Validation of a Lysis Buffer Containing 4 M Guanidinium Thiocyanate (GITC)/ Triton X-100 for Extraction of SARS-CoV-2 RNA for COVID-19 Testing: Comparison of Formulated Lysis Buffers Containing 4 to 6 M GITC, Roche External Lysis Buffer and Qiagen RTL Lysis Buffer

Martina F. Scallan*^[1], Catherine Dempsey*^[2], John MacSharry[1,3,4], Isabelle O'Callaghan[2], Paula M. O'Connor[4,5], Conor P. Horgan[6], Edel Durack[7], Paul D. Cotter[4,5], Sarah Hudson[8], Humphrey A. Moynihan[6] & Brigid Lucey[9,10]

***Joint first and corresponding authors: ^m.scallan@ucc.ie; ^Catherine.dempsey@hse.ie**

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[1]School of Microbiology, University College Cork, College Road, Cork, Ireland; [2]Department of Clinical Microbiology, Cork University Hospital, Wilton, Cork; [3]School of Medicine, UCC; [4]APC Microbiome Ireland, UCC; [5]Teagasc Food Research Centre, Moorepark, Co. Cork; [6]Eli Lilly Kinsale Ltd, Kinsale, Co. Cork; [7]Bernal Institute, University of Limerick, Limerick; [8]Department of Chemical Sciences, University of Limerick; [9]Department of Biological Sciences, Cork Institute of Technology, Bishopstown, Cork; [10]Academy of Clinical Science & Laboratory Medicine, Old Kilmainham, Dublin 8. Ireland

Abstract

The COVID-19 pandemic has resulted in increased need for diagnostic testing using reverse transcriptase real-time PCR (RT-PCR). An exponential increase in demand has resulted in a shortage of numerous reagents in particular those associated with the lysis buffer required to extract the viral RNA. Herein, we describe a rapid collective effort by hospital laboratory scientists, academic researchers and the biopharma industry to generate a validated lysis buffer. We have formulated a 4M Guanidinium thiocyanate (GITC)/ Triton X-100 Lysis buffer which provides comparable results with the recommended reagents. This buffer will ease the burden on hospital labs in their heroic efforts diagnose a large population of patients.

Introduction

The global demand for reagents for real-time reverse transcriptase PCR (RT-PCR) diagnostic tests for COVID-19 has caused a bottle neck across Ireland and the UK in efforts to follow the World Health Organisation's advice to "*Test, Test, Test*".

Expansion of PCR testing is critical to gain control over the pandemic spread of COVID-19 and the world-wide shortage of lysis buffer is a rate limiting step. Here we disseminate details of a lysis buffer formulation that came into existence as a consequence of a rapidly formed multi-centre collaboration. A replacement lysis buffer for extraction of viral nucleic acids from respiratory samples was desperately needed by diagnostic laboratories. A panel of new lysis buffer formulations was generated and validated for use in COVID-19 testing by virus-specific RT-PCR and are being distributed throughout Ireland. It is hoped that by sharing the formulation and validation, we can make it useful to others in the national and international scientific community.

The key chemical constituent of the lysis buffer (guanidinium thiocyanate) has become scarce, scientists from the School of Microbiology, University College Cork, combined their long-held knowledge of lysis buffers for RNA extraction, with insights from published work relating to extraction of RNA using magnetic glass beads (Chomczynski & Sacchi, 1987; Hui He *et al.*, 2017). Ultimately, they formulated a lysis buffer, which uses much less guanidinium thiocyanate than standard formulations appear to use (4 M compared to 6 M). Simultaneously, scientists at Teagasc, Fermoy were working on a different experimental formulation for lysis buffer (Boom *et al.,* 1990). Four laboratory preparations in total were tested for efficacy by medical scientists based in the Clinical Microbiology Department at Cork University Hospital (CUH), who conducted validation experiments using positive and negative samples. After validation, pharma-based scientists, generously prepared a large volume of the much needed 4 M GITC lysis buffer for distribution by CUH.

Virology and RNA biology expertise: Dr Martina Scallan & Dr John Mac Sharry

Buffer preparation: Dr John Mac Sharry & Dr Martina Scallan; Prof Paul Cotter & Paula O'Connor

Evaluation of buffer preparations and validation for use for in-vitro diagnosis of patients tested for Covid-19: Senior medical scientists Catherine Dempsey & Isabelle O'Callaghan

Risk Assessment for buffer preparation: Dr Edel Durack & Dr Sarah Hudson

Co-ordination of scaling of preparation for distribution by CUH: Dr Humphrey Moynihan & Dr Conor Horgan

Co-ordination/preparation of paper: Dr Brigid Lucey

It should be noted that the following chemical preparation, after validation was selected for use with named commercial platforms and testing kits. We recommend that each centre adopting it in their laboratories should first validate it for use with their own systems. It is also advisable to provide a lot number with each batch prepared for traceability.

The 4 M GITC lysis buffer was developed based on the published method of Chomczynski & Sacchi (1987), adapted for magnetic glass bead extraction (Hui He *et al.*, 2017), and validated against recognised standards using positive and negative controls. Details of the composition of 4 M GITC lysis buffer and for the preparation of 1 litre are given below.

The lysis procedure used in the diagnostic laboratory mixes lysis buffer and sample 1:1, this generates a working concentration of 2 M GITC during the lysis step as reported to be optimal by Hui He *et al.,* 2017.

Buffer Composition:

4 M guanidinium thiocyanate (GITC)
55 mM* Tris-HCl
25 mM EDTA (Ethylenediaminetetraacetic acid)
3 % (v/v) Triton X-100
0.01 % (w/v) Bromophenol blue

(*NOTE: calculated from the total amount of 0.1 M Tris pH 7.6 added, diluted by the degree of volume expansion observed when the GITC goes into solution).

Preparation of one litre of 4M Guanidinium thiocyanate (GITC)/ Triton X-100 Lysis buffer (please also read risk assessment):

1. 472.75 g of GITC is brought into solution initially by adding 400 ml of 0.1 M Tris HCl pH 7.6. This will require heating in a 65°C water bath and some shaking of the vessel (but with lid well secured). In our hands, once fully dissolved the volume of the solution was 600 ml.
2. Make up to 750 ml with 0.1 M Tris HCl pH 7.6.
3. Add 50 ml of 0.5 M EDTA, mix.
4. Add 30 ml Triton-X-100, mix
5. Volume made up to 1 L with 0.04 % (w/v) bromophenol blue (DEPC-treated water can be used instead)

Notes:

- See Appendix 1 for chemical agent risk assessment sheet
- Use laboratory coat, safety goggles, gloves, chemical respirator dust mask (N95 mask)
- Open containers of guanidinium thiocyanate powder should be handled in a fume cabinet
- The balance for weighing should be positioned outside the fume cabinet (but close by). Receiving vessel must have a lid that can be sealed for weighing
- Significant chemical or physical hazards notable when preparing buffer: poisoning and environmental hazards
- Buffers made up with DEPC-treated molecular biology grade water
- A significant increase in volume is observed upon GITC dissolution and the degree of volume expansion was found to vary between preparations performed at different sites. This may reflect variations related to the manufacture of GITC and/or storage conditions of the chemical and accentuates the need for individual site validation before use diagnostically.

Validation of the preparation was conducted using two different automated extraction instruments and two different detection instruments.

The validation consisted of a comparison of the efficacy of extraction with four laboratory-prepared buffers and both a Qiagen (RLT lysis buffer from an RNeasy kit Cat. No./ID: 79216) and a Roche external lysis buffer when testing positive (amplifying two Covid-19 specific targets) and negative controls. (In each case the extraction volumes were 200 µl of buffer:200 µl transport medium.) Both positive and negative controls incorporated an internal control. Detection of targets was compared for crossing point values during real time Polymerase Chain Reactions (RT PCR).

Results

Detection of targets was compared for crossing point values (Cp) for the four prepared buffers and the Roche external lysis buffer as shown in **Table 1**. Automated extraction was conducted using the Roche MagNa pure LC and the PCR was performed on the Roche z480 RT PCR instrument. On a second day, the 4 M GITC buffer was tested in comparison with Roche and Qiagen buffers, using the IndiMag48 extraction system and RT PCR was conducted on a Roche LightCycler II 480 (**Table 2**). On the basis of these combined data, the buffer containing 4 M GITC was confirmed as suitable for use with the diagnostic systems in CUH. **Figure 1** shows the relative performance of all buffers tested, measured using Cp values

Table 1. Comparison of the efficacy of four formulations of lysis buffer intended for the extraction of viral RNA from respiratory samples for Covid-19 testing when compared with the Roche external lysis buffer

Sample type	SARS CoV2 Cp^	B beta CoV Cp^	Internal Control Cp^	Lysis Buffer
Positive sample	22.37	22.96	29.46	4 M GITC (pH7.6) with Triton
Negative sample	Not Detected	Not Detected	31.42	4 M GITC (pH7.6) with Triton
Positive sample	22.36	23.36	29.62	5.4 M GITC (pH6.4) with Triton
Negative sample	Not Detected	Not Detected	31.18	5.4 M GITC (pH6.4) with Triton
Positive sample	22.29	23.25	29.44	6 M GITC (pH7.6) with Triton
Negative sample	Not Detected	Not Detected	32.98	6 M GITC (pH7.6) with Triton
Positive sample	22.57	21.69	28.82	4.75 M GITC (pH 7.6) without Triton
Negative sample	Not Detected	Not Detected	30.76	4.75 M GITC (pH 7.6) without Triton
Positive sample	20.85	21.62	27.32	Roche Lysis buffer
Negative sample	Not Detected	Not Detected	28.59	Roche Lysis buffer

^Cp = Crossing point during the test reaction cycles at which test is denoted positive; The complete formulations for the 5.4 M, 6 M GITC preparations with Triton X-100 and the 4.75 M GITC formulation without Triton are not currently listed. pH values refer to the pH of 0.1 M Tris-HCl used in buffer preparation.

Table 2. Comparison of the efficacy of 4 M GITC (with 3% Triton X-100) with Qiagen and Roche lysis buffers

Sample type	SARS CoV2 Cp^	B beta CoV Cp^	Internal Control Cp^	Lysis buffer
Positive sample	22.40	22.36	27.48	Roche
Negative sample	Not Detected	Not Detected	29.55	Roche
Positive sample	21.98	22.50	27.61	Qiagen
Negative sample	Not Detected	Not Detected	31.53	Qiagen
Positive sample	22.79	21.78	28.32	4 M GITC 3% Triton
Negative sample	Not Detected	Not Detected	31.56	UCC

^Cp = Crossing point during the test reaction cycles at which test is denoted positive

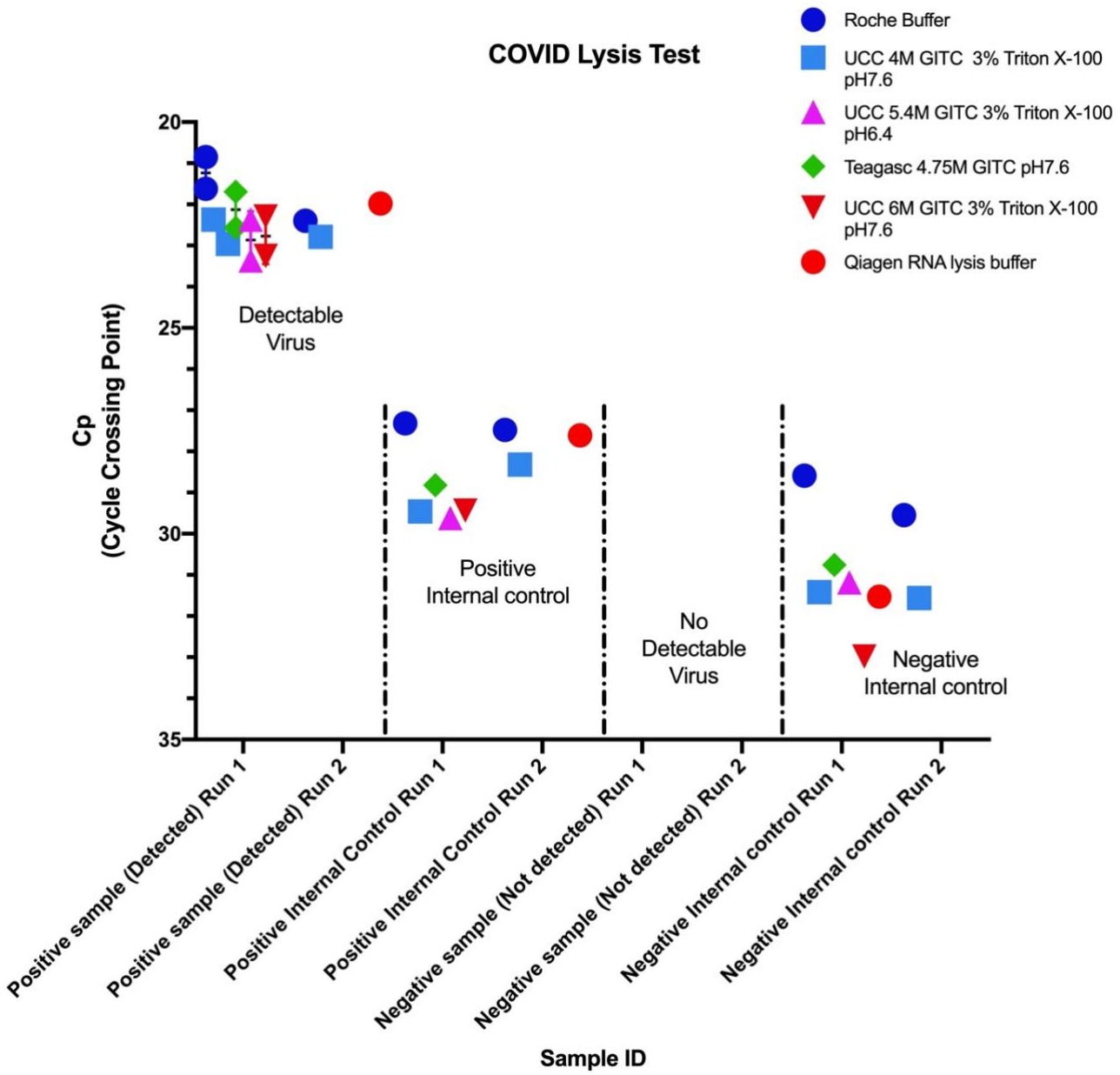


Figure 1. Comparison of the performance of commercial and non-commercial buffers as indicated by Cp values (y-axis) when tested against positive and negative sample controls during real-time RT-PCR diagnostic test for Covid-19.

Based on these data, the 4 M GITC lysis buffer was selected for future preparation and use when extracting respiratory samples for Covid-19.

Discussion

A 4 M GITC lysis buffer with 3 % (v/v) Triton X-100 has been shown to work very well in COVID-19 RT-PCR, it includes a detergent which helps to disintegrate the virus during extraction, bromophenol blue as a visual aid for addition of lysis buffer to clinical samples and has the advantage of using less of a scarce ingredient. The knowledge that 4 M guanidinium thiocyanate (GITC) is sufficient in this lysis buffer means the straitened global supply of this chemical can be shared more effectively to make more lysis buffer to perform more tests around the world and help the world to take back control against this pandemic virus.

Heeding Dr Michael J. Ryan's advice "Perfection is the enemy of the good when it comes to emergency management", we do not want to delay in communicating our findings to the wider world. Further variations of the 4 M GITC lysis buffer

formulation can be tested, in particular formulations with graded increases in % w/v of Triton X-100. Work is ongoing to test if variations in the molarity of Tris-HCl affect lysis buffer performance. Of highest priority is to get the message out that this 4 M GITC lysis buffer works well. In addition we observed that Qiagen's RLT lysis buffer from RNeasy kits worked very well with the systems and processes in place in the diagnostic laboratory at Cork University Hospital.

When Covid-19 testing began, the individual diagnostic microbiology hospital laboratories in Ireland agreed not to stockpile any of their resources from any of the other Irish hospital laboratories. Through their professional body, the Academy of Clinical Science and Laboratory Medicine, they had daily meetings (using WhatsApp) to problem solve as a group where necessary.

Normally, hospital laboratories buy in reagents that allow them to concentrate on the business of diagnosis of patients and the preparation of their own reagents is rare. However, a matter of particular concern was the unexpected short supply of one reagent (lysis buffer) needed to lyse the virus causing COVID-19, to facilitate RNA extractions and subsequent detection. In straitened times, society needs to pool its resources and expertise to best impact and this case study and validation represents a generous and rapid response from the scientific community, when called upon by the Academy of Clinical and Laboratory Medicine, at a time when large international commercial companies were unable to supply this vital reagent.

In the midst of this combined effort, research and medical scientists in other parts of Ireland are communicating their validation results in turn. Part of the group effort in the case being described here is that of the two scientists in the University of Limerick, who prepared the risk assessment documentation for the report, while simultaneously making up buffer and supplying their nearest hospital laboratory at University Hospital Limerick.

In an urgent situation, Irish scientists, academics and biopharma have worked collectively to pool expertise, technical know-how, chemicals from their research laboratories, their facilities, time, co-ordination and communication skills along with determination to develop a suitable replacement lysis buffer at scale in less than two weeks. Like hospital laboratories, third level research laboratories rely on buying ready-to-go kits and reagents to speed up their research. Fortunately, retention of the knowedge regarding the chemical composition of the buffers among the scientists allowed them to make up their own reagents from raw materials.

Acknowledgements

The authors wish to acknowledge colleagues in Sligo Institute of Technology and in the Department of Clinical Microbiology at University College Hospital, Limerick for their valuable assistance during consideration of lysis buffer formulations. Also, Dr John O'Callaghan, Senior Technical Officer, School of Microbiology, UCC for technical assistance during the UCC stages of development and preparation (and for providing the Qiagen buffer), Dr Ewen Mullins of Teagasc for sourcing and sharing reagents, and Dr Darren Crowley and Dr Eoghan Casey who were involved in the scaled-up buffer preparation at Eli Lilly.

References

Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J. Rapid and simple method for purification of nucleic acids. Journal of Clinical Microbiology. 1990;28(3): 495–503.

Chomczynski P & Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry 1987;162,(1):156-159.

Hui He, Rongqun Li, Yi Chen *et al.,* Integrated DNA and RNA extraction using magnetic beads from viral pathogens causing acute respiratory infections. Scientific Reports 2017; 7:45199.

Appendix 1. Risk Assessment of agents used in the preparation of lysis buffer containing Guanidine Thiocyanate, Trizma Base, EDTA disodium salt, Triton-X and Bromophenol Blue.

UNIVERSITY *of* LIMERICK

CHEMICAL AGENT RISK ASSESSMENT SHEET

TITLE OF ACTIVITY: Preparation of L6 buffer

ACADEMIC / PROJECT SUPERVISOR: Sarah Hudson

LOCATION: AD3-041 (Room No.)

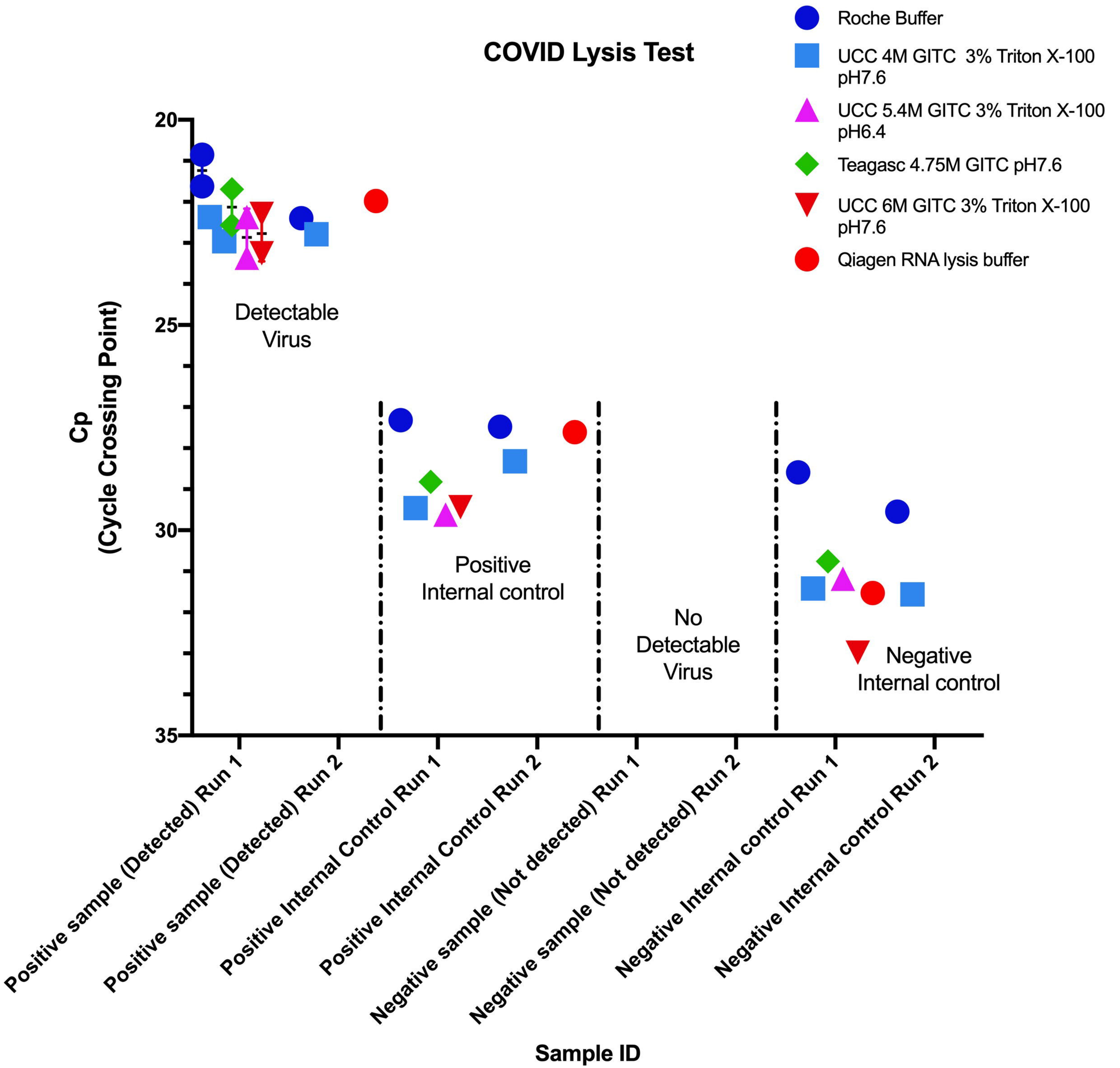
MODULE CODE: N/A (if relevant)

ASSESSMENT UNDERTAKEN BY: Edel Durack

ASSESSMENT DATE: 30/03/2020

ASSESSMENT REVIEW DATE: 30/03/2021

1. (Use GHS Safety Data Sheets (SDS) & Chem. Agent CoP to complete this table.)	HEALTH HAZARD CATEGORIES											OTHER HAZARDS								EXPOSURE								
LIST CHEMICAL NAMES	Toxic Cat 1,2, or 3	Toxic Cat 4	Corrosive Cat 1	Corrosive Cat 2	Sensitising Resp.	Sensitising Skin	Carcinogenicity Cat 1 or 2	Mutagenicity Cat 1 or 2	Reproductive Toxicity Cat 1 or 2	Specific Target Organ Toxicity	Aspiration Toxicity	Flammable Sol,Liq or Gas,	Self-Reactive Organic Peroxide	Pyrophoric/ Self-heating	Oxidising (Sol /Liq or Gas)	Explosive Div 1.1-1.6	Gas under Pressure	Haz. To Aquatic Env.	Ozone Layer Hazard	**Inhalation**	**Skin**	**Eyes**	**Ingestion**	Amount Used. (L or Kg)	Dustiness/ Volatility (High, Med, Low)	Duration of Contact(Hr/Day)	No. of People Exposed.	OELV/BLV
GHS Pictogram																												
Guanidine thiocyanate (Sigma Aldrich G6693)		X	X	X	X						X									X	X	X	X	<1kg	H	<1h	1-3	
Trizma Base (Sigma Aldrich T1503)																				X		X	X	<1kg	L	<1h	1-3	
EDTA disodium dihyrdate (Duchefa Biochemie)																				X	X	X	X	<1kg	L	<1h	1-3	
Triton X-100 (Sigma Aldrich T8787)		X	X	X		X															X	X	X	mls	N/A	<1h	1-3	
Hydrochloric Acid (Sigma Aldrich)		X	X	X		X														X	X	X	X	mls	N/A	<1h	1-3	
Bromophenol Blue sodium salt (Sigma Aldrich 318744)																								mls	N/A	<1h	1-3	


COVID Lysis Test
Roche Buffer
UCC 4M GITC 3% Triton X-100 pH7.6
UCC 5.4M GITC 3% Triton X-100 pH6.4
Teagasc 4.75M GITC pH7.6
UCC 6M GITC 3% Triton X-100 pH7.6
Qiagen RNA lysis buffer
Cp (Cycle Crossing Point)
20
25
30
35
Detectable Virus
Positive Internal control
No Detectable Virus
Negative Internal control
Positive sample (Detected) Run 1
Positive sample (Detected) Run 2
Positive Internal Control Run 1
Positive Internal Control Run 2
Negative sample (Not detected) Run 1
Negative sample (Not detected) Run 2
Negative Internal control Run 1
Negative Internal control Run 2
Sample ID