Complete Project Report

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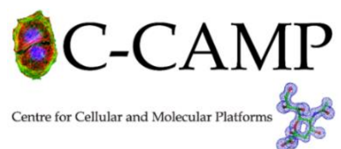

Title: Functional testing of Dx/ Dt Phi(Ø)Script Reverse transcriptase enzyme using two step RT-qPCR.	
RID:	5887 (C-CAMP Technology Platform Services)
Name of the sample(s):	Dx/ Dt Phi(Ø)Script RT enzyme Batch 1 (Cat# R6401) and Batch 2 (Cat# R6101)
Requester organization:	Rubizon Private Limited
Requester name:	Vikram N. Prabhu
Name of the CCAMP-InDx CoE:	CCAMP-InDx, CoE-Quality
CCAMP-InDx CoE lead:	Dr. Mohammad Nasiruddin
CCAMP-InDx CoE scientist(s):	Urvashi Chandola
CCAMP-InDx project manager:	Dr. Mayooreshwar Rajankar
CCAMP-InDx S&T lead:	Dr. Mitali Samaddar
Project receipt date: 04.07.23	Project completion date:

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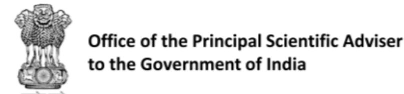

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Prepared, Reviewed and Approved by 13

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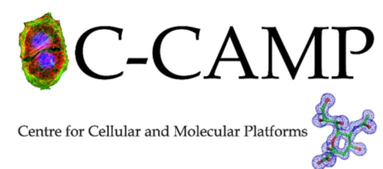
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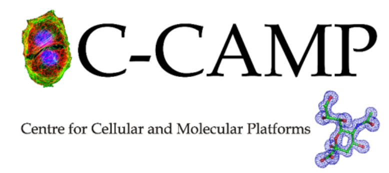


TPS RID: 5887

Summary data

Method: Two step RT- qPCR
Global marketed product: Revert Aid RT Thermo Scientific (Cat No EP0441)
Sample: Dx/ Dt Phi(Ø)Script Reverse transcriptase Batch 1 (Cat No R6401) and Batch 2 (Cat No R6101)

1 Test 1

Functional testing of Dx/ Dt Phi(Ø)Script Reverse transcriptase enzyme using two step RT-qPCR method for human RNase P gene.

Summary data Table 1: Cycle threshold (Ct) values for *RNase P* target gene amplification for benchmark and batch 1 and batch 2 sample.

	n1								
Template (ng)	Revert Aid RT			Dx/ Dt Batch 1			Dx/ Dt Batch 2		
	Ct (r1)	Ct (r2)	%RSD	Ct (r1)	Ct (r2)	%RSD	Ct (r1)	Ct (r2)	%RSD
1500	23.54	23.83	0.86	23.99	23.56	1.27	23.26	23.26	0.00
150	27.00	27.11	0.28	26.85	26.99	0.36	27.03	27.0	0.07
15	31.25	31.31	0.13	31.43	31.37	0.14	31.07	31.25	0.035
1.5	34.16	33.93	0.07	34.28	34.51	0.07	34.21	34.19	0.01
NTC	Und	Und	-	Und	Und	-	Und	Und	-

n: no. of independent experiments, %RSD: percent relative standard deviation, NTC: No template control

Summary Table 2: Statistical analysis of linear regression plot & compliance with acceptance criteria for n1

		n1		
Criteria	**Acceptable range**	**Revert Aid RT**	**Dx/ Dt Batch 1**	**Dx/ Dt Batch 2**
Coefficient of determination (R^2)	(0.98 to 1.00)	0.99	0.99	0.99
Slope	(3.1 to 3.6)	-3.5	-3.6	-3.6
Reaction Efficiency **Efficiency = $-1 + 10^{(-1/slope)}$**	(90 to 110%)	100 %	90 %	90 %
Criteria compliance (YES/NO)		**Yes**	**Yes**	**Yes**

Summary data Table 3: Cycle threshold (Ct) values for *RNase P* target gene amplification for benchmark and batch 1 and batch 2 sample.

Template (ng)	n2								
	Revert Aid RT			Dx/ Dt Batch 1			Dx/ Dt Batch 2		
	Ct (r1)	Ct (r2)	%RSD	Ct (r1)	Ct (r2)	%RSD	Ct (r1)	Ct (r2)	%RSD
1500	23.77	23.49	0.83	23.51	23.54	0.09	23.66	23.49	0.50
150	26.88	26.94	0.15	27.08	26.66	1.10	27.12	26.79	0.8
15	31.23	31.27	0.09	31.16	31.32	0.36	31.2	31.13	0.15
1.5	34.13	34.14	0.02	34.03	34.00	0.06	34.14	34.17	0.06
NTC	Und	Und	-	Und	Und	-	Und	Und	-

Summary data Table 4: Statistical analysis of linear regression plot & compliance with acceptance criteria for n2.

		n2		
Criteria	**Acceptable range**	Revert Aid RT	Dx/ Dt Batch 1	Dx/ Dt Batch 2
Coefficient of determination (R^2)	(0.98 to 1.00)	0.99	0.99	0.99
Slope	(3.1 to 3.6)	-3.58	-3.58	-3.59
Reaction Efficiency Efficiency = $-1 + 10^{(-1/slope)}$	(90 to 110%)	90.25 %	90. 25 %	90 %
Criteria compliance (YES/NO)		**Yes**	**Yes**	**Yes**

2 Summary Conclusion:

1. The reverse transcription activity of Revert Aid RT and Dx/ Dt Phi(Ø)Script Reverse transcriptase of Batch 1 and Batch 2 enzyme were found to be comparable in RT qPCR method using TaqMan Probe.
2. The optimal performance of Revert Aid RT and Dx/ Dt Phi(Ø)Script Reverse transcriptase of Batch 1 and Batch 2 enzyme were found to be similar as demonstrated by Ct values across the linear range.
3. The functional activity of Dx/ Dt Phi(Ø)Script Reverse transcriptase of Batch 1 and Batch 2 enzyme was found to be optimal as demonstrated by the values of Regression, Slope and Amplification efficiency.

Test 1: Functional testing of Dx/ Dt Phi(Ø)Script Reverse transcriptase enzyme using two step RT- qPCR method.

1 Study objective

functional testing of Dx/ Dt Phi(Ø)Script Reverse Transcriptase of Batch 1 and Batch 2 enzyme using two step RT- qPCR.

2 Study design

Functional testing of Reverse Transcriptase enzyme using two step RT-qPCR for human RNase P gene. Linear range of cDNA is used as a template to check the functional activity of reverse transcriptase enzyme using TaqMan probe.

Two batches of the Dx/ Dt Phi(Ø)Script Reverse Transcriptase enzyme were tested using two step Rt-qPCR. Following prerequisites is essential for the experiment which are,

- A standard curve to represent the linearity of template.
- At least two independent experiments to be done to derive the conclusion.

3 Materials and Methods

3.1 Instruments

- Real Time PCR machine (Make: BIO-RAD) – CFX Opus 96 Real-Time PCR (EQP No: C-CAMP 0675)
- Laminar Air flow machine (Make: CRUMA) – (EQP No: C-CAMP 0673)
- Laminar Air flow machine (Make: CRUMA) – (EQP No: C-CAMP 0674)
- Pipettes (Make: Eppendorf) – (Pipette No: 144661K, 144685K, J33051K, L70794K, L61057K, L60953K)

3.2 Materials

3.2.1 Chemicals and reagents

- 10mM dNTP (Thermo Scientific, Cat No R0191)
- RT Buffer (Thermo Scientific, Cat No EP0441)
- Revert Aid RT enzyme (Thermo Scientific, Cat No EP0441)
- 10X Dream Taq Buffer (Thermo Scientific, Cat No EP0701)
- Dream Taq Enzyme (Thermo Scientific, Cat No EP0701)
- Trizol reagent (Takara Biotech Cat No 9109)
- Chloroform (Merck Cat No UN1888)
- Isopropanol (Fisher Scientific Prod. No 13825)
- Ethanol (Merck Cas No 64-11-5)
- Ultrapure PCR grade H_2O (DNAse, RNAse free) (Invitrogen, Cat No 10977-015)
- PCR tubes, flat cap. Clear and PCR tube strip, flat cap

3.2.2 Standard/ Reference material description

Benchmark Description

Revert Aid RT, conc. 200 U/ µl, (Cat No EP0441) (Lot no. # 01274452) with 5X buffer sourced by Thermo Scientific, Expiry: 09/25, is used as a market comparator (CoA attached in the reference section).

Sample description

Dx/ Dt Phi(Ø)Script Reverse transcriptase (Conc. – 200 U/µl) is supplied with 5 X assay buffer (Tris – HCl, KCl, EDTA, DTT, Glycerol) (pH - 8) sourced by Rubizon Pvt Ltd.

Reference Material

HEK 293T total RNA was used as template for internal control.

3.2.3 Primers and Probes

Primers & probes were sourced from Integrated DNA Technologies (IDT). The internal control gene primers & probe for human *RNase P* were custom-made (linear mRNA ACCESSION NM_005837). Primers & probes were synthesized at 200 nm scale and were HPLC purified. The primers and probe concentrations were standardized for optimum performance.

RNase P, Homo sapiens, ribonuclease P/MRP subunit (POP7) LOCUS: NM_005837, 850bp, mRNA, linear ACCESSION NM_005837

ATGGGACTTCAGCATGGCGGTGTTTGCAGATTTGGACCTGCGAGCGGGTTCTGACCTGAAGGCTCTGCGCGG ACTTGTGGAGACAGCCGCTCACCTTGGCTATTCAGTTGTTGCTATCAATCATATCGTTGACTTTAAGGAAAAGA Human *RNase P* (internal control)

Forward Primer (RP-F): 5’ -AGATTTGGACCTGCGAGCG-3’ (19 bases)

Reverse Primer (RP-R): 5’ -GAGCGGCTGTCTCCACAAGT-3’ (20 bases)

RNase P (CY5): 5’- CY5- TTCTGACCTGAAGGCTCTGCGCG-BHQ3-3’ (25 bases)

3.3 Procedure/Protocol

RT – PCR (reverse transcription polymerase chain reaction) involves two steps: the RT reaction and PCR amplification. RNA is first reverse transcribed into cDNA using a reverse transcriptase and the resulting cDNA is used as templates for subsequent PCR amplification using primers specific for one or more genes.

3.3.1 Preparation of reagents

- **<u>Preparation of stock solution for Human RNase P Forward Primer</u>**
  The stock concentration of Human RNase P forward primer was 100 µM. From the main stock (100 µM), working solution (4 µM) was prepared using ultrapure PCR grade H2O.

- **<u>Preparation of stock solution for Human RNase P Reverse Primer</u>**
  The stock concentration of Human RNase P reverse primer was 100 µM. From the main stock (100 µM), working solution (4 µM) was prepared using ultrapure PCR grade H2O.

- **<u>Preparation of stock solution for Human RNase P Probe</u>**
  The stock concentration of Human RNase P probe was 100 µM. From the main stock (100 µM), working solution (4 µM) was prepared using ultrapure PCR grade H2O.

3.3.2 Preparation of total RNA from HEK293T cells

Total RNA was isolated from HEK 293 cell line using Trizol- Chloroform method as follows:

i. Add 1000 µl of Trizol for two to three million of cells.

ii. Vortex and then slowly triturate to disperse all cells.

iii. Incubate the tube at room temperature for minimum of 15 minutes (vortex the tube each after 5 minutes).

iv. Add 200 to 300 µl of Chloroform (be careful while pipetting and adding the chloroform, because chloroform will drip down from the tip) and vortex for 5 seconds and then centrifuge at 13000 rpm for 20 minutes at 4°C.

v. Collect the clean aqueous solution into a new microfuge tube.

vi. Add two volumes of Isopropanol and mix well by inverting.

vii. Incubate 5 minutes at room temperature. Centrifuge at 13000 rpm for 15 minutes at 4°C.

viii. Remove the supernatant and wash the pellet with 70% ethanol (prepared in DEPC water) twice by centrifuging at 13000 rpm for 15 minutes at 4°C.

ix. Remove the supernatant and air dry the pellet manually.

x. Resuspended the pellet in DEPC water (amount of water is based on the size of the pellet) by tapping the tube thoroughly and then slowly triturate with 10 µl tip.

xi. Quantify the extracted RNA using Nanodrop (260/280 = 1.8- 2.00 and 260/230 = 2.0- 2.20).

3.3.3 Step 1 of RT PCR Method: Preparation of cDNA

cDNA was prepared from RNA (2µg) by Reverse transcription. Reverse Transcription reaction was set up as outlined in the table below:

TABLE 1: cDNA preparation

Reagents	Volume (µl)	Final Concentration	Reaction Conditions
HEK293T total RNA	1.3	2 µg	Incubate at 70°C for 5 min and then quickly put on ice
10 µM oligo dT primer	2	0.8 µM	
Ultrapure PCR grade H2O	11.7		
5X RT Buffer	5	1X	25°C for 5 minutes (Priming) 42°C for 60 minutes (Reverse transcription) 95°C for 2 minutes (RT inactivation) Hold at 4°C
10mM dNTPs	1	0.4 mM	
Revert Aid RT (Benchmark) (200U/µl)	0.5	100U for Revert Aid RT	
Ultrapure PCR grade H2O	3.5		
Total Volume	25		

Reverse transcription reaction was performed using the following conditions:

25°C for 5 minutes (Priming)
42°C for 60 minutes (Reverse transcription)
95°C for 2 minutes (RT inactivation)
Hold at 4°C (Optional step)

Quantity the cDNA solution using Nanodrop, dilute it with ultrapure PCR grade H2O to the tube to make up approximate concentration of cDNA solution 20ng/µl. Aliquot 10 µl of the cDNA solution in PCR tubes and stored at -20˚C.

3.3.4 Step 2 of RT PCR Method: PCR using cDNA as template.

Table 2: Master mix Preparation

Reagent	Volume per reaction (µl)	Final conc.
10 mM dNTPs	0.4	0.2 mM
Dream Taq Enzyme (5U/µl)	0.25	1.25 U
10X Dream Taq Buffer	2	1 X
RNase P Forward Primer (4 µM)	4	0.8 µM
RNase P Reverse Primer (4 µM)	4	0.8 µM
RNase P Probe	2	0.4 µM

Setting up qPCR for the different dilution range (1500 ng to 1.5 ng) using 10 – fold dilution:

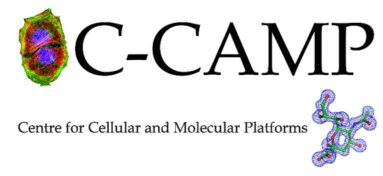
Table 3: Linear range for cDNA template ranging from 1500 – 1.5 ng.

Template (ng)	**Dilution Factor (D.F)**	**Log 10 (D.F)**	**Template (µl)**	**NFW (µl)**
1500	1	0	5 µl from stock	-
150	10	-1	5 µl from stock	46
15	100	-2	1µl from 150 ng	49
1.5	1000	-3	1µl from 15 ng	49

Mix the master mix thoroughly and dispense15 µl appropriate volumes into PCR tubes.
Add 5 µl template from each dilution series to the individual tubes containing the master mix.
Seal the plate with sealer. Spin the plate at 3500 – 4000 rpm for 1 min.

Table4: PCR Cycling condition.

Step	**Stage**	**Temp.**	**Time**
1.	Initial denaturation	95°C	5 min
2.	Denaturation	95°C	30 seconds
3.	Annealing	55°C	30 seconds
4.	Extension	72°C	30 seconds x 45 cycles



4 Results

4.1 Specification, System suitability and Acceptance Criteria

4.1.1 Specifications

RNase P Forward Primer (RP-F): 5’ -AGATTTGGACCTGCGAGCG-3’
RNase P Reverse Primer (RP-R): 5’ -GAGCGGCTGTCTCCACAAGT-3’
RNase P (CY5): 5’- CY5- TTCTGACCTGAAGGCTCTGCGCG-BHQ3-3’

4.1.2 System Suitability

System suitability indicates essential prerequisites for the experiment that are discussed as follows:

- **Compliance to the expected Ct range value determined from previous experiments.** Revert Aid RT enzyme was used as global benchmark. In this study, we have tested only one batch of the Revert Aid RT (Lot no. # 01274452). The following analysis of Ct values helps contribute towards a greater understanding of selecting Revert Aid as Benchmark/ Standard for our study. We have compared the Ct values for standard with repeated experiments done previously as given in the table below.

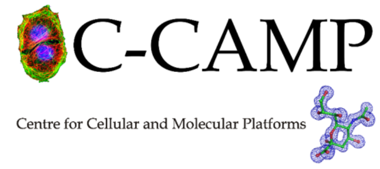
Table 5: Cycle threshold (Ct) values for *RNase P* target gene amplification for standard

Template (ng)	n1			n2			n3		
	Ct (r1)	Ct (r2)	%RSD	Ct (r1)	Ct (r2)	%RSD	Ct (r1)	Ct (r2)	%RSD
1500	23.82	23.62	0.59	24.66	24.71	0.14	23.42	23.01	1.24
150	27.07	27.13	0.15	28.54	27.32	3.08	26.53	26.95	1.11
15	33.02	32.16	1.86	31.82	32.72	1.97	30.31	29.62	1.62
1.5	33.20	32.95	0.53	34.21	33.88	0.68	32.57	32.42	0.32
NTC	Und	Und		Und	Und		Und	Und	

n: no. of independent experiments, %RSD: percent relative standard deviation, NTC: No template control, r: technical duplicates.

Through summation of experiments and analysis of Ct values, it was found that the Ct values for standard were comparable and an acceptance criterion for the acceptable Ct range for standard is given in the section 3.

- **Linearity of the standard curve:** Performance parameters like Slope, Coefficient of determination and Amplification efficiency are considered important to appraise the linearity of the assay.



4.1.3 Acceptance criteria

Following performance parameters are considered in defining the acceptance criteria for two step RT-qPCR.

- **Acceptance criteria for Standard:**

The Ct value should comply with the acceptance criteria for Ct range. The acceptance criteria for acceptable Ct range for the standard is given in the table below.

Table 6: Acceptance criteria for Standard

Specification Acceptance criteria (Ct value range)		
Template (ng)	Min. Ct value	Max. Ct value
1500	23.01	24.71
150	26.53	28.54
15	29.62	33.02
1.5	32.42	34.25

- **Acceptance criteria for standard curve:**

The Test compliance is determined YES when the linear regression statistics are within the acceptable range of criteria for two step RT- qPCR.

Table 7: Acceptance criteria

Criteria	**Acceptable range**
Coefficient of determination (r^2)	0.98 to 1.00
Slope	-3.1 to -3.6
Amplification Efficiency = $-1 + 10^{(-1/slope)}$	90 to 110%
Acceptance Criteria Complies (Yes/No)	

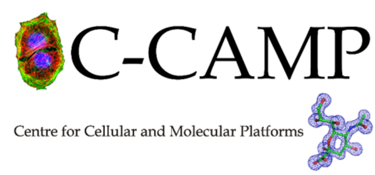
5 Data

5.1 Data for Experiment 1 (n1):

Table 8: Cycle threshold (Ct) values for RNase P target gene amplification for Revert Aid RT, Dx/ Dt Batch 1 and Dx/ Dt Batch 2 sample.

cDNA (ng/µl)	**Revert Aid RT**		**Dx/ Dt Batch 1**		**Dx/ Dt Batch 2**	
	Ct		**Ct**		**Ct**	
	(r1)	(r2)	(r1)	(r2)	(r1)	(r2)
1500	23.54	23.83	23.99	23.56	23.26	23.26
150	27.00	27.11	26.85	26.99	27.03	27.0
15	31.25	31.31	31.43	31.37	31.07	31.25
1.5	34.16	33.93	34.28	34.51	34.21	34.19
NTC	Udt	Udt	Udt	Udt	Udt	Udt

n: no. of experiments, r: Technical replicate, NTC: no template control, Und: undetermined, % RSD: percentage relative standard deviation.



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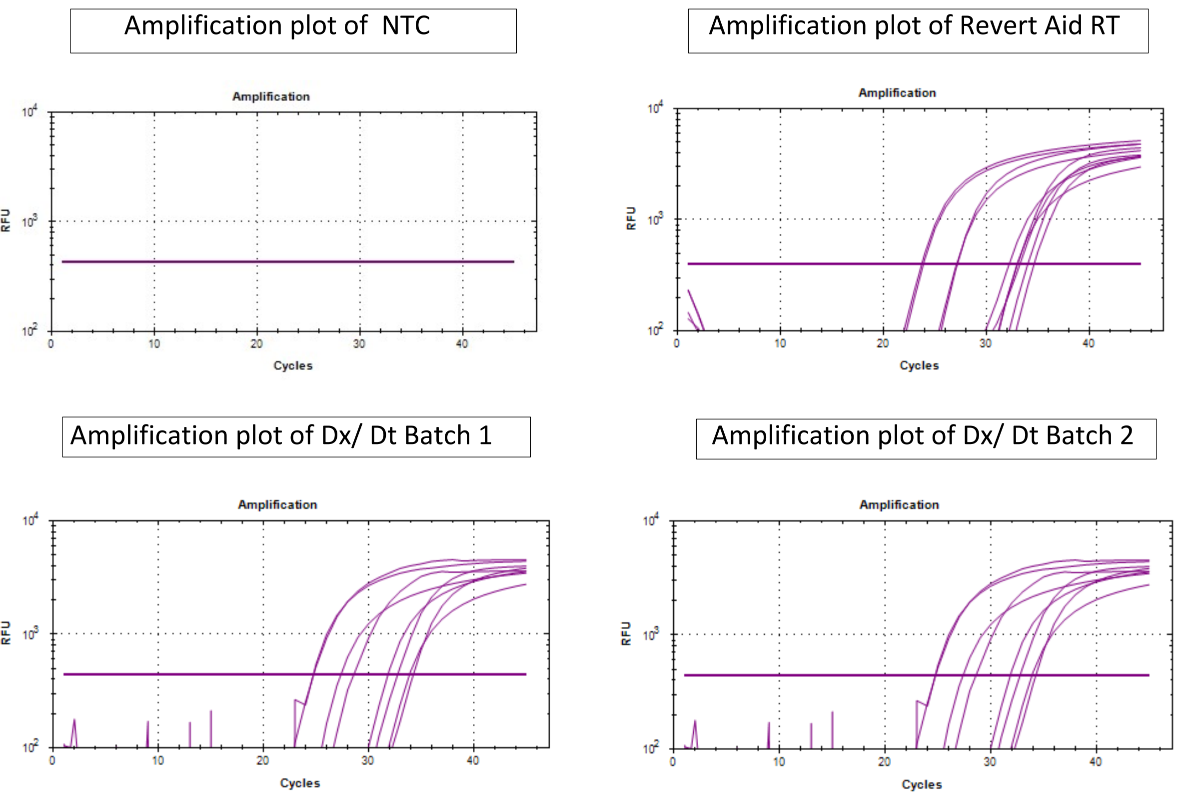

Fig. 1: Amplification plots of cDNA template 10 fold serially diluted ranging from 1500 to 1.5 ng using TaqMan probes for standard and samples.

5.2 Data analysis and interpretation

We have determined linear range using GraphPad Prism 9.0.2 for line fitting with linear regression and interpolation of unknown module. (https://www.graphpad.com/guides/prism/latest/curvefitting/reg_interpolating_from_a_standard_.htm)

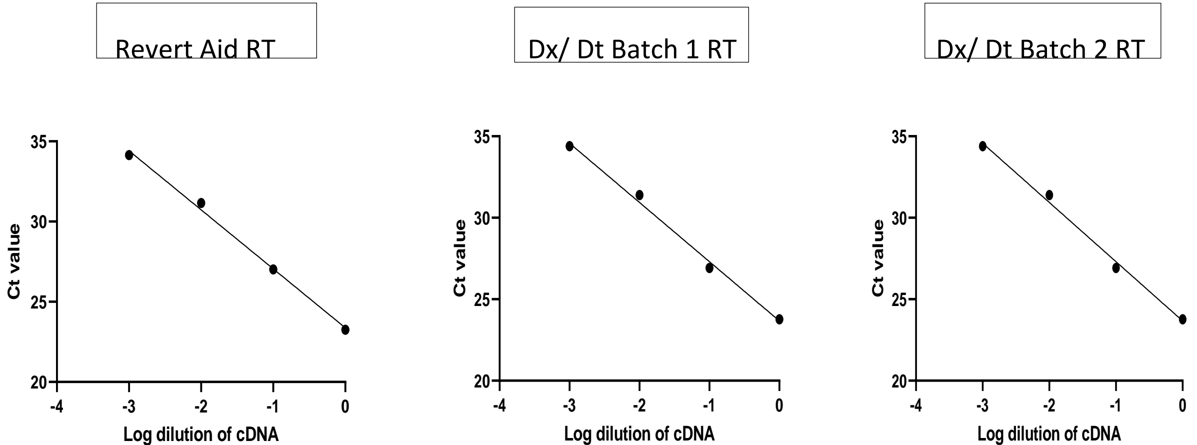

Fig. 2: Linear regression analysis of 10-fold diluted range of cDNA template for RNase P target gene

TABLE 9: Linear regression analysis & compliance with acceptance criteria

Criteria	Acceptable range	Revert Aid RT	Dx/ Dt Batch 1	Dx/ Dt Batch 2
Coefficient of determination (R^2)	(0.98 to 1.00)	0.99	0.99	0.99
Slope	(-3.1 to -3.6)	-3.5	-3.6	-3.6
Reaction Efficiency **Efficiency = $-1 + 10^{(-1/slope)}$**	(90 to 110%)	100 %	90 %	90 %
Criteria compliance (YES/NO)		**Yes**	**Yes**	**Yes**

5.3 Observations:

- Amplification plot and cycle threshold (Ct) values obtained for RNase P.
- RT- qPCR complies with the acceptance criteria for performance.
- Ct values between both the batches of Rt are comparable.

5.4 Data for Experiment 2 (n2):

TABLE 10: Cycle threshold (Ct) values for *RNase P* target gene amplification for Revert Aid RT, Dx/ Dt Batch 1 and Dx/ Dt Batch 2.

cDNA (ng/µl)	**Revert Aid RT**		**Dx/ Dt Batch 1**		**Dx/ Dt Batch 2**	
	Ct		**Ct**		**Ct**	
	(r1)	(r2)	(r1)	(r2)	(r1)	(r2)
1500	23.77	23.49	23.51	23.54	23.66	23.49
150	26.88	26.94	27.08	26.66	27.12	26.79
15	31.23	31.27	31.16	31.32	31.2	31.13
1.5	34.13	34.14	34.03	34.00	34.14	34.17
NTC	Udt	Udt	Udt	Udt	Udt	Udt

n: no. of experiments, r: Technical replicate, NTC: no template control, Und: undetermined, % RSD: percentage relative standard deviation.

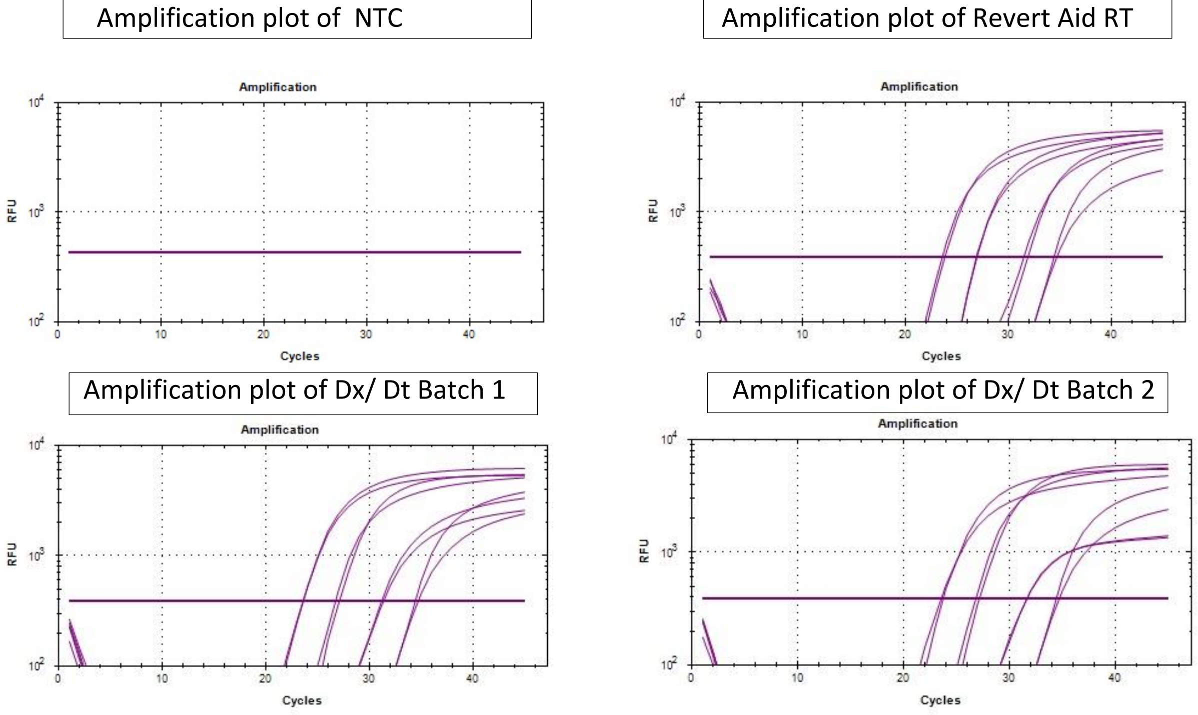

Fig. 1: Amplification plots of cDNA template 10 fold serially diluted ranging from 1500 to 1.5 ng using TaqMan probes for standard and samples.

5.5 Data analysis and interpretation

We have determined linear range using GraphPad Prism 9.0.2 for line fitting with linear regression and interpolation of unknown module.
(https://www.graphpad.com/guides/prism/latest/curvefitting/reg_interpolating_from_a_standard_.htm)

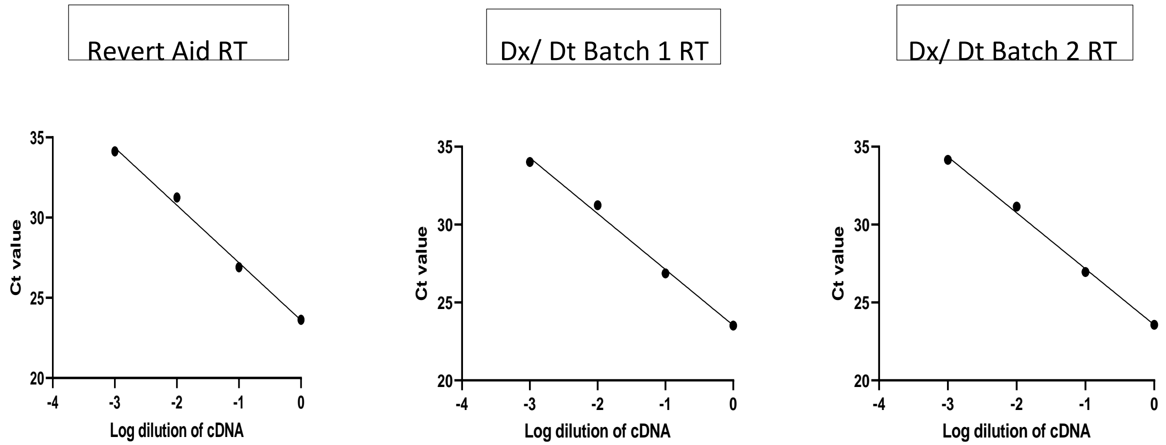

Fig. 2: Linear regression analysis of 10-fold diluted range of cDNA template for RNase P target gene

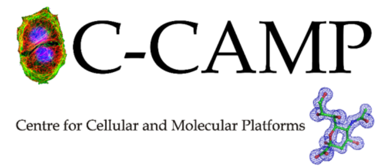

TABLE 11: Linear regression analysis & compliance with acceptance criteria

Criteria	Acceptable range	Revert Aid RT	Dx/ Dt Batch 1RT	Dx/ Dt Batch 2 RT
Coefficient of determination (R^2)	(0.98 to 1.00)	0.99	0.99	0.99
Slope	(-3.1 to -3.6)	-3.58	-3.58	-3.59
Reaction Efficiency **Efficiency = $-1 + 10^{(-1/slope)}$**	(90 to 110%)	90.25 %	90. 25 %	90 %
Criteria compliance (YES/NO)		**Yes**	**Yes**	**Yes**

5.6 Observations:

- Amplification plot and cycle threshold (Ct) values obtained for RNase P.
- RT- qPCR complies with the acceptance criteria for performance.
- Ct values between both the batches of Rt are comparable.

6 Conclusions

- The reverse transcription activity of Revert Aid RT and Dx/ Dt Phi(Ø)Script Reverse transcriptase of Batch 1 and Batch 2 enzyme were found to be comparable in RT qPCR method using TaqMan Probe.
- The optimal performance of Revert Aid RT and Dx/ Dt Phi(Ø)Script Reverse transcriptase of Batch 1 and Batch 2 enzyme were found to be similar as demonstrated by Ct values across the linear range.
- The functional activity of Dx/ Dt Phi(Ø)Script Reverse transcriptase Batch 1 and Batch 2 enzyme was found to be optimal as demonstrated by the values for Regression, Slope and Amplification efficiency.

7 References

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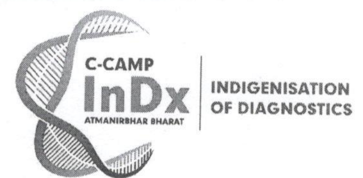

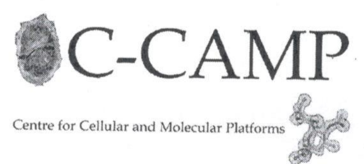

Prepared, Reviewed and Approved by

Project Role	Name	Signature and Date
CCAMP-InDx CoE scientist(s)	Urvashi Chandola	[signature]
CCAMP-InDx CoE lead	Dr. Mohd. Nassirudin	M. Nasiruddin 31/08/2023
CCAMP-InDx S&T lead	Dr. Mitali Samaddar	for MR 31/08/2023
CCAMP-InDx project manager	Dr. Mayooreshwar Rajankar	MR 31/08/2023
CCAMP-InDx COO	Mr. Lalith Kishore	[signature]