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
Author	[redacted]
Owner	[redacted]
Authoriser	[redacted]
Revision #	2

TRIM link to SOP	D21-2063055	**Date of assay**	**17/11/2021**
TRIM link to PCR template	D21-2061428	TRIM link to assay data file	D21-3344781
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	**166026648**	**2022-03-15**	
Buffer AVL	Qiagen, 52906	**166025080**	**2022-03-15**	
Buffers AW1 and AW2	Qiagen, 52906	**166025919 & 166021395**	**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 & 2004106	**N/A**	
TaqPath 1-step RT-qPCR Master Mix, CG	Applied BioSystems, A15299	2293147	**2022-01-30**	
ModRNA1525 RT-PCR forward primer working stock		**00711497-0162-M06**	-	**see last page for probe and primer details**
ModRNA1525 RT-PCR reverse primer working stock		**00711497-0162-M04**	-	
ModRNA1525 RT-PCR probe working stock		**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	18	Volume of master mix per well	15µL	Total volume of master mix required (incl.10% overage)	283	Volume of sample per well	5µL

Component	**Volume per well**	**Total Volume** (incl. 10% overage)
Mastermix	10 µL	**190**
Forward Primer	1 µL	**19**
Reverse Primer	1 µL	**19**
Probe	1 µL	**19**
RT-PCR grade water	2 µL	**36**
Total volume of reaction mixture		**283**

Reaction mixture notes:
Mastermix used: ~~TaqPath 1-step RT-qPCR Master Mix, CG~~
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	**2111004115**	**17.120, 16.998, 16.718**	**confirmed**
Sample B	**2111004218**	**16.447, 16.519, 16.427**	**confirmed**
Sample C			
Sample D			

Assay Validity Data

Criterion	Required value	Observed values	Validity
Ct of each Negative Extraction Control well	> 28 or undetermined	**31.205, 32.264, 29.730**	**Valid**
Ct of each No Template Control well	> 28 or undetermined	**35.549, 35.872, 31.445**	**Valid**
Ct of each PCR Positive Control well	< 28	**13.277, 13.486, 13.483**	**Valid**
Ct of each Positive Extraction Control well	< 28	**18.392, 18.347, 18.568**	**Valid**

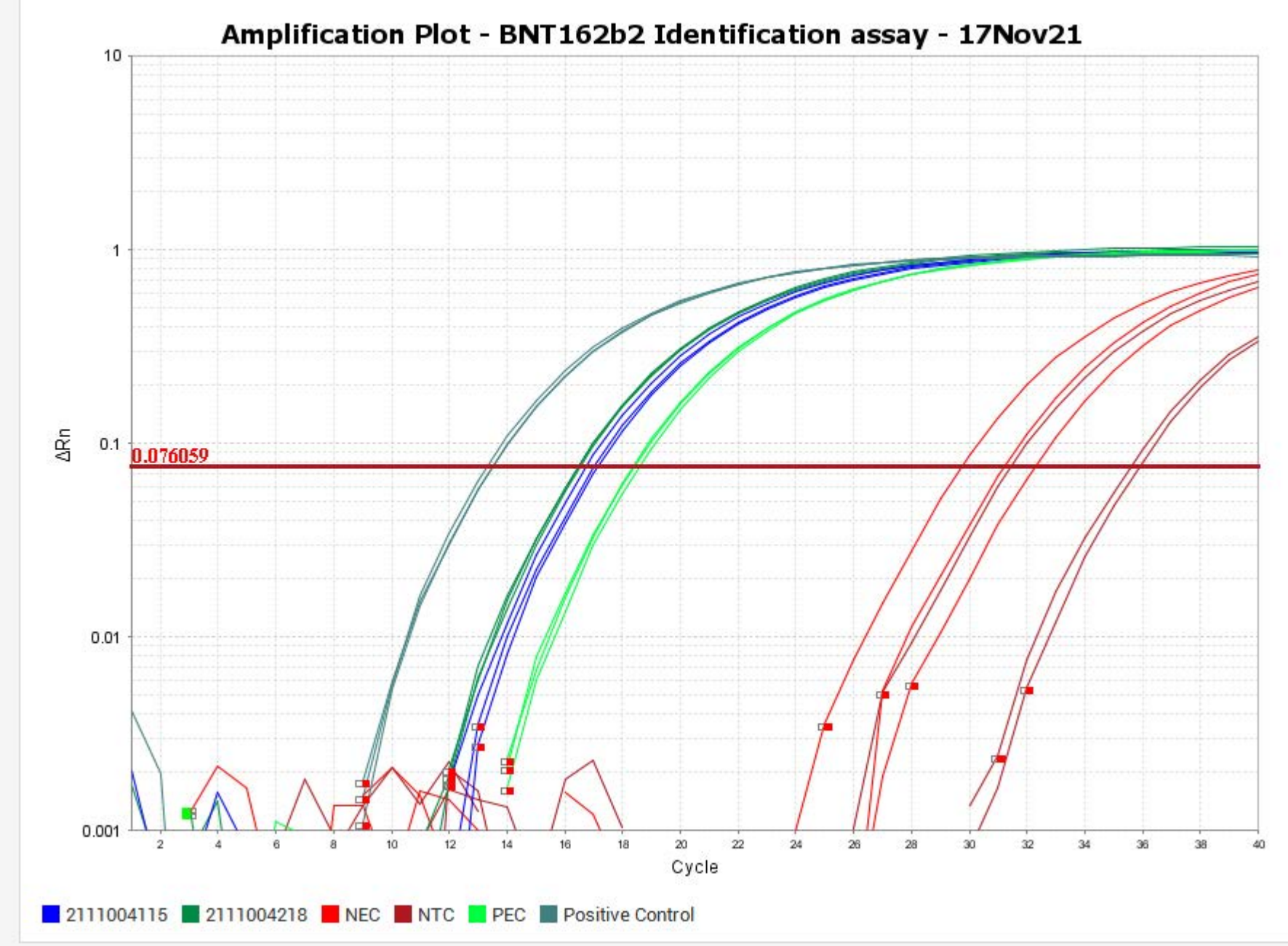
Sample interpretation

Sequence identity of the mRNA is considered confirmed if all test sample replicates show amplification curves with a Ct value of < 28.0000. If all test sample replicates show amplification curves with a Ct value of > 28.0000 the identity is considered not confirmed. If a mixture of results (with Ct values both greater and less than 28.0000) 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):
Ct of No Template Control (NTC) more than 8 Ct higher than Ct of PCR Positive Control:


Amplification Plot - BNT162b2 Identification assay - 17Nov21
10
1
0.1
0.01
0.001
ΔRn
0.076059
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
Cycle
2111004115
2111004218
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_21.eds
Run Started:	11-17-2021 17:49:50 AEDT
Run Finished:	11-17-2021 18:56:01 AEDT
Run Duration:	66 minutes 10 seconds
Date EDS Modified:	11-18-2021 10:43:02 AEDT
Date EDS Created:	01-11-2021 15:21:26 AEDT
User Name:	
Number of Wells Used:	18
Number of Wells with Results:	18
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:	

Reagent Information

Type	Name	Part Number	Lot Number	Expiration Date

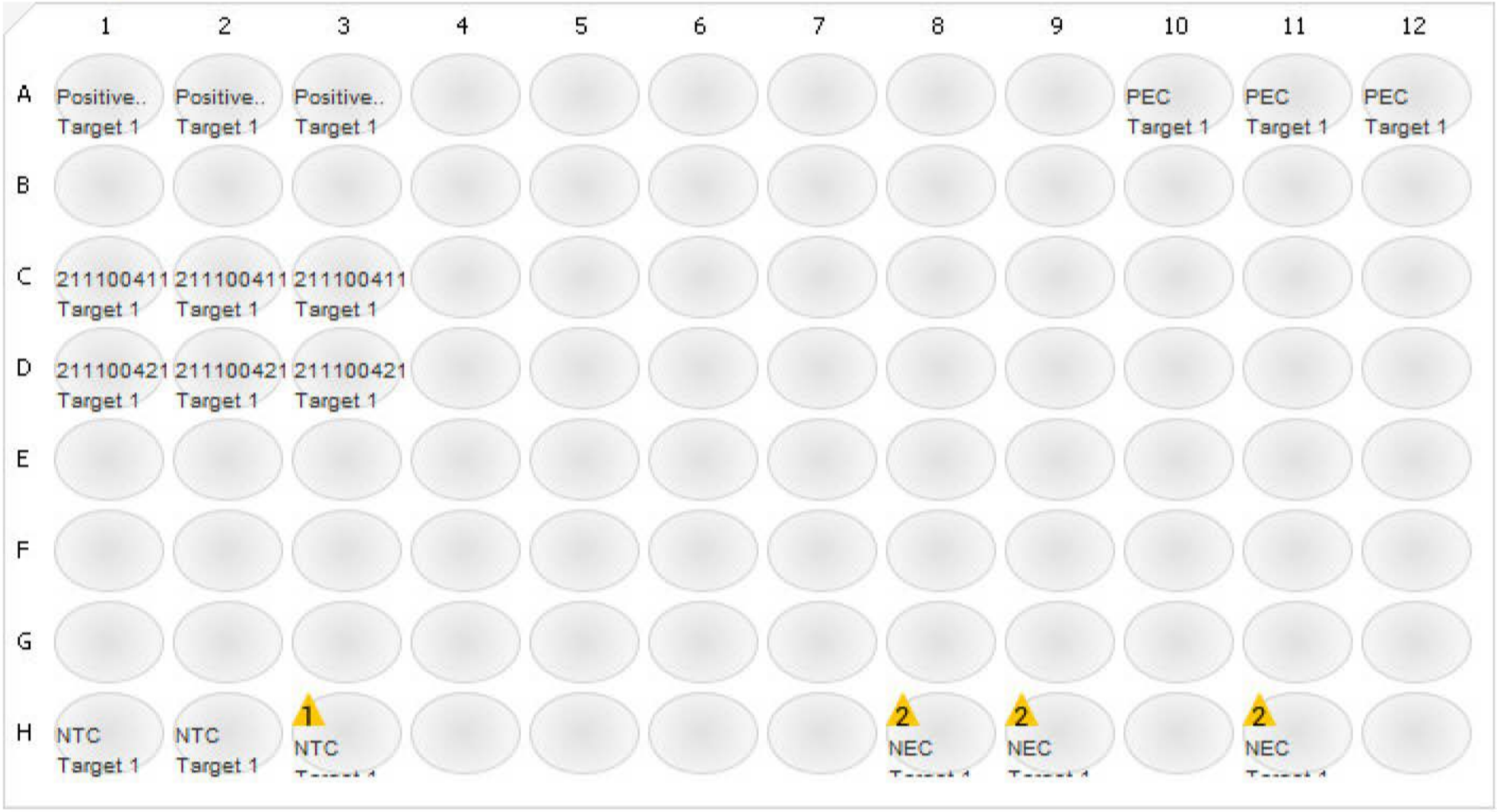
Experiment Results Report

Results Summary

Sample	Target	Qty Mean	Qty SD	CT Mean	CT SD
2111004115	Target 1			16.945	0.207
2111004218	Target 1			16.464	0.048
NEC	Target 1				
NTC	Target 1				
PEC	Target 1			18.436	0.117
Positive Control	Target 1			13.415	0.120

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	13.277	13.415	0.120				0.076	3	9	0.938
A2	Positive Control	Target 1	U	13.486	13.415	0.120				0.076	3	9	0.943
A3	Positive Control	Target 1	U	13.483	13.415	0.120				0.076	3	9	0.957
H11	NEC	Target 1	N	29.730	31.066	1.273				0.076	3	25	0.974
A10	PEC	Target 1	U	18.392	18.436	0.117				0.076	3	14	0.952
A11	PEC	Target 1	U	18.347	18.436	0.117				0.076	3	14	0.949
A12	PEC	Target 1	U	18.568	18.436	0.117				0.076	3	14	0.947
C1	2111004115	Target 1	U	17.120	16.945	0.207				0.076	3	13	0.948
C2	2111004115	Target 1	U	16.998	16.945	0.207				0.076	3	13	0.949
C3	2111004115	Target 1	U	16.718	16.945	0.207				0.076	3	12	0.953
D1	2111004218	Target 1	U	16.447	16.464	0.048				0.076	3	12	0.947
D3	2111004218	Target 1	U	16.427	16.464	0.048				0.076	3	12	0.964
D2	2111004218	Target 1	U	16.519	16.464	0.048				0.076	3	12	0.946
H1	NTC	Target 1	N	35.549						0.076	3	31	0.924
H9	NEC	Target 1	N	32.264	31.066	1.273				0.076	3	28	0.982
H8	NEC	Target 1	N	31.205	31.066	1.273				0.076	3	26	0.967
H3	NTC	Target 1	N	31.445						0.076	3	27	0.965

Experiment Results Report

Well	Sample	Target	Task	CT	CT Mean	CT SD	Qty	Qty Mean	Qty SD	CT Threshold	Baseline Start	Baseline End	Cq Conf
H2	NTC	Target 1	N	35.872						0.076	3	32	0.922

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

Experiment Results Report

QC Summary

Total Wells:96 Processed Wells:18 Targets Used:1 Well Setup:18 Flagged Wells:4 Samples Used:6

Flag	Description	Frequency	Locations
AMPNC	Amplification in negative control	4	H11, H9, H8, H3
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	3	H11, H9, H8
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	