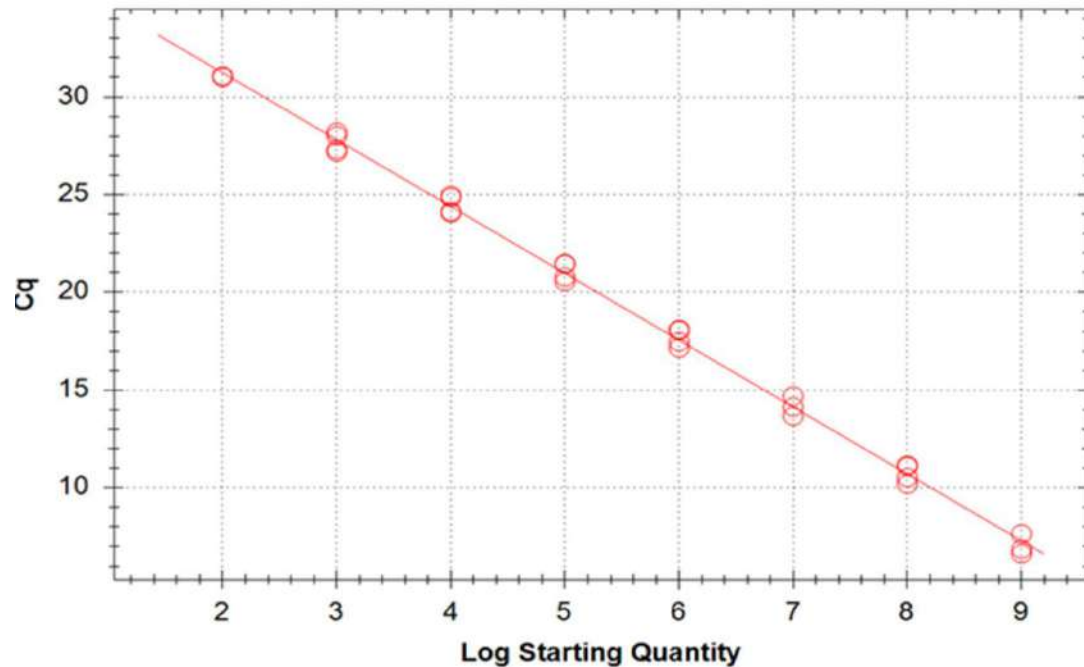
NGS



ACTGCTGA

Diagnóstico molecular

qPCR



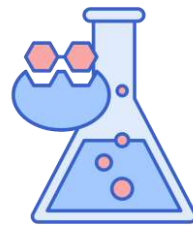
Cq

NGS



ACTGCTGA

Diagnóstico molecular

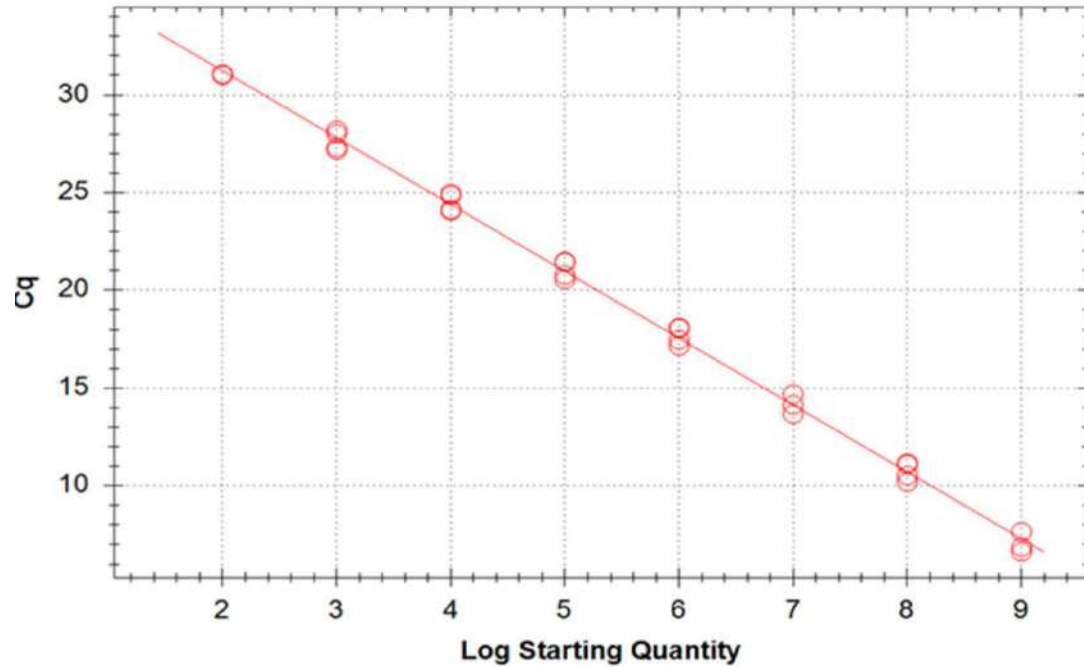


Enzimologia



Biologia sintética

qPCR



Cq

NGS



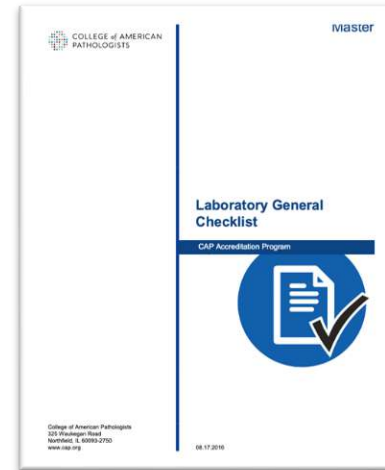
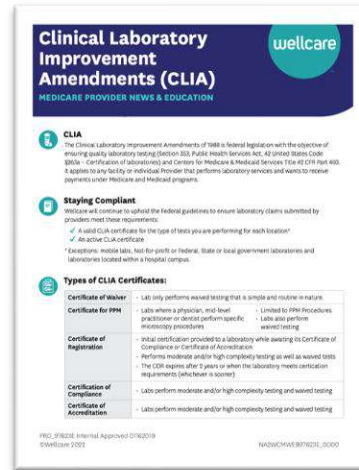
ACTGCTGA

Regras do jogo – métodos próprios (LDT)

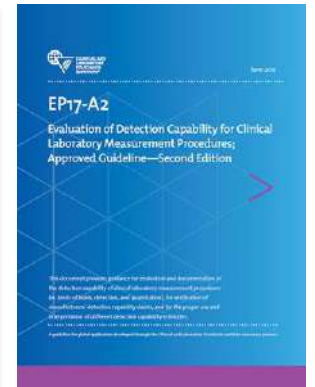
Regulamentação
“Autoriza a fazer”



Certificações
“O que fazer”

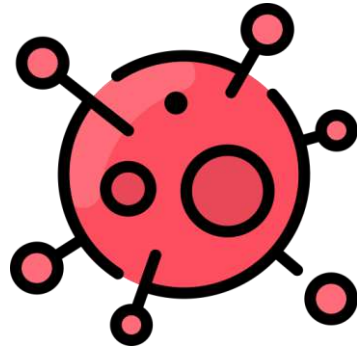


Guidelines e referências
“Como fazer”

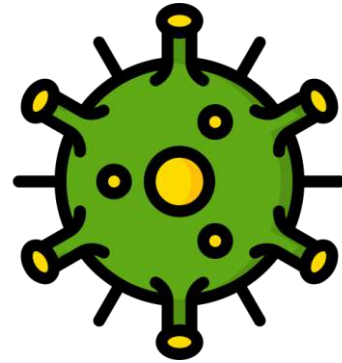


Métodos próprios - Surtos, epidemias e genômica

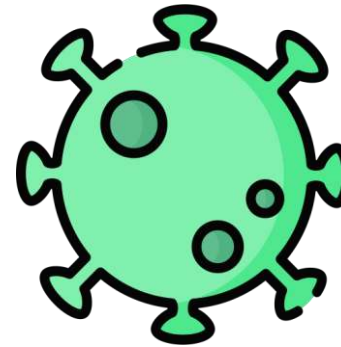
qPCR



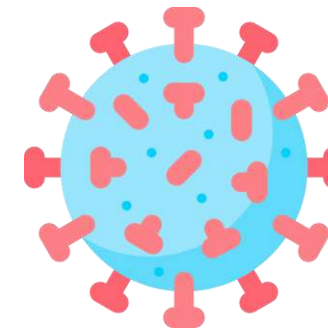
H1N1
2009



CHIKV
2015



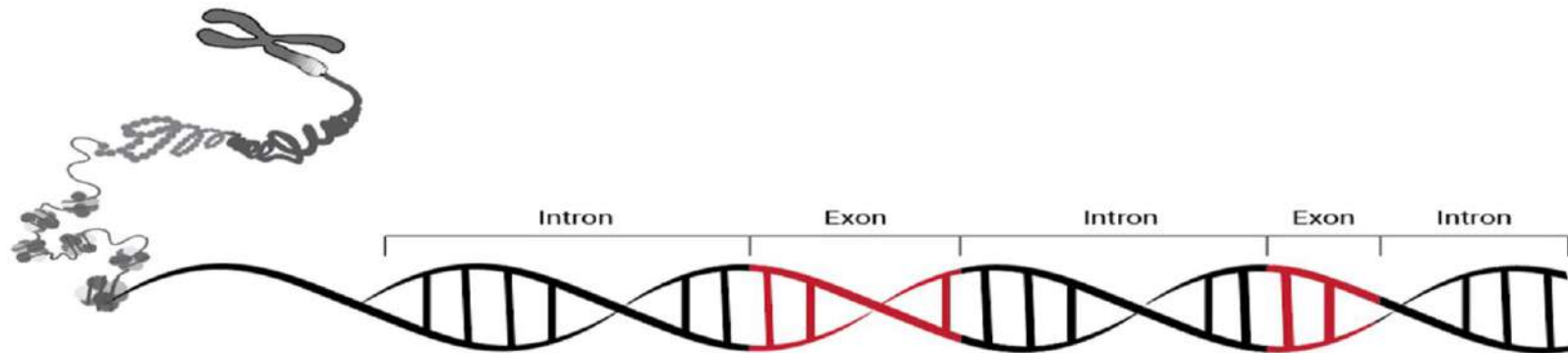
ZIKV
2016



SARS-COV2
2019

AE

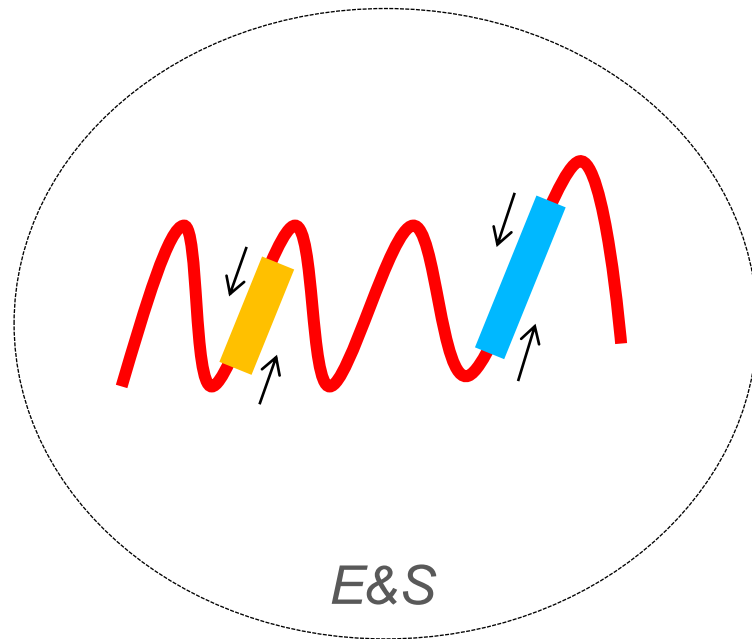
NGS



BM

Ensaaios independentes

qPCR

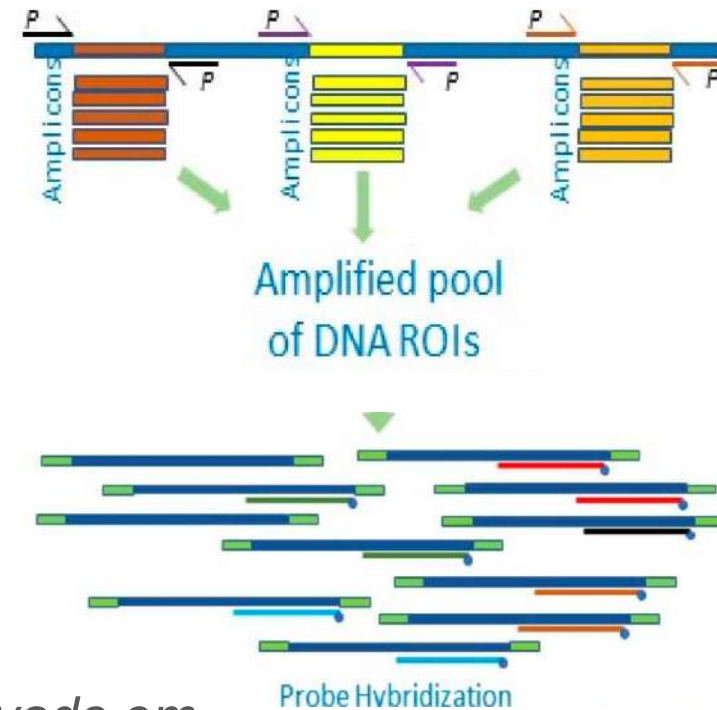


Primers

Concordância
T, P, e N

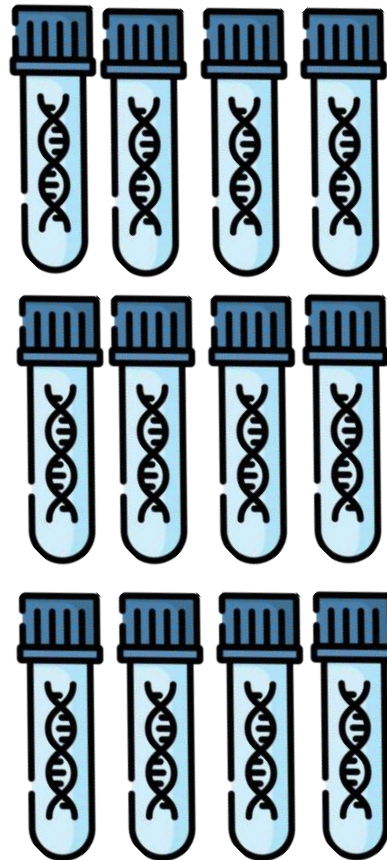
“Se mesmo resultado for observado em pelo menos dois ensaios independentes e o método destes ensaios já é estabelecido o resultado é considerado verdadeiro”

NGS



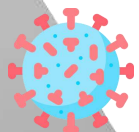
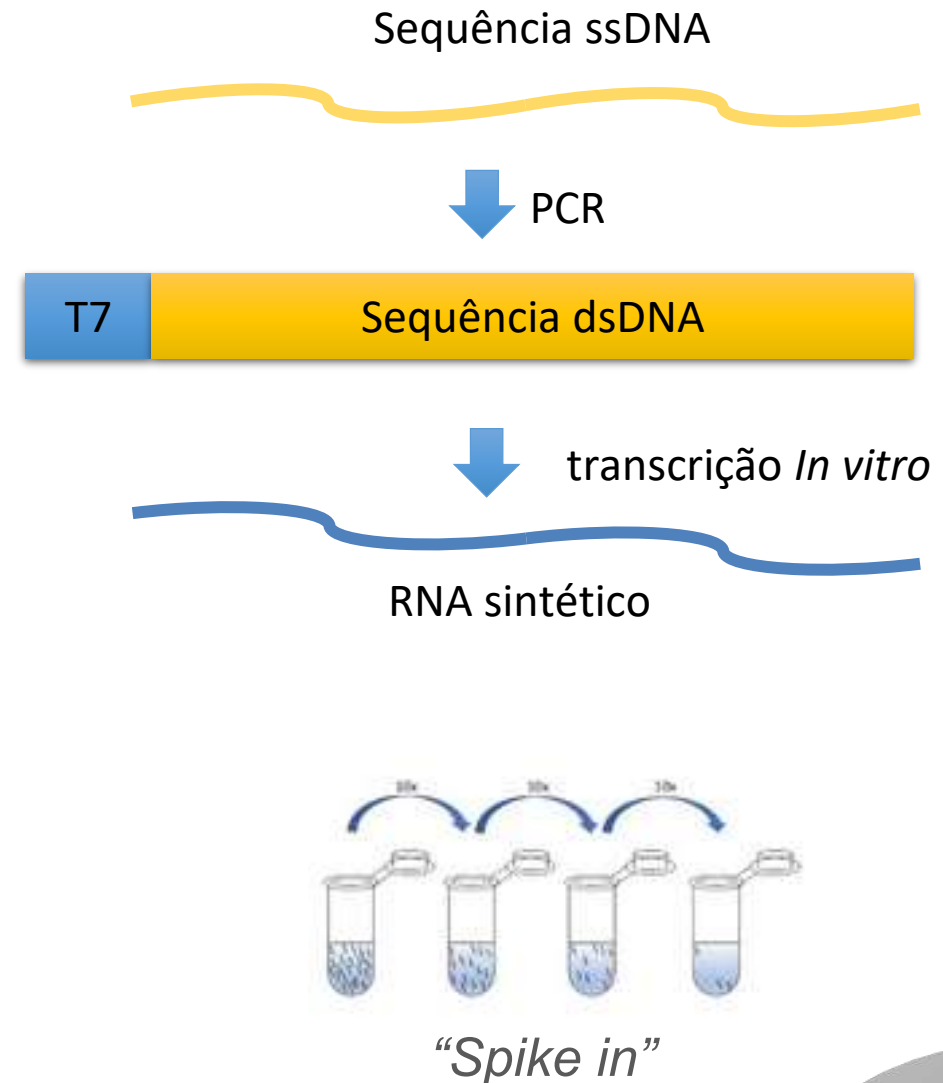
Diagnostics **2022**, 12(7), 1539;

Material biológico e fase pré-analítica



N=60

Amostras com resultado conhecido
Conservante

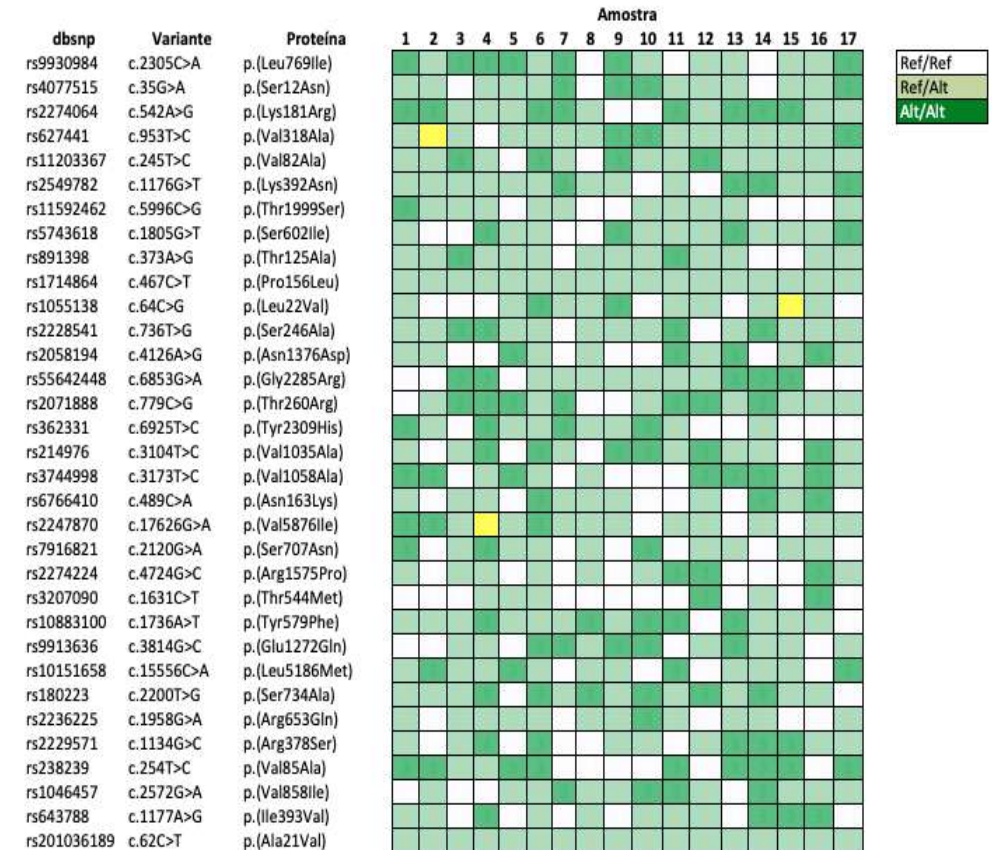


Quantidade de amostras (n)

qPCR

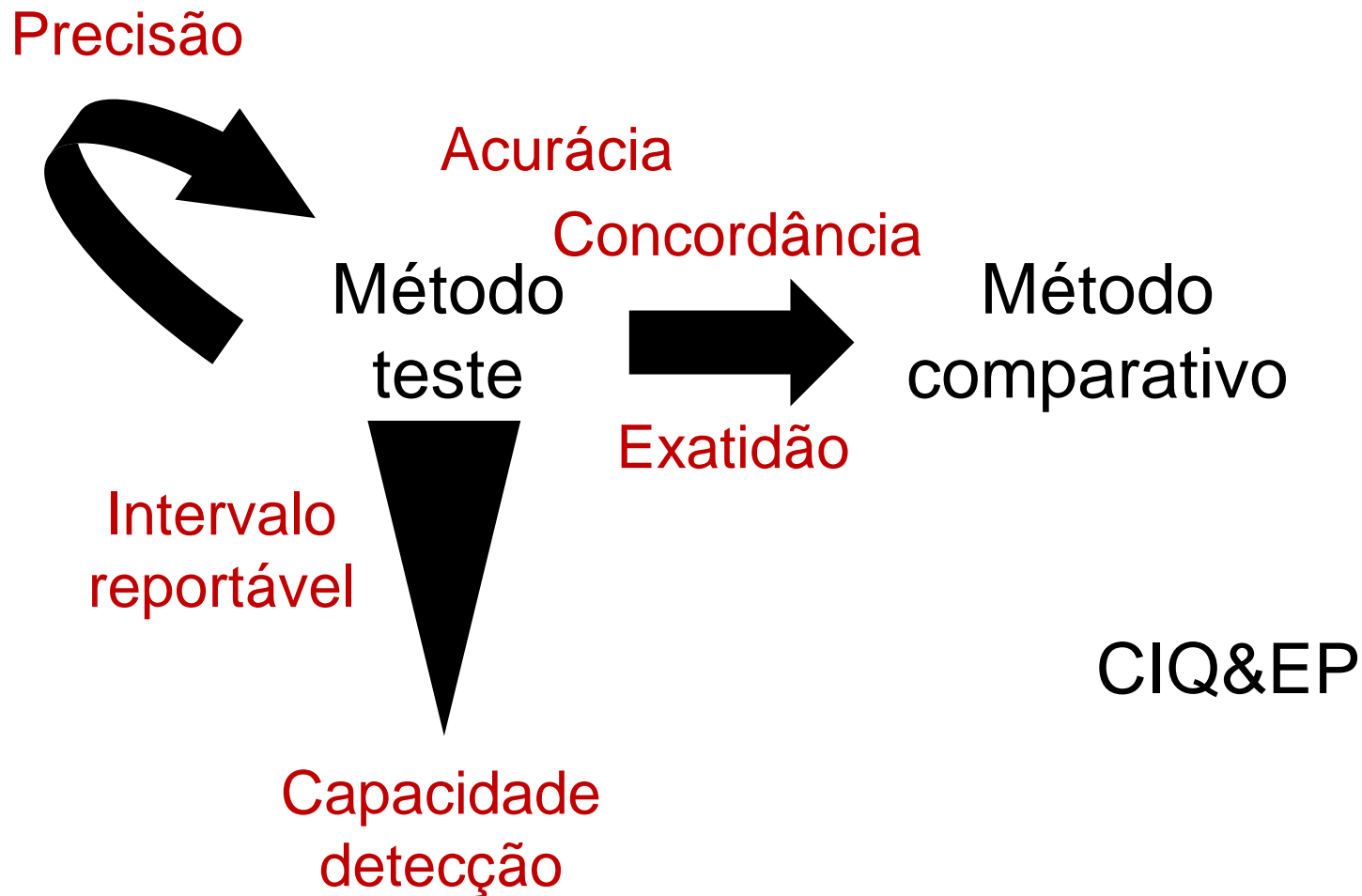
Tamanho amostral (n)	Concordância	Limite Inferior (95% CI)
10	100%	69,15%
20	100%	83,16%
30	100%	88,43%
40	100%	91,24%
50	100%	92,87%
60	100%	94,04%
300	100%	98,78%

NGS



n = 558/561 - 99,4% - (95%CI 98,4-99,9%)

Parâmetros de desempenho



Validação ensaio molecular qualitativo - exemplo

Open Access

Article

Analytical Sensitivity and Specificity of Two RT-qPCR Protocols for SARS-CoV-2 Detection Performed in an Automated Workflow

by  Gustavo Barcelos Barra ^{*,†}  ,  Ticiane Henriques Santa Rita [†] ,  Pedro Góes Mesquita [†] ,
 Rafael Henriques Jácomo  and  Lídia Freire Abdalla Nery 

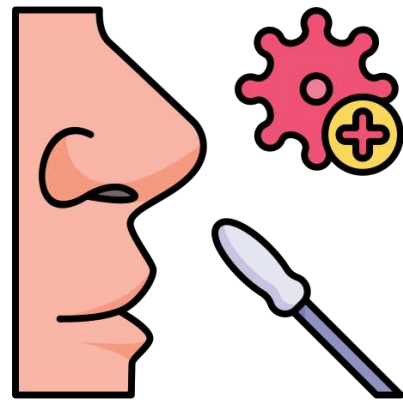
Research and development department, Sabin diagnostic medicine, Brasilia 70.632, Brazil

* Author to whom correspondence should be addressed.

† These authors contributed equally to this work.

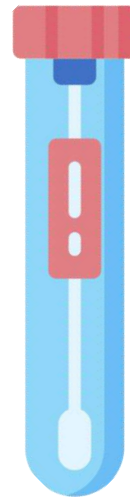
Genes **2020**, *11*(10), 1183; <https://doi.org/10.3390/genes11101183>

Amostras



Swab Nasal
(n=60, saudáveis)

“Spike in”



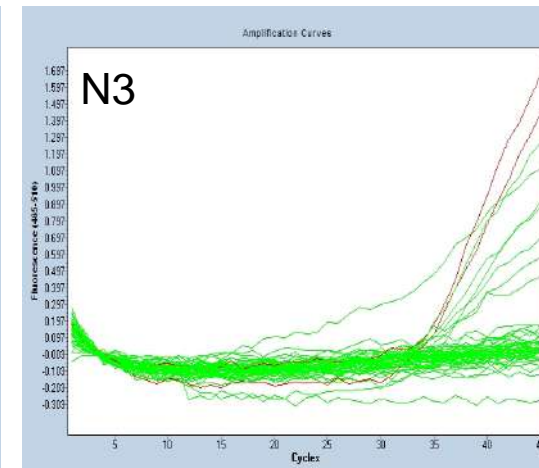
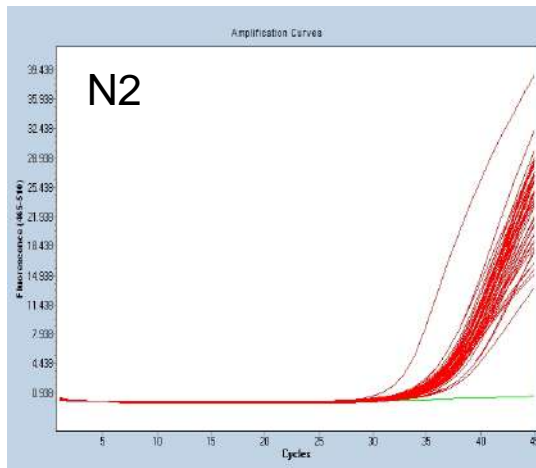
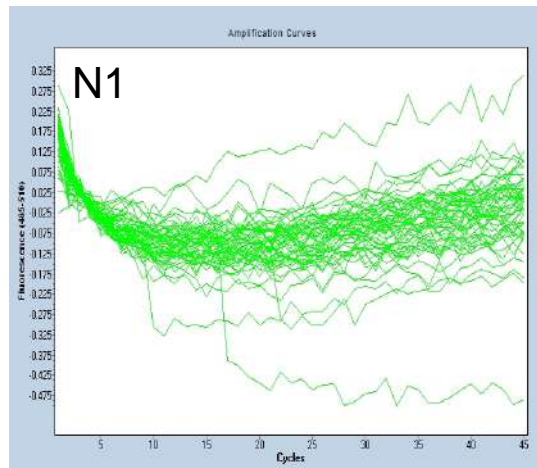
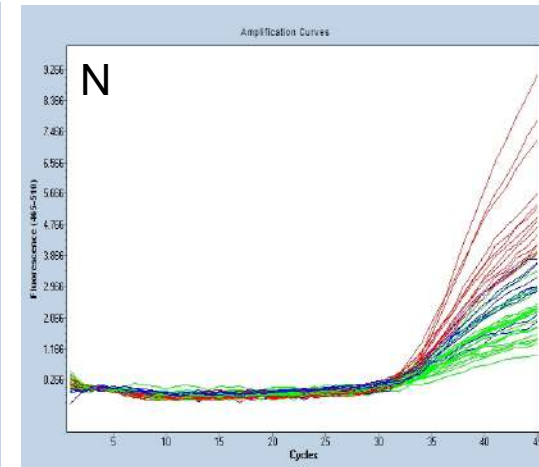
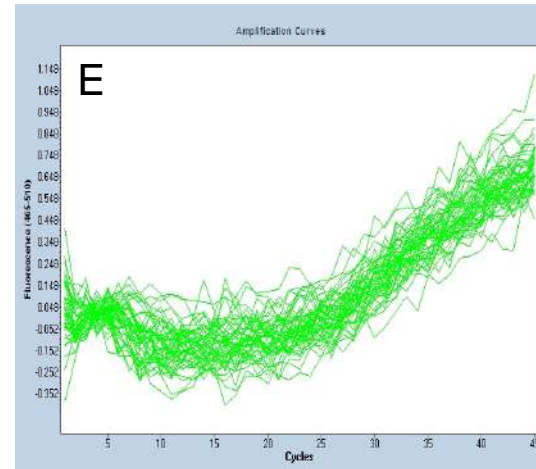
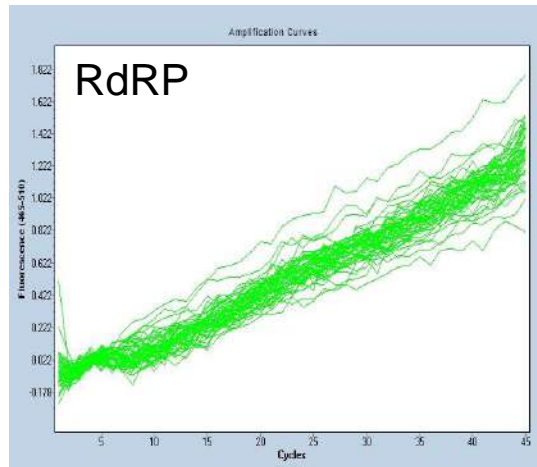
T7 promoter

RdRP

TAATACGACTCACTATAGGGGTGAAATGGTCATG
 TGTGGCGGTTCACTATATGTTAAACCAGGTGGAA
 CCTCATCAGGAGATGCCACAACCTGCTTATGCTAA
 TAGTGTTTTTAACATTTGACAGGTACGTTAATAG
 TTAATAGCGTACTTCTTTTTCTTGCTTTCGTGGT
 E ATTCTTGCTAGTTACACTAGCCATCCTTACTGCG
 CTTGATTGTGTGCGTACTGCTGCAATATGACCC
 N1 CAAAATCAGCGAAATGCACCCCGCATTACGTTTG
 GTGGACCCTCAGATTCAACTGGCAGTAACCAGAT
 N2 TACAAACATTGGCCGCAAATTGCACAATTTGCC
 CCAGCGCTTCAGCGTTCTTCGGAATGTCGCGCGG
 GAGCCTTGAATACACCAAAGATCACATTGGCAC
 CCGCAATCCTGCTAACAAATGCTGCAATCGTGCTA
 N and N3 CAACTTCCTCAAGGAACAACATTGCCAAAAGGCT
 TCTACGCAGAAGGGAGCAGAGGGCGGCAGTCAAGC
 CTCTTCTCGTTCCTC

Quantidade (cópias)

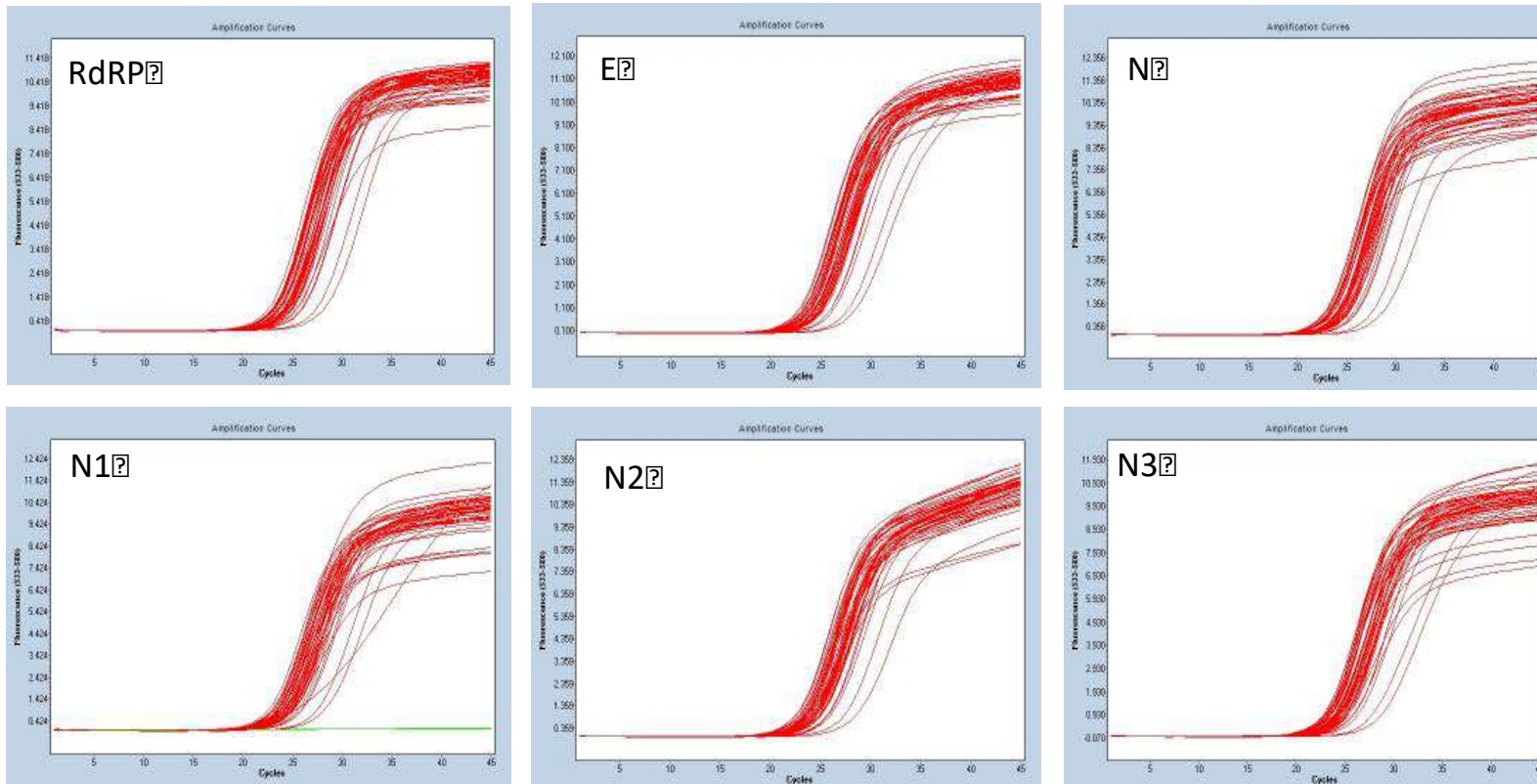
Especificidade analítica - 6 ensaios



n=60, saudáveis

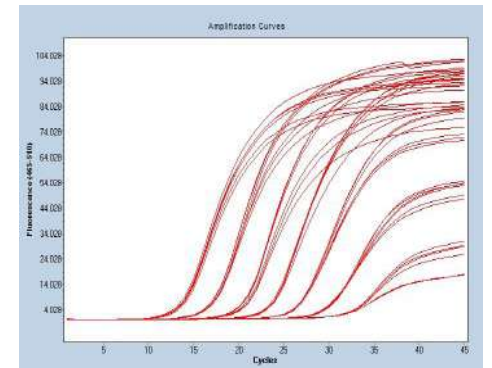
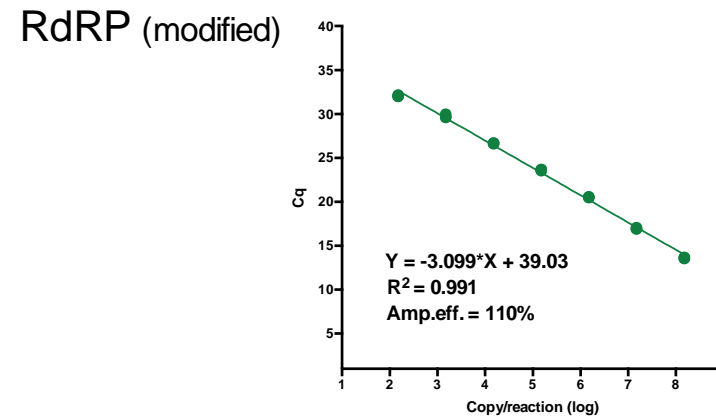
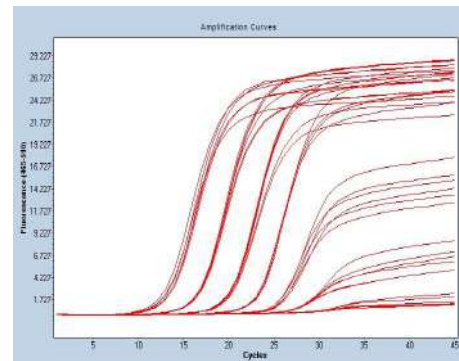
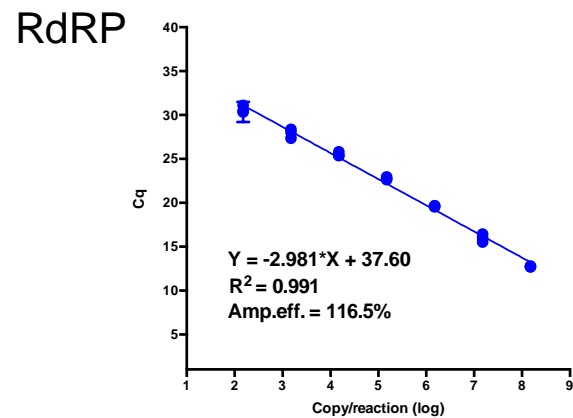
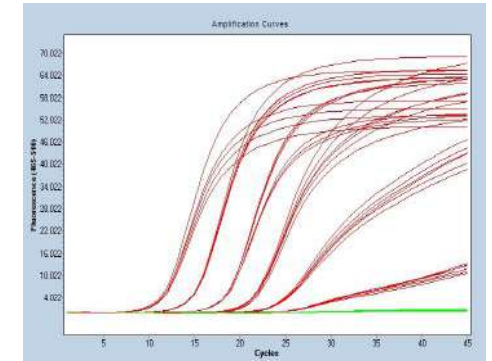
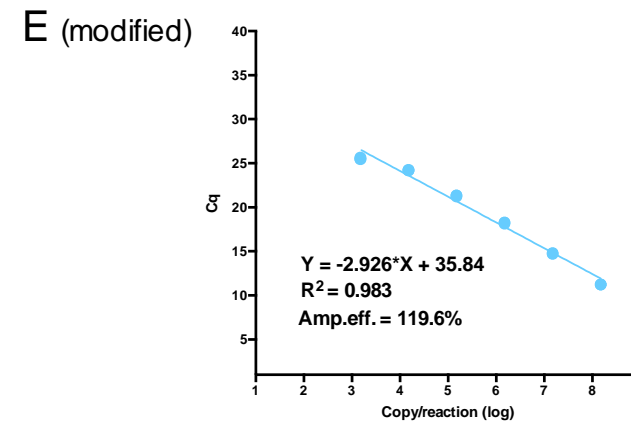
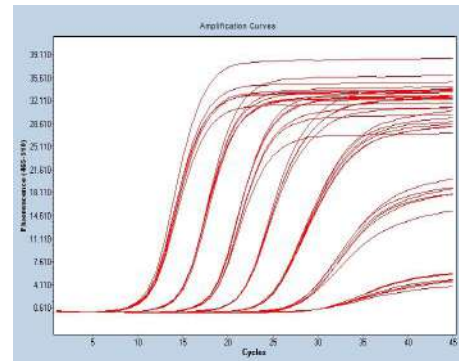
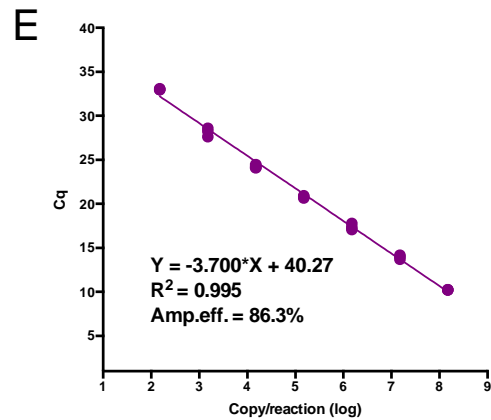
Controle de reação – RNase P

Sample Control (RPP30) (HEX)



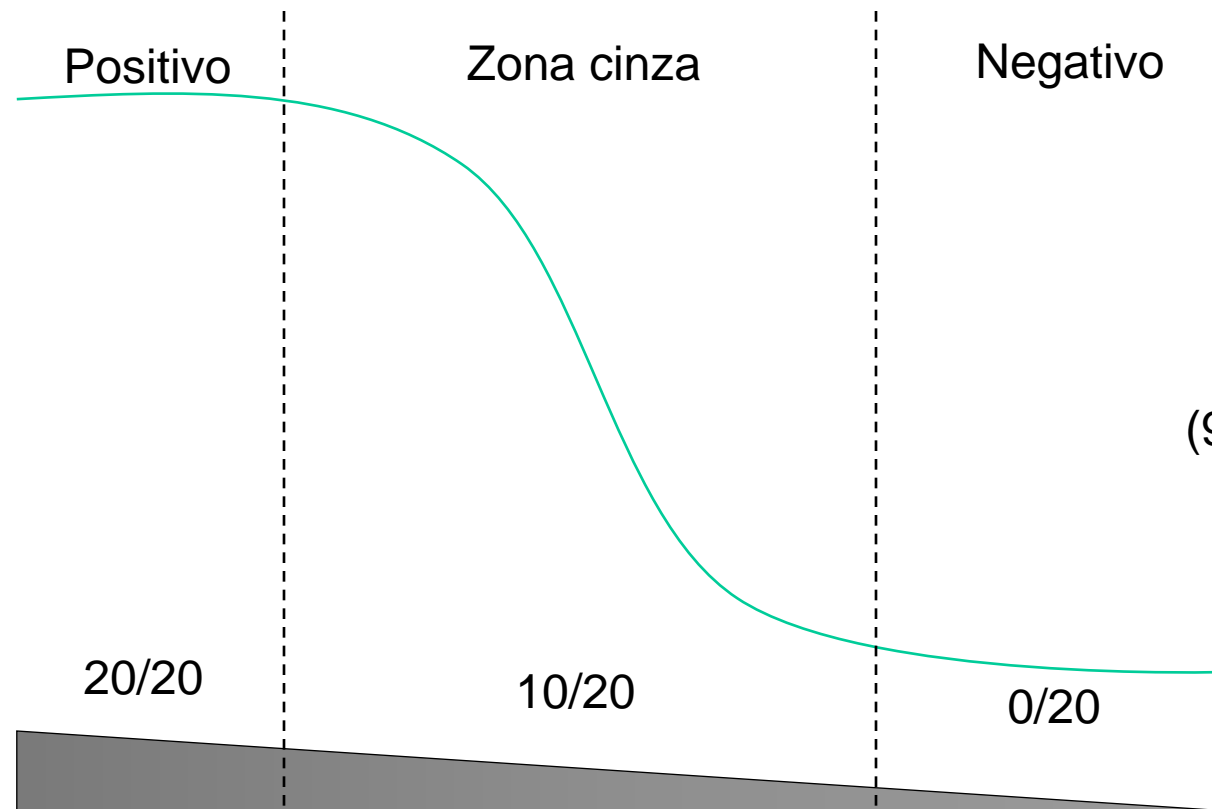
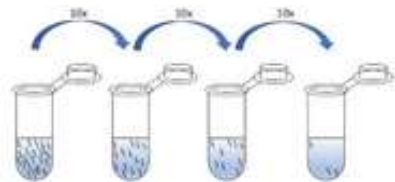
n=60, saudáveis

Intervalo reportável – 1:10



Alto, médio, baixo

LOD e precisão - Qualitativo

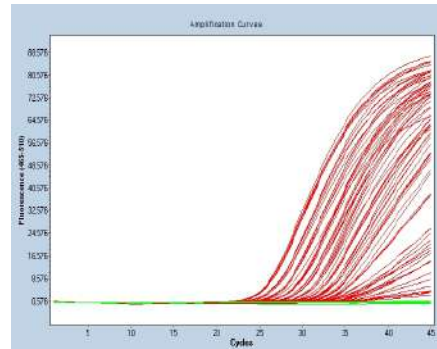
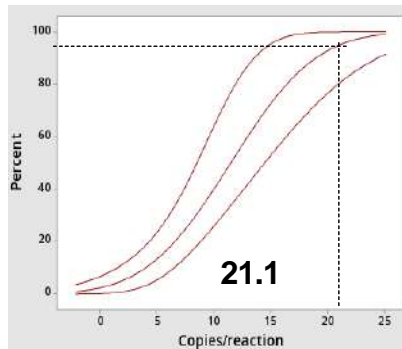


Regressão probit
LOD 95
25.6 cópias/PCR
(95% CI - 14-85 cópias/PCR)

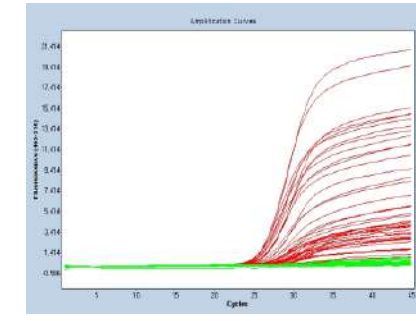
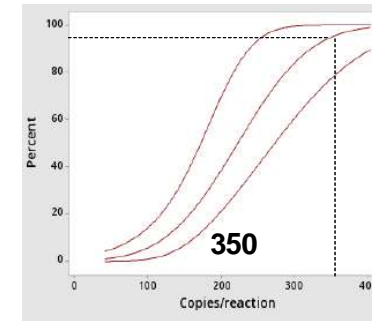
1x LOD	72	36	18	9	4,5	2,25	1,12	0,66	Cópias/PCR	Diluição 1:2
4 x 5 Precisão	72				7,2			0,72	Cópias/PCR	Diluição 1:10

LOD e precisão - Qualitativo

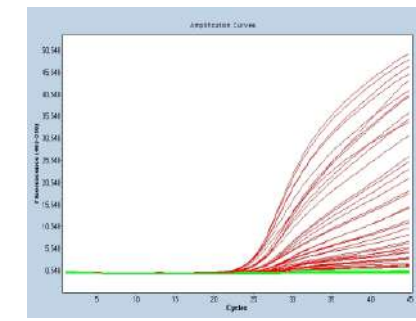
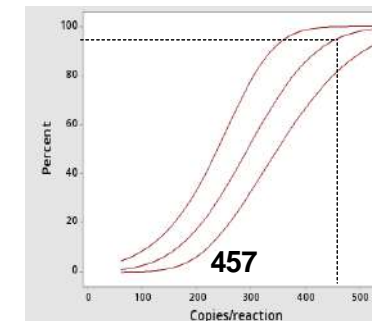
N1



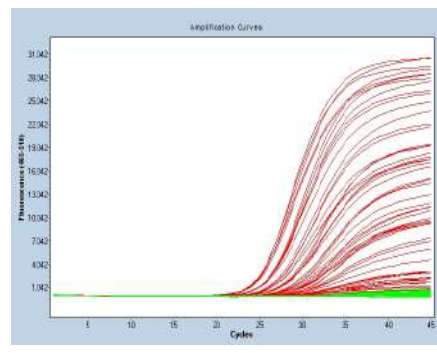
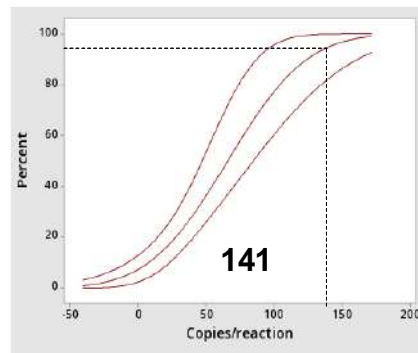
RdRP



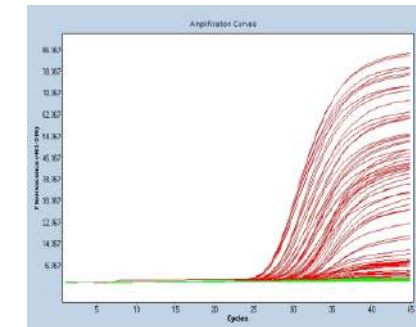
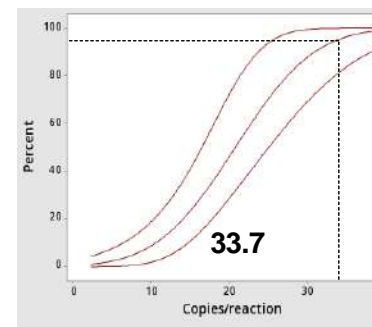
E (modified)



E



RdRP (modified)



Regressão probit

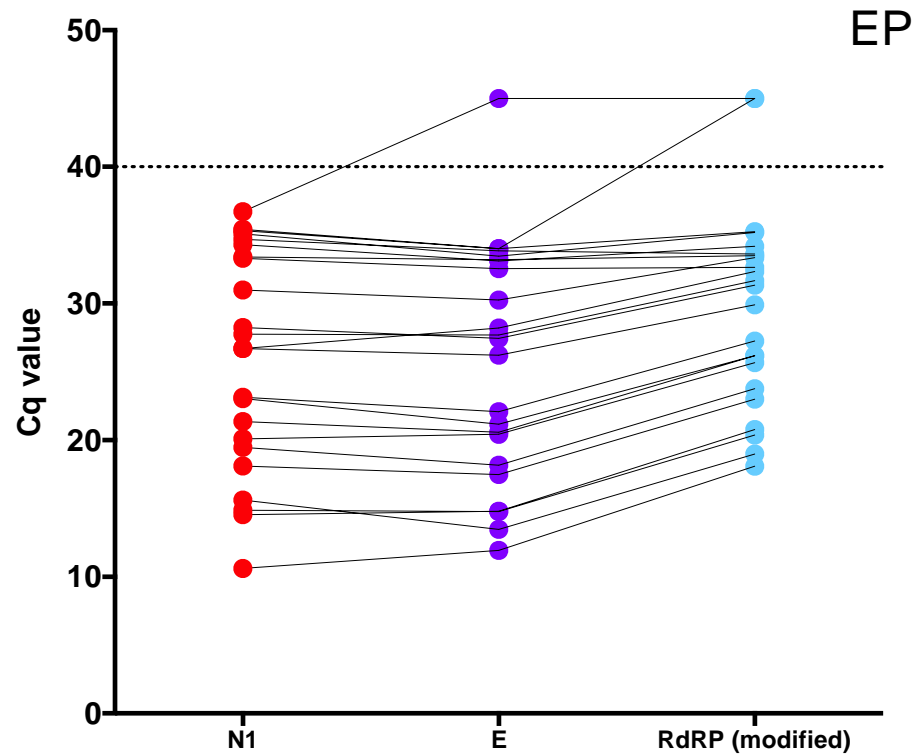
Diluição 1:2

LOD e precisão - Qualitativo

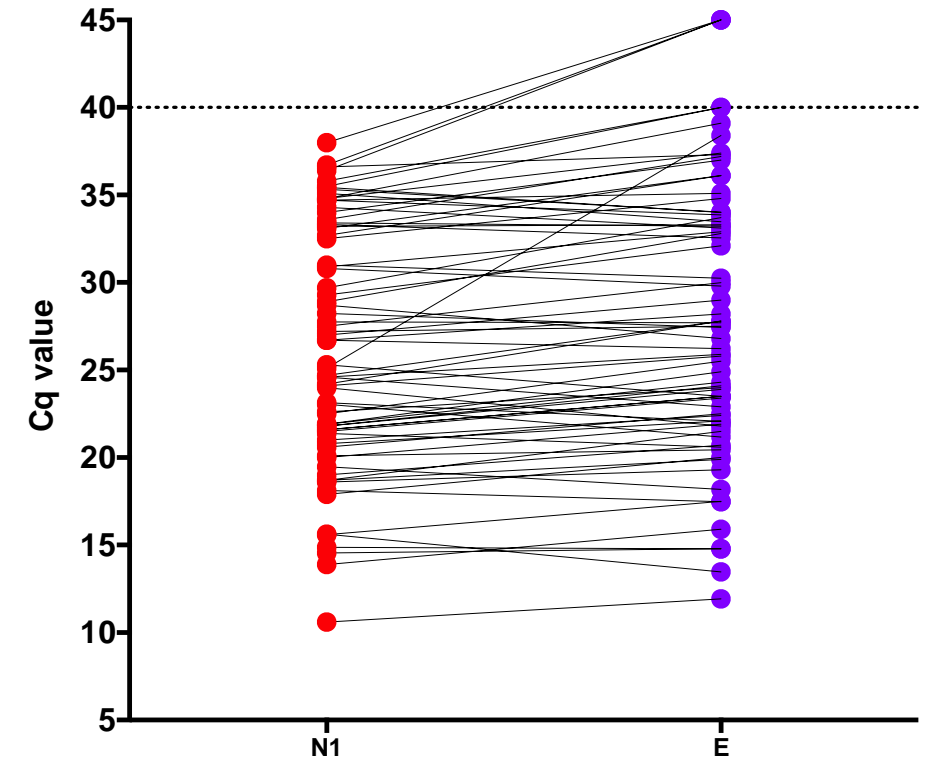
SARS-CoV-2 diagnostic RNA Copies/Reaction	Tested replicates	Number of positive replicates				
		N1	E	E (modified)	RdRP	RdRP (modified)
26473,3	12	12	12	12	12	12
13236,6	12	12	12	12	12	12
6618,3	12	12	12	12	12	12
3309,2	12	12	12	12	12	12
1654,6	12	12	12	12	12	12
827,3	12	12	12	12	12	12
413,6	12	12	12	10	12	12
206,8	12	12	12	4	4	12
103,4	12	12	8	0	2	12
51,7	12	12	7	0	0	12
25,9	12	12	2	0	0	8
12,9	12	6	4	0	0	4
6,5	12	4	2	0	0	0
3,2	12	1	1	0	0	0
1,6	12	0	0	0	0	0
0,8	12	1	0	0	0	0
0,4	12	0	0	0	0	0
0,0	12	0	0	0	0	0

CIQ&EP

Concordância *on-going*



		+		-	
+	N1		21	1	
	E		20	2	
-	N1		0	942	
	E		0	942	



		+		-	
+	N1		72 (96%)	3	
	E		0	2120 (99,8%)	

Overcoming Supply Shortage for SARS-CoV-2 Detection by RT-qPCR

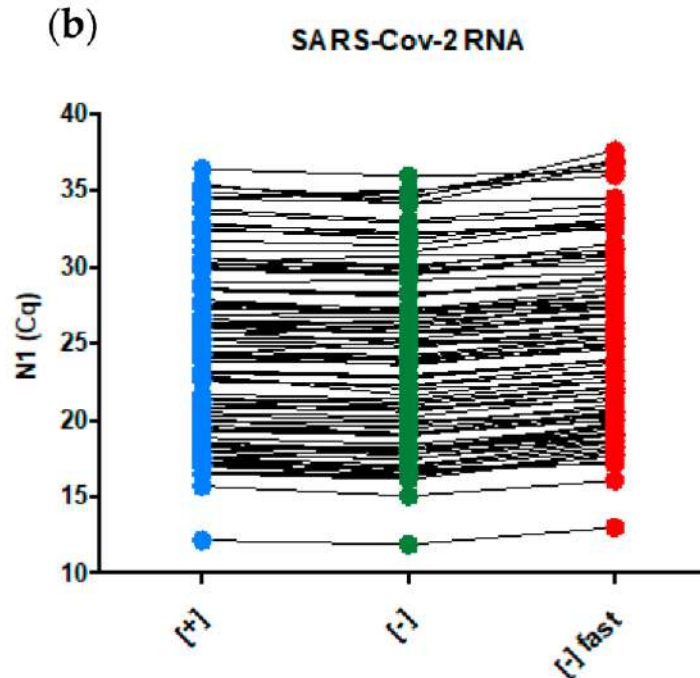
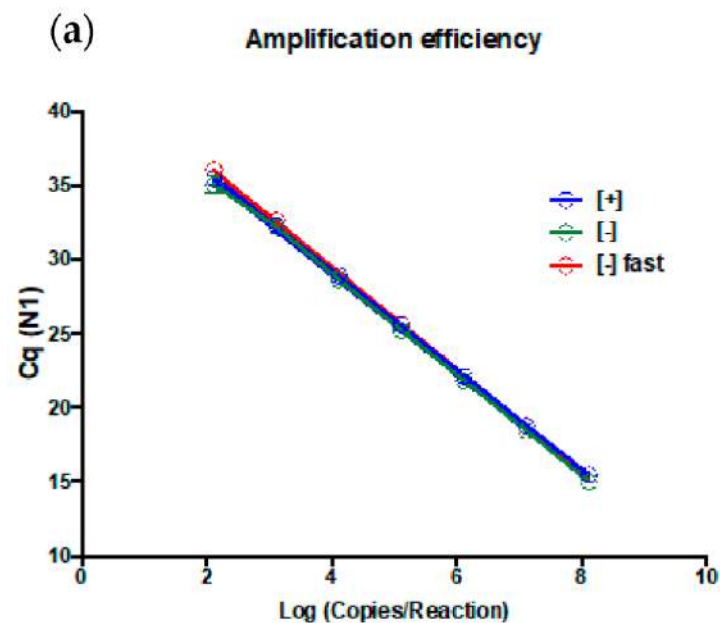
by  Gustavo Barcelos Barra ^{*},†  ,  Ticiane Henriques Santa Rita [†] ,  Pedro Góes Mesquita [†] ,
 Rafael Henriques Jácomo  and  Lídia Freire Abdalla Nery 

Research and Development Section, Sabin Medicina Diagnóstica, 70632-340 Brasília, Brazil

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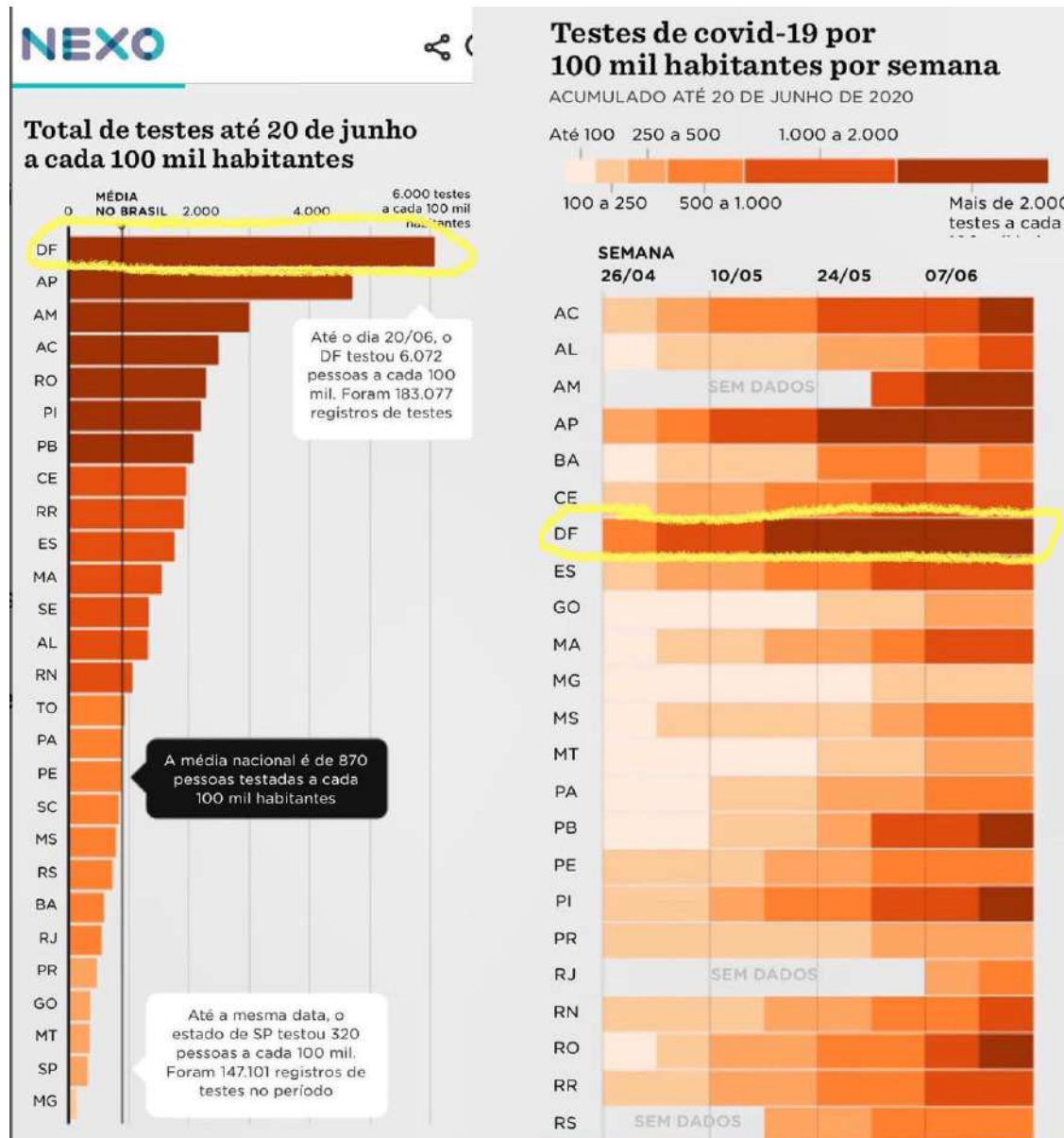
† These authors contributed equally to this work.

Genes **2021**, *12*(1), 90; <https://doi.org/10.3390/genes12010090>

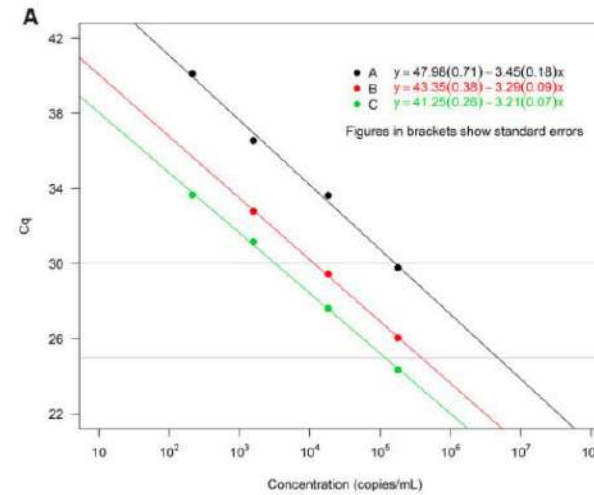
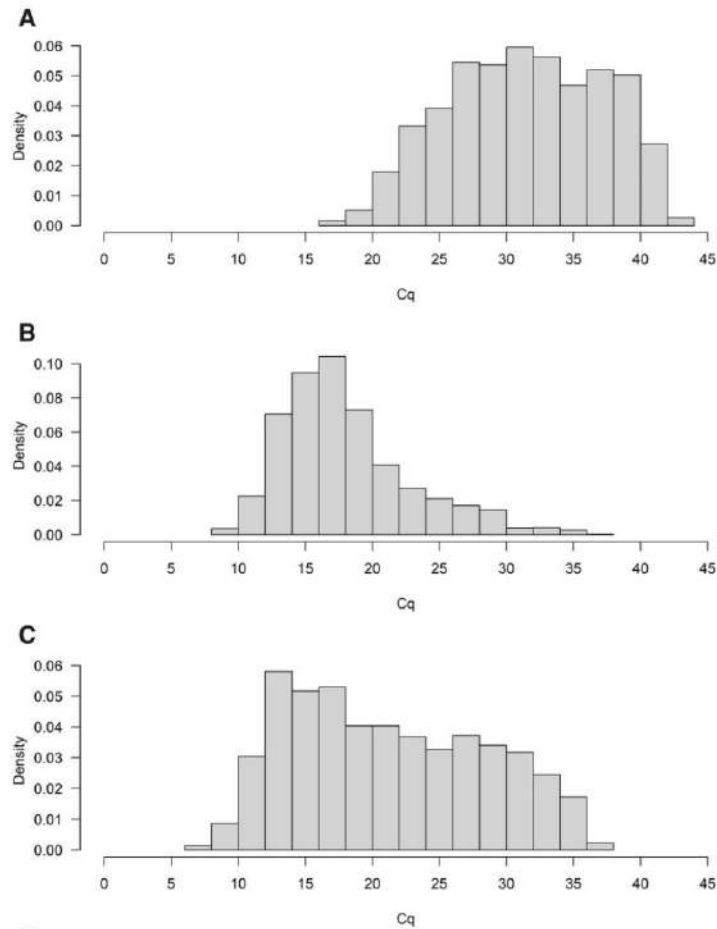


Produção > 30%
Primer < 500%
Probes < 250%

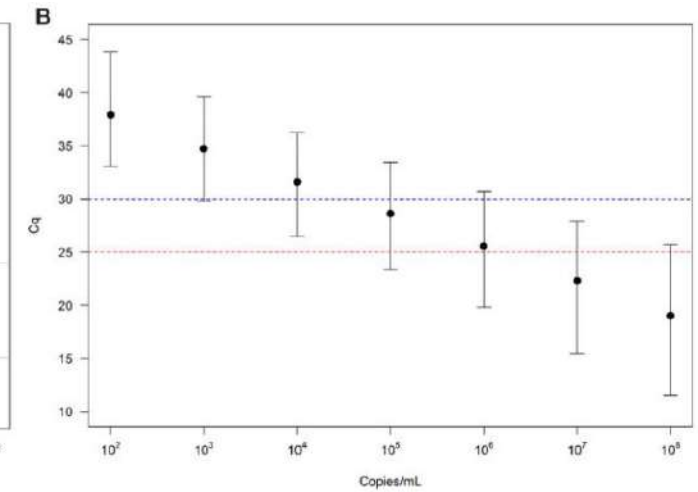
Importância dos métodos próprios



Cq



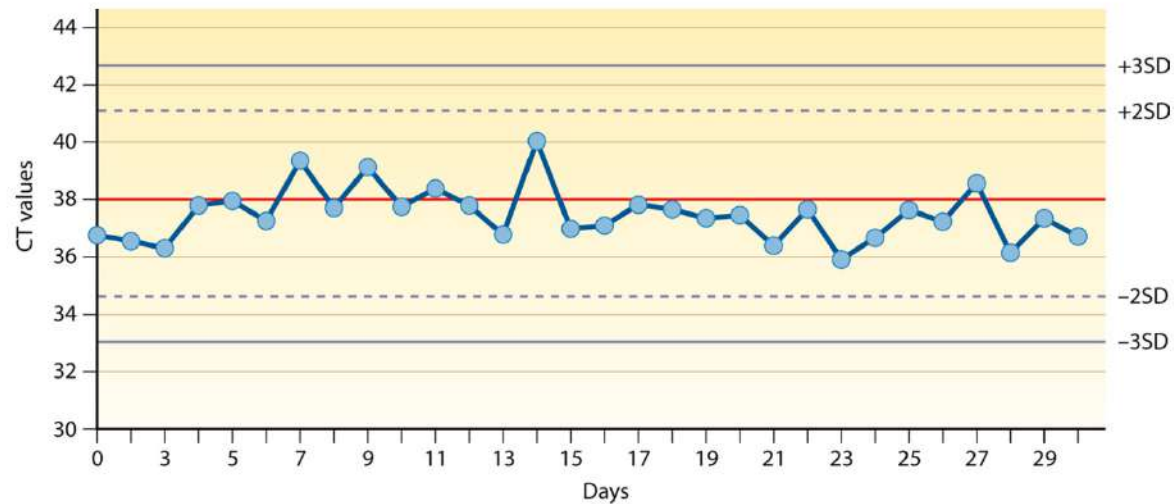
Intervalo (99% CI) do valor
Cq - EQA.



Intervalo (99% CI) do valor
Cq - EQA.

CIQ

Valor



Monitoramento contínuo do Cq do controle positivo baixo usando gráfico de Levey-Jennings plots.

Desfecho

#	Negativo	500 cópias/ml	Cq
1	Não detectado	Detectado	35,7
2	Não detectado	Detectado	36
3	Não detectado	Detectado	35,3
4	Não detectado	Detectado	37
5	Não detectado	Detectado	38
6	Não detectado	Não detectado	-
7	Detectado	Detectado	39/36

Monitoramento contínuo do desfecho do controle positivo baixo usando tabela simples

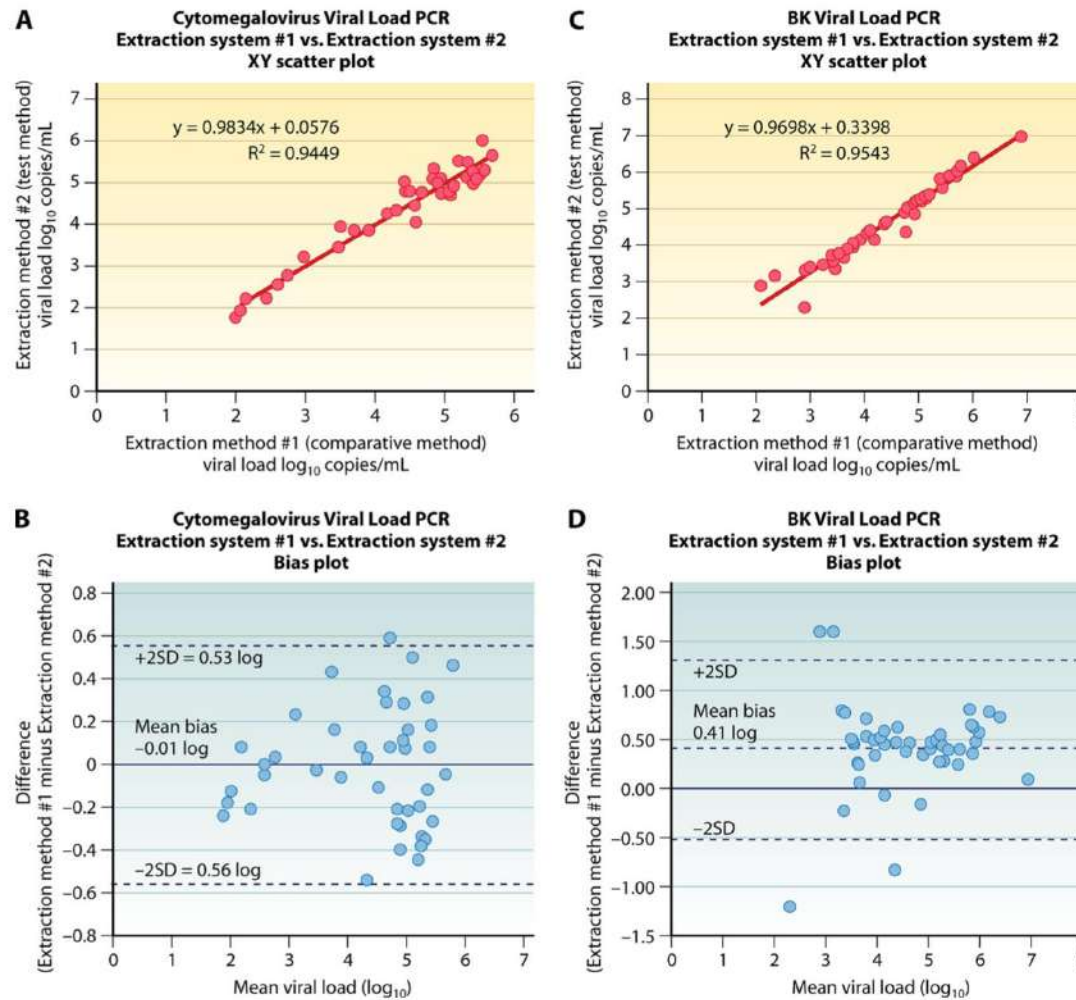
Ensaaios quantitativos

TABLE 2. Possible protocol for determining reportable range, analytical sensitivity, and precision in combined experiments based on current CLSI guidelines

Performance characteristic	Analyte concentration tested ^a											Comment(s)
	Low					Medium			High			
	1	2	3	4	5	6	7	8	9	10	11	
Reportable range (for quantitative assays)	×	×	×	×	×	×	×	×	×	×	×	7-11 concentrations across anticipated measuring range; 2-4 replicates on same day
Analytical sensitivity (LOD)	×	×	×	×	×							8-12 replicates of 4-5 samples at the low concentration end over 5 days
Precision												
Qualitative assay	×	×	×									Use concentrations at LOD, 20% above LOD, and 20% below LOD; test in duplicate over 15 days (include data from analytical sensitivity runs to provide data over 20 days)
Quantitative assay		×			×					×		Use high, low, and LOD concentrations; test in duplicate over 19 days (include data from reportable range study as day 1 to provide data over 20 days)

^a ×, the concentration is tested. The reportable range is from concentration 2 to concentration 10; the LOD, LLOQ, and upper limit of linearity are at concentrations 2, 4, and 10, respectively.

Ensaio quantitativos - Exatidão



Ensaio de proficiência

LETTER TO EDITOR

J Bras Patol Med Lab, v. 53, n. 3, p. 194-195, June 2017

Creation of a comprehensive proficiency testing program for molecular diagnosis in Brazil

Criação de um amplo programa de ensaios de proficiência para o diagnóstico molecular no Brasil

Gustavo B. Barra¹; Rafael H. Jácomo¹; Jéssica S. Gomes²; Rafael M. Lopes²

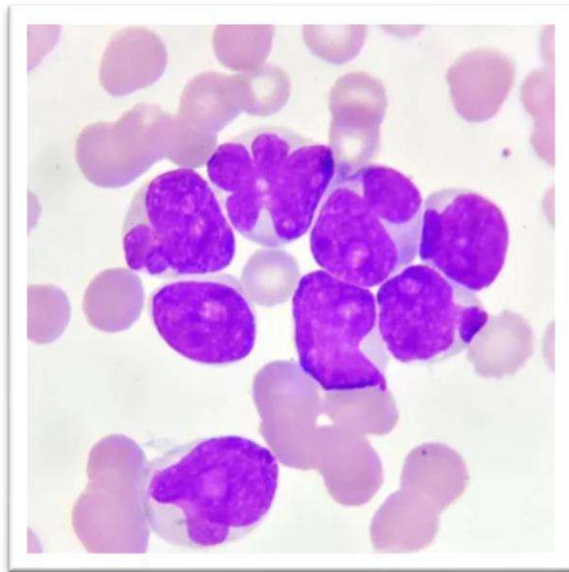
1. Laboratório Sabin de Análises Clínicas, Brasília, DF, Brazil. 2. Controllab, Rio de Janeiro, Brazil.

Key words: laboratory proficiency testing; molecular pathology.

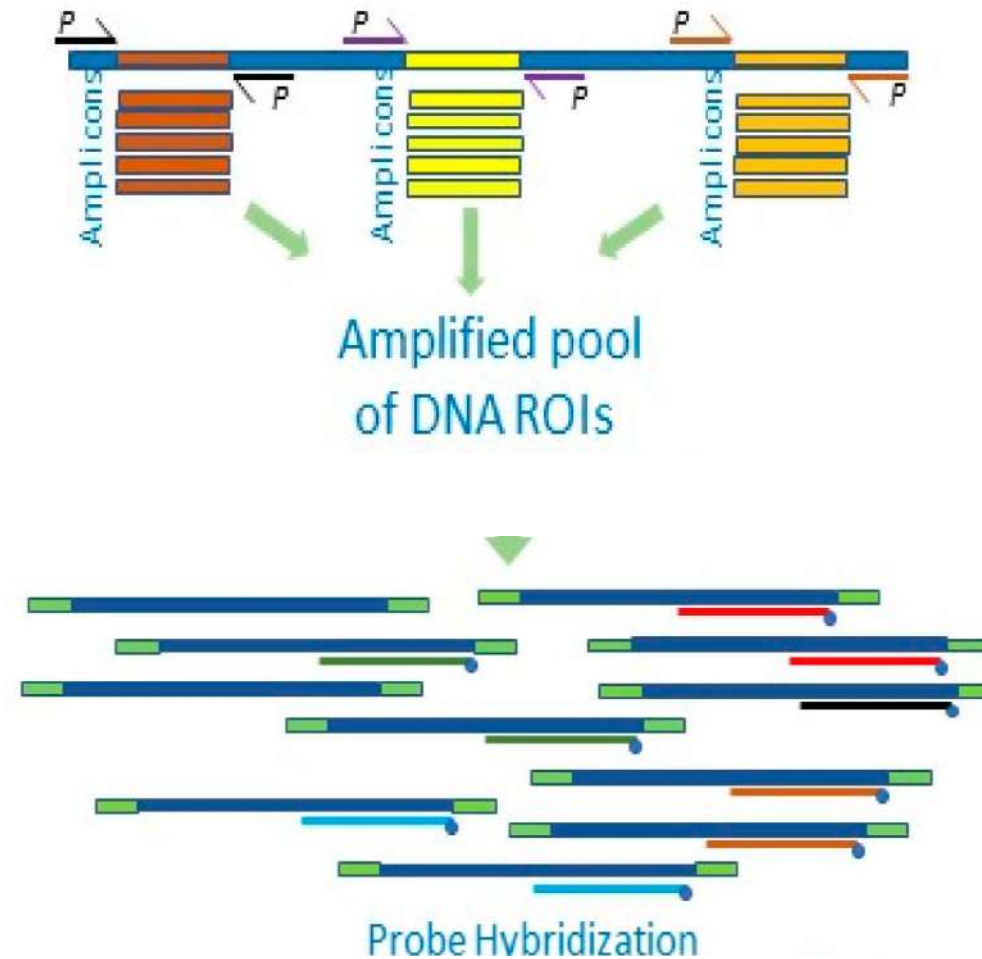
10.5935/1676-2444.20170030

NGS – Painel mielóide

Painel mieloide - amostras e ensaios



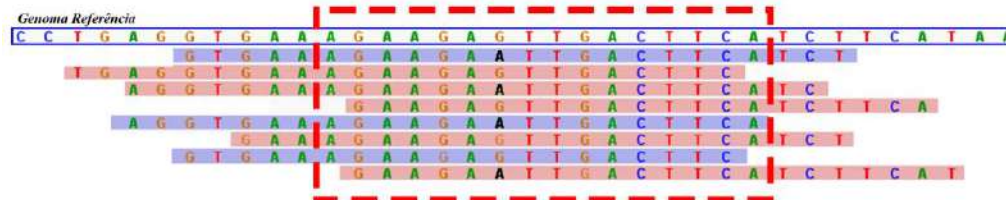
N=31, LMA



Painel mieloide - intervalo reportável

40 genes

Gene	100X (%)	250X (%)	500X (%)
ABL1	100	100	100
ASXL1*	100	99,26	99,25
BCOR*	97,46	97,46	95,93
BRAF	100	89,37	82,55
CALR*	100	100	100
CBL	100	100	100
CEBPA*	100	100	100
CSF3R	100	100	100
DNMT3A	100	100	100
ETV6*	100	100	96,2
EZH2*	99,98	96,01	94,94
FLT3	100	100	100
FLT3ITD	100	100	100
FLT3TKD	100	100	100
GATA2	100	100	100
HRAS	100	100	100
IDH1	100	100	100
IDH2	100	100	100
IKZF1*	100	100	100
JAK2	100	100	100
KIT	100	100	100
KRAS	100	100	100
MPL	100	100	100
MYD88	100	100	100
NF1*	100	100	100
NPM1	100	100	100
NRAS	100	100	100
PHF6*	100	100	100
PRPF8*	97,86	97,09	97,09
PTPN11	100	100	100
RB1*	96,52	96,52	96,5
RUNX1*	100	100	100
SETBP1	100	100	100
SF3B1	100	100	100
SH2B3*	100	96,85	91,36
SRSF2	100	100	100
STAG2*	97,33	97,33	93,66
TET2*	100	100	100
TP53*	100	99,68	99,36
U2AF1	100	100	100
WT1	100	100	100
ZRSR2*	92,91	92,91	92,91



Painel mieloide – Concordância T vs C

	Variante 1	T	C	Variante 2	T	C	Variante 3	T	C	Variante 4	T	C	Variante 5	T	C
1	-														
2	TET2 Ala1458fs	0,49	0,46	ASXL1 Cys594fs	0,49	0,46									
3	-														
4	-														
5	-														
6	TET2 Ser407fs	0,09	0,11												
7	BCOR Glu1518Lys	0,50	0,47												
8	-														
9	JAK2 Val617Phe	0,92	0,93												
10	NPM1 Trp288fs	0,23	0,21	TET2 Tyr1255fs	0,42	0,43									
11	DNMT3 Arg882His	0,47	0,47	IDH2 Arg140Gln	0,46	0,47	RUNX1 Ala324fs	0,43	0,4	CSF3R Ser783fs	0,06	NC			
12				FLT3 Asp835Glu	0,07	0,07	NRAS Gly12Asp	0,14	0,13						
13				FLT3 Asp835Tyr	0,36	0,39									
14	WT1 Arg385fs	0,42	0,43	FLT3-ITD	0,19	0,2									
15	NPM1 Trp288fs	0,25	0,28	TET2 Gln758*	0,47	LC	TET2 Gln769*	0,46	LC	TET2 His922fs	0,42	LC	ASXL1 Glu635fs	0,53	0,33
16	NPM1 Trp288fs	0,41	0,38	FLT3 Asp835Ala	0,08	0,07	NRAS Gly12Asp	0,16	0,16	FLT3 Ile836del	0,05	0,06	TET2 Tyr1560*	0,5	0,5
17	CEBPA ArgAsp300GlnTyr	0,89	0,88												
18	NPM1 Trp288fs	0,39	0,43	WT1 Tyr407*	0,48	0,41	DNMT3A Leu508fs	0,47	0,48	FLT3 Val592Asp	0,41	0,48			
19	IDH1 Arg132Cys	0,48	0,53	KRAS Cys186fs	0,34	0,46									
20	IDH1 Arg132Cys	0,37	0,48	TET2 Ala1341fs	0,52	0,55									
21	NPM1 Trp288fs	0,41	0,45	DNMT3A Arg882His	0,45	0,46									
22	FLT3 Ile836Leu	0,10	0,09	DNMT3A Arg882His	0,23	0,21									
23	TP53 Ser94*	0,83	0,88												
24	-														
25	TP53 Val216Met	0,44	0,50												
26	RUNX1 Arg107Leu	0,51	0,48												
27	RUNX1 Arg201Gln	0,48	0,47	RUNX1 Ser141*	0,44	0,47									
28	-														
28	-														
29	-														
30	-														
31	-														

Amostras 31
 Positivas 21/21
 Negativas 10/10

Variantes 44
 39/44 T & C (88%)
 3 LC → C
 1 NC → C

n=31, 21 positivas (44 variantes) e 10 negativas (sem variantes)

Painel mieloide - Precisão

	Variante 1	T1	T2	Variante 2	T1	T2	Variante 3	T1	T2	Variante 4	T1	T2	Variante 5	T1	T2
1	-														
2															
3															
4															
5															
6															
7															
8															
9															
10															
11	DNMT3 Arg882His	0,47	0,46	IDH2 Arg140Gln	0,46	0,46	RUNX1 Ala324fs	0,43	0,43	CSF3R Ser783fs	0,06	0,06			
12	FLT3 Asp835Glu	0,07	0,07	NRAS Gly12Asp	0,14	0,15									
13															
14	WT1 Arg385fs	0,42	0,39	FLT3-ITD	0,19	0,19									
15	NPM1 Trp288fs	0,25	0,22	TET2 Gln758*	0,47	0,46	TET2 Gln769*	0,46	0,46	TET2 His922fs	0,42	0,44	ASXL1 Glu635fs	0,53	0,6
16	NPM1 Trp288fs	0,41	0,36	FLT3 Asp835Ala	0,08	0,09	NRAS Gly12Asp	0,16	0,16	FLT3 Ile836del	0,05	0,05	TET2 Tyr1560*	0,45	0,46
17															
18	NPM1 Trp288fs	0,39	0,44	WT1 Tyr407*	0,48	0,43	DNMT3A Leu508fs	0,47	0,4	FLT3 Val592Asp	0,41	0,4			
19															
20															
21	NPM1 Trp288fs	0,41	0,43	DNMT3A Arg882His	0,45	0,46									
22															
23															
24															
25															
26															
27															
28															
28															
29	-														
30															

Amostras 9
Positivas 7
Negativas 2

Variantes 24
24/24 T1 & T2

On-going

n=9, 7 positivas (24 variantes) e 2 negativas (sem variantes)

Painel mieloide - LOD

	Variante 1			Variante 2			Variante 3			Variante 4			Variante 5							
	1	1:4	1:8	1	1:4	1:8	1	1:4	1:8	1	1:4	1:8	1	1:4	1:8					
1	-																			
2	TET2 Ala1458fs	0,49	0,07	0,04	ASXL1 Cys594fs	0,49	0,1	0,06												
3																				
4																				
5																				
6																				
7																				
8																				
9	JAK2 Val617Phe	0,92	0,14	0,10																
10																				
11	DNMT3 Arg882His	0,47	0,20	0,10	IDH2 Arg140Gln	0,46	0,17	0,07	RUNX1 Ala324fs	0,43	0,13	0,07	CSF3R Ser783fs	0,06	0,02	ND				
12	FLT3 Asp835Glu	0,07	0,02	ND	NRAS Gly12Asp	0,14	0,04	ND												
13	FLT3 Asp835Tyr	0,36	0,11	0,06																
14																				
15	NPM1 Trp288fs	0,25	0,12	0,06	TET2 Gln758*	0,47	0,1	0,06	TET2 Gln769*	0,46	0,1	0,06	TET2 His922fs	0,42	0,09	0,005	ASXL1 Glu635fs	0,53	0,12	0,07
16																				
17																				
18	NPM1 Trp288fs	0,39	0,12	0,06	WT1 Tyr407*	0,48	0,1	0,05	DNMT3A Leu508fs	0,47	0,15	0,08	FLT3 Val592Asp	0,41	0,09	0,05				
19																				
20	IDH1 Arg132Cys	0,37	0,09	0,03	TET2 Ala1341fs	0,52	0,14	0,04												
21	NPM1 Trp288fs	0,41	0,08	0,05	DNMT3A Arg882His	0,45	0,09	0,07												
22	FLT3 Ile836Leu	0,10	0,02	ND	DNMT3A Arg882His	0,3	0,20	0,10												
23	TP53 Ser94*	0,83	0,17	0,05																
24																				
25	TP53 Val216Met	0,44	0,08	0,03																
26	RUNX1 Arg107Leu	0,51	0,13	0,06																
27	RUNX1 Arg201Gln	0,48	0,22	0,15	RUNX1 Ser141*	0,44	0,24	0,15												
28																				
28																				
29																				
30	-																			
31																				

Amostras 16
Positivas 14
Negativas 2

Variantes 30
1→1:4→1:8 (VAF)
49→7→4
92→14→10
7→2→ND
44→8→3
6→2→ND
52→14→4
47→15→8
53→12→7
37→9→3

n=16, 14 positivas (30 variantes) e 2 negativas (sem variantes)

Lista de orientação em diagnóstico molecular SBPC/ML





EP

**Obrigado e
até a próxima!**

Gustavo Barra

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