Australian Government
Department of Health
Therapeutic Goods Administration

Laboratories Branch

Operations	Biotherapeutics Laboratories Operations Manual
Procedure	Identification of the mRNA in modRNA BNT162b2 (1525) using RT-PCR Assay
Written	[redacted]
Authorised	[redacted]
Date issued	03 February 2021
Revision #	1

TRIM link to SOP	D21-2063055	**Date of assay**	**29/10/2021**
TRIM link to PCR template	D21-2061428	TRIM link to assay data file	el://D21-3274840?db=A7&open
Operator	[redacted]	**Checked by**	[redacted]

Reaction mixture preparation: Prepare 15µL of reaction mixture per well plus 10% overage
Primers and probe are provided by Pfizer and are stored in aliquots at working concentration (x10)
Each assay requires a total of 12 wells for PCR and extraction controls plus 3 wells per test sample.

Reporting: Record the sample LIMS numbers in the table below prior to the assay. Once the assay and analysis are complete, record the assay validity criteria parameters on the worksheet below noting whether the assay is valid. For a valid assay, record the sample Ct values and whether identity has been confirmed in the table below.
Using the Quantstudio Design & Analysis Software, update the assay results file to include the LIMS numbers of each sample (replacing the placeholder letter designation), using the "Advanced Setup" pane of the "Plate" tab. Save the updated file in the assay specific data folder in TRIM (E21-219384).
Following completion of the assay, convert this worksheet into a .pdf and append to it the following: .pdf copies of amplification curve plots, a .pdf experiment report as generated by the Quanstudio Design & Analysis Software. Combine these into a single .pdf and file it in the assay specific data folder (E21-219384) along with the Quantstudio data file.

Reagent information

Reagent	Manufacturer and Catalogue Number	Lot Number	Expiry	Notes
QIAamp Viral RNA Mini Kit	Qiagen, 52906	166024851	2022-03-09	
Buffer AVL	Qiagen, 52906	166024291	2022-04-21	
Buffers AW1 and AW2	Qiagen, 52906	166023682 & 166019545	N/A	
Ethanol 96-100%	Supelco	K50375083828	2023/07/31	
TE Buffer	Life Technologies, AM9849	01063124	N/A	
RT-PCR-grade water	Life Technologies, AM9935	2104121	N/A	
TaqPath 1-step RT-qPCR Master Mix, CG	Applied BioSystems, A15299	2293147	2022-01-30	Opened 26/10
ModRNA1525 RT-PCR forward primer working stock	Pfizer, 4304972	00711497-0162-M06	-	
ModRNA1525 RT-PCR reverse primer working stock	Pfizer, 4304972	00711497-0162-M04	-	see last page for probe and primer details
ModRNA1525 RT-PCR probe working stock	Pfizer, 4316032	00711497-0162-M02	-	

Sample Preparation and Dilution

Positive PCR Control	Positive Extraction Control	Negative Extraction Control	Test Samples
Dilution 1: 10µl DS RM (10µg/mL) + 90µL RT-PCR-grade water = 1µg/mL Dilution 2: 10µL Dilution 1 (1ug/mL) + 190µL RT-PCR-grade water = 50ng/mL = 50 pg/µL 5µL Dilution 2 per RT-PCR well	Prepare with QIAamp Viral RNA Mini Kit. Lysis step composition: 140µL undiluted DP RM + 560 QIAamp Buffer AVL, without carrier RNA. Follow kit protocol Elute in 60µL Buffer Dilution 1: 10µL eluate + 990µL RT-PCR-grade water Dilution 2: 10µL Dilution 1+ 990µL RT-PCR-grade water 5µL Dilution 2 per RT-PCR well	Prepare with QIAamp Viral RNA Mini Kit. Lysis step composition: 140µL TE buffer + 560 QIAamp Buffer AVL, without carrier RNA Follow kit protocol Elute in 60µL Buffer Dilution 1: 10µL eluate + 990µL RT-PCR-grade water Dilution 2: 10µL Dilution 1+ 990µL RT-PCR-grade water 5µL Dilution 2 per RT-PCR well	Prepare with QIAamp Viral RNA Mini Kit. Lysis step composition: 140µL undiluted test sample + 560 QIAamp Buffer AVL, without carrier RNA Follow kit protocol Elute in 60µL Buffer Dilution 1: 10µL eluate + 990µL RT-PCR-grade water Dilution 2: 10µL Dilution 1+ 990µL RT-PCR-grade water 5µL Dilution 2 per RT-PCR well

RT-PCR Reaction Mixture

Number of wells required	15	Volume of master mix per well	15µL	Total volume of master mix required (incl.10% overage)	240	Volume of sample per well	5µL

Component	**Volume per well**	**Total Volume** (incl. 10% overage)
Mastermix	10 µL	**160**
Forward Primer	1 µL	**16**
Reverse Primer	1 µL	**16**
Probe	1 µL	**16**
RT-PCR grade water	2 µL	**32**
Total volume of reaction mixture		**240**

Reaction mixture notes:
Mastermix used: ~~Taqman Fast Advanced / Taqman Universal II~~
4x TaqPath 1-step Master Mix, CG

PCR Plate layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	PCR Positive Control	PCR Positive Control	PCR Positive Control							Positive Extraction Control	Positive Extraction Control	Positive Extraction Control
B												
C	**Sample A**	**Sample A**	**Sample A**									
D	~~**Sample B**~~	~~**Sample B**~~	~~**Sample B**~~									
E	~~**Sample C**~~	~~**Sample C**~~	~~**Sample C**~~									
F	~~**Sample D**~~	~~**Sample D**~~	~~**Sample D**~~									
G												
H	No Template Control	No Template Control	No Template Control							Negative Extraction Control	Negative Extraction Control	Negative Extraction Control

Sample	**Sample LIMS#:**	**Ct Value**	**Identity results** (*confirmed/not confirmed*) (Ct must be < 32 for all sample replicates)
Sample A	**2110003905**	**16.997, 17.126, 16.792**	**Confirmed**
Sample B			
Sample C			
Sample D			

Assay Validity Data

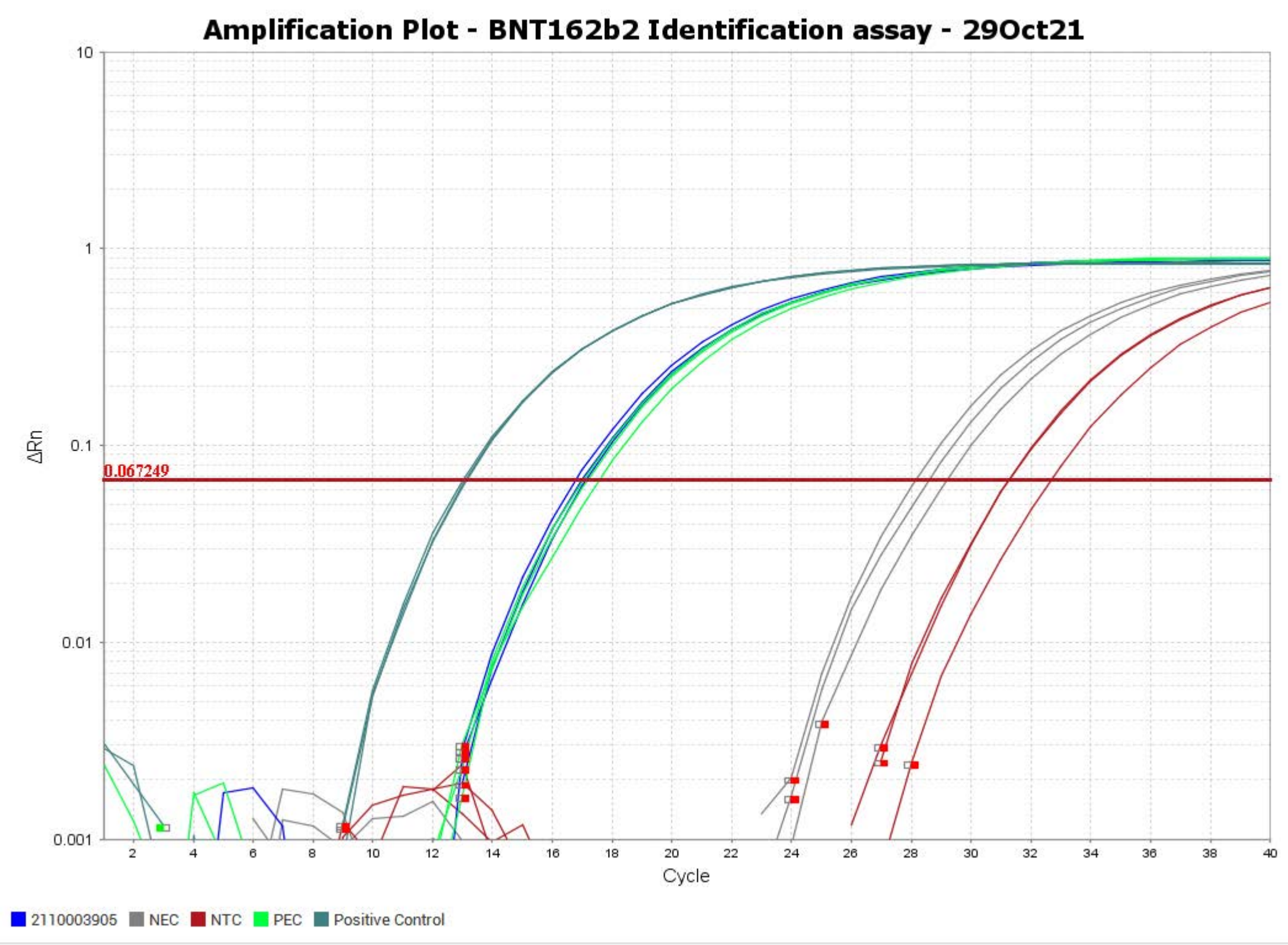
Criterion	Required value	Observed values	Validity
Ct of each Negative Extraction Control well	> 32 or undetermined	**29.162, 28.569, 28.119**	**Valid – see assay validity criteria below page**
Ct of each No Template Control well	> 32 or undetermined	**31.726, 31.260, 32.655**	**Valid – see assay validity Criteria below page**
Ct of each PCR Positive Control well	< 32	**13.600, 13.403, 13.317**	**Valid**
Ct of each Positive Extraction Control well	< 32	**17.018, 17.344, 17.226**	**Valid**

Sample interpretation

Sequence identity of the mRNA is considered confirmed if all test sample replicates show amplification curves with a Ct value of < 32.0000. If all test sample replicates show amplification curves with a Ct value of > 32.0000 the identity is considered not confirmed. If a mixture of results (with Ct values both greater and less than 32.0000) is found for a test sample it must be repeated.

Pipettes used: **Extraction step**: 30006, 33190, 33166
PCR step: 5646, 5653, 33087

Batch details for probes and primers:
For Primer: (Merck) Sigma, Ref# VC00021, SY21020241530-088, Lot# 3026595983-000020
Rev Primer: (Merck) Sigma, Ref# VC00021, SY21020241529-078, Lot# 3026595983-000030
100 µM stock prepared as per manufacturer's instructions, 20 µL of 100 µM added to 91 µL RT-PCR
Grade water to make 18 µM working stock, MJ 06Sep21
Probe: (Applied Biosystems) ThermoFisher, Lot# 7495294-1 C1, Ref# 4316034
100 µM stock prepared as per manufacturer's instructions; 5 µL 100 µM added to 95 µL RT-PCR Grade
Water to make 5 uM working stock, MJ 27Sep21
NOTE: Validity criteria for assay has been altered as per recommendation in D21-2294359.
Criteria used to assess validity of assay are as follows:
Ct of Negative Extraction Control (NEC) more than 8 Ct higher than Ct of Positive Extraction Control (PEC):
Lowest NEC Ct value: 28.119; highest PEC Ct value: 17.559. Difference = 10.56 -Valid
Ct of No Template Control (NTC) more than 8 Ct higher than Ct of PCR Positive Control:
Lowest NTC Ct value: 31.260 ; highest PCR Positive Control value: 13.110. Difference = 18.15 – Valid


Amplification Plot - BNT162b2 Identification assay - 29Oct21
10
1
0.1
0.01
0.001
ΔRn
0.067249
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
Cycle
2110003905
NEC
NTC
PEC
Positive Control

Experiment Results Report

Experiment Summary

Experiment Name:	2021-01-11_133749
Experiment Type:	Standard Curve
Chemistry:	TaqMan® Reagents
BarCode:	
File Name:	ID Pfizer_17.eds
Run Started:	10-29-2021 11:25:40 AEDT
Run Finished:	10-29-2021 12:31:54 AEDT
Run Duration:	66 minutes 13 seconds
Date EDS Modified:	10-29-2021 13:35:44 AEDT
Date EDS Created:	01-11-2021 15:21:26 AEDT
User Name:	
Number of Wells Used:	15
Number of Wells with Results:	15
Instrument Name:	QS3
Instrument Type:	QuantStudio™ 3 System
Instrument Serial Number:	272322852
Model/Block Type:	QuantStudio™ 3 System / 96-Well 0.2-mL Block
Quantification Cycle Setting:	Cт
Stage/Step for Analysis:	Stage2, Step2
Comments:	

Experiment Results Report

Reagent Information

Type	Name	Part Number	Lot Number	Expiration Date

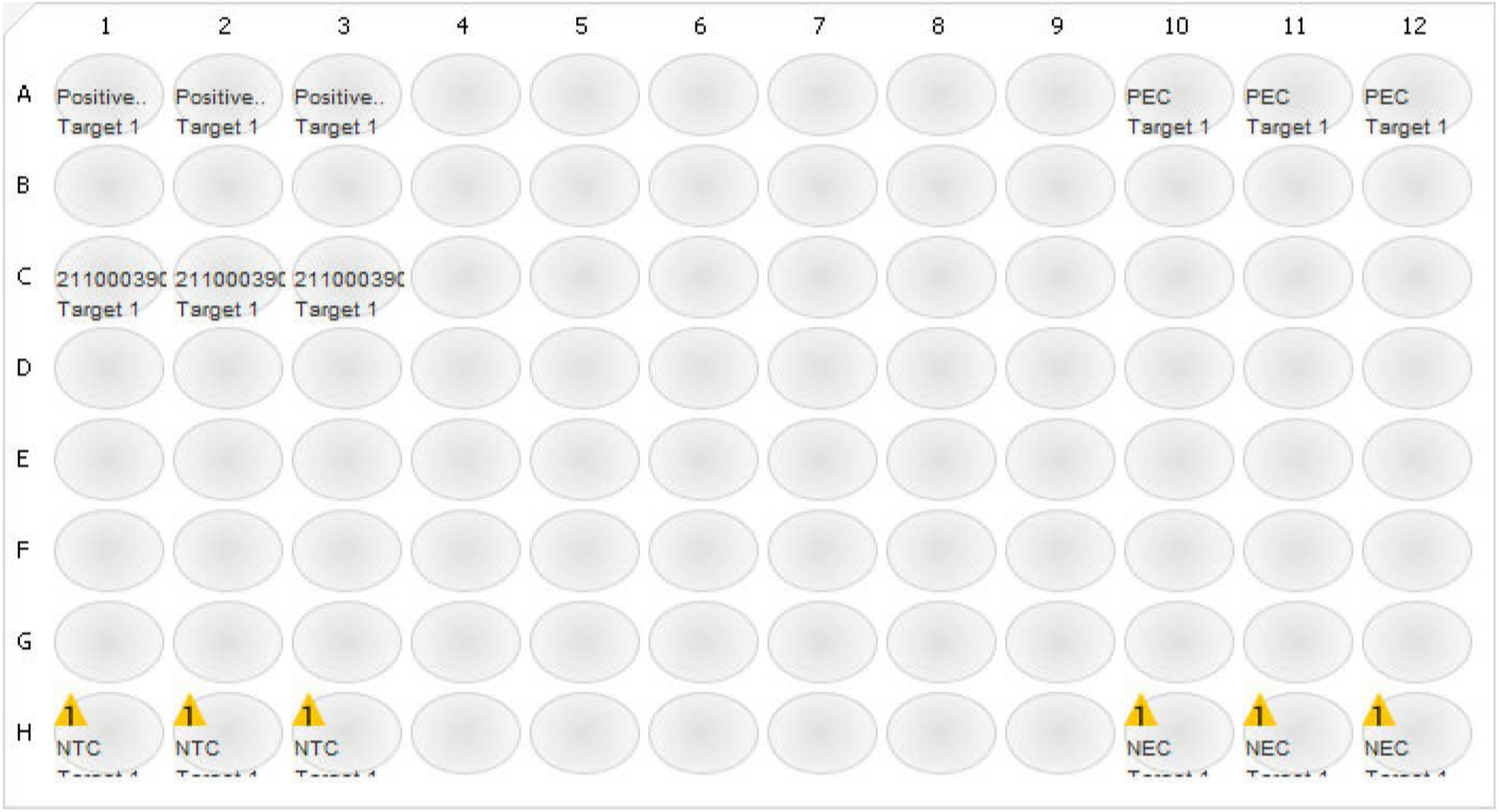
Experiment Results Report

Results Summary

Sample	Target	Qty Mean	Qty SD	CT Mean	CT SD
2110003905	Target 1			16.972	0.168
NEC	Target 1				
NTC	Target 1				
PEC	Target 1			17.254	0.274
Positive Control	Target 1			13.068	0.055

Experiment Results Report

Plate Layout



Experiment Results Report

Results Table

Well	Sample	Target	Task	CT	CT Mean	CT SD	Qty	Qty Mean	Qty SD	CT Threshold	Baseline Start	Baseline End	Cq Conf
A1	Positive Control	Target 1	U	13.088	13.068	0.055				0.067	3	9	0.936
A2	Positive Control	Target 1	U	13.006	13.068	0.055				0.067	3	9	0.937
A3	Positive Control	Target 1	U	13.110	13.068	0.055				0.067	3	9	0.924
H11	NEC	Target 1	N	29.162						0.067	3	25	0.964
A10	PEC	Target 1	U	17.176	17.254	0.274				0.067	3	13	0.949
H10	NEC	Target 1	N	28.569						0.067	3	24	0.971
A11	PEC	Target 1	U	17.027	17.254	0.274				0.067	3	13	0.946
A12	PEC	Target 1	U	17.559	17.254	0.274				0.067	3	13	0.958
H12	NEC	Target 1	N	28.119						0.067	3	24	0.968
C1	2110003905	Target 1	U	16.997	16.972	0.168				0.067	3	13	0.952
C2	2110003905	Target 1	U	17.126	16.972	0.168				0.067	3	13	0.944
C3	2110003905	Target 1	U	16.792	16.972	0.168				0.067	3	13	0.931
H1	NTC	Target 1	N	31.276						0.067	3	27	0.953
H3	NTC	Target 1	N	31.260						0.067	3	27	0.958
H2	NTC	Target 1	N	32.655						0.067	3	28	0.951

Legend: S = Standard, N = Non Template Control, U = Unknown, UND. = Undetermined

Experiment Results Report

QC Summary

Total Wells:96 Processed Wells:15 Targets Used:1 Well Setup:15 Flagged Wells:6 Samples Used:5

Flag	Description	Frequency	Locations
AMPNC	Amplification in negative control	6	H11, H10, H12, H1, H3, H2
BADROX	Bad passive reference signal	0	
BLFAIL	Baseline algorithm failed	0	
CQCONF	Low Cq confidence	0	
CTFAIL	Cт algorithm failed	0	
DRNMIN	Define acceptable delta Rn based on Cт range	0	
EXPFAIL	Exponential algorithm failed	0	
HIGHSD	High standard deviation in replicate group	0	
NOAMP	No amplification	0	
NOISE	Noise higher than others in plate	0	
NOSIGNAL	No signal in well	0	
OFFSCALE	Fluorescence is offscale	0	
OUTLIERRG	Outlier in replicate group	0	
PRFDROP	Passive reference signal changes near Cт	0	
PRFLOW	Low passive reference signal	0	
SPIKE	Noise spikes	0	
THOLDFAIL	Thresholding algorithm failed	0	