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Multi-pathogen detection & quantification using digital PCR V.1

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Wastewater Genomics S...

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We use this protocol and it's working

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Disclaimer

Parts of this protocol are derived from the QIAcuity OneStep Advanced Probe Kit User Manual.

Abstract

This protocol is optimised for quantifying RNA and DNA targets using the QIAcuity OneStep Advanced Probe Kit with hydrolysis probes in singleplex or multiplex (up to two targets) reactions on QIAGEN's QIAcuity digital PCR (dPCR) instruments. It also includes instructions for preparing in-house 20× primer–probe assays.

Materials

1.5 mL Eppendorf tubes
Standard 96-well plate
Microseal Adhesive Seal
QIAcuity 8.5K, 96-well Nanoplate
Pipettes of different sizes
Multichannel pipettes
QIAcuity OneStep Advanced Probe Kit

Before start

- Refer to the QIAcuity User Manual and QIAcuity User Manual Extension for guidance on assay design and experimental setup for the QIAcuity platform.
- The QIAcuity OneStep Advanced Probe Kit has been specially formulated with a hot-start RT enzyme, allowing users to assemble reactions at room temperature
- The Enhancer GC is recommended for use with all Applied Biosystems TaqMan assays, amplicons >150 bp in length, GC-rich amplicons, and RNA targets containing challenging secondary structures.

Assay set-up in a clean-room

1 Place the following on ice to thaw:
- 100x OneStep Advanced Reverse Transcription Mix,
- 4x QIAcuity OneStep Advanced Probe Master Mix
- Template RNA
- Primer-probe Mix
- Enhancer GC
- RNase-Free Water

2 Vigorously mix the individual solutions and centrifuge the tubes briefly to settle the liquids

3 Using the calculations in Table 1, prepare a master mix in a clean 1.5 mL Eppendorf tube. Remember to add 10% of each component to account for pipetting errors.

4 **Table 1:** Assay composition

	Component	x1 sample	x? samples	Final concentration
	4x OneStep Advanced Probe Master Mix	3 µL	µL	1x
	100x OneStep Advanced RT Mix	0.12 µL	µL	1x
	20x primer-probe mix 1	0.6 µL	µL	0.4 µM forward primer 0.4 µM reverse primer 0.2 µM probe
	20x primer-probe mix 2* (for multiplex)	0.6 µL	µL	0.4 µM forward primer 0.4 µM reverse primer 0.2 probe
	Enhancer GC	1 µL	µL	-
	Total reaction volume	**5.32 µL**	**µL**	**-**

? = number of samples + controls + 10%, ***** = substitute with RNase-Free water for singleplex assay

5 Vortex the reaction mix thoroughly and briefly centrifuge. Dispense 5.32 µL of the reaction mix into each well of a standard 96-well PCR pre-plate (according to your experiment layout).

Note

The pre-plate may be assembled at room temperature.

Add nucleic acids in PCR-room

6 Add 8 µL template RNA to wells containing the reaction mix. Thoroughly mix the template RNA with the reaction mix by pipetting up and down.

7 Seal the pre-plate with a microseal adhesive suitable for standard 96-well plates and briefly centrifuge (1000 rpm for 30 seconds) to collect any droplets.

8 Transfer 13.32 µL from each well of the pre-plate to a 96-well QIAcuity Nanoplate, taking care to avoid introducing bubbles.

Note

Always place the Nanoplate on a Nanoplate Tray when handling to protect the sensitive imaging surface at the bottom. Do not place the Nanoplate on ice at any time—work at room temperature.

9 Seal the Nanoplate with the seal provided in the kit, load it into the QIAcuity instrument, and initiate the one-step RT-dPCR cycling program below.

Note

This cycling program works for all* different primer-probe combinations, despite their different melting temperatures. This is possible because of

QIAcuity RT-dPCR cycling program

10 **Table 2:** QIAcuity RT-dPCR cycling program

	Step	Time	Temperature
	Reverse Transcription	40 min	50°C
	RT Enzyme Inactivation	2 min	95°C

	Step	Time	Temperature
	2-step cycling (45 cycles)	-	-
	Denaturation	5 sec	95°C
	Combined annealing & Extension	1 min	60°C

Preparing in-house 20x primer-probe assay

11 If you choose not to purchase QIAcuity pathogen detection assays from QIAGEN, you may source your own primers and probes and prepare them as 100 µM stock solutions.

12 Thaw your primers and probe on ice and prepare a 20x assay as indicated in Table 3 below.

13 **Table 3:** Preparing 20x primer-probe assays

	Component	Final Conc. in 20x assay	Volume to add
	Forward primer (100 µM)	8 µM	8 µL
	Reverse primer (100 µM)	8 µM	8 µL
	Probe (100 µM)	4 µM	4 µL
	Nuclease-free water	-	80 µL
	Total	**-**	**100 µL**

Our working assays

14 All primers and probes are sourced from IDT through Whitehead Scientific. The reporters, internal quenchers, and 3'-end quenchers used in the assays below may be proprietary to IDT. When designing assays, carefully consider the compatibility of the selected reporters and quenchers, and exercise particular caution when planning multiplex assays to avoid cross-reactivity between chemistries.

14.1 Influenza A assay

	Primer/probe	Sequence (5'-3')
	InfA For1	CAAGACCAATCYTGTCACCTCTGAC
	InfA Rev1	GCATTYTGGACAAAVCGTCTACG
	InfA Probe	/56-FAM/TGCAGTCCT/ZEN/CGCTCACTGGGCACG/3IABkFQ/

Research Use Only CDC Influenza SARS-CoV-2 (Flu SC2) Multiplex Assay Primers and Probes

14.2 Influenza B assay

	Primer/probe	Sequence (5'-3')
	InfB For	TCCTCAAYTCACTCTTCGAGCG
	InfB Rev	CGGTGCTCTTGACCAAATTGG
	InfB Probe	/5YakYel/CCAATTCGA/ZEN/GCAGCTGAAACTGCGGTG/3IABkFQ/

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14.3 Pan-measles assay

	Primer/probe	Sequence (5'-3')
	MVN1139F	TGGCATCTGAACTCGGTATCAC
	MVN1213R	TGTCCTCAGTAGTATGCATTGCAA
	MVNP1163Probe-2	/56-TAMN/CCGAGGATGCAAGGCTTGTTTCAGA/3BHQ_1/

Hummel, K.B., Lowe, L., Bellini, W.J. and Rota, P.A., 2006. Development of quantitative gene-specific real-time RT-PCR assays for the detection of measles virus in clinical specimens. *Journal of virological methods*, *132*(1-2), pp.166-173.

14.4 Measles vaccine strain assay

	Primer/probe	Sequence (5'-3')
	VMA168F	GAGATTGGGGGGCAAGGAAGAT

	Primer/probe	Sequence (5'-3')
	VMA242R	GCATCACTTGCTCTGCTGGGCC
	VMA190P	FAM-AGGAGGGTC/BMN-Q535/AAACAGAGTCGA-MGB

Ndlovu, N., Mabasa, V., Sankar, C., Msomi, N., Phalane, E., Singh, N., Gwala, S., Els, F., Macheke, M., Maposa, S. and Yousif, M., 2024. Wastewater testing during the South African 2022-2023 measles outbreak demonstrates the potential of environmental surveillance to support measles elimination. *medRxiv*, pp.2024-09.

14.5 Rubella assay

	Primer/probe	Sequence (5'-3')
	RV98	GGCAGTTGGGTAAGAGACCA
	RV251r	CGTGGAGTGCTGGGTGAT
	RV159rp	/5Cy5/ACATCGCGCACTTCCCACG/3IAbRQSp/

World Health Organization, 2007. Manual for the laboratory diagnosis of measles and rubella virus infection. In *Manual for the laboratory diagnosis of measles and rubella virus infection*.

14.6 Hepatitis A assay

	Primer/probe	Sequence (5'-3')
	HAV-68-F	TCACCGCCGTTTGCCTAG
	HAV-240-R	GGAGAGCCCTGGAAGAAAG
	HAV-150-Probe	/56-FAM/TTAATTCCT/ZEN/GCAGGTTCAGG/3IABkFQ/

Costafreda, M.I., Bosch, A. and Pintó, R.M., 2006. Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. *Applied and environmental microbiology*, *72*(6), pp.3846-3855.

14.7 Hepatitis E assay

	Primer/probe	Sequence (5'-3')
	JVHEVF	GGTGGTTTCTGGGGTGAC
	JVHEVR	AGGGGTTGGTTGGATGA A
	JVHEVPmod-2	/5TexRd-XN/TGATTCTCAGCCCTTCGC/3IAbRQSp/

Jothikumar, N., Cromeans, T.L., Robertson, B.H., Meng, X.J. and Hill, V.R., 2006. A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. *Journal of virological methods*, *131*(1), pp.65-71.

14.8 Mpox generic assay

	Primer/probe	Sequence (5'-3')
	N3R-F	TCCAATTATGACCTTTGATGGAA
	N3R-R	TGTTTAATTGTAGGACGGTTGT
	N3R-P	/56-FAM/TCCATCTAT/ZEN/CTCGTTCATGGTCGGT/3IABkFQ/
	B18R-F	TGGTTGCCGGATCTACGTATT
	B18R-R	ACGTTCTTAGTCGGTGCATT
	B18R-P	/5Cy5/CTAGTTGCTAGATCTCTCGCTATCATAG/3BHQ-3/

Fan, Z., Xie, Y., Huang, B., Zhao, F., Hu, Y., Huang, Y., Mei, S., Wei, L., Wang, L., Wang, L. and Gao, Z., 2024. Development of a multiplex real-time PCR assay for the simultaneous detection of mpox virus and orthopoxvirus infections. *Journal of Virological Methods*, *328*, p.114957.

14.9 *Mycobacterium complex* assay (IS6110)

	Primer/probe	Sequence (5'-3')
	TB_For	TCCACGGCCTGGGCTACCA
	TB_Rev	CATCGCGCACGCATCGGG
	TB_Probe	/56-ROXN/CACTCCGCCGATCGTTTTTCC/3IABkFQ/

14.10 Mycobacterium tuberculosis assay (Glycosyltransferase)

	Primer/probe	Sequence (5'-3')
	Forward	TCCACGGCCTGGGCTACCA
	Reverse	CATCGCGCACGCATCGGG
	Probe	/56-ROXN/CACTCCGCCGATCGTTTTTCC/3IABkFQ/

Park, J., Kwak, N., Chae, J.C., Yoon, E.J. and Jeong, S.H., 2023. A two-step real-time PCR method To identify Mycobacterium tuberculosis infections and six dominant nontuberculous mycobacterial infections from clinical specimens. *Microbiology spectrum*, *11*(4), pp.e01606-23.

14.11 Assays ordered ready-made from Qiagen

	Assay name	GeneGlobe ID	Reporter
	SARS-CoV-2 (N1)	DMA00710	ROX
	Vibrio Cholerae	DMA00340	TAMRA
	Dengue virus (pan)	DMA00788	FAM
	Hepatitis B	DMA00808	FAM

Protocol references

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