PlexPCR® RespiVirus

Multiplex real-time RT-PCR assay for the detection of Influenza A, Influenza B, Rhinoviruses A, B and C, Respiratory Syncytial Viruses A and B, Human metapneumovirus, Adenoviruses B and C, and Human parainfluenza viruses 1, 2, 3 and 4

CE 0123 IVD UKCA
IVDR Certified

Product	Platform	Size (reactions)	Catalogue no.
PlexPCR® RespiVirus$_{(610)}$	LC480 II	100	REF **1201001**
PlexPCR® RespiVirus$_{(610)}$	LC480 II	192	REF **1201192**

Accessory products – Analysis software

PlexPCR® RespiVirus (LC480)	REF **99011**

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FOR PROFESSIONAL USE ONLY
Not for sale in the USA

Contents

1 Product description

The ***Plex****PCR*® RespiVirus kit is a qualitative, 2-well, one-step, reverse transcription real-time PCR (RT-qPCR) assay for the detection of Influenza A (FluA), Influenza B (FluB), Rhinoviruses (RhV), Respiratory Syncytial Viruses A and B (RSV A and B), Human metapneumovirus (HMPV), Adenoviruses B and C (AdV B and C) and Human parainfluenza viruses 1, 2, 3 and 4 (HPIV 1-4). Well 1 consists of 5 readouts: Readout 1) FluA, Readout 2) FluB, Readout 3) RSV A and B, Readout 4) RhV and Readout 5) Internal control (to monitor extraction efficiency and RT-qPCR inhibition). Well 2 consists of 3 readouts: Readout 1) HMPV, Readout 2) AdV B and C, and Readout 3) HPIV 1-4. The ***Plex****PCR*® RespiVirus kit utilises ***Plex****Zyme*® technology for specificity and superior multiplexing capability.

The assay is compatible with samples extracted using the NucliSENS® easyMAG® (bioMérieux), MagNA Pure 96 System (Roche), liquid handling using the ***Plex****Prep*® (SpeeDx), and real-time detection on the LightCycler® 480 II Instrument (LC480 II, Roche).

2 Intended use

The ***Plex****PCR*® RespiVirus kit is an *in vitro* diagnostic reverse transcriptase real-time PCR (RT-qPCR) test for the qualitative detection of FluA, FluB, RhV, RSV A and B, HMPV, and HPIV 1-4 RNA and AdV B and C DNA.

The ***Plex****PCR*® RespiVirus kit is intended to aid in the diagnosis of FluA, FluB, RhV, RSV A and B, HMPV, AdV B and C and HPIV 1-4 and should be used in conjunction with clinical and other laboratory information.

The ***Plex****PCR*® RespiVirus kit may be used with the following specimen types: nasopharyngeal swabs only.

The ***Plex****PCR*® RespiVirus kit is intended be used in professional settings such as hospitals, or reference or state laboratories. It is not intended for self-testing, home use, or point-of-care use.

3 Pathogen information

Influenza viruses belong to the family *Orthomyxoviridae* and have a single stranded RNA genome[1]. The influenza viruses are classified into types A, B, and C on the basis of their core proteins, with only type A (FluA) and B (FluB) causing human disease of any concern. FluA viruses are further divided into subtypes according to the specific variety and combinations of two proteins that occur on the surface of the virus, the hemagglutinin or “H” protein and the neuraminidase or “N” protein[1]. In 2009 a novel FluA H1N1 strain emerged and spread across the world causing the 2009 H1N1 pandemic[2]. FluB can be divided into 2 main groups (lineages), referred to as B/Yamagata and B/Victoria lineages[3]. Influenza infections are characterized by a sudden onset of fever, cough (usually dry), headache, muscle and joint pain, severe malaise, sore throat and a runny nose[3]. Most people recover from fever and other symptoms within a week without requiring medical attention, but influenza can cause severe illness or death especially in elderly, in infants and young children, and in immunocompromised hosts[3].

Human parainfluenza viruses are enveloped single-stranded RNA viruses belonging to the *Paramyxoviridae* family, and are divided into types 1 (HPIV 1), 2 (HPIV 2), 3 (HPIV 3) and 4 (HPIV 4)[4]. HPIV 1 and HPIV 2 both cause croup, with HPIV 1 most often identified as the cause in children[4]. Both can also cause upper and lower respiratory illness and cold-like symptoms. HPIV 3 is most commonly associated with bronchiolitis and pneumonia in infants and young children[4]. HPIV 4 is recognized less often but can cause mild to severe respiratory illnesses[4].

Human rhinoviruses are single-stranded RNA viruses, belonging to the *Picornaviridae* family with most serotypes falling into one of three main species: rhinovirus A, rhinovirus B, or rhinovirus C[5]. Rhinoviruses frequently cause the common cold in adults and children. Rhinoviruses are also associated with lower respiratory illness and with a significant burden of disease in infants and young children[5]. Rhinoviruses are frequently associated with exacerbations of asthma and chronic obstructive pulmonary disease in adults[6].

Respiratory syncytial virus (RSV) is a single-stranded RNA genome belonging to the *Paramyxoviridae* family *Pneumovirus* genus. Two subgroups exist, RSV A and RSV B. RSV causes significant morbidity and mortality in infants worldwide, and accounts for as much as 70% of all childhood respiratory infections[8]. In children up to 18 months old, RSV infection is associated with moderate to severe upper and lower respiratory tract infections, with symptoms including acute bronchiolitis, reactive airway disease, and excessive mucus production[9]. While RSV is mostly regarded as a childhood virus, adults are also susceptible to infection.

Like RSV, Human metapneumovirus (HMPV) is a single-stranded RNA genome belonging to the *Paramyxoviridae* family, *Pneumovirus* genus[9]. Approximately 5–15% of all respiratory tract infections of infants and toddlers are caused by HMPV, making it the second most common cause of hospitalisation of young children after RSV[8]. HMPV also causes acute respiratory tract infections that are similar to RSV[9]. Although HMPV mainly targets the young, it can also be associated with immunocompromised adults and the elderly, with serious infections sometimes leading to respiratory failure[8].

Adenoviruses (AdV) are non-enveloped double-stranded DNA viruses that can cause an array of clinical diseases, including pneumonia, conjunctivitis, gastroenteritis, hepatitis, and myocarditis[10]. AdV account for at least 5-10% of paediatric, and 1-7% of adult respiratory tract infections[11]. Gastrointestinal symptoms may be present concomitantly, particularly in children[11]. AdV infections affect infants and young children much more frequently than adults. Severe, disseminated AdV infection can occur in immunocompromised patients[11].

4 Kit contents

Table 1. *PlexPCR*® RespiVirus(610) kit contents				
Cap colour	**Contents**	**Description**	**Quantity (100 reactions)**	**Quantity (192 reactions)***
Yellow	RV1 (610) mix, 20x	Well 1 mix containing oligonucleotides^ for amplification and detection of FluA, FluB, RhV, RSV & RNA internal control assay on the LC480 II	1 x 100 µL	1 x 150 µL
Brown	RV2 (610) mix, 20x	Well 2 mix containing oligonucleotides^ for amplification and detection of AdV, HMPV & HPIV 1-4	1 x 100 µL	1 x 150 µL
Green	***Plex*** Mastermix, 2x	Mastermix containing components necessary for qPCR including dNTPs, $MgCl_2$, DNA polymerase and buffer	2 x 1 mL	2 x 1.2 mL
Neutral	RTase, 100x	Reverse transcriptase enzyme for generating complementary DNA (cDNA) from RNA template	2 x 20 µL	1 x 90 µL
Black	RNase Inhibitor, 50x	RNase inhibitor	2 x 40 µL	1 x 135 µL
Purple	Internal Control RNA#	Internal control micelles containing internal control RNA template to monitor extraction and amplification efficiency	1 x 200 µL	1 x 200 µL
Blue	Nuclease Free Water	PCR grade water	1 x 1 mL	1 x 1 mL

Store template tubes separately from oligo mixes, i.e. template or nucleic acid handling room

^ Oligonucleotides are PCR primer pairs, ***Plex****Zyme*® enzymes and fluorescent probes

* Sufficient for 192 x 10 µL tests. Additional volume supplied for compatibility with liquid handling instrumentation, validated with ***Plex****Prep*® (SpeeDx).

5 Shipping and storage

- The components of the ***Plex****PCR*® RespiVirus kits are shipped on dry ice or ice gel packs. All components should be stored between -25°C and -15°C upon receipt. It is recommended that freeze/thaw cycles are limited to 15.
- When stored under the recommended conditions and handled correctly, activity of the kit is retained until the expiry date stated on the label. Do not use past expiry date.

6 Warnings and precautions

6.1 General

- For *in vitro* diagnostic use only.
- Carefully read these Instructions for Use prior to use. Closely follow procedures as described to ensure reliability of test results. Any deviation from these procedures may affect test performance.
- Users should be adequately trained in the use of the ***Plex****PCR*® RespiVirus assay.
- Any serious incident shall be reported to the manufacturer and competent authority of the Member State in which user and/or patient is established

6.2 Laboratory

- It is recommended to perform sample preparation/extraction, mastermix preparation, sample addition and thermocycling in spatially separated spaces. At a minimum the PCR instrument should ideally be in a separate room to areas where reactions are prepared.
- It is recommended to follow routine laboratory precautions. Wear appropriate personal protective equipment such as gloves, protective eye wear and laboratory coat when handling reagents.
- Pathogenic organisms may be present in clinical specimens. Treat all biological specimens as potentially infectious and follow your institution's safety procedures for handling chemicals and biological samples.
- Follow your institution's hazardous waste disposal procedures for proper disposal of specimens, reagents and other potentially contaminated materials.

6.3 Specimen handling

- Specimens should be collected, transported and stored using standard laboratory techniques or according to collection kit instructions.

6.4 Assay

- Basic precautions for preventing contamination of PCR reactions include the use of sterile filter pipette tips, use of a new pipette tip for every pipetting action, and separation of workflow.
- PCR tests are prone to contamination from previous PCR products. Never open reaction vessels after the completion of PCR.

6.5 Safety precautions

- Safety Data Sheets (SDS) are available on request. Please contact tech@speedx.com.au for more information.

6.6 Assay Plugins: Warnings/Precautions/Limitations

- SpeeDx software can only control the analysis of raw data generated from the test kit when used with its respective PCR instrument. It does not control the preparation of samples, reactions, programming of equipment or delivery of treatment.
- Users should be adequately trained in the use of the ***Plex****PCR*® RespiVirus analysis software and the access should be limited to each assigned single user
- It is recommended to implement user authentication access and cybersecurity controls such as anti-virus software or use of a firewall within the IT system and infrastructure which uses the software
- Upon detection of a cybersecurity incident such as unauthorised access and ransomware attacks, please contact tech@speedx.com.au for further support.

7 Associated Products and Consumables

Positive Control Material

- ***Plex**PCR*® RespiVirus Positive Control kit (SpeeDx, Cat no 95002)

General lab consumables

- Gloves and clean lab coats
- Vortex mixer
- Benchtop centrifuge for 0.5 mL and 1.5 mL tubes
- Micropipettors
- Sterile aerosol-resistant pipette tips
- 0.5 mL tubes and 1.5 mL tubes (PCR-grade)
- 2.0 mL tubes (for pre-dilution of internal control cells)

For NucliSENS® easyMAG® instrument

- 1x Phosphate Buffered Saline (PBS)
- NucliSENS® easyMAG® Lysis Buffer (Biomerieux, Cat no 280134)
- NucliSENS® easyMAG® Magnetic Silica (Biomerieux, Cat no 280133)
- NucliSENS® easyMAG® Extraction buffer 1 (Biomerieux, Cat no 280130)
- NucliSENS® easyMAG® Extraction buffer 2 (Biomerieux, Cat no 280131)
- NucliSENS® easyMAG® Extraction buffer 3 (Biomerieux, Cat no 280132)
- NucliSENS® easyMAG® Disposables (Biomerieux, Cat no 280135)
- 8-well premix strips (for dispensing magnetic silica/Internal Control pre-mix)

For MagNA Pure 96 Instrument

- 1x Phosphate Buffered Saline (PBS)
- MagNA Pure 96 Internal Control Tube (Roche, Cat no 06374905001)
- MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche, Cat no 06543588001)
- MagNA Pure 96 System Fluid (external) (Roche, Cat no 06640729001)
- MagNA Pure 96 Processing Cartridge (Roche, Cat no 06241603001)
- MagNA Pure 96 Pure tip 1000 µL (Roche, Cat no 6241620001)
- MagNA Pure 96 Output Plate (Roche, Cat no 06241611001)
- MagNA Pure Sealing Foil (Roche, Cat no 06241638001)

For LightCycler® 480 Instrument II

- ***Plex**PCR*® Colour Compensation (CC) kit (SpeeDx, Cat no 90001)
- LightCycler® 480 Multiwell Plate 96 (Roche, Cat no 04729692001)
- LightCycler® 480 Multiwell Plate 384 (Roche, Cat no 04729749001)
- LightCycler® 480 Sealing Foil (Roche, Cat no 04729757001)

*For SpeeDx **PlexPrep**® liquid handling instrument*

- ***PlexPrep***® 8 position deck equipped with 2 independent channels and an 8-Probe Head (Part no 6600200-01)
- 4x Framed tip rack modules (Cat no HMT-6600533-01)
- 4x 24 position tube module (Cat no HMT-6600555-01)
- 1x 24 position small tube module (Cat no HMT6600409-01)
- 50 µL conductive filtered tips (Cat no HMT-235948)
- 30 µL conductive filtered tips (Cat no HMT-235903)
- 1000 µL conductive filtered tips (Cat no HMT-235905)

Sample Collection Devices

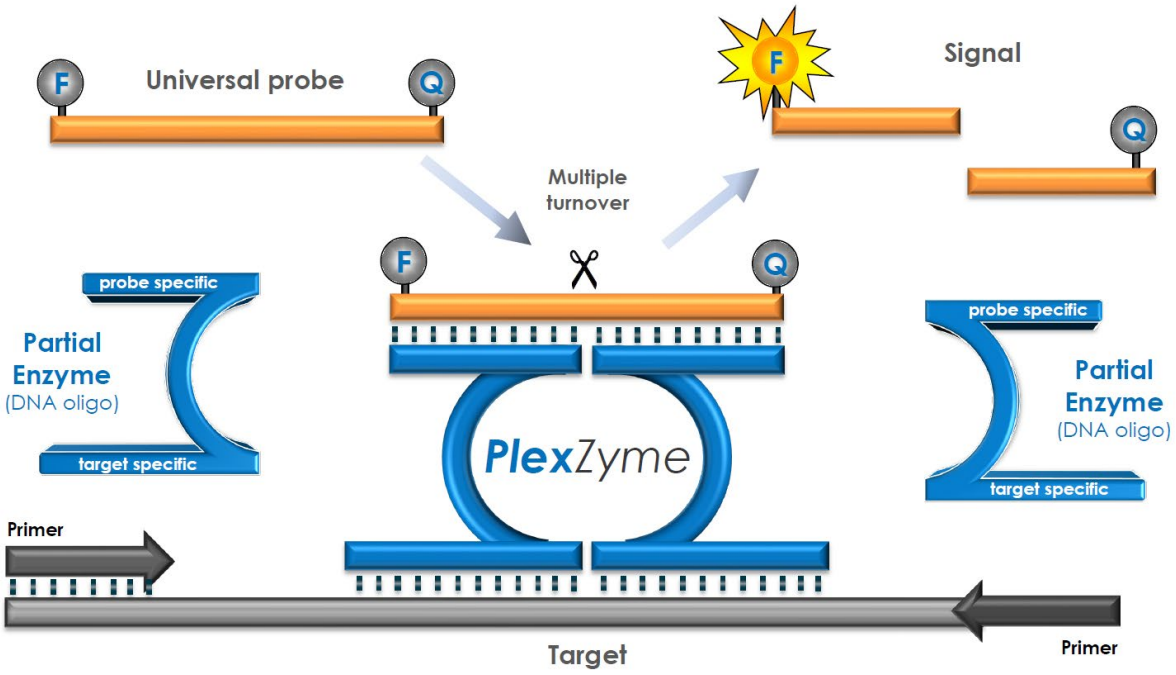
- Copan FLOQSwab® - *compatible with 3mL UTM Tube or Pouch*
- Ultra Minitip Flocked Swab with 100 mm Breakpoint in Peel Pouch (Copan, Cat no 503CS01)
- Ultra Minitip Flocked Swab with 100 mm Breakpoint in Dry Tube (Copan, Cat no 516C)
- Minitip Flocked Swab with 100 mm Breakpoint in Dry Tube (Copan, Cat no 518C)
- Minitip Flocked Swab with 100 mm Breakpoint in Peel Pouch (Copan, Cat no 518CS01)
- Flexible Minitip Flocked Swab with 100 mm Breakpoint in Dry Tube (Copan, Cat no 553C)

8 Principle of the technology

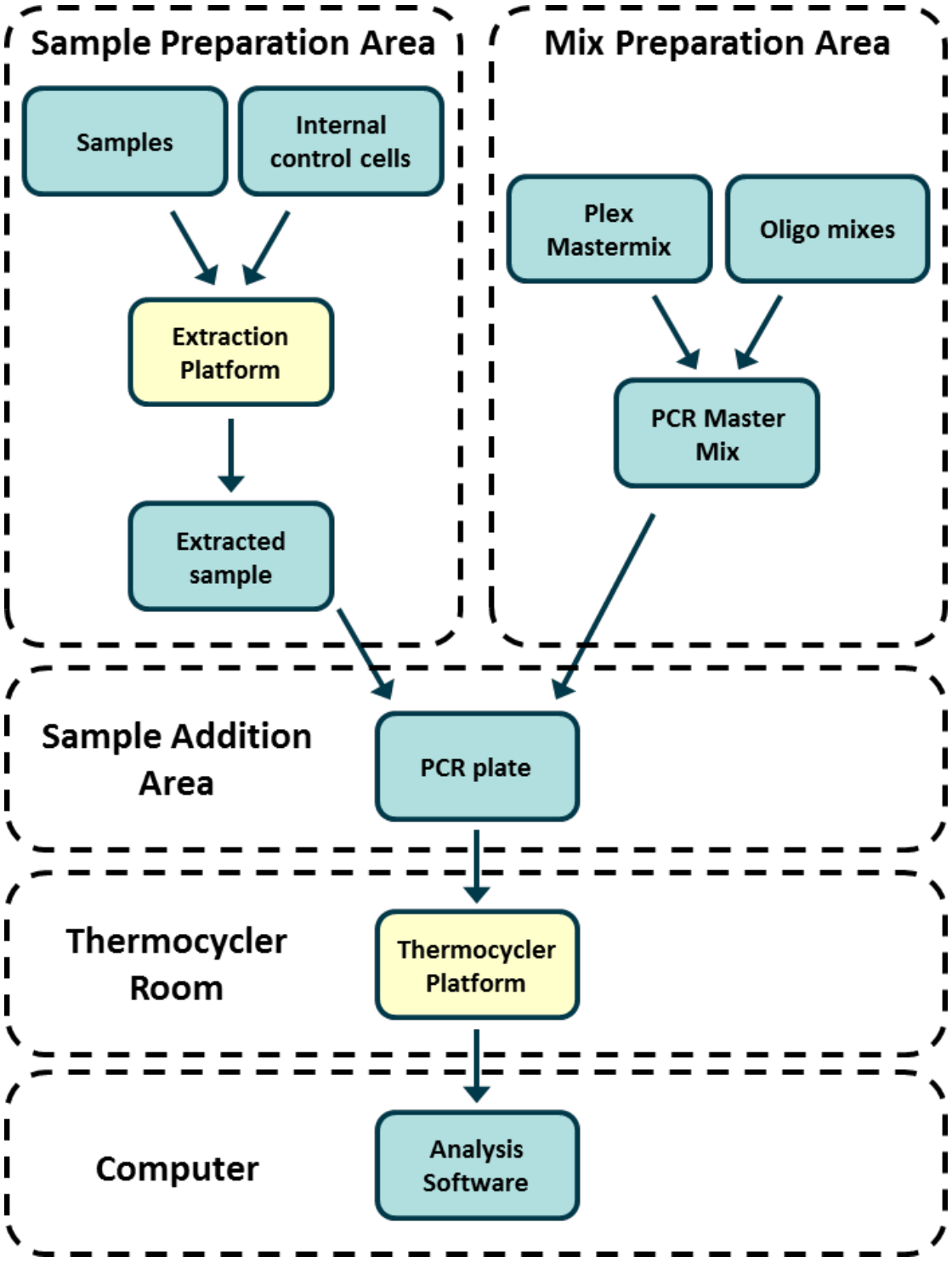
Real-time PCR (qPCR) can be used to amplify and detect specific target nucleic acids from pathogens. ***Plex****PCR*® is a qPCR technology utilising ***Plex****Zyme*® enzymes that detect and report the amplified product through the generation of a fluorescent signal (**Figure 1**).

Plex*Zyme*® enzymes are catalytic DNA complexes composed of two DNA oligos referred to as "Partial Enzymes". Each Partial Enzyme has a target-specific region, a catalytic core and a universal probe binding region. When the target product is present, the two Partial Enzymes bind adjacently to form the active ***Plex****Zyme*® which has catalytic activity to cleave a labelled probe. Cleavage separates the fluorophore and quencher dyes, producing a fluorescent signal that can be monitored in real time. ***Plex****Zyme*® enzymes have additional specificity compared to alternate detection technologies, since two Partial Enzymes are required to bind for detection. ***Plex****Zyme*® enzymes are also multiple turnover enzymes, and multiple probes can be cleaved during each PCR cycle, resulting in a strong and sensitive signal. ***Plex****Zyme*® assays are highly sensitive and specific and are ideally suited for the multiplexed detection of pathogens.

Figure 1. Schematic representation of *PlexZyme*® detection and universal signalling



9 Procedure overview



10 Detailed procedure

Note: Provided reagents are named in italics and colour of the tube cap follows in brackets.

10.1 Sample collection, transport and storage

Inadequate or inappropriate specimen collection, storage and transport are likely to yield false test results. Proper training in specimen collection is highly recommended to ensure specimen quality and stability.

Follow specimen collection device manufacturer instructions for proper collection methods.

Prior to any collection method, trained staff must ensure proper understanding of the device and methodology. At minimum, review the test description for the following: indication of specimen type, sufficient volume, procedure(s), necessary collection materials, patient preparation, and proper handling and storage instructions.

Clinical material collected from patient must be placed in a 16 x 100 mm tube containing a minimum of 3mL of suitable transport medium (e.g., Universal Transport Medium or Viral Transport Medium).

10.1.1 Nasopharyngeal Collection and Handling:

Always follow specimen collection device manufacturer instructions for proper collection methods. Directions are summarized below for the collection of specimens with Copan FLOQSwabs® in pouch that require transportation for analysis.

1. Open the pouch from the side indicated by the arrow and remove the swab taking care not to touch anything with the swab tip.
2. Collect the sample. During sampling, the swab tip must only come in contact with the area from which the specimen is to be taken in order to reduce contamination risks.
3. Check that the test tube and swab sizes are compatible.
4. Insert the swab into the test tube by aligning the swab breakpoint, with the edge of the test tube. The moulded breaking point is detectable as a narrowing of the stick at about 100 mm from the tip.
5. Gently bend the swab until it breaks.
6. Close the test tube by securely screwing on the cap.
7. Discard the broken off part of the swab in a dedicated container.
8. All potentially infectious material must be treated as if it is infectious and transported in sealed biohazard bag(s).
9. Refer to manufacturer's instructions for specific storage temperatures requirements.

10.2 Sample processing

The ***PlexPCR***® RespiVirus kit has been validated on the following extraction instruments in **Table 2**.

See **Section 10.3** for instructions to use the Internal Control.

See **Section 15** for instructions on how to use the ***PlexPCR***® RespiVirus Positive Control kit (Cat no 95002).

Table 2. Validated extraction protocols

Instrument	Extraction kit	Sample volume	Protocol	Elution volume
NucliSENS® easyMAG® [a]	NucliSENS® easyMAG® reagents	400 µL	Generic 2.01 total nucleic acid	50 µL
MagNA Pure 96 [b]	MagNA Pure 96 DNA and Viral NA Small Volume Kit	200 µL	Pathogen Universal 200	50 µL

[a] See 10.3.1 for how to use the internal control on the NucliSENS® easyMAG®

[b] See 10.3.2 for how to use the internal control on the MagNA Pure 96

10.2.1 Storage of extracted samples

The recommended storage of extracted RNA samples is up to 30 days at -70°C.

10.3 Internal Control (IC)

The kit includes an internal control to monitor extraction efficiency and RT-qPCR inhibition. It consists of an internal control assay, which is contained in the RV1 (610) mix (**YELLOW**) and *Internal Control RNA* (**PURPLE**). The RV1 mix is added to the PCR Master Mix (**Table 8**). The *Internal Control RNA* is diluted and processed as below for specific extraction instrument. The internal control RNA template is therefore co-extracted with the sample and co-amplified in the reaction.

10.3.1 Internal Control on the NucliSENS® easyMAG®

Perform a two-step dilution to prepare the *Internal Control RNA* (**PURPLE**) to a final concentration of 1 in 5000 in 1x PBS (**Table 3** and **Table 4**). For additional samples adjust the volume as required using the same dilution factor. Using the diluted internal control RNA and NucliSENS® easyMAG® Magnetic Silica, prepare a 'pre-mix' for the required number of samples (**Table 5**). 100 µL of pre-mix silica is required per sample.

Note: Do NOT store diluted Internal Control RNA

Table 3. Initial dilution of Internal Control RNA for the NucliSENS® easyMAG® (1 in 100 dilution)

Internal Control RNA (PURPLE) (µL)	1x PBS (µL)	Total volume (µL)	Dilution Factor 1
20	1980	2000	100

Table 4. Second dilution of Internal Control RNA for the NucliSENS® easyMAG® (for final 1 in 5000 dilution)

Volume of diluted IC RNA (Initial dilution)*(µL)	1x PBS (µL)	Total volume (µL)	Dilution Factor 2
40	1960	2000	50

* Prepared in **Table 3**

Table 5. Pre-mix of NucliSENS® easyMAG® Magnetic Silica and diluted IC RNA

Number of samples	Volume of diluted IC RNA (Second dilution)* (µL)	Volume of Magnetic silica (µL)	Total volume of mixture
1	50	50	100

* Prepared in **Table 4**

Samples are extracted using the "on-board" workflow on the NucliSENS® easyMAG®. Refer to the NucliSENS® easyMAG® user manual for more information.

"On-board" workflow

Transfer specimens into the sample vessel.

Load sample vessels onto the easyMAG®.

Program the following Extraction Requests:

Protocol: Generic 2.0.1 (for software version 2.0)

Matrix: Other

Volume (mL): 0.400

Eluate (µL): 50 µL

Type: Primary

After on-board lysis, add 100 µL of pre-mix silica to each sample.

Continue extraction process.

10.3.2 Internal Control on the MagNA Pure 96

Dilute the *Internal Control RNA* (**PURPLE**) 1 in 100 in 1x PBS (**Table 6**). Adjust volume as required using the same dilution factor (see extraction kit manual for minimum volume for required number of samples). The diluted internal control RNA is loaded into the Internal Control Tube on the MagNA Pure 96 and 20 µl is automatically added to each sample (default).

Note: Do NOT store diluted Internal Control RNA

Table 6. Dilution of Internal Control Cells for the MagNA Pure 96 (1 in 100 dilution)			
Internal Control RNA **(PURPLE) (µL)**	**1x PBS (µL)**	**Total volume (µL)**	**Volume added to sample (µL)**
36	3564	3600	20

10.4 Preparation of real-time PCR

Note: Before use of reagents, thaw completely, and mix thoroughly by briefly vortexing.

The ***Plex****PCR*® RespiVirus kit can be tested at a final reaction volume of 20 µL in 96-well plates on the LC480 II or at a reaction volume of 10 µL in 384-well plates on the LC480 II. Refer to **Section 22** for instructions on testing a final reaction volume of 10 µL. The ***Plex****PCR*® RespiVirus$_{(610)}$ kit 192 pack size has appropriate dead volume for use with liquid handling systems and has been validated with the SpeeDx ***Plex****Prep*®. Contact tech@speedx.com.au for assistance with protocols.

Refer to **Table 1** for description of kit contents.

10.4.1 Master Mix preparation

Make up the Master Mix as outlined in (**Table 7**).

For a 20 µL reaction volume, 15 µL Master Mix and 5 µL sample is required.

- Make up the Master Mix as outlined in **Table 7**, and then make up the RV1 (well 1) and RV2 (well 2) reaction mixes as outlined in **Table 8** and **Table 9**.
- Pipette the RV1 and RV2 reaction mixes into the PCR plate and then add extracted sample to both reactions.
- Positive and negative controls should be run on each plate.
- Seal, then centrifuge the plate and transfer to thermocycler.

Table 7. Master Mix setup for 20 µL reaction volume		
Reagent	**Concentration**	**Volume per 20 µL reaction (µL)**
Nuclease Free Water (**BLUE**)	N/A	3.4
Plex Mastermix (**GREEN**)	2x	10.0
RTase (**NEUTRAL**)	100x	0.2
RNase Inhibitor (**BLACK**)	50x	0.4
Total volume (µl)		14.0

Table 8. RV1 reaction mix for 20 µL reaction volume		
Reagent	**Concentration**	**Volume per 20 µL reaction (µL)**
Master Mix	N/A	14.0
RV1 (610) mix (**YELLOW**)	20x	1.0
Total volume (µL)		15.0
Add 5 µL sample for a final volume of 20 µL		

Table 9. RV2 reaction mix for 20 µL reaction volume		
Reagent	**Concentration**	**Volume per 20 µL reaction (µL)**
Master Mix	N/A	14.0
RV2 Mix (**BROWN**)	20x	1.0
Total volume (µL)		15.0
Add 5 µL sample for a final volume of 20 µL		

11 Programming and analysis

Details for programming and analysis are described in the **Sections 19 - 20**.

The RV1 Mix uses five channels for detection of FluA, FluB, RhV, RSV & the Internal Control (**Table 10**).

The RV2 Mix uses three channels for detection of AdV, HMPV & HPIV 1-4 (**Table 10**).

Table 10. Channels for *PlexPCR*® RespiVirus targets					
RV1 Mix					
	CHANNEL A	**CHANNEL B**	**CHANNEL C**	**CHANNEL D**	**CHANNEL E**
Instrument	**RhV**	**RSV**	**FluA**	**Internal Control**	**FluB**
LC480 II	465-510	533-580	533-610	533-640	618-660
RV2 Mix					
	CHANNEL A	**CHANNEL B**	**CHANNEL C**	**CHANNEL D**	**CHANNEL E**
Instrument	**HMPV**	**AdV**	**HPIV 1-4**	**n/a**	**n/a**
LC480 II	465-510	533-580	533-610	-	-

Details for programming the LightCycler 480 II instrument and analysis are described in the **Appendices**.

12 Interpretation of results

Data interpretation requires the ***PlexPCR***® RespiVirus analysis software. The ***PlexPCR***® RespiVirus analysis software automates the data interpretation of amplification results and streamlines workflow. Instructions for how to use the analysis software are described in **Section 21**.

See **Table 11** for the appropriate analysis software. The analysis software can be supplied on request. Please contact tech@speedx.com.au for more information.

Table 11. *PlexPCR*® RespiVirus analysis software		
Cat no	**Analysis software***	**Real-time PCR instrument**
99011	***PlexPCR***® RespiVirus (LC480)	LC480 II

* Refer to the website https://plexpcr.com/products/respiratory-infections/plexpcr-respivirus/#resources to ensure you are using the most current version of analysis software.

13 Limitations

- The ***PlexPCR***® RespiVirus assay should only be performed by personnel trained in the procedure and should be performed in accordance to these Instructions for Use.
- Reliable results are dependent on adequate specimen collection transport, storage, and processing. Failure to observe proper procedures in any one of these steps can lead to incorrect results.
- The ***PlexPCR***® RespiVirus assay is a qualitative assay and does not provide quantitative values or information about organism load.
- Results from the test must be correlated with the clinical history, epidemiological data, laboratory data and any other data available to the clinician.
- Prevalence of viral targets will affect the positive and negative predictive values for the assay.
- Negative results do not exclude the possibility of infection due to improper specimen collection, technical error, presence of inhibitors, specimen mix up, or low numbers of organisms in the clinical specimen.
- False positive results may occur due to cross-contamination by target organisms, their nucleic acids or amplified product.
- A false positive Adenovirus B and C result may result from cross-reactivity with Adenovirus groups A, D, E and F (which may also be implicated in respiratory illness) and the enteroviruses (Coxsackievirus B5, Enterovirus 71 and Echovirus) (refer to **Section 16.2.4**).
- Clinical specimens may not be detected by the ***PlexPCR***® RespiVirus analysis software if they contain a very high viral load. Users must check all curves before proceeding. When a high load specimen exceeds the detection limit, specimens should be diluted and re-tested.

14 Quality control

The ***PlexPCR***® RespiVirus kit includes an internal control to monitor extraction efficiency and qPCR inhibition (**Section 10.3**).

The ***PlexPCR***® RespiVirus Positive Control kit (Cat no 95002) is recommended as positive control material for nucleic acid amplification. Refer to **Section 15** for instructions to use the ***PlexPCR***® RespiVirus Positive Control kit. A known negative specimen is recommended to be used as a negative control.

15 *PlexPCR*® RespiVirus Positive Control instructions

The ***PlexPCR***® RespiVirus Positive Control kit contains positive control material for AdV B, AdV C, FluA, FluB, HMPV, RhV, RSV A, RSV B, and HPIV 1 to 4 (**Table 12**).

A known negative specimen can be used as a negative control.

Table 12. Contents for *PlexPCR*® RespiVirus Positive Control kit (cat no 95002)

Cap colour	Contents#	Amplification control targets	Quantity
Orange	RV PC1	Flu A, Flu B, RhV, RSV A, HMPV, AdV B, HPIV 1	1 x 100 µL
Blue	RV PC2	RSV B, AdV C, HPIV 2	1 x 100 µL
White	RV PC3	HPIV 3	1 x 100 µL
Brown	RV PC4	HPIV 4	1 x 100 µL

Store contents separately from mastermix setup area

15.1 Instructions for use

Prepare qPCR reactions as described in **Section 10.4** using positive control material as sample.

Data interpretation requires the ***PlexPCR***® RespiVirus analysis software, refer to **Section 21** for example results.

16 Performance characteristics

16.1 Clinical performance

16.1.1 Clinical Study 1

A retrospective clinical study was conducted at Canterbury Health Laboratories (CHL), Christchurch, New Zealand, on characterised, archived samples from 2016-2017. 322 positive and 35 negative samples were selected for inclusion in the study. The 322 positive samples consisted of 145 nasopharyngeal swabs, 90 nasal swabs, 4 throat swabs, 5 bronchial alveolar lavage samples, 4 bronchial wash samples, 2 tracheal aspirate samples and 72 site unspecified samples. Samples were extracted using the NucliSENS® easyMAG® (bioMérieux) extraction platform using the generic 2.01 total nucleic acid protocol. 400 µL of sample was extracted and the final elution volume was 50 µL. Samples were tested with the ***Plex****PCR*® RespiVirus$_{(610)}$ kit on the LC480 II. A sub-set of 327 samples (295 positive and 32 negative) were also tested on the LC480 II, using a 10 µL final reaction volume (2.5 µL sample) with the ***Plex****PCR*® RespiVirus$_{(610)}$ kit.

The performance of the ***Plex****PCR*® RespiVirus$_{(610)}$ kit were compared to the Fast Track Diagnostics (FTD) Respiratory Pathogens 21 assay. Discrepant samples were re-tested with the FTD Respiratory 21 assay and a qPCR assay[12, 13]. In addition, AdV samples were typed by PCR and Sanger sequencing, and discrepant samples that could not be sequenced or did not contain AdV B/C were excluded from the analysis. Resolved results and sensitivity and specificity of the ***Plex****PCR*® RespiVirus$_{(610)}$ kit are shown in **Table 13** (FluA, FluB, RSV and RhV) and **Table 14** (hMPV, AdV, HPIV) for 20 µL reaction volume and **Table 15** (FluA, FluB, RSV and RhV) and **Table 16** (hMPV, AdV, HPIV) for 10 µL reaction volume.

Table 13. Clinical evaluation of the *PlexPCR*® RespiVirus$_{(610)}$ kit (20 µL reaction volume)

		FTD Respiratory pathogens 21 multiplex assay multiplex PCR assay							
		FluA		FluB		RSV		RhV	
		Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
***PlexPCR*® RespiVirus$_{(610)}$ 96 well**	**Positive**	47	1	48	2	50	0	54	5
	Negative	4	305	3	304	6	301	6	292
Total		51	306	51	306	56	301	60	297
Sensitivity		92.16% CI 81.12% to 97.82%		94.12% CI 83.76% to 98.77%		89.29% CI 78.12% to 95.97%		90.00% CI 79.49% to 96.24%	
Specificity		99.67% CI 98.19% to 99.99%		99.35% CI 97.66% to 99.92%		100.00% CI 98.76% to 100.00%		98.32% CI 96.12% to 99.45%	

CI – 95% confidence interval

Table 14. Clinical evaluation of the *PlexPCR*® RespiVirus$_{(610)}$ kit (20 µL reaction volume) (cont.)

		FTD Respiratory pathogens 21 multiplex assay multiplex PCR assay					
		hMPV		AdV*		HPIV	
		Positive	Negative	Positive	Negative	Positive	Negative
***PlexPCR*® RespiVirus$_{(610)}$ 96 well**	**Positive**	27	0	23	3	82	1
	Negative	0	330	0	323	0	274
Total		27	330	23	326	82	275
Sensitivity		100% CI 87.23% to 100.00%		100.00% CI 85.18% to 100..00%		100.00% CI 95.60% to 100.00%	
Specificity		100.00% CI 98.89% to 100.00%		99.08% CI 97.33% to 99.81%		99.64% CI 97.99% to 99.99%	

CI – 95% confidence interval

* The *Plex*PCR® RespiVirus kit is designed to detect AdV B and C; discrepant sample that could not be sequenced or did not contain AdV B/C (other AdV groups) were excluded from analysis

Table 15. Clinical evaluation of the *PlexPCR*® RespiVirus(610) kit (10 µL reaction volume)									
		FTD Respiratory pathogens 21 multiplex assay multiplex PCR assay							
		FluA		FluB		RSV		RhV	
		Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
PlexPCR® RespiVirus (610) 384 well	Positive	47	2	50	2	24	2	51	5
	Negative	4	274	1	274	5	296	6	265
Total		51	276	51	276	29	298	57	270
Sensitivity		92.16% CI 81.12% to 97.82%		98.04% CI 89.55% to 99.95%		82.76% CI 64.23% to 94.15%		89.47% CI 78.48% to 96.04%	
Specificity		99.28% CI 97.41% to 99.91%		99.28% CI 97.41% to 99.91%		99.33% CI 97.60% to 99.92%		98.15% CI 95.73% to 99.40%	

CI – 95% confidence interval

Table 16. Clinical evaluation of the *PlexPCR*® RespiVirus(610) kit (10 µL reaction volume) (cont.)							
		FTD Respiratory pathogens 21 multiplex assay multiplex PCR assay					
		hMPV		AdV*		HPIV	
		Positive	Negative	Positive	Negative	Positive	Negative
PlexPCR® RespiVirus (610) 384 well	Positive	27	0	22	0	79	0
	Negative	0	300	1	296	2	246
Total		27	300	23	296	81	246
Sensitivity		100% CI 87.23% to 100.00%		95.65% CI 78.05% to 99.89%		97.50% CI 91.36% to 99.70%	
Specificity		100.00% CI 98.78% to 100.00%		100.00% CI 98.76% to 100.00%		100.00% CI 98.51% to 100.00%	

CI – 95% confidence interval

* The ***PlexPCR***® RespiVirus kit is designed to detect AdV B and C; discrepant sample that could not be sequenced or did not contain AdV B/C (other AdV groups) were excluded from analysis

16.1.2 Clinical Study 2

A retrospective clinical study was conducted at Centre of Infectious Diseases and Microbiology Laboratory Services (CIDMLS), Institute of Clinical Pathology and Medical Research (ICPMR). Samples were collected between December 2018- June 2019 and included a total of 367 specimens of which 170 were collected from males and 197 from females. The following specimen types were included: 2 eye swabs, 8 lavage, 4 mouth swabs, 60 nasopharyngeal aspirates, 88 nasopharyngeal swabs, 3 nasopharynx samples, 60 nose swabs, 13 nose/throat aspirates, 4 pharynx swabs, 2 sputum samples, 46 throat swabs, 62 unknown samples, 7 unknown aspirates and 8 miscellaneous samples. A total of 32 samples were excluded from further analysis as they were invalid and could not be repeated due to insufficient re-testing volume. Samples were extracted using the MagNA Pure 96 (MP96) extraction platform using the MagNA Pure 96 DNA and Viral NA Small Volume Kit and Pathogen Universal 200 protocol. 200 µL of sample was extracted and the final elution volume was 50 µL. Samples were tested in 20 µL reactions on the LC480 II instrument using the ***PlexPCR***® RespiVirus(610).

The performance of the ***PlexPCR***® RespiVirus(610) kit was compared to results with a testing site's validated 4 well in-house Respiratory Virus[14] assay on the LightCycler 480 II (Roche) or the Xpert® Xpress Flu/RSV assay on the GeneXpert. The in-house assay has common targets with the ***PlexPCR***® RespiVirus assay: FluA, FluB, RSV A/B, RhV, hMPV, and HPIV1-3. The in-house Respiratory

Assay detects all Adenovirus species but does not target HPIV4, while ***PlexPCR***® RespiVirus$_{(610)}$ targets AdV groups B and C, and includes HPIV4 in the grouped HPIV(1-4) readout. Analysis of results in accordance to specimen type is shown in **Table 17**. The overall sensitivity and specificity of the ***PlexPCR***® RespiVirus (610) kit for Flu A is listed in **Table 18,** Flu B in **Table 19**, RSV in **Table 20**, RhV in **Table 21**, hMPV in **Table 22**, AdV in **Table 23** and HPIV in **Table 24** .

Table 17. Clinical result analysis in accordance to specimen type #

Specimen	Expected Flu A positive	Expected Flu B positive	Expected RSV positive	Expected RhV positive	Expected HMPV positive	Expected AdV positive	Expected HPIV positive
Eye	-	-	-	-	-	2/2	-
Lavage	-	-	1/1	1/1	-	-	-
Mouth swab	0/1	-	-	0/1	-	-	1/1
Nasopharyngeal aspirate	4/5	2/3	17/17	15/17	4/4	11/11	9/9
Nasopharyngeal swab	10/10	16/16	8/8	6/7	7/7	3/3	9/9
Nasopharynx	1/2	-	-	1/1	-	-	-
Nose swab	8/9	3/3	8/8	7/9	2/2	5/5	7/7
Nose/Throat aspirate	-	-	6/6	1/3	-	2/2	3/3
Pharynx swab	1/1	3/3	-	-	-	-	-
Sputum	-	-	-	0/1	-	-	-
Throat swab	9/10	5/5	5/5	7/7	1/1	2/2	3/3
Unknown	10/10	15/15	2/2	9/11	3/3	3/3	8/8
Unknown aspirate	-	-	-	4/4	-	-	3/3
Miscellaneous	1/1	-	1/1	2/2	1/1	2/2	-

Table 18. Clinical performance of the PlexPCR® RespiVirus (610) Flu A

		Reference FluA detection (In-house PCR assay)	
		Positive	**Negative**
PlexPCR® RespiVirus (610)	**Positive**	44	0
	Negative	5	261
	Total	49	261
	Sensitivity	89.8% (95% CI 77.8 – 96.6%)	
	Specificity	100% (95% CI 98.6 – 100%)	

Table 19. Clinical performance of the PlexPCR® RespiVirus (610) Flu B and diagnostic result							
		Reference Flu B detection (In-house PCR assay)		Reference Flu B detection (Xpert® Xpress Flu/RSV)		Reference Flu B detection (Combined)	
		Positive	Negative	Positive	Negative	Positive	Negative
PlexPCR® RespiVirus (610)	Positive	21	0	23	0	44	0
	Negative	1[a]	288	0[b]	2	1[c]	290
	Total	22	288	23	2	45	290
Sensitivity		95.5% (95% CI 77.2 – 99.9%)		100% (95% CI 85.2 – 100%)		97.8% (95% CI 88.2 – 99.9%)	
Specificity		100% (95% CI 98.7 – 100%)		100% (95% CI 15.8 – 100%)		100% (95% CI 98.7 – 100%)	

[a] 2/3 Flu B false negative samples (2 nose swabs) were confirmed negative when retested with the in-house reference method.
[b] 2/2 Flu B false negative samples (1 nasopharyngeal swab and 1 unknown sample) were confirmed negative when tested with the in-house reference method.
[c] 4/5 Flu B false negative samples (2 nose swabs, 1 nasopharyngeal swab and 1 unknown sample) were confirmed negative when retested with the in-house reference method.

Table 20. Clinical performance of the PlexPCR® RespiVirus (610) RSV			
		Reference RSV detection (In-house PCR assay)	
		Positive	Negative
PlexPCR® RespiVirus (610)	Positive	48	1
	Negative	0	261
	Total	48	262
Sensitivity		100% (95% CI 92.6 – 100%)	
Specificity		99.6% (95% CI 97.9 – 100%)	

Table 21. Clinical performance of the PlexPCR® RespiVirus (610) RhV			
		Reference RhV detection (In-house PCR assay)	
		Positive	Negative
PlexPCR® RespiVirus (610)	Positive	53	10
	Negative	11[#]	236
	Total	64	246
Sensitivity		82.8% (95% CI 71.3 – 91.1%)	
Specificity		95.9% (95% CI 92.7 – 98.0%)	

[#] 2/13 RhV False negative samples (1 nose aspirate and 1 unknown sample) were confirmed negative when retested with the in-house reference method

Table 22. Clinical performance of the PlexPCR® RespiVirus (610) hMPV			
		Reference hMPV detection (In-house PCR assay)	
		Positive	Negative
PlexPCR® RespiVirus (610)	Positive	18	11
	Negative	0[#]	281
	Total	18	292
	Sensitivity	100% (95% CI 81.5 – 100%)	
	Specificity	96.2% (95% CI 93.4 – 98.1%)	

[#] 3/3 hMPV false negative samples (1 nasopharyngeal aspirate, 1 nasopharyngeal swab and 1 lavage) were confirmed negative when retested with the in-house reference method.

Table 23. Clinical performance of the PlexPCR® RespiVirus (610) AdV			
		Reference AdV detection (In-house PCR assay)	
		Positive	Negative
PlexPCR® RespiVirus (610)	Positive	30	7[^]
	Negative	0	273
	Total	30	280
	Sensitivity	100% (95% CI 88.4 – 100%)	
	Specificity	97.5% (95% CI 94.9 – 99.0%)	

[^] 2/9 AdV false positive samples (2 nasopharyngeal aspirates) were confirmed positive when retested with the in-house reference method.

Table 24. Clinical performance of the PlexPCR® RespiVirus (610) HPIV			
		Reference HPIV detection (In-house PCR assay)	
		Positive	Negative
PlexPCR® RespiVirus (610)	Positive	43	7
	Negative	0	260
	Total	43	267
	Sensitivity	100% (95% CI 91.8 – 100%)	
	Specificity	97.4% (95% CI 94.7 – 98.9%)	

16.2 Analytical performance

16.2.1 Reproducibility

The reproducibility of the ***PlexPCR***® RespiVirus kit was assessed using quantified genomic template for FluA, FluB, RSV A, RSV B, RhV, HMPV, AdV B, AdV C and HPIV 1-4, tested at 3x LOD. Experiments were performed on the LC480 II utilising 20 µL reaction volume in 96-well plates.

To determine lot-to-lot variability, two lots were tested, run on one machine performed by one operator (**Table 25**). The two lots showed good reproducibility with coefficient of variation (%CV) between 0.68-1.66%.

Table 25. Lot-to-lot variability

	Average Cq	STDEV	%CV	# Samples
FluA 180 genomes	24.2	0.28	1.18	12/12
FluB 150 genomes	24.5	0.41	1.66	12/12
RSV A 90 genomes	22.7	0.15	0.68	12/12
RSV B 135 genomes	23.4	0.28	1.12	12/12
RhV 150 genomes	25.2	0.25	1.01	12/12
HMPV 90 genomes	22.2	0.30	1.34	12/12
AdV B 105 genomes	21.2	0.34	1.58	12/12
AdV C 105 genomes	21.1	0.24	1.14	12/12
HPIV 1 48 genomes	21.8	0.30	1.37	12/12
HPIV 2 60 genomes	21.6	0.24	1.12	12/12
HPIV 3 48 genomes	23.2	0.22	0.93	12/12
HPIV 4 72 genomes	22.5	0.33	1.45	12/12

To determine day-to day variability, testing was performed over three days by one operator on the same machine (**Table 26**). The three runs showed good reproducibility between different days with coefficient of variation (%CV) between 0.86-2.66%.

Table 26. Day-to-day variability

	Average Cq	STDEV	%CV	# Samples
FluA 180 genomes	24.4	0.52	2.14	18/18
FluB 150 genomes	23.8	0.63	2.66	18/18
RSV A 90 genomes	22.8	0.34	1.50	18/18
RSV B 135 genomes	23.6	0.52	2.21	18/18
RhV 150 genomes	25.7	0.50	1.96	18/18
HMPV 90 genomes	23.2	0.26	1.13	18/18
AdV B 105 genomes	22.0	0.40	1.83	18/18
AdV C 105 genomes	22.3	0.23	1.03	18/18
HPIV 1 48 genomes	23.2	0.30	1.28	18/18
HPIV 2 60 genomes	22.5	0.24	1.06	18/18
HPIV 3 48 genomes	24.3	0.29	1.19	18/18
HPIV 4 72 genomes	23.5	0.20	0.86	18/18

To determine run-to-run variability, three qPCR runs were compared, run on the same day by the same operator (**Table 27**). The three runs showed good reproducibility with coefficient of variation between 0.90-2.18%.

Table 27. Run-to-run variability				
	Average Cq	STDEV	%CV	# Samples
FluA 180 genomes	24.8	0.54	2.17	18/18
FluB 150 genomes	23.9	0.32	1.33	18/18
RSV A 90 genomes	23.3	0.51	2.18	18/18
RSV B 135 genomes	24.2	0.32	1.34	18/18
RhV 150 genomes	26.0	0.27	1.04	18/18
HMPV 90 genomes	22.9	0.31	1.36	18/18
AdV B 105 genomes	21.5	0.26	1.20	18/18
AdV C 105 genomes	22.0	0.20	0.90	18/18
HPIV 1 48 genomes	23.1	0.44	1.91	18/18
HPIV 2 60 genomes	22.4	0.29	1.30	18/18
HPIV 3 48 genomes	24.1	0.31	1.27	18/18
HPIV 4 72 genomes	23.3	0.35	1.51	18/18

To determine operator variability, two runs were compared from two operators (**Table 28**). The two runs from different operators showed good reproducibility with coefficient of variation between 1.38-2.67%.

Table 28. Operator variability				
	Average Cq	STDEV	%CV	# Samples
FluA 180 genomes	24.6	0.50	2.04	12/12
FluB 150 genomes	23.9	0.36	1.50	12/12
RSV A 90 genomes	23.1	0.32	1.38	12/12
RSV B 135 genomes	23.8	0.48	2.02	12/12
RhV 150 genomes	26.0	0.44	1.68	12/12
HMPV 90 genomes	22.7	0.60	2.66	12/12
AdV B 105 genomes	22.0	0.59	2.67	12/12
AdV C 105 genomes	22.1	0.42	1.89	12/12
HPIV 1 48 genomes	22.6	0.53	2.33	12/12
HPIV 2 60 genomes	22.1	0.40	1.80	12/12
HPIV 3 48 genomes	23.9	0.49	2.06	12/12
HPIV 4 72 genomes	23.2	0.41	1.77	12/12

To determine instrument variability, two runs from two machines were compared, performed by the same operator (**Table 29**). The runs from different instruments showed good reproducibility with coefficient of variation between 0.86-1.51%.

Table 29. Instrument variability				
	Average Cq	**STDEV**	**%CV**	**# Samples**
FluA 180 genomes	24.1	0.27	1.12	12/12
FluB 150 genomes	23.3	0.24	1.03	12/12
RSV A 90 genomes	22.5	0.31	1.36	12/12
RSV B 135 genomes	23.0	0.24	1.04	12/12
RhV 150 genomes	25.8	0.30	1.18	12/12
HMPV 90 genomes	22.3	0.27	1.22	12/12
AdV B 105 genomes	21.6	0.27	1.28	12/12
AdV C 105 genomes	21.7	0.19	0.86	12/12
HPIV 1 48 genomes	22.2	0.33	1.51	12/12
HPIV 2 60 genomes	21.9	0.23	1.07	12/12
HPIV 3 48 genomes	23.5	0.27	1.14	12/12
HPIV 4 72 genomes	22.7	0.20	0.90	12/12

16.2.2 Analytical sensitivity

The analytical sensitivity of the ***PlexPCR***® RespiVirus kit was established on the LC480 II using 20 ul reaction volumes on 96-well plates. A limited dilution series was performed using DNA/RNA template for FluA, FluB, RSV A, RSV B, RhV, HMPV, AdV B, AdV C and HPIV 1-4. The LOD of each target was confirmed using an additional 20 replicates. The sensitivity for each target was determined as the lowest number of genomes of template per reaction that could be detected with ≥95% of replicates performed (**Table 30**). The sensitivity of each target for 10 µL reaction volumes was performed on the LC480 II using 384-well plates and was determined when ≥95% of replicates performed could be detected at the stated concentration (**Table 30**).

Table 30. Analytical sensitivity		
Target	**Limit of detection (genomes/reaction)**	
	10 µL reaction volume	**20 µL reaction volume**
FluA*	40	60
FluB	32.5	50
RSV A	15	30
RSV B	25	45
RhV	25	50
HMPV	17.5	30
AdV B	15	35
AdV C	17.5	35
HPIV 1	8	16
HPIV 2	10	20
HPIV 3	8	16
HPIV 4	12	24

*FluA H5N1 serotype

16.2.3 Inclusivity

Additional strains were tested for inclusivity using 20 µL reaction volumes on the LC480 II. Each strain of FluA was tested at 1x LOD genomes per reaction. All strains achieved ≥ 95% detection (**Table 31**).

Table 31. Analytical inclusivity

Strain	20 µL reaction volume
	# Replicates
H1N1 strain A/Virginia/ATCC/2009	19/20
H3N2 strain A/Aichi/2/68	20/20

16.2.4 Analytical specificity

The ***Plex****PCR*® RespiVirus kit was designed to be specific by checking for homology to non-target organisms in public sequence databases. Specificity testing of the ***Plex****PCR*® RespiVirus kit was performed on the LC480 II utilising 20 µL reaction volumes in 96-well plates. Cross-reactivity was observed with Adenovirus groups A, D, E and F and the enteroviruses (Coxsackievirus B5, Enterovirus 71 and Echovirus) at the stated concentrations; all other selected organisms did not show cross-reactivity at the stated concentrations (**Table 32**).

Table 32. Analytical specificity

Organism	Test concentration (genomes/reaction)	Results
Adenovirus A	10^6	Detected*
Adenovirus D	10^6	Detected*
Adenovirus E	10^6	Detected*
Adenovirus F	10^4	Detected*
Bordetella parapertussis	10^4	Not detected
Bordetella pertussis	10^4	Not detected
Candida albicans	10^4	Not detected
Chlamydophila pneumoniae	10^6	Not detected
Coxsackievirus B5	10^4	Detected**
Cytomegalovirus	10^4	Not detected
Enterovirus 71	10^4	Detected**
Echovirus 5	10^4	Detected**
Epstein-Barr virus	10^4	Not detected
Escherichia coli	10^6	Not detected
Haemophilus influenzae	10^6	Not detected
Herpes Simplex Virus Type 1	10^4	Not detected
Herpes Simplex Virus Type 2	10^4	Not detected
Herpes virus 6 DNA	10^4	Not detected
Human Parvovirus B19	10^6	Not detected
Klebsiella pneumoniae	10^6	Not detected
Legionella longbeachae	10^6	Not detected
Legionella pneumophila	10^4	Not detected

Table 32. Analytical specificity		
Organism	**Test concentration (genomes/reaction)**	**Results**
Measles	10^4	Not detected
Moraxella catarrhalis	10^4	Not detected
Mumps	10^4	Not detected
Mycoplasma avium subspecies *paratuberculosis*	10^6	Not detected
Mycoplasma genitalium	10^3	Not detected
Mycoplasma hominis	10^4	Not detected
Mycoplasma pneumoniae	10^4	Not detected
Mycoplasma tuberculosis	10^6	Not detected
Neisseria gonorrhoeae strain FA 1090	10^6	Not detected
Neisseria meningitidis	10^6	Not detected
Parechovirus	10^4	Not detected
Salmonella typhi	10^4	Not detected
Staphylococcus aureus (mecA-)	10^4	Not detected
Streptococcus agalactiae	10^6	Not detected
Streptococcus pneumoniae	10^6	Not detected
Streptococcus salivarius	10^6	Not detected
Ureaplasma urealyticum	10^3	Not detected
Varicella zoster virus	10^4	Not detected

* Adenoviridae family detected by AdV B/C

** Enterovirus genus detected by RhV

16.2.5 Competitive interference

To examine the potential for competitive interference between co-amplified targets of the ***PlexPCR***® RespiVirus kit, contrived samples to simulate co-infections were prepared. Detection of FluA, FluB, RSV A, RSV B, RhV, HMPV, AdV B, AdV C and HPIV 1-4 at low concentrations (3x LOD) was compared to detection in mixed samples spiked with a high concentration of another target. Experiments were performed on the LC480 II utilising 20 µL reaction volume in 96-well plates. All targets were correctly detected, indicating that competitive interference was not observed in any of the combinations tested (**Table 33** and **Table 34**).

Table 33. Competitive interference					
Low concentration target		**Competitive interferent (high concentration)**		**# Samples detected**	
Target	**3x LOD (genomes/reaction)**	**Target**	**Genomes/reaction**	**Number correctly detected**	**% Correctly detected**
FluA	180	--	--	3/3	100
		FluB	10,000	3/3	100
		RSV A	10,000	3/3	100
		RSV B	10,000	3/3	100
		RhV	10,000	3/3	100
FluB	150	--	--	3/3	100
		FluA	10,000	3/3	100
		RSV A	10,000	3/3	100
		RSV B	10,000	3/3	100
		RhV	10,000	3/3	100
RSV A	90	--	--	3/3	100
		FluA	10,000	3/3	100
		FluB	10,000	3/3	100
		RhV	10,000	3/3	100
RSV B	135	--	--	3/3	100
		FluA	10,000	3/3	100
		FluB	10,000	3/3	100
		RhV	10,000	3/3	100
RhV	150	--	--	3/3	100
		FluA	10,000	3/3	100
		FluB	10,000	3/3	100
		RSV A	10,000	3/3	100
		RSV B	10,000	3/3	100

Table 34. Competitive interference					
Low concentration target		**Competitive interferent (high concentration)**		**# Samples detected**	
Target	**Genomes/reaction (3x LOD)**	**Target**	**Genomes/reaction**	**Number correctly detected**	**% Correctly detected**
HMPV	90	--	--	3/3	100
		AdV B	10,000	3/3	100
		AdV C	10,000	3/3	100
		HPIV 1	10,000	3/3	100
		HPIV 2	10,000	3/3	100
		HPIV 3	10,000	3/3	100
		HPIV 4	10,000	3/3	100
AdV B	105	--	--	3/3	100
		HMPV	10,000	3/3	100
		HPIV 1	10,000	3/3	100
		HPIV 2	10,000	3/3	100
		HPIV 3	10,000	3/3	100
		HPIV 4	10,000	3/3	100
AdV C	105	--	--	3/3	100
		HMPV	10,000	3/3	100
		HPIV 1	10,000	3/3	100
		HPIV 2	10,000	3/3	100
		HPIV 3	10,000	3/3	100
		HPIV 4	10,000	3/3	100
HPIV 1	48	--	--	3/3	100
		HMPV	10,000	3/3	100
		AdV B	10,000	3/3	100
		AdV C	10,000	3/3	100
HPIV 2	60	--	--	3/3	100
		HMPV	10,000	3/3	100
		AdV B	10,000	3/3	100
		AdV C	10,000	3/3	100
HPIV 3	48	--	--	3/3	100
		HMPV	10,000	3/3	100
		AdV B	10,000	3/3	100
		AdV C	10,000	3/3	100
HPIV 4	72	--	--	3/3	100
		HMPV	10,000	3/3	100
		AdV B	10,000	3/3	100
		AdV C	10,000	3/3	100

16.2.6 Potentially interfering substances

The effect of potential interfering substances on the ***Plex****PCR*® RespiVirus kit was assessed in contrived samples through the performance of the Internal Control, which monitors extraction and RT-qPCR inhibition. The potential inhibitory substances tested included substances that may be found in clinical specimens. These included whole blood, Listerine® mouth wash (Johnson & Johnson Pacific Pty Ltd), Otrivin® nasal spray (GlaxoSmithKline Consumer Healthcare Australia Pty Ltd), Alanase nasal spray (Mylan New Zealand Ltd), Mucin or bovine submaxillary gland, type I-S (Sigma-Aldrich Inc) and Difflam® throat lozenge (iNova Pharmaceuticals (Aust) Pty Ltd). Each inhibitory substance was spiked into negative nasopharyngeal swab specimens and extracted with Internal Control RNA and assessed by the Internal Control performance, run on the LC480 II using 20 µL reaction volume in 96-well plates. None of the potential inhibitory substances affected the performance of the Internal Control assay (**Table 35**).

Table 35. Potentially interfering substances

Substance	Concentration	IC Average Cq	STDEV	# samples detected
--	--	23.57	0.12	3/3
Whole Blood	2% v/v	23.31	0.38	3/3
Listerine® mouth wash	1% v/v	23.69	0.20	3/3
Otrivin® nasal spray	1% v/v	23.74	0.42	3/3
Alanase nasal spray	1.5% v/v	23.56	0.33	3/3
Mucin (bovine submaxillary gland, type I-S)	1% w/v	23.44	0.68	3/3
Difflam® throat lozenge	1% w/v	23.65	0.05	3/3

16.3 External quality assessment programmes

The performance of the ***Plex****PCR*® RespiVirus kit was assessed on quality assurance panels from Quality Control for Molecular Diagnostics (QCMD) and Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP). For detailed results or further information please contact tech@speedx.com.au.

17 Customer and technical support

Please contact Technical Support for questions on reaction setup, cycling conditions and other enquiries.

Tel: +61 2 9209 4170, Email: tech@speedx.com.au

18 References

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19 Appendix 1a: LightCycler® 480 Instrument II programming for reaction volume of 20 µl

The following information is based on LightCycler 480 Software (version 1.5).

The ***PlexPCR***® RespiVirus$_{(610)}$ kit contains dyes for the LightCycler® 480 Instrument II. The ***PlexPCR***® Colour Compensation kit (Cat no 90001) must be run and applied for LC480 II analysis (see **Section 19.2**). This kit can be supplied on request.

19.1 Programming the LightCycler® 480 Instrument II (LC480 II)

Detection Format

Create a custom **Detection Format**

Open Tools > **Detection Formats**

Create a New Detection Format, and name '**SpeeDx PlexPCR**' (may be created during the generation of SpeeDx Colour Compensation file) (See **Figure 2**)

For **Filter Combination Selection** select the following (Excitation-Emission) shown in **Table 36**:

Table 36. Filter combinations^

LC480 II	440-488	465-510	533-580	533-610	533-640	618-660

^ These Filter Combinations are the default names for the channels

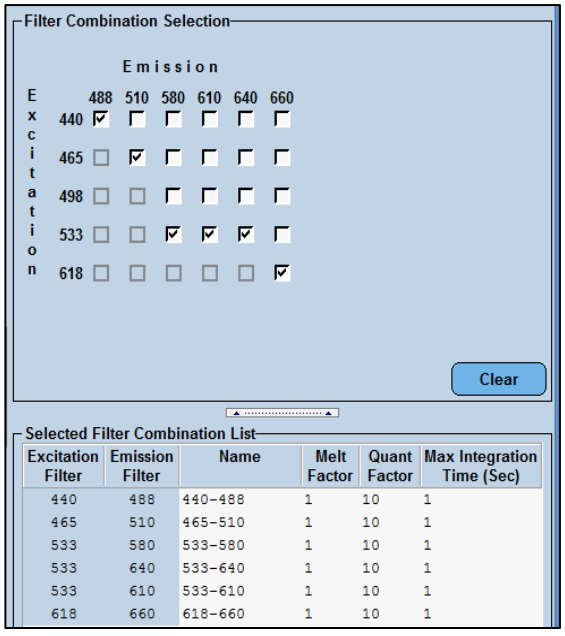
Set the **Selected Filter Combination List** for all channels as:

Melt Factor: 1

Quant Factor: 10

Max Integration Time (sec): 1

Figure 2. Custom SpeeDx Detection Format



Instrument Settings

Create a custom **Detection Format**

Open Tools > **Instruments**

For **Instrument Settings** > select **Barcode Enabled**

Experiment setup

Select **New Experiment**

In the **Run Protocol** tab

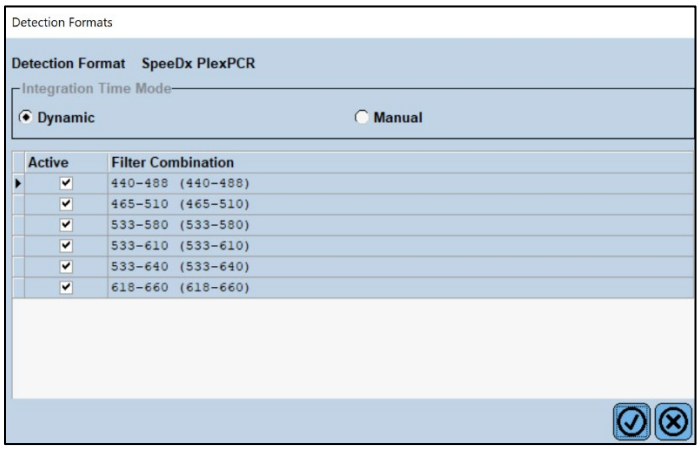
For **Detection Format** select the custom '**SpeeDx PlexPCR**' (**Figure 3)**

Select **Customize** >

Select **Integration Time Mode** > **Dynamic**

Select all Active **Filter Combinations** as shown in **Figure 3** and **Table 36**

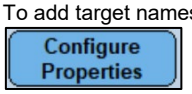
Figure 3. Customize Detection Format



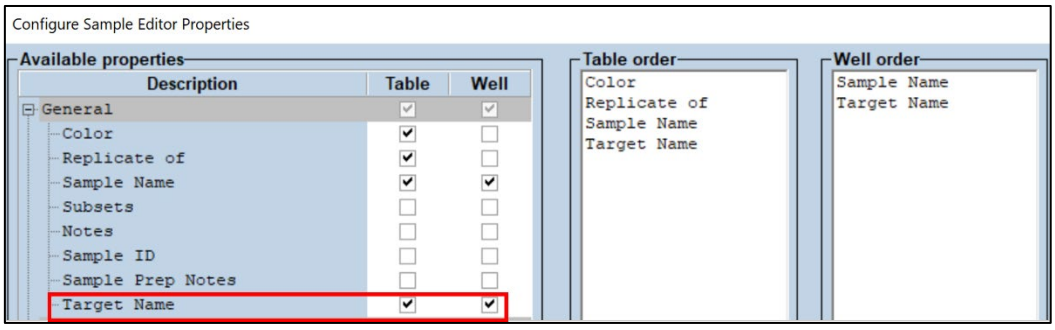
To enable automated sample detection in the analysis software, add target names and assign nametags to the wells on the plate (see **Section 21.3**)

Open the **Sample Editor** module

To add target names, select **Configure Properties**



Select the tick boxes next to 'Target Name' and accept



Edit the Target Name for each channel to match the Target Instrument Reference defined in the Lab Configuration > Assays menu of the analysis software and shown in **Table 37**.

Well 1 containing the RV1 (610) mix will be recognised by the 5 Target names (RhV / RSV / FluA / FluB / IC), Well 2 containing the RV2 (610) mix will be recognised by the 3 Target names (HMPV / AdV B/C / HPIV) in their corresponding filter combinations.

Table 37. Channels for *PlexPCR*® RespiVirus$_{(610)}$ targets

Channel	465-510	533-580	533-610	533-640	618-660
RV1 (610) mix target name	RhV	RSV	FluA	IC	FluB
RV2 (610) mix target name	HMPV	AdV B/C	HPIV	N/A	N/A

To assign nametags, select the well

Edit **Sample Name** to match nametag defined in the Lab Configuration > Assays menu of the analysis software (see **Section 21.3**)

Samples should be labelled with the nametag as a Prefix. Default nametags are provided for the control reactions (as shown in **Table 38** and **Figure 4**) The sample name will also need to be identical for the 2 wells containing the same specimen.

NOTE: Sample nametags are case sensitive. The nametag must match exactly to those assigned in the run file.

Table 38. Sample nametags for analysis software

Sample type	Mix Name	Default Prefix_ (in analysis software)
Regular sample	RV1	No default – user defined
Regular sample	RV2	No default – user defined
Negative Control	RV1	NC
Negative Control	RV2	NC
No Template Control	RV1	NTC
No Template Control	RV2	NTC
Positive control (RV PC1)	RV1	P1A
Positive control (RV PC1)	RV2	P1A
Positive control (RV PC2)	RV1	P1B
Positive control (RV PC2)	RV2	P1B
Positive control (RV PC3)	RV1	P1C
Positive control (RV PC3)	RV2	P1C
Positive control (RV PC4)	RV1	P1D
Positive control (RV PC4)	RV2	P1D

Figure 4. Sample Editor – Assigning nametags to wells

Pos	Filter combination	Color	Repl Of	Sample Name	Target Name
A1	465-510 (465			NC	RhV
A1	533-580 (533			NC	RSV
A1	533-610 (533			NC	FluA
A1	533-640 (533			NC	IC
A1	618-660 (618			NC	FluB
A2	465-510 (465			NC	HMPV
A2	533-580 (533			NC	AdV B/C
A2	533-610 (533			NC	HPIV
A2	533-640 (533			NC	
A2	618-660 (618			NC	
A3	465-510 (465			NTC	RhV
A3	533-580 (533			NTC	RSV
A3	533-610 (533			NTC	FluA
A3	533-640 (533			NTC	IC
A3	618-660 (618			NTC	FluB
A4	465-510 (465			NTC	HMPV
A4	533-580 (533			NTC	AdV B/C
A4	533-610 (533			NTC	HPIV
A4	533-640 (533			NTC	
A4	618-660 (618			NTC	
A5	465-510 (465			P1A	RhV
A5	533-580 (533			P1A	RSV
A5	533-610 (533			P1A	FluA
A5	533-640 (533			P1A	IC
A5	618-660 (618			P1A	FluB
A6	465-510 (465			P1A	HMPV
A6	533-580 (533			P1A	AdV B/C
A6	533-610 (533			P1A	HPIV
A6	533-640 (533			P1A	
A6	618-660 (618			P1A	

For 20 µL qPCR reaction.

Set **Reaction Volume** > 20 µL

Create the following Program (**Table 39,** shown in more detail in **Figure 5 - Figure 9**):

Table 39. Thermocycling program

Program name	Cycles	Target °C	Hold	Ramp rate (°C/s)≠
Reverse transcription	1	48°C	10 min	4.4
Polymerase activation	1	95°C	2 min	4.4
Touch down cycling$^{\delta}$: Step down -0.5°C/cycle	10	95°C	5 s	4.4
		61°C – 56.5°C$^{\delta}$	30 s	2.2
Quantification cycling^{+}: Acquisition/Detection	40	95°C	5 s	4.4
		52°C^{+}	50 s	2.2
Cooling	1	40°C	30 s	2.2

≠ *Default ramp rate (96 well plate)*

$^{\delta}$ ***Step size:*** *-0.5°C/Cycle,* ***Sec Target:*** *56°C*

+ ***Analysis mode:*** *Quantification,* ***Acquisition mode****: Single*

Figure 5. Thermocycling program (20 µL reaction) – Reverse transcription

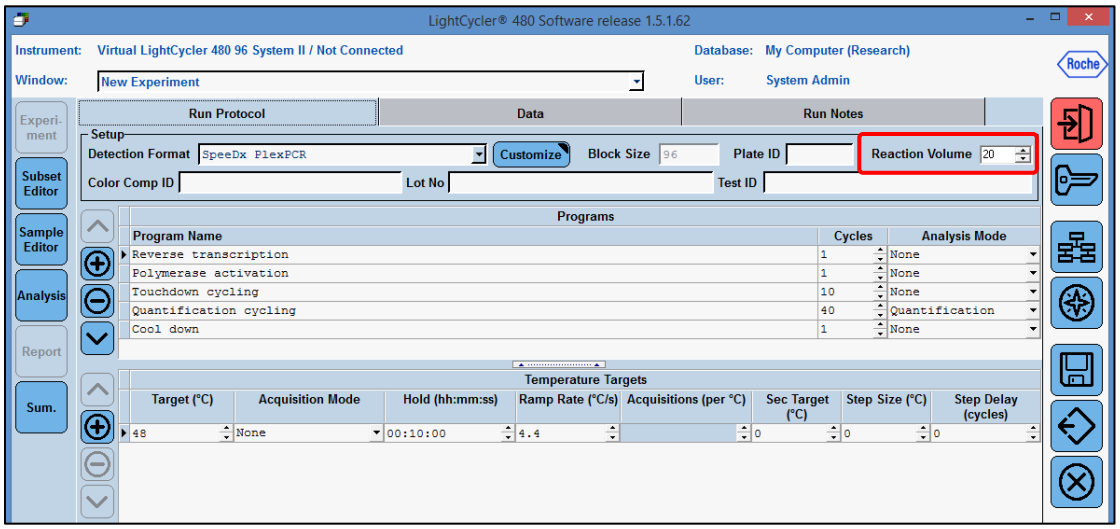


Figure 6. Thermocycling program (20 µL reaction) – Polymerase activation

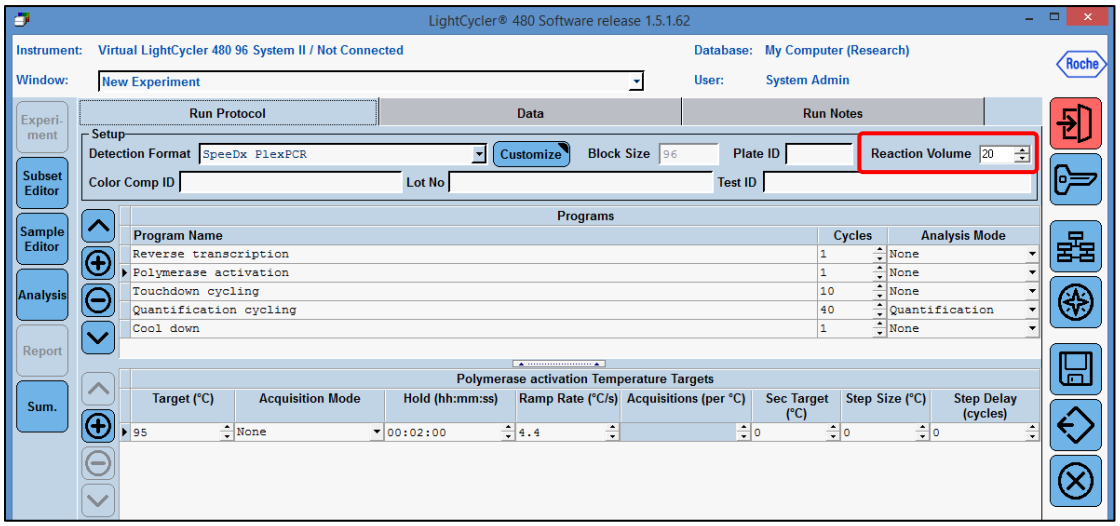


Figure 7. Thermocycling program (20 µL reaction) – Touchdown cycling

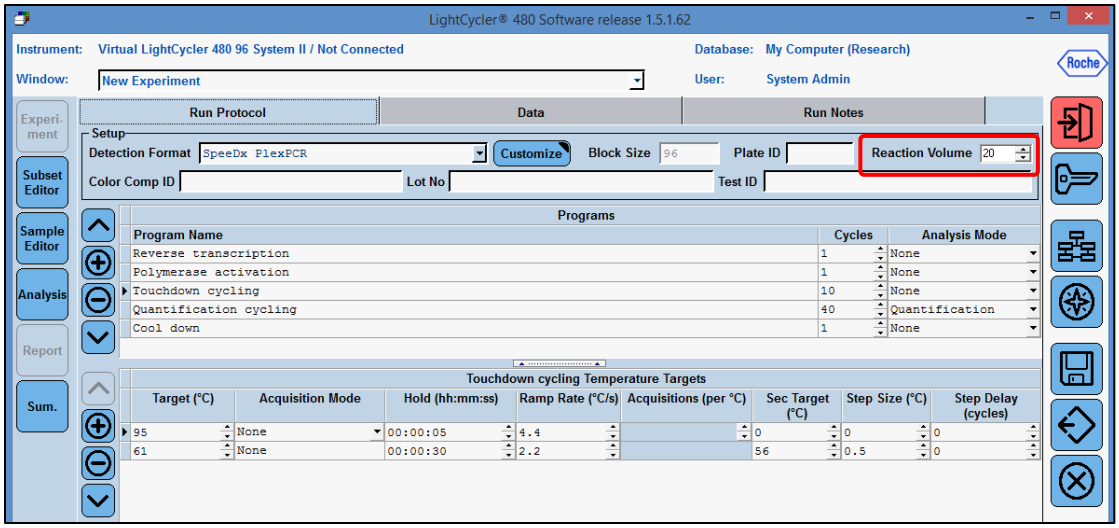


Figure 8. Thermocycling program (20 µL reaction) – Quantification cycling

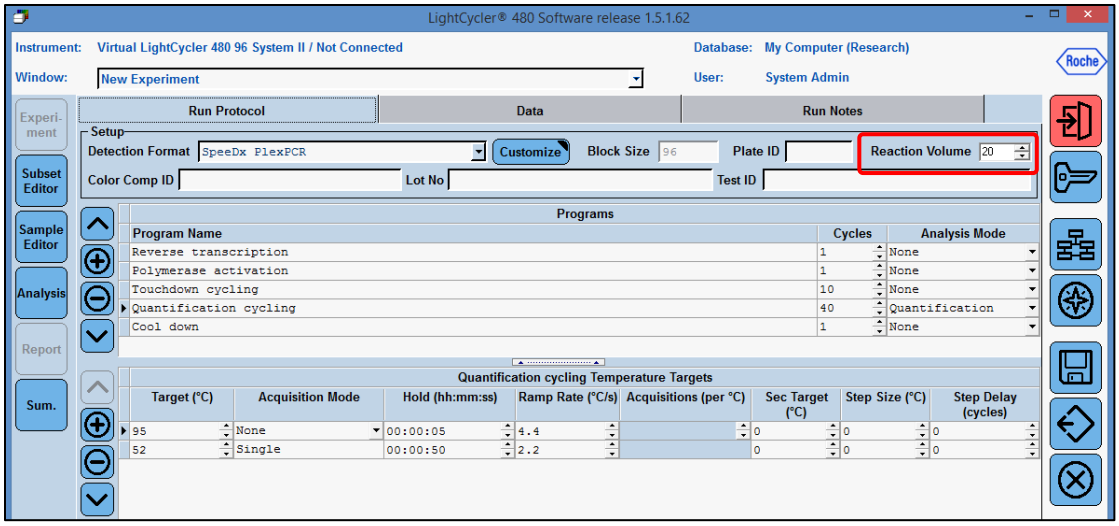
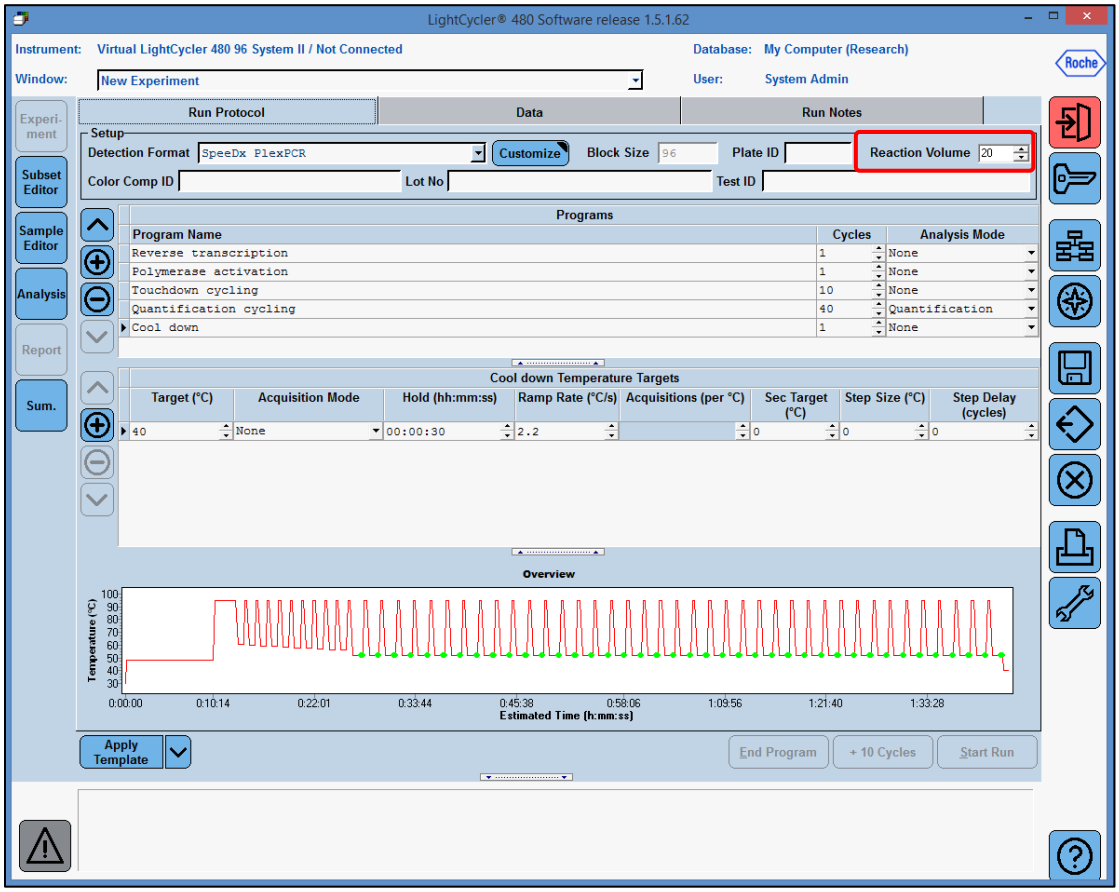


Figure 9. Thermocycling program (20 µL reaction) – Cooling



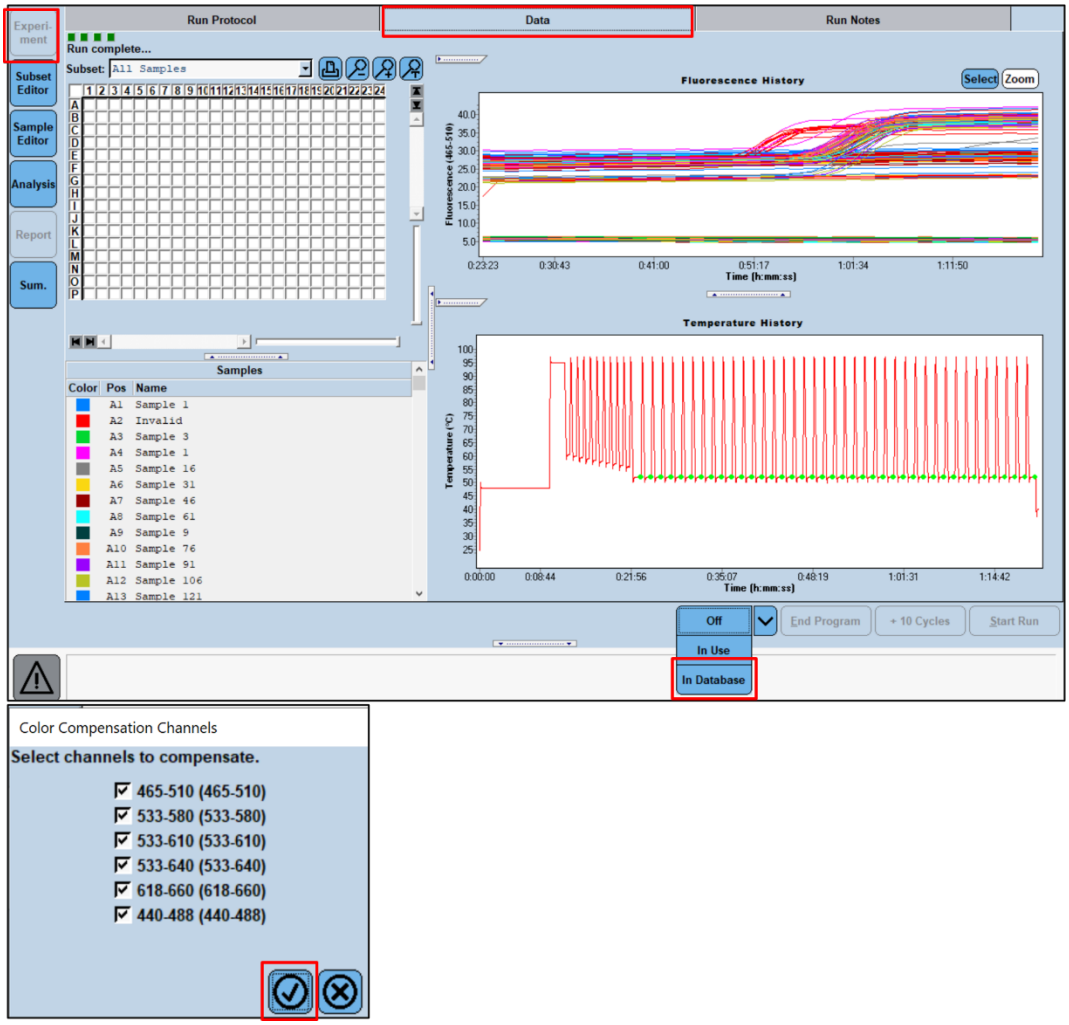
> **Start Run**

When the cycling program has finished, attach the CC object to the run file as shown in **Figure 10** and export as a .IXO file for analysis in the ***PlexPCR***® RespiVirus analysis software. Refer to **Section 19.2** for instructions on how to create the CC Object and store this within the LightCycler 480 software database.

Select **Experiment > Data**

Click the drop-down arrow next to **Colour Comp (Off)** and select **In Database**

Figure 10. Attaching the CC object to the run file



Select the appropriate CC Object, ensure all channels are selected and select the **tick** icon 

Select the **Save** icon 

Select the **Export** icon 

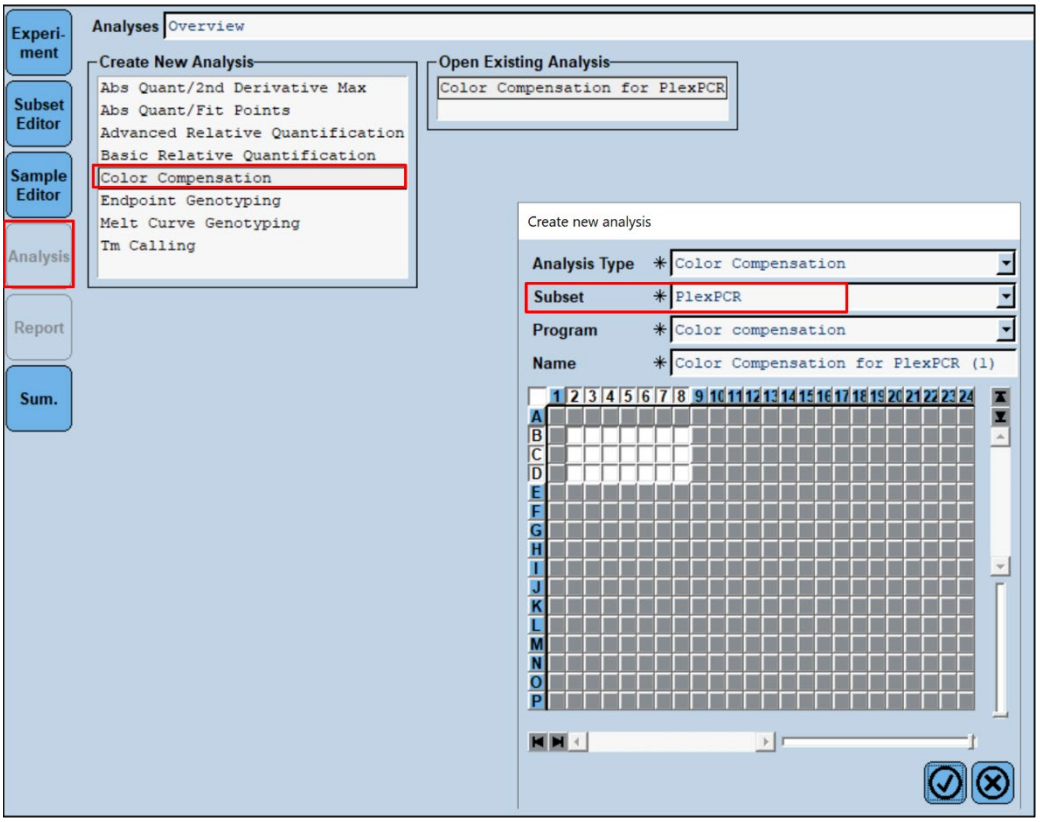
Save in an easily identifiable location

19.2 Colour Compensation for LightCycler® 480 Instrument II

The ***PlexPCR***® Colour Compensation (Cat no 90001) kit must be run and applied for LC480 II analysis. This kit can be supplied on request.

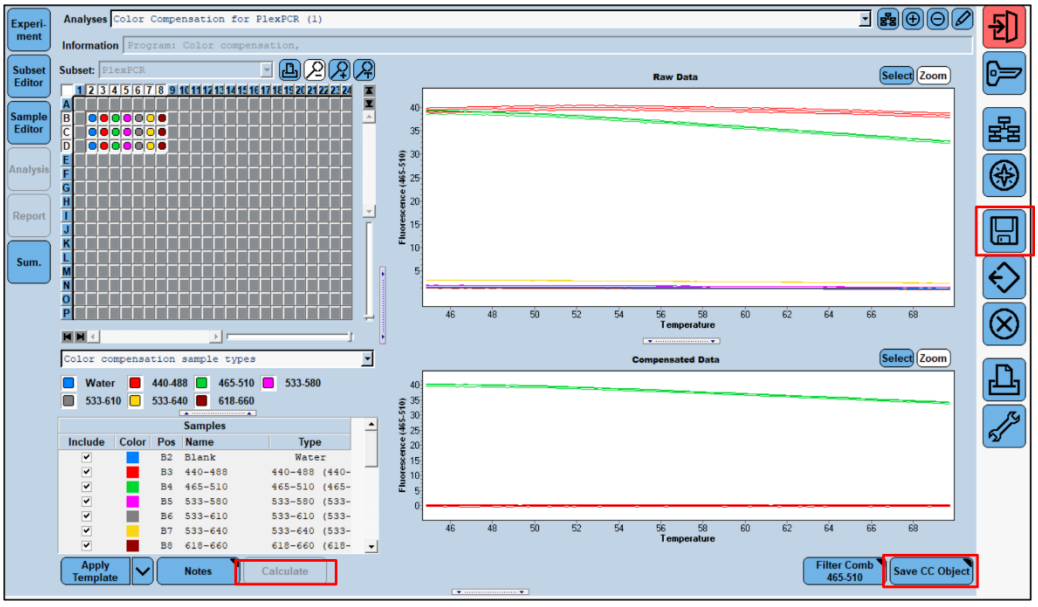
Analyse the Colour Compensation file via **Analysis > Colour Compensation** and select the correct subset, shown in **Figure 11.**

Figure 11. Analysis – Colour Compensation



Select **Calculate (Figure 12)**

Figure 12. Calculate and save CC Object



Refer to the PlexPCR Colour Compensation Instructions for Use (IF-IV001) for further details to ensure the Colour Compensation file has been created correctly

Select **Save** 

Select **Save CC Object** to save the CC object within the LightCycler 480 software database

19.3 Interpretation of results

Data interpretation requires the ***Plex****PCR*® RespiVirus (LC480) analysis software. The analysis software can be supplied on request. Please contact tech@speedx.com.au for more information.

Refer to **Section 21** for instructions for using the ***Plex****PCR*® RespiVirus (LC480) analysis software.

20 Appendix 1b: LightCycler® 480 Instrument II programming for reaction volume of 10 µl

The following information is based on LightCycler 480 Software (version 1.5).

The ***PlexPCR***® RespiVirus$_{(610)}$ kit contains dyes for the LightCycler® 480 Instrument II. The ***PlexPCR***® Colour Compensation kit (Cat no 90001) must be run and applied for LC480 II analysis (see **Section 19.2**). This kit can be supplied on request.

20.1 Programming the LightCycler® 480 Instrument II (LC480 II)

Detection Format

Create a custom **Detection Format**

Open Tools > **Detection Formats**

Create a New Detection Format, and name '**SpeeDx PlexPCR'** (may be created during the generation of SpeeDx Colour Compensation file) (See **Figure 13**)

For **Filter Combination Selection** select the following (Excitation-Emission):

	Table 40. Filter Combinations^					
LC480 II	440-488	465-510	533-580	533-610	533-640	618-660

^ These Filter Combinations are the default names for the channels

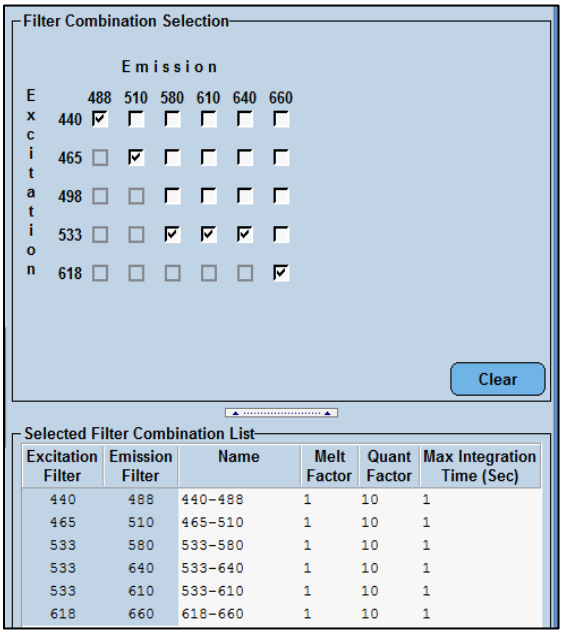
Set the **Selected Filter Combination List** for all channels as:

Melt Factor: 1

Quant Factor: 10

Max Integration Time (sec): 1

Figure 13. Custom SpeeDx Detection Format



Instrument Settings

Create a custom **Detection Format**

Open Tools > **Instruments**

For **Instrument Settings** > select **Barcode Enabled**

Experiment setup

Select **New Experiment**

In the **Run Protocol** tab

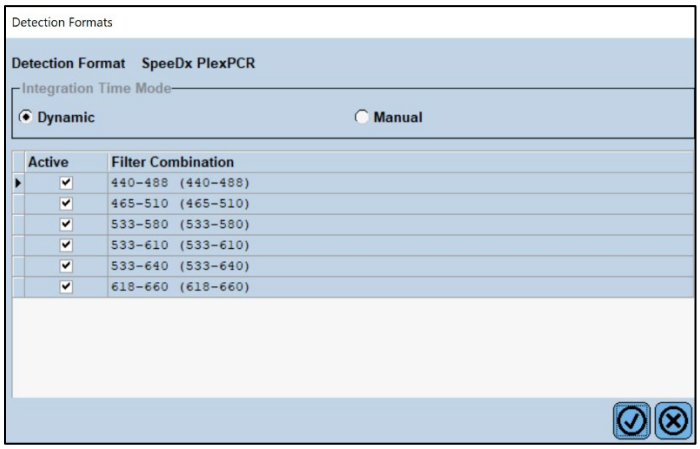
For **Detection Format** select the custom 'SpeeDx PlexPCR' (**Figure 14**)

Select **Customize** >

Select **Integration Time Mode** > **Dynamic**

Select all Active **Filter Combinations** as shown in **Figure 14** and **Table 40**

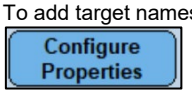
Figure 14. Customize Detection Format



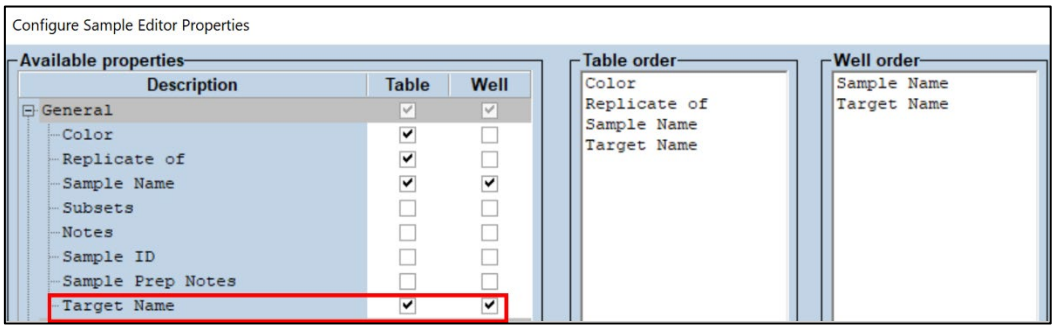
To enable automated sample detection in the analysis software, add target names and assign nametags to the wells on the plate (see **Section 21.3**)

Open the **Sample Editor** module

To add target names, select **Configure Properties**



Select the tick boxes next to 'Target Name' and accept



Edit the Target Name for each channel to match the Target Instrument Reference defined in the Lab Configuration > Assays menu of the analysis software and shown in **Table 41**.

Well 1 containing the RV1 (610) mix will be recognised by the 5 Target names (RhV / RSV / FluA / FluB / IC), Well 2 containing the RV2 (610) mix will be recognised by the 3 Target names (HMPV / AdV B/C / HPIV) in their corresponding filter combinations.

Table 41. Channels for *PlexPCR*® RespiVirus$_{(610)}$ targets

Channel	465-510	533-580	533-610	533-640	618-660
RV1 (610) mix target name	RhV	RSV	FluA	IC	FluB
RV2 (610) mix target name	HMPV	AdV B/C	HPIV	N/A	N/A

To assign nametags, select the well

Edit **Sample Name** to match nametag defined in the Lab Configuration > Assays menu of the analysis software (see **Section 21.3**)

Samples should be labelled with the nametag as a Prefix. Default nametags are provided for the control reactions (as shown in **Table 42** and **Figure 15**) The sample name will also need to be identical for the 2 wells containing the same specimen.

NOTE: Sample nametags are case sensitive. The nametag must match exactly to those assigned in the run file.

Table 42. Sample nametags for analysis software

Sample type	Mix Name	Default Prefix_ (in analysis software)
Regular sample	RV1	No default – user defined
Regular sample	RV2	No default – user defined
Negative Control	RV1	NC
Negative Control	RV2	NC
No Template Control	RV1	NTC
No Template Control	RV2	NTC
Positive control (RV PC1)	RV1	P1A
Positive control (RV PC1)	RV2	P1A
Positive control (RV PC2)	RV1	P1B

Table 42. Sample nametags for analysis software		
Sample type	**Mix Name**	**Default Prefix_ (in analysis software)**
Positive control (RV PC2)	RV2	P1B
Positive control (RV PC3)	RV1	P1C
Positive control (RV PC3)	RV2	P1C
Positive control (RV PC4)	RV1	P1D
Positive control (RV PC4)	RV2	P1D

Figure 15. Sample Editor – Assigning nametags to wells

Pos	Filter combination	Color	Repl Of	Sample Name	Target Name
A1	465-510 (465			NC	RhV
A1	533-580 (533			NC	RSV
A1	533-610 (533			NC	FluA
A1	533-640 (533			NC	IC
A1	618-660 (618			NC	FluB
A2	465-510 (465			NC	HMPV
A2	533-580 (533			NC	AdV B/C
A2	533-610 (533			NC	HPIV
A2	533-640 (533			NC	
A2	618-660 (618			NC	
A3	465-510 (465			NTC	RhV
A3	533-580 (533			NTC	RSV
A3	533-610 (533			NTC	FluA
A3	533-640 (533			NTC	IC
A3	618-660 (618			NTC	FluB
A4	465-510 (465			NTC	HMPV
A4	533-580 (533			NTC	AdV B/C
A4	533-610 (533			NTC	HPIV
A4	533-640 (533			NTC	
A4	618-660 (618			NTC	
A5	465-510 (465			P1A	RhV
A5	533-580 (533			P1A	RSV
A5	533-610 (533			P1A	FluA
A5	533-640 (533			P1A	IC
A5	618-660 (618			P1A	FluB
A6	465-510 (465			P1A	HMPV
A6	533-580 (533			P1A	AdV B/C
A6	533-610 (533			P1A	HPIV
A6	533-640 (533			P1A	
A6	618-660 (618			P1A	

For 10 µL qPCR reaction.

Set **Reaction Volume** > 10 µL

Create the following Program (shown in more detail in **Figure 16- Figure 20**):

Table 43. Thermocycling program				
Program name	**Cycles**	**Target °C**	**Hold**	**Ramp rate (°C/s)≠**
Reverse transcription	1	48°C	10 min	4.8
Polymerase activation	1	95°C	2 min	4.8
Touch down cycling$^{\delta}$: Step down -0.5°C/cycle	10	95°C	5 s	4.8
		61°C – 56.5°C$^{\delta}$	30 s	2.5
Quantification cycling^{+}: Acquisition/Detection	40	95°C	5 s	4.8
		52°C^{+}	50 s	2.5
Cooling	1	40°C	30 s	2.5

≠ Default ramp rate (384 well plate)

$^{\delta}$ **Step size:** -0.5°C/Cycle, **Sec Target:** 56°C

$^{+}$ **Analysis mode:** Quantification, **Acquisition mode**: Single

> **Start Run**

Figure 16. Thermocycling program (10 µL reaction) – Reverse transcription

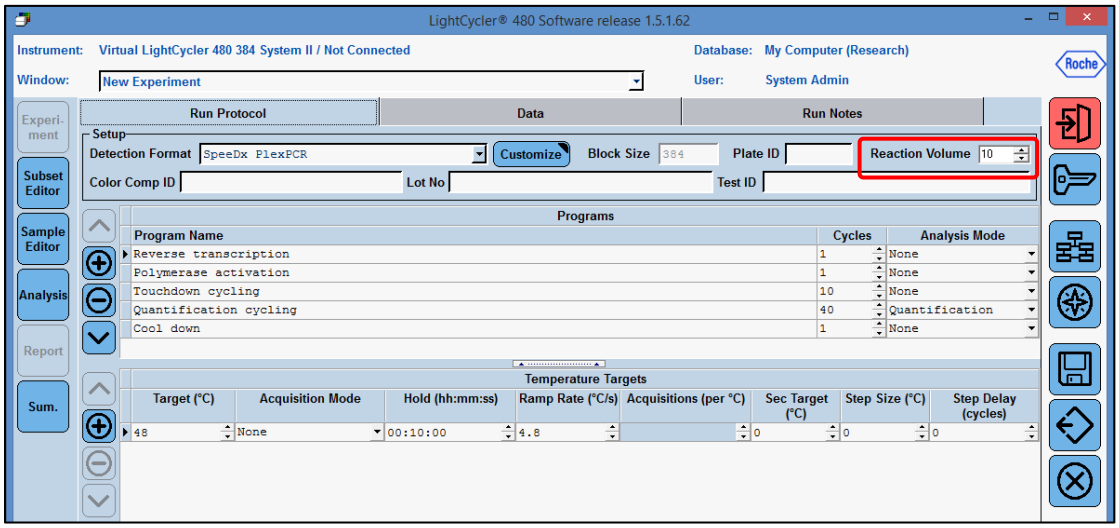


Figure 17. Thermocycling program (10 µL reaction) – Polymerase activation

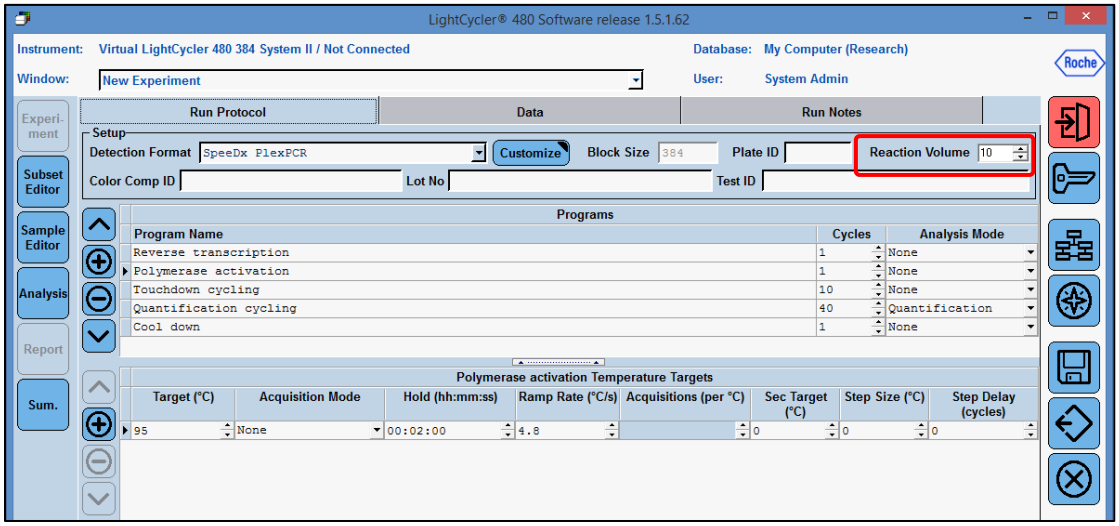


Figure 18. Thermocycling program (10 µL reaction) – Touchdown cycling

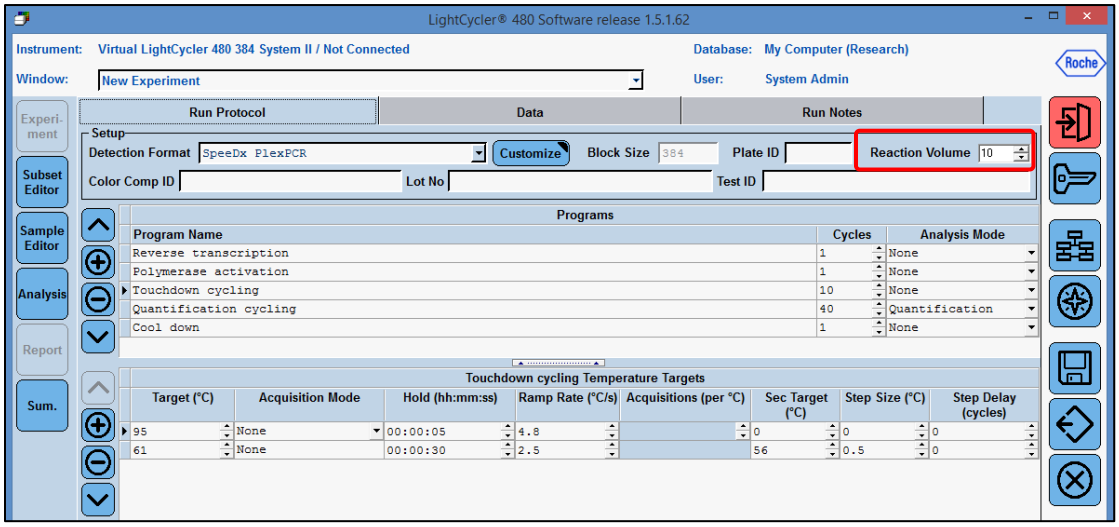


Figure 19. Thermocycling program (10 µL reaction) – Quantification cycling

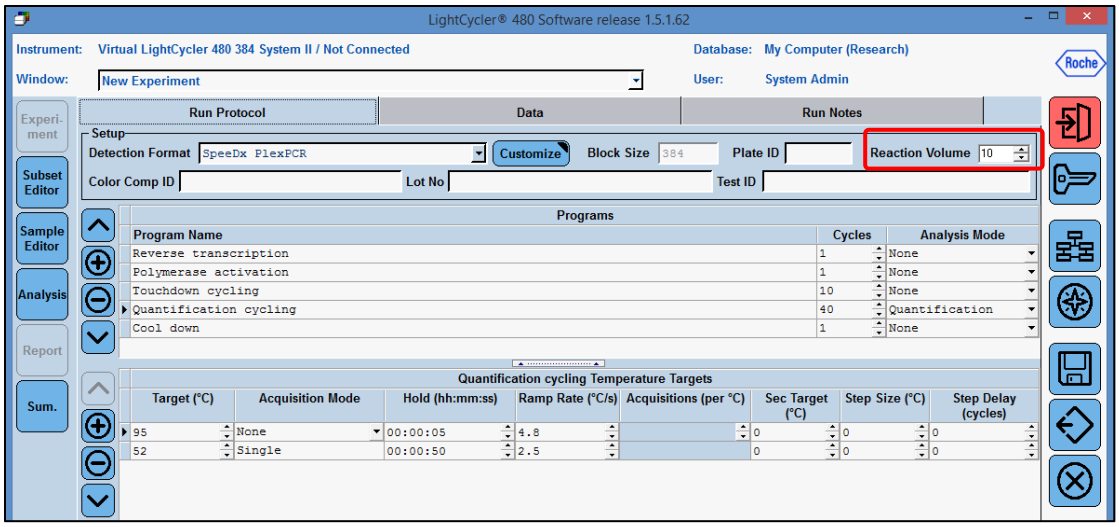
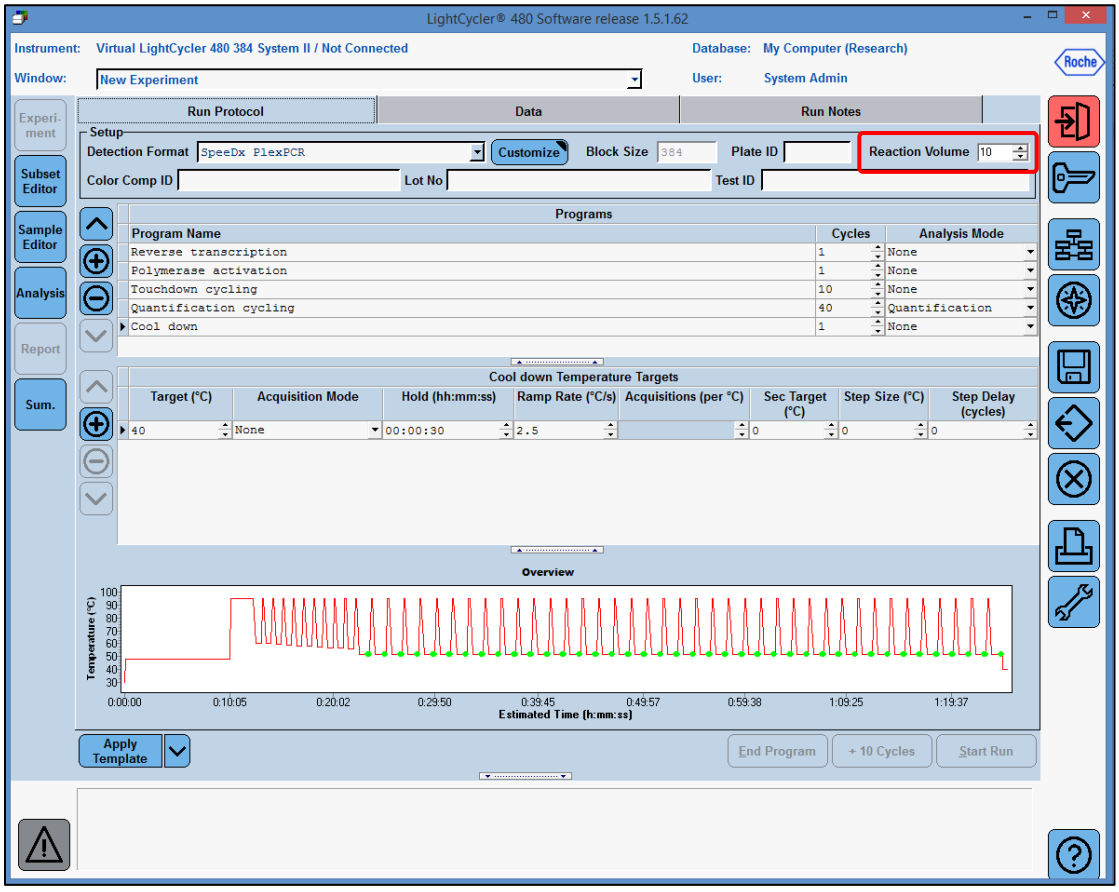


Figure 20. Thermocycling program (10 µL reaction) – Cooling

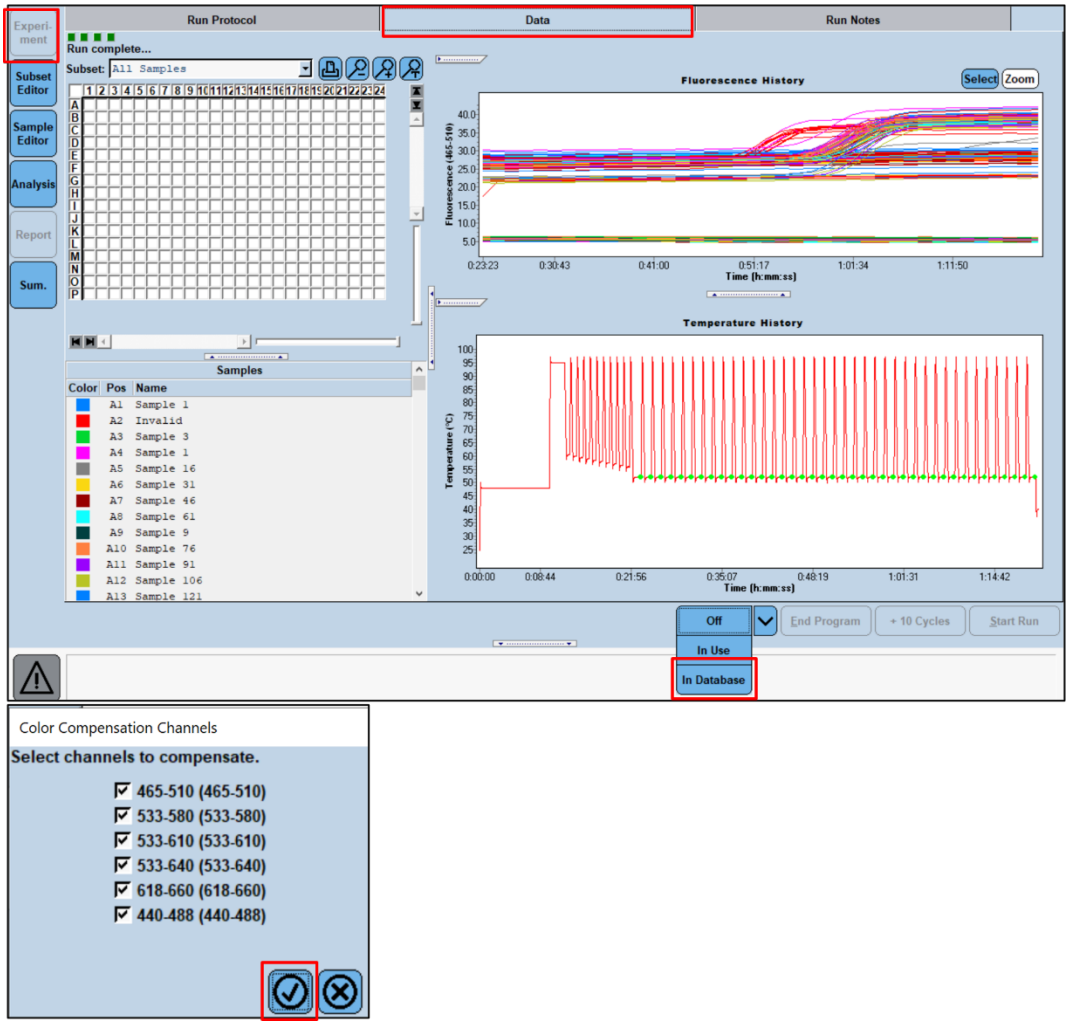


When the cycling program has finished, attach the CC object to the run file as shown in **Figure 21** and export as a .IXO file for analysis in the ***PlexPCR***® RespiVirus analysis software. Refer to **Section 20.2** for instructions on how to create the CC Object and store this within the LightCycler 480 software database.

Select **Experiment > Data**

Click the drop-down arrow next to **Colour Comp (Off)** and select **In Database**

Figure 21. Attaching the CC object to the run file



Select the appropriate CC Object, ensure all channels are selected and select the **tick** icon 

Select the **Save** icon 

Select the **Export** icon 

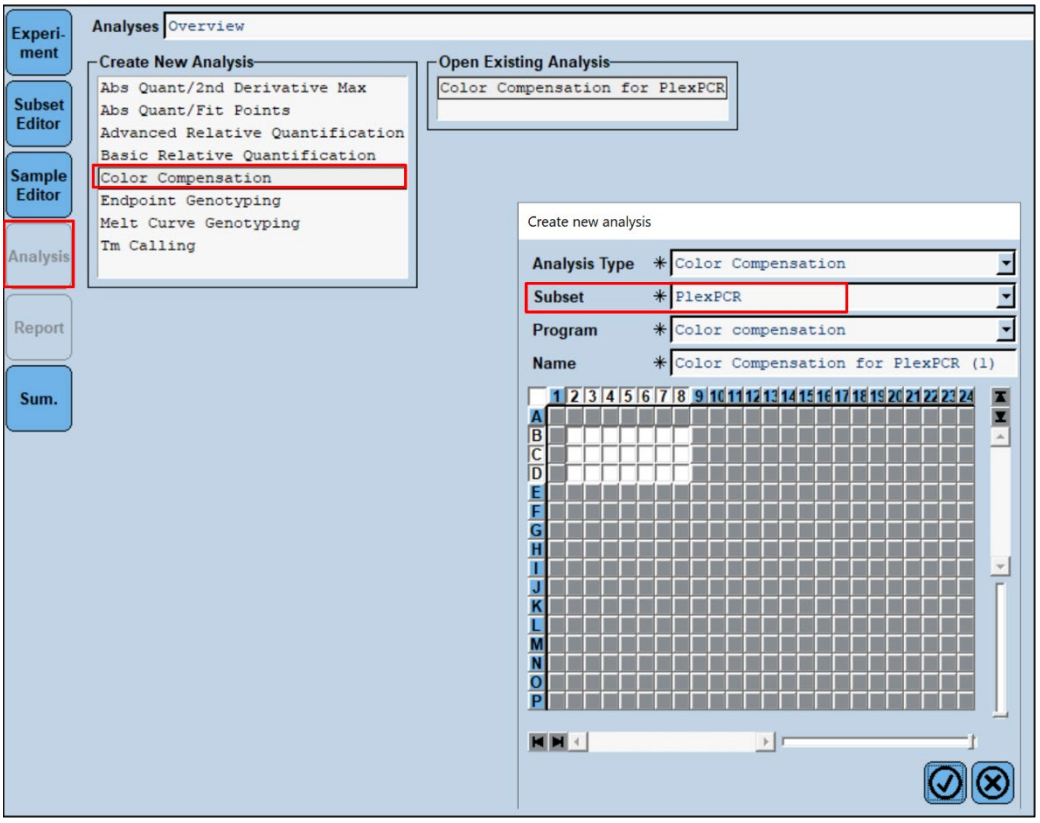
Save in an easily identifiable location

20.2 Colour Compensation for LightCycler® 480 Instrument II

The ***PlexPCR***® Colour Compensation (Cat no 90001) kit must be run and applied for LC480 II analysis. This kit can be supplied on request.

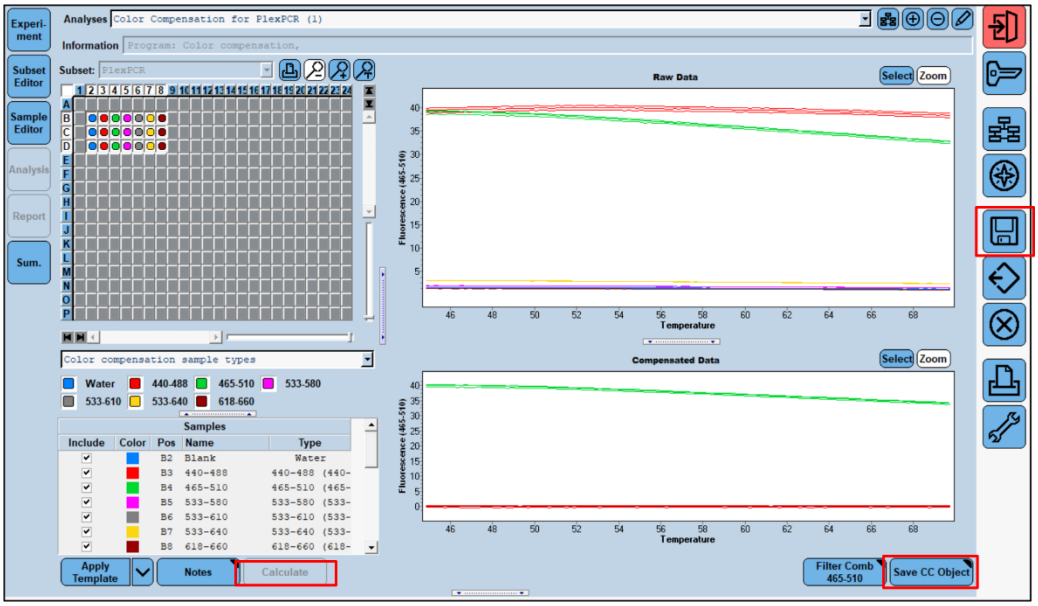
Analyse the Colour Compensation file via **Analysis > Colour Compensation** and select the correct subset, shown in **Figure 22.**

Figure 22. Analysis – Colour Compensation



Select **Calculate (Figure 23)**

Figure 23. Calculate and save CC Object



Refer to the PlexPCR Colour Compensation Instructions for Use (IF-IV001) for further details to ensure the Colour Compensation file has been created correctly

Select **Save** 

Select **Save CC Object** to save the CC object within the LightCycler 480 software database

20.3 Interpretation of results

Data interpretation requires the ***Plex****PCR*® RespiVirus (LC480) analysis software. The analysis software can be supplied on request. Please contact tech@speedx.com.au for more information.

Refer to **Section 21** for instructions for using the ***Plex****PCR*® RespiVirus (LC480) analysis software.

21 Appendix A: Result interpretation

Data interpretation requires the ***Plex****PCR*® RespiVirus analysis software. The ***Plex****PCR*® RespiVirus analysis software automates the data interpretation of amplification results and streamlines workflow.

See **Table 44** or the appropriate analysis software for the specific kit and qPCR instrument. The analysis software can be supplied on request. Please contact tech@speedx.com.au for more information.

Table 44. *Plex*PCR® RespiVirus analysis software

Cat no	Analysis software*	Real-time PCR instrument
99011	***Plex****PCR*® RespiVirus (LC480)	LC480 II

* Refer to the website https://plexpcr.com/products/respiratory-infections/plexpcr-respivirus/#resources to ensure you are using the most current version of analysis software.

NOTE: Follow standard laboratory practices for transfer, reporting and storage of results to prevent loss of sample information.

21.1 FastFinder platform - Minimum IT requirements

The analysis software is available within the FastFinder platform (https://www.ugentec.com/fastfinder/analysis). It is recommended that customers access the software platform from a secure and trusted network and computer. The minimum IT requirements for access and use of the FastFinder platform are listed below.

Hardware requirements

Internet Connection Cable or DSL

Min. screen resolution: 1366x768 pixels, optimal 1920 x 1080 pixels or higher

Supported browsers

- Microsoft Edge 88 or newer
- Firefox 83 or newer
- Google Chrome 88 or newer.

Firewall requirements

The following hosts must be reachable over HTTPS (port 443):

*.ugentec.app

*.fastfinder.app

*.pendo.io

*.fonts.gstatic.com

*.googleapis.com

*.msecnd.net

*.visualstudio.com

*.browser-update.org

*.blob.core.windows.net

*.powerbi.com

*.analysis.windows.net

*.pbideldicated.windows.net

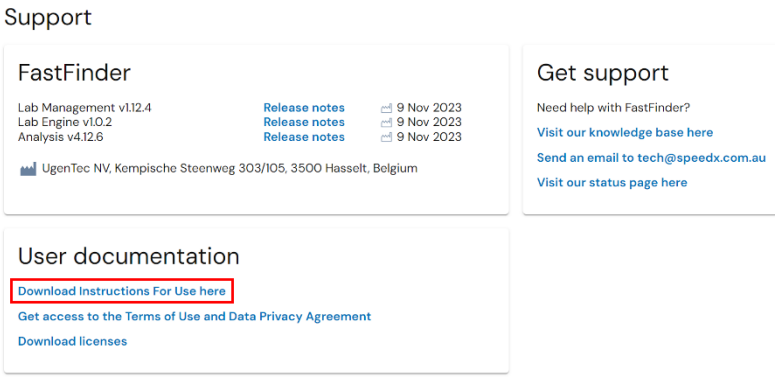
*.content.powerapps.com

If required, firewall exceptions will have to be configured for these hosts. In order to access all content of in-app user guides, the host *.player.vimeo.com must also be reachable.

For further detailed instructions on the **FastFinder** platform, refer to the **FastFinder Instructions For Use** accessible from the **Support** menu.

To access the Support menu

- Select Support from the list of menu options on the left-hand side panel
- Select **Download Instructions For Use here** within the **User Documentation** section



21.2 Assay plug-in (new user)

Refer to the **FastFinder Instructions For Use** for detailed instructions to set up assays, accessible from the **Support** menu

Open **FastFinder.**

FastFinder can be accessed directly through a web browser by logging in with your unique username and password at https://customer.fastfinder.app.

- Select **Lab Configuration > Assays** from the left-hand menu
- Select **Add New Assay**
  - > For LC480 II > Select **PlexPCR RespiVirus (LC480)** from the list
- Select **Import Selected**

To activate or de-activate versions of the assay plug-in

> In **General tab**

> Navigate to the Status

> Select Active to activate or deactivate the version of the assay

21.3 Sample Naming

Sample nametags can be assigned to an assay plug-in to automate detection of wells and sample types for analysis.

Select Lab **Configuration > Assays** from the left-hand menu

- In the **General tab**, navigate to the **Sample types** table nametags (prefix), select ⊕ to add a new nametag
  - > Add desired word, acronym or letter to text box
  - > Default nametags are provided for the controls. These can be removed by selecting the × next to the nametag
- In the instrument software (before or after run is completed) assign the same nametag to appropriate wells
  - > For **LC480 II** see **Section 19** for instructions on programming sample nametags in the run file

NOTE: Sample nametags are case sensitive. The nametag must match exactly to those assigned in the run file.

21.4 Analysis

Select **Analyses** from the left-hand menu to start a new analysis

Select **+ Create New Analysis** from the top right of the screen

Search for the file to be uploaded for analysis from a specified directory

- Select run (data) file from the relevant folder
  - > Select **Open**

The analysis will appear within the **Open Tab** as a new row within the table

- If all nametags have been applied and read correctly, the status will appear as **Ready for review**
- If the assay information needs to be manually assigned to the wells, the status will appear as **Manual PCR setup required**

Assign the assay information to the plate manually if sample naming has not been set up in the Lab **Configuration > Assays** menu or sample names/targets have not been applied in the instrument software

Select the runfile from the **Open tab** within the **Analyses** menu

The Plate Configuration will be displayed within the **PCR setup tab** of the open analysis

- For **LC480 II** > Select **PlexPCR RespiVirus (LC480)**

- Select wells and assign as:
  - > Regular Sample (S)
    - RV1 (S1) or RV2 (S2) mix
  - > Negative Control (Na)
    - RV1 (N1a) or RV2 (N2a) mix
  - > No Template Control (Nb)
    - RV1 (N1b) or RV2 (N2b) mix
  - > Positive Control RV PC1 (Pa)
    - RV1 (P1a) or RV2 (P2a) mix
  - > Positive Control RV PC2 (Pb)
    - RV1 (P1b) or RV2 (P2b) mix
  - > Positive Control RV PC3 (Pc)
    - RV1 (P1c) or RV2 (P2c) mix

- Positive Control RV PC4 (Pd)
    - RV1 (P1d) or RV2 (P2d) mix

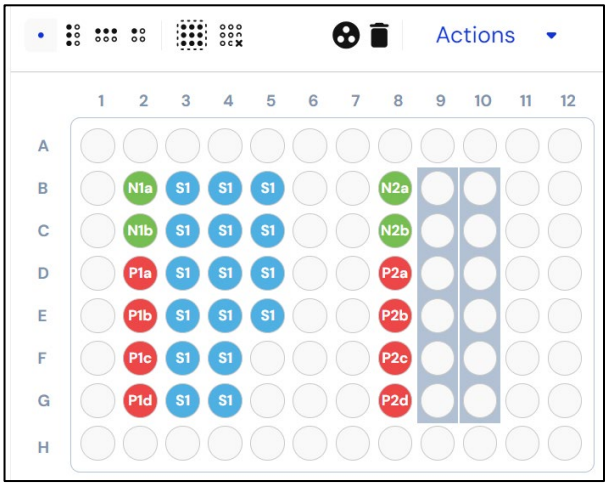
In order for the software to recognize both wells corresponding to the same specimen, the sample name will need to be identical for the two wells (see **Sections 19 and 20**). In addition, the appropriate RVI or RV2 label should be assigned

For example: the same specimen containing:

- RV1 mix → assign with 'S1'
- RV2 mix → assign with 'S2'

To assign wells on the plate either:

- Click and drag the coloured symbols to place them on the plate
- Select one or multiple wells (use Ctrl and shift keys) and then click the relevant colored symbols to assign to selection.



Select **Analyze**

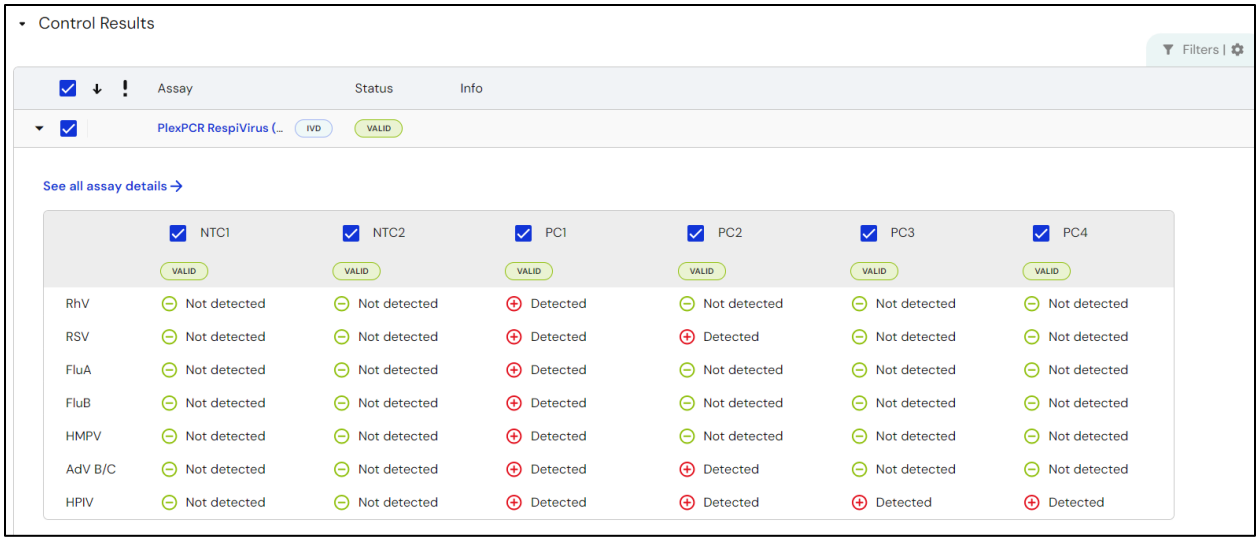
21.5 Results

See **Table 46** for a summary of possible reported sample results.

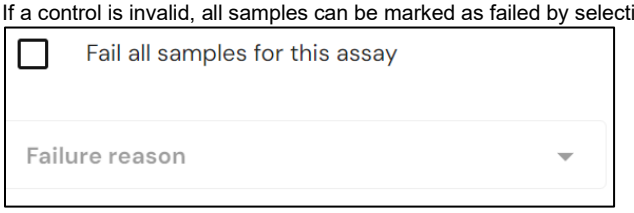
NOTE: It is highly recommended that amplification curves should be visually inspected and confirmed for all positive samples.

21.5.1 Summary Tab

Control results for every assay are shown at the top-left of the Summary tab, allowing evaluation of control validity for the run. More details can be found by expanding this block, displaying the details per control.

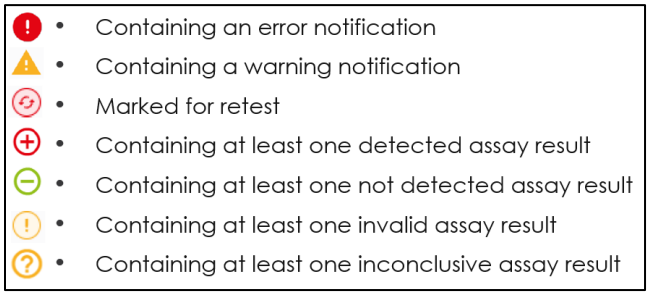


If a control is invalid, all samples can be marked as failed by selecting **Fail all samples for this assay**

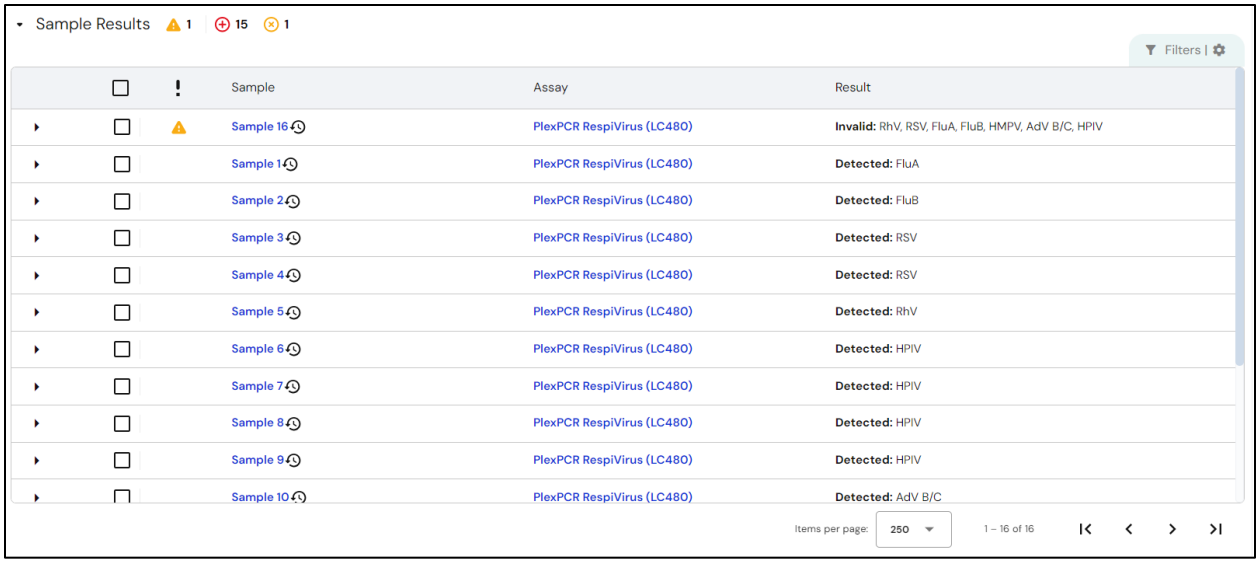


A failure reason needs to be chosen from the dropdown menu

Sample results are shown at the bottom-left of the Summary tab. Next to the header, additional icons may provide a high-level overview of the analysis results as well as indicating the total number of samples corresponding to a particular icon.

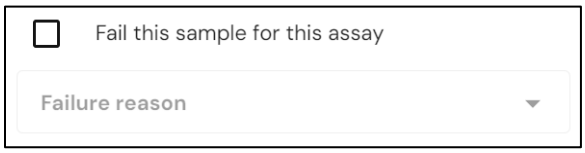


Each sample is displayed as a row within the sample results table



The drop-down menu offers more details on each target result and Cq per sample (Refer to the examples shown in **Section 21.9** and **Section 21.10**.

Individual samples can be marked as failed if desired (e.g. if the sample is Invalid) by selecting **Fail this sample for this assay**



A failure reason needs to be chosen from the dropdown menu

Fluorescence graphs can be viewed at the top-right of the Summary tab

A plate layout can be viewed at the bottom-right of the Summary tab

Example information and warning notifications are summarized below in **Table 45**.

Table 45. Example information and warning notifications for the PlexPCR® RespiVirus analysis software*

Sample Type	Error	Notification
Assay target notifications		
Regular Sample	Invalid – IC failure	Warning: IC invalid. Re-extract and re-test sample.
	Valid but control invalid – Invalid control warning on regular sample with valid result	Warning: Invalid control present. Re-extract and re-test the sample.
Negative Control	Invalid - Contamination	Warning: Possible contamination detected.
No Template Control		
Gene target notifications		
Regular Sample	Target Cq outside cut-off	Info: Cq outside cutoff
Positive Control	Invalid – Target not detected	Warning: Expected reaction did not occur in control.
Negative Control	Invalid - Contamination	Warning: Possible contamination

Table 45. Example information and warning notifications for the PlexPCR® RespiVirus analysis software*		
Sample Type	**Error**	**Notification**
	Invalid – IC not detected	Warning: IC not detected
	Invalid – IC Cq outside cut-off	Warning: Cq outside cutoff
No Template Control	Invalid - Contamination	Warning: Possible contamination
Regular Sample or Control	Uncertain fluorescence signal	Warning: Uncertain fluorescence signal. Review required.
	Cq detected with low fluorescence	dRn end fluorescence below cut-off

*The examples listed here may not be applicable for all assay plug-ins. Refer to the FastFinder Instructions For Use for all possible notifications, accessible from the Support menu

21.5.2 Details Tab

All targets are shown for each sample as separate rows within the table on the left-hand side. Selecting one or more rows will display the corresponding fluorescence curves on the graph at the top-right and will also highlight the wells within the plate layout shown at the bottom-right.

Select **Filters** to display results according to parameters such as assay name, sample type, target and result.

To finalise analysis and prevent further user edits

> Select **Authorize**

> Select **Authorize** again to confirm

- To assign a second review

> Select **Actions**, **Assign label** and **Second Review**

- To assign the analysis to a different user

> Select **Actions** and **Assign User**

> Select the appropriate user from the drop-down list

- To reject the analysis

> Select **Actions** and **Discard Analysis**

> Add a comment and select **Discard** to confirm

21.6 Reference curve

A reference curve can be saved and used to compare to samples on the same or across different plates

- Select the sample of interest in either the **Summary** or **Details** tab

- From the amplification graph menu > Select 

> Select the check box for the curve of interest and select **Mark as reference**

This reference curve will now appear linked to the assay in the **Lab Configuration > Assays** menu within the **PCR tab** and can be inactivated at any time.

21.7 Exporting Results

- To export results from an individual authorised run as either a CSV or PDF file:
  - > Select **Actions > Downloads** in the top-right corner
  - > Select either of the following report types: **Analysis (CSV)** or **Analysis (PDF)**
- To export results from previously authorised runs as a CSV file:
  - > Navigate to the **Archive > Sample Results** menu
  - > Use the filters at the top of the page to display the results of interest (the CSV file is limited to a maximum of 10,000 results)
  - > Select **Export CSV** in the top-right corner

21.8 Accessing a previous analysis via the Archive menu

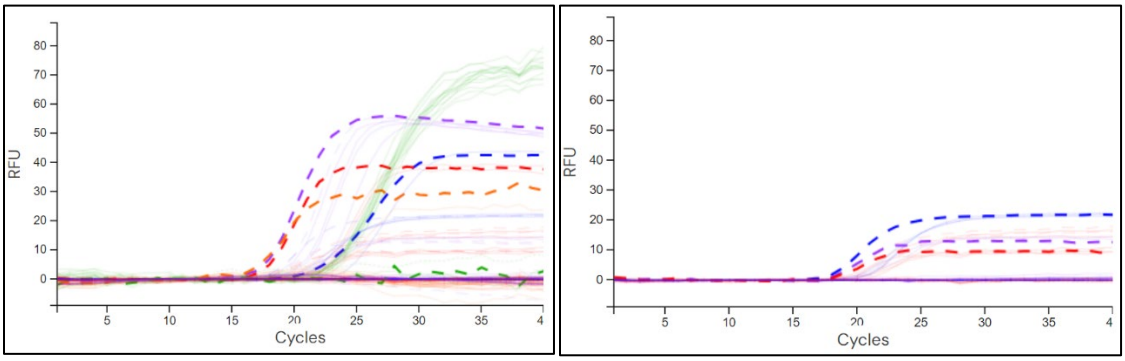
- All authorized analyses are available by selecting **Archive > Analysis Results**. Select a row to return to the results overview for that particular analysis
- All authorized regular samples are stored within the **Archive > Sample Results** menu. Selecting a sample will display additional information including the analysis name and the result details
- The individual target results for all authorized regular samples and controls are stored within the **Archive > Target Results** menu. Selecting a target will highlight this on the fluorescence graph. Selecting the Analysis Name will return to the results overview for that particular analysis.

21.9 Controls example graphs

The following examples show the amplification curves (baseline-corrected amplification curves) and the Results overview from the **PlexPCR RespiVirus (LC480)** analysis software for control sample types.

21.9.1 Positive control RV PC 1

RV1 mix **RV2 mix**



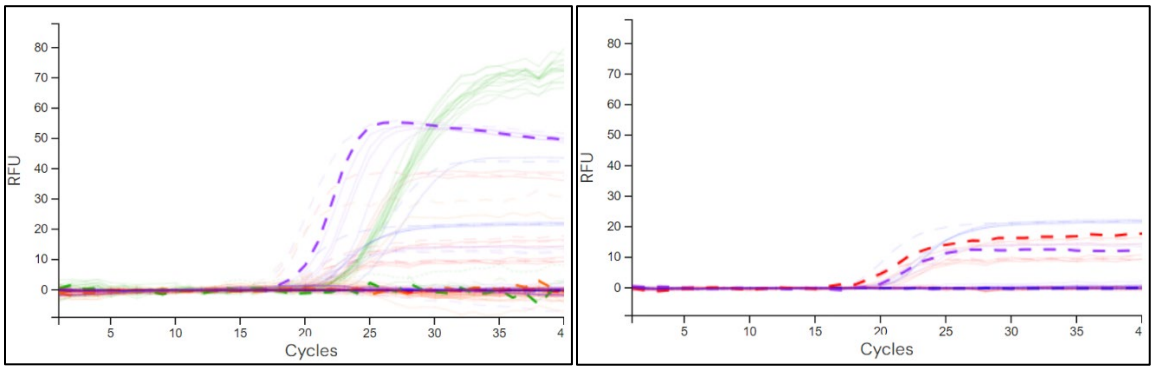
RhV RSV FluA FluB IC **HMPV AdV B/C HPIV**

Sample	Assay	Result
RV PC1	PlexPCR RespiVirus (LC480)	**Valid**

RhV	⊕ Detected
RSV	⊕ Detected
FluA	⊕ Detected
FluB	⊕ Detected
HMPV	⊕ Detected
AdV B/C	⊕ Detected
HPIV	⊕ Detected

21.9.2 Positive control RV PC 2

RV1 mix **RV2 mix**

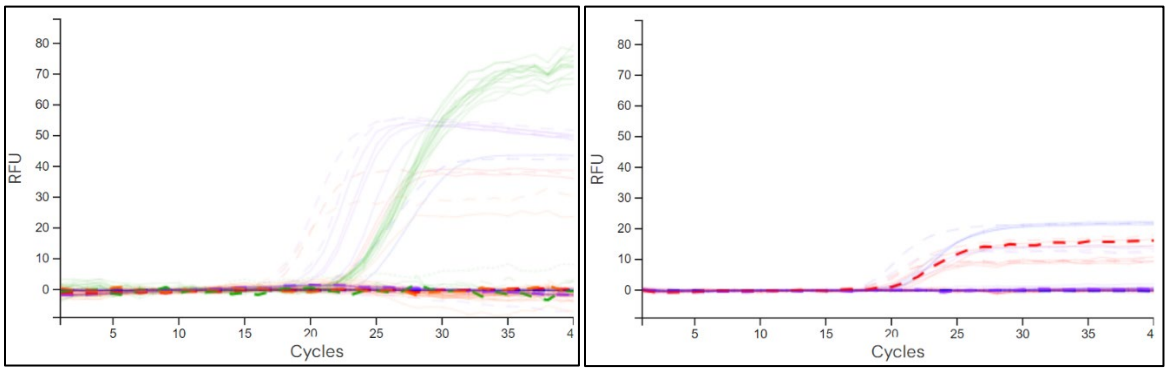


RhV RSV FluA FluB IC **HMPV AdV B/C HPIV**

Sample	Assay	Result
RV PC2	PlexPCR RespiVirus (LC480)	**Valid**
	RhV ⊖ Not detected	
	RSV ⊕ Detected	
	FluA ⊖ Not detected	
	FluB ⊖ Not detected	
	HMPV ⊖ Not detected	
	AdV B/C ⊕ Detected	
	HPIV ⊕ Detected	

21.9.3 Positive control RV PC 3

RV1 mix **RV2 mix**

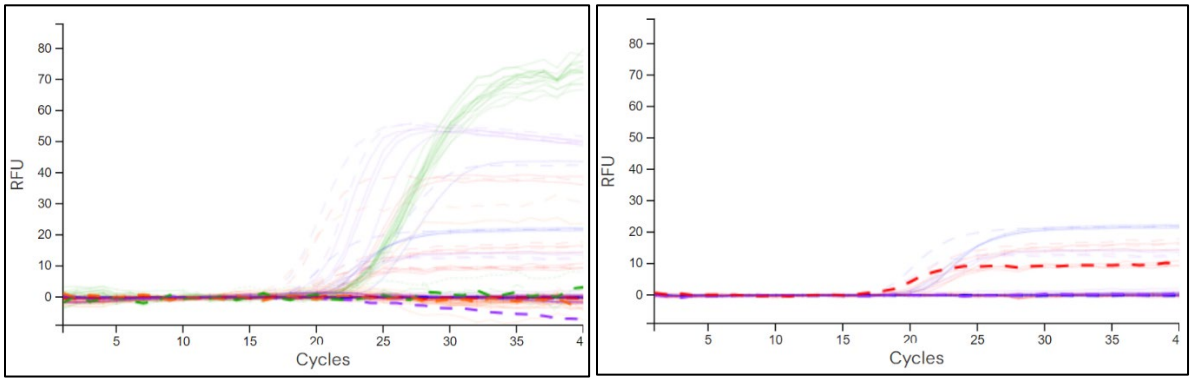


RhV RSV FluA FluB IC HMPV AdV B/C HPIV

Sample	Assay	Result
RV PC3	PlexPCR RespiVirus (LC480)	**Valid**
	RhV ⊖ Not detected	
	RSV ⊖ Not detected	
	FluA ⊖ Not detected	
	FluB ⊖ Not detected	
	HMPV ⊖ Not detected	
	AdV B/C ⊖ Not detected	
	HPIV ⊕ Detected	

21.9.4 Positive control RV PC 4

RV1 mix **RV2 mix**

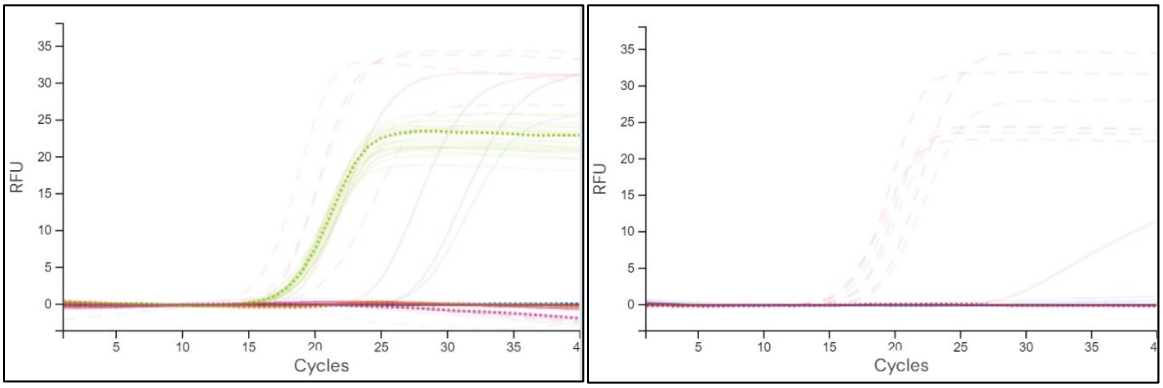


RhV RSV FluA FluB IC HMPV AdV B/C HPIV

Sample	Assay	Result
RV PC2	PlexPCR RespiVirus (LC480)	**Valid**
	RhV ⊖ Not detected	
	RSV ⊖ Not detected	
	FluA ⊖ Not detected	
	FluB ⊖ Not detected	
	HMPV ⊖ Not detected	
	AdV B/C ⊖ Not detected	
	HPIV ⊕ Detected	

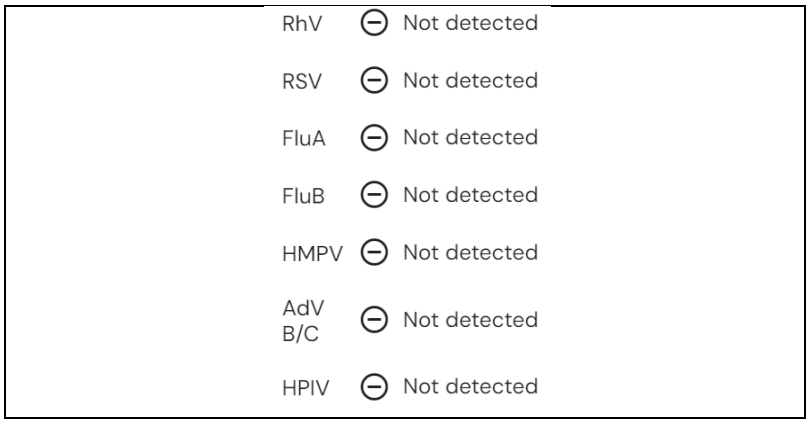
21.9.5 Negative control (negative specimen)

RV1 mix **RV2 mix**



RhV RSV FluA FluB IC **HMPV AdV B/C HPIV**

Sample	Assay	Result
NC	PlexPCR RespiVirus (LC480)	**Valid**



21.10 Examples

Example results for the ***PlexPCR***® RespiVirus analysis software are shown in **Table 46.**

Table 46. Example results for interpretation of PlexPCR® RespiVirus analysis software

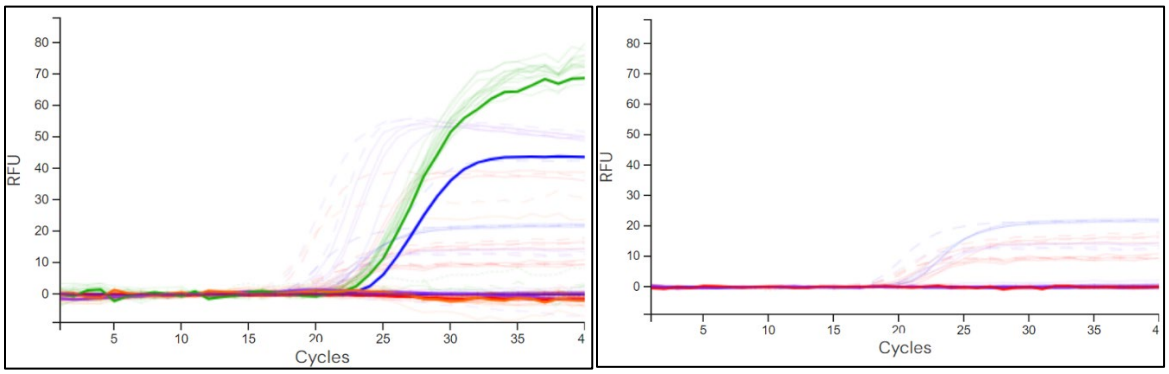
Sample	Assay	Result
Sample 101	PlexPCR RespiVirus (LC480)	**Not detected**
Sample 102	PlexPCR RespiVirus (LC480)	**Detected: FluA**
Sample 103	PlexPCR RespiVirus (LC480)	**Detected: FluB**
Sample 104	PlexPCR RespiVirus (LC480)	**Detected: RSV**
Sample 105	PlexPCR RespiVirus (LC480)	**Detected: RhV**
Sample 106	PlexPCR RespiVirus (LC480)	**Detected: HMPV**
Sample 107	PlexPCR RespiVirus (LC480)	**Detected: AdV B/C**
Sample 108	PlexPCR RespiVirus (LC480)	**Detected: HPIV**
[1]Sample 109	PlexPCR RespiVirus (LC480)	**Invalid: RhV, RSV, FluA, FluB**

[1] A sample interpreted as Invalid will be flagged with ⚠

The following examples show the amplification curves (baseline-corrected amplification curves) and the Results overview from the **PlexPCR RespiVirus (LC480)** analysis software for regular/unknown sample types.

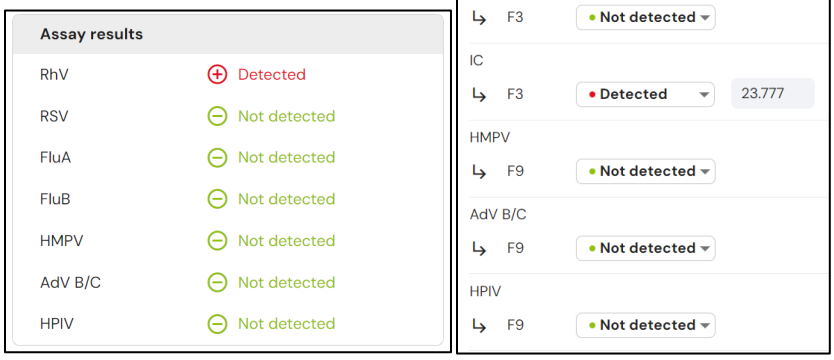
21.10.1 Example 1. Positive sample – single target detected

RV1 mix **RV2 mix**



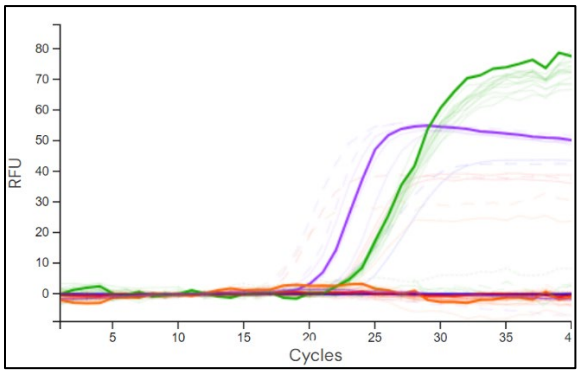
RhV RSV FluA FluB IC **HMPV AdV B/C HPIV**

Sample	Assay	Result
Sample 110	PlexPCR RespiVirus (LC480)	**Detected:** RhV



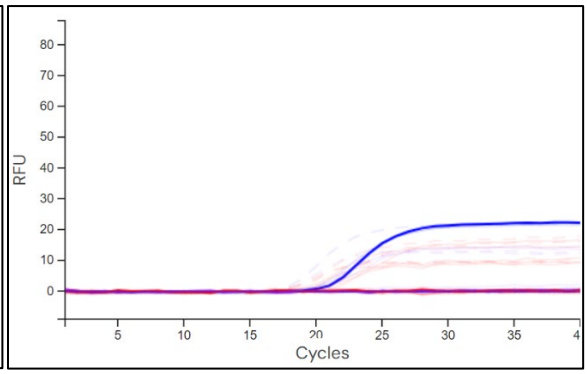
21.10.2 Example 2. Positive sample – multiple targets detected

RV1 mix



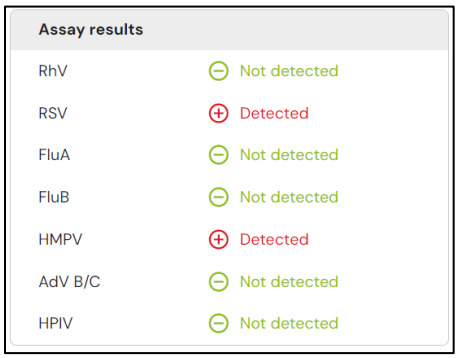
RhV RSV FluA FluB IC

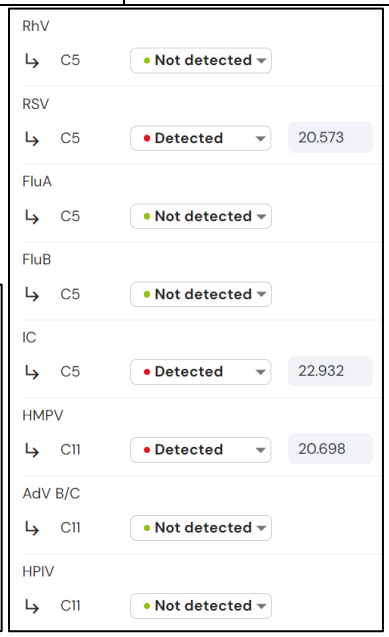
RV2 mix



HMPV AdV B/C HPIV

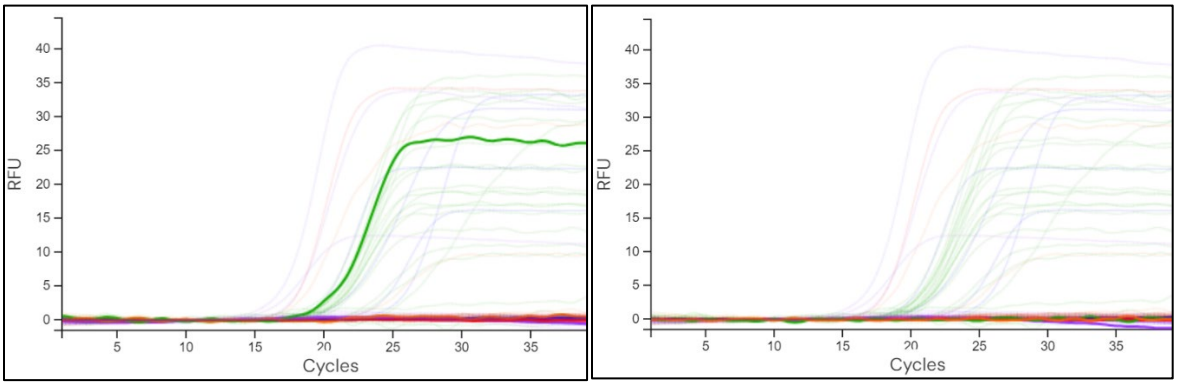
Sample	Assay	Result
Sample 111	PlexPCR RespiVirus (LC480)	Detected: RSV, HMPV





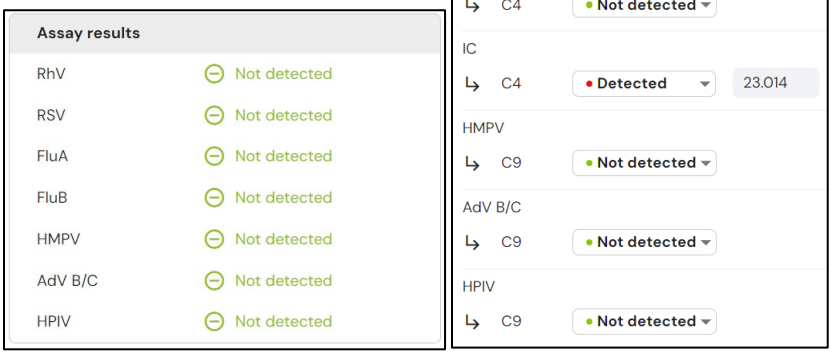
21.10.3 Example 3. Negative sample

RV1 mix **RV2 mix**



RhV RSV FluA FluB IC HMPV AdV B/C HPIV

Sample	Assay	Result
Sample 112	PlexPCR RespiVirus (LC480)	**Not detected**



22 Appendix B: PlexPCR® RespiVirus at 10 µL reaction volume

The ***Plex****PCR*® RespiVirus$_{(610)}$ kit 192 pack size has appropriate dead volume for use with liquid handling systems and has been validated with SpeeDx ***Plex****Prep*® for 10 µL reactions in 384-well plates on the LC480 II. Contact tech@speedx.com.au for assistance with protocols.

Refer to **Section 10.4** for instructions on testing a final reaction volume of 20 µL.

22.1 Master Mix preparation - 10 µL qPCR reaction volume

NOTE: Before use of the reagents, thaw completely, and mix thoroughly by briefly vortexing

For a 10 µl reaction volume, 7.5 µL Master Mix and 2.5 µLsample is required.

- Make up the Master Mix as outlined in **Table 47**, and then make up the RV1 (well 1) and RV2 (well 2) reaction mixes as outlined in **Table 48** and **Table 49**.
- Pipette the RV1 and RV2 reaction mixes into the PCR plate and then add extracted sample to both reactions.
- Positive and negative controls should be run on each plate.
- Seal, then centrifuge the plate and transfer to thermocycler.

Table 47. Master Mix setup for 10 µL reaction volume

Reagent	Concentration	Volume per 10 µL reaction (µL)
Nuclease Free Water (**BLUE**)	N/A	1.7
Plex Mastermix (**GREEN**)	2x	5.0
RTase (**CLEAR**)	100x	0.1
RNase Inhibitor (**BLACK**)	50x	0.2
Total volume (µL)		7.0

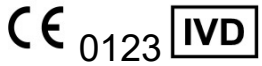
Table 48. RV1 (610) reaction mix for 10 µL reaction volume

Reagent	Concentration	Volume per 10 µL reaction (µL)
Master Mix	N/A	7.0
RV1 (610) mix (**YELLOW**)	20x	0.5
Total volume (µL)		7.5
Add 2.5 µL sample for a final volume of 10 µL		

Table 49. RV2 (610) reaction mix for 10 µL reaction volume

Reagent	Concentration	Volume per 10 µL reaction (µL)
Master Mix	N/A	7.0
RV2 (610) mix (**BROWN**)	20x	0.5
Total volume (µL)		7.5
Add 2.5 µL sample for a final volume of 10 µL		

23 Glossary



European Conformity
For *In Vitro* Diagnostic Use

Catalogue number

Batch code

Authorised Representative
In the European Community

Manufacturer

Date of manufacture

Temperature limitation

Contains sufficient for
xxx determinations

Use by Date

Importer

United Kingdom Conformity Assessment Mark

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