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Working

Binary synthetic template oligonucleotide positive control for in-house diagnostic real-time RT-PCR

Version 3

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ABSTRACT

This protocol details how to make and use a pair of synthetic template oligonucleotides (STOs) for use as real-time reverse transcription polymerase chain reaction (RT-rPCR) controls. The method can also be used for PCR controls. This binary positive control approach is easy to replicate once mastered, quick to produce, results in enough RNA stock for years of PCR runs, and provides several benefits over the use of a wild-type positive control such as the nucleic acids extracted from virus culture or from a known previously-positive clinical specimen.

The method is an adaptation of a previsouly published approach [Ref 4; see Steps section] and has recently been described in some publications.[Ref 5]

Our approach to a positive control requires that each PCR run includes two additional reactions.

1. one controls for primer function (**PRIMER** control). This STO includes hybridisation sites for the specific target assay's primers bouding a generic probe hybridisation target site
2. one controls for probe function (**PROBE** control). This STO includes a hybridisation site for the specific target assay's probe(s), bounded by a pair of generic primer hybridisation target sites

EXTERNAL LINK

https://www.ncbi.nlm.nih.gov/pubmed/27643685

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Hall-Mendelin S, Pyke AT, Moore PR, Mackay IM, McMahon JL, Ritchie SA, Taylor CT, Moore FA, van den Hurk AF. Assessment of Local Mosquito Species Incriminates Aedes aegypti as the Potential Vector of Zika Virus in Australia. PLoS Negl Trop Dis. 2016 Sep 19;10(9):e0004959 https://www.ncbi.nlm.nih.gov/pubmed/27643685

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

This protocol presumes expertise in laboratory work, molecular biology, laboratory safety and PCR.

MATERIALS

NAME	CATALOG #	VENDOR
RNeasy Mini Kit	74104	Qiagen
TURBO DNA-free™ Kit	AM1907	Thermo Scientific
Riboprobe(R) System - T7	P1440	Promega
1.5mL Self-Standing Screw Cap Tubes O-ring	14-222-581	Fisher Scientific
Water Molecular Grade RNase DNase and protease-free	786-293	

NAME	CATALOG #	VENDOR
Ribonucleotide triphosphates (rNTPs) 4x 0.5ml each at 10mM	P1221	Promega

SAFETY WARNINGS

BEFORE STARTING

Before making this control you will need:

- an RT-rPCR assay specific to your target or interest
- *in vitro* transciption reagents
- a dedicated work area that is distinct from where you usually prepare PCR mixes and load templates. Ideally with separate air handling, reagents, pipettes, PPE, disposables and cold storage (High concentration clean olignonucleotide ["HiCCO room"]
- label printer such as https://www.barcodes.com.au/zebra-gc420t-label-printer/
- A Class II biosafety cabinet (BSCII)

Overview

1 This protocol describes the design and use of a binary positive control system for use in clinical biospecimen testing. An earlier version of this method was previously published, as was this more recent version [Ref.1, Ref.2] The end products avoid the need to handle infectious wild-type virus or rely on previously positive clinical specimens for a PCR positive control source.

Ours is a public health virology laboratory, so the example below uses a specific virus, but it could easily be adapted for non-viral targets. I have chosen an RNA virus (enterovirus, EV-D68) and a pre-existing real-time reverse transcription polymerase chain reaction (RT-rPCR) test. I've included comments on how to adapt this protocol for rPCR of a DNA virus.

We will create two synthetic template oligonucleotides (STOs) for inclusion alongside a non-template (water) control in *every* RT-rPCR run we perform. Each template challenges either the virus test's primers (PRIMER control) or the virus test's probe (PROBE control), is run in a separate reaction mix and will generate distinct fluorescent signals when amplified in the presence of an appropriate reaction mix.

This work is conducted in a physically distinct room that is separate from biospecimen extraction, template addition and post-PCR activities. After working in the HiCCO (high concentration clean oligonucleotide) room, the scientist may no longer enter extraction or template addition rooms until the following day after a shower and clothing change.

The benefits of this approach include:

1. reducing the risk of contamination of PCR reactions with wild-type virus nucleic acid control material
2. eliminating the risk of infection from handling new, emerging or exotic infectious material
3. only need a target region's nucleotide sequence, removing difficulties and delays due to procuring hard to obtain and/or dangerous materials from overseas or in the early stages of an outbreak
4. create an easy-to-renew, long-life source of test control material
5. permitting independent verification of primer or probe performance in each test run
6. verifying the RT and PCR reactions are performing within specifications
7. creating quantified reagents for internal quality assurance programs

Nucleotide sequences and how they are used

2
- As an example we will use enterovirus species D genotype 68 (EV-D68) for the test target. We have previously described this assay here. This assay uses 2 forward primers to account for virus variation, but they hybridise to the same site so they are treated as a single oligo in the design of our PRIMER control STO.
- The PRIMER control STO design incorporates the EV-D68 primer hybridisation site sequences and a non-viral probe hybridisation site sequence
- The PROBE control STO design incorporates the EV-D68 probe hybridisation site sequence and a pair of non-viral primer hybridisation site sequences
- We use human gene sequences from the human E2 ubiquitin conjugating enzyme E2 D2 (UBE2D2; [Ref.4] GenBank accession U39317) for our non-viral primer and probe sequences.

Oligonucleotide name	Role	Sequence (5' - 3')
UBE2D2_01.2	UBE2D2[1] forward primer	TGAAGAGAATCCACAAGGAATTGA
UBE2D2_02.2	UBE2D2 reverse primer	CAACAGGACCTGCTGAACACTG

UbcH5B_TM	UBE2D2 probe	CAL FLUOR ORG 560[2] - TGATCTGGCACGGGACCCTCCA - BHQ1
EV-D68-For1	EV-D68 forward primer	TGTTYCCACGGTTGAAAAYAA
EV-D68-For2	EV-D68 forward primer	TTCCCACGGTTGAAARNYRAC
EV-D68-Rev	EV-D68 reverse primer	CAAGCTACACACGGGTTAGT
EV-D68-FAM-TM2018	EV-D68 probe	FAM[2] - CCGTTAWCCGCTATAGTACTTCGAGAAACC - BHQ1

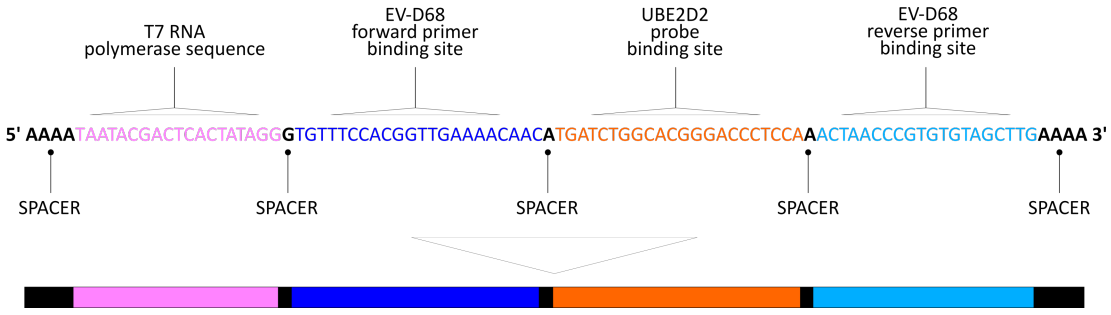
[1]UBE2D2 oligo sequences originate from [Ref.5]; fluorophore, oligo concentrations and cycling conditions differ from publication; the assay skips 3 exons or ~ 65,240nt so genomic DNA is too large to amplify and only spliced RNA will provide a wild target for the complete assay if used in extracts containing human mRNA. NB: the complete assay = UBE2D2 primers + probe which are never combined in our binary positive control system.
[2]FAM is the preferred green channel fluorophore for a virus target probe. CAL FLUOR ORANGE 560 is preferred for the UBE2D2 probe but many other coloured fluorophores are available and may be substituted.
[3]BHQ-1 is a black hole or "dark" quencher and is preferred to TAMRA. Different proprietary dark quenchers are offered by different suppliers and are sometimes specific to the fluorophore. It is important to check the appropriate fluorophore/dark quencher pair is selected when choosing to use other than FAM/BHQ1 or CALFLUOR560/BHQ1

STO design

3 We next design the two single-stranded DNA STOs, PRIMER control and PROBE control.
These are designed to be rPCR-amplifiable and detectable templates. This means they each incorporate forward primer, reverse primer and probe hybridisation sequences in appropriate orientation. We will design these for an RT-PCR so they will include a 5' T7 (could also use SP6) transcription initiation site [Ref.3] preceding the primer-probe-primer sequence.
If you are designing a DNA virus test, you can leave off the T7 primer sequence and skip over the *in vitro* transcription step as you won't need to make RNA.

PRIMER CONTROL DESIGN

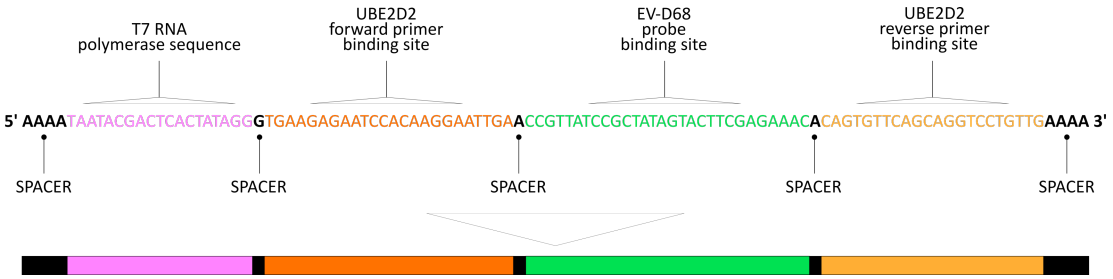
- a single amplifiable STO target sequence (this one is 94nt long) is designed (using Word, Excel or a sequence analysis software package like Geneious) by laying the oligonucleotides next to each other, separated by spacer sequences (bold).
- I find it helps to visualise the STO and use colour to identify the various hybridisation targets
- the single-strand is only as long as the primers+probe hybridisation sites, plus the T7 transcription initiation site (pink).
- the PRIMER control tests the virus assay *primers,* not the probe, so it contains virus primer hybridisation sites (blue) plus that for the UBE2D2 probe (orange).



Schematic of the 5' to 3' design of the EV-D68 PRIMER control STO, ready for ordering as a single DNA strand for later use in an *in vitro* transcription reaction to create RNA copies. The reverse primer hybridsation sequence is added in the reverse complement of the primer sequence.

PROBE CONTROL DESIGN

- a single amplifiable STO target sequence (this one is 106nt long) is designed (using Word, Excel or a sequence analysis software package like Geneious) by laying the oligonucleotides next to each other, separated by spacer sequences (bold).
- the single-strand is only as long as the primers+probe hybridisation sites, plus the T7 transcription initiation site (pink).
- the PROBE control only tests the virus assay *probe,* not the primers, so it contains a virus probe hybridisation site (green) plus those for the UBE2D2 primers (orange).



Schematic of the 5' to 3' design of the EV-D68 PROBE control STO, ready for ordering as a single DNA strand for later use in an *in vitro* transcription reaction to create RNA copies. The reverse primer hybridisation sequence is added in the reverse complement of the primer sequence.

Purchase

4 Once designed, the STOs can be ordered from your manufacturer of choice.

One example product is IDT's "Ultramer" at 4nmole scale. Each comes dry and suitably purified to be used in the next *in vitro* transcription step after resuspension in molecular grade water.

We recommend ordering STO DNA from a facility that is different to the one from which you purchase the diagnostics primers and probes. If that isn't possible, at least order the two set of reagents a week apart, not in a single batch.

In vitro transcription

5 Reconstitute each lyophilised STO DNA to a final concentration of 200µM (equivalent to 200 picomoles per microliter; pmol/µl), in their original tube, using nuclease-free water.

Affix a printed label to the tube stating the date of reconstitution and final concentration. Add another label to the STO datasheet as well as reconstitution details, and file.

Freeze unused reconstituted STO at -20°C.

In a 200µl (0.2ml) PCR tube, assemble the following reaction mix on the bench at room temperature (only thaw frozen reagents as needed, keep them in a cold brick, then immediately return them to the freezer when finished with them):

Reagent	Volume (µl)
Transcription Optimized 5X Buffer[1]	20
100mM DTT	10
rRNasin (40U/µl)	2.5
rNTPs (A, G, C, U) at 2.5mM each[2]	20
2,000-5,000ng of FWD strand[2] STO DNA	3
30-40U T7 RNA polymerase (kit has 500U or ~20rxn at 1µl/@10-20U/rxn	2
H_2O	42.5
Total volume	100

[1]T7 *in vitro* transcription Riboprobe® System-T7, Promega (P1440); [2]If kit stock is exhausted, rNTPs can be purchased separately (P1221)

Incubate the tube in a 96-well conventional thermocycler using with the following conditions:

- 37°C 60min
- 4°C hold

DNA template removal from *in vitro* transcribed RNA

6 Stop the thermocycler and remove the tube.

To this tube add the following reaction mix on the bench at room temperature (only thaw frozen reagents as needed, keep them in a cold brick, then immediately return them to the freezer when finished with them):

Reagent	Volume (µl)
ivtRNA preparation from 5	**100**
10X TURBO DNase Buffer[1] (0.1X total volume)	**12**
TURBO DNase (4U; removes up to 20µg of genomic DNA)	**2**
H_2O	**6**
Total volume	120

[1]TURBO DNA-free™ Kit, AM1907

Split by transferring 60µl into a new 200µl tube.

Incubate both tubes in a 96-well conventional thermocycler using with the following conditions:

- 37ºC 30min
- 4ºC hold

Remove each tube, add 1µl of TURBO DNase, mix by pipetting, return the tubes to the thermocycler and incubate them using the following cycling conditions:

- 37ºC 30min
- 4ºC hold

Remove the tubes, combine contents into a fresh 1.5ml tube and add **14µl** of thawed DNase Inactivation Reagent (10X) to each 0.2ml tube.
- Incubate at room temperature for 5min, flicking 2-3 times during incubation
- Centrifuge at 10,000xg for 1.5min
- Use a micropipette to carefully transfer the supernatant into a new nuclease-free 1.5ml tube

Purification of *in vitro* transcribed RNA

7

To purify RNA, we have summarized an RNeasy kit method below performed on the bench at room temperature:

- Add **250µl** 100% ethanol to supernatant from 6, mix by pipetting up and down and transfer total to an RNeasy spin column
- Spin at 10,000rpm for 15sec
- Transfer RNeasy column to a fresh collection tube, discard old collection tube
- Add **500µL** Buffer RPE
- Spin at 10,000rpm for 15sec
- Transfer RNeasy column to a fresh collection tube, discard old collection tube
- Add **500µL** Buffer RPE
- Spin at 10,000rpm for 2min
- Transfer RNeasy column to a nuclease-free 1.5ml tube (included), discard old collection tube
- Add **50µl** RNase-free water
- Spin at 10,000rpm for 1min to elute STO RNA
- Transfer eluate to a labelled screw cap tube

Storage of *in vitro* transcribed RNA

8 Divide the undiluted *in vitro* STO RNA preparation into two labelled tubes.

Obtain an optical density on one aliquot of the undiluted material. Store in separate locations as a safeguard for freezer failure or access limitations during laboratory maintenance etc.. Store at -80°C where they will remain amplifiable for years. Location details should be recorded in full.

Titration, testing and storing of dilutions

9 PCR and RT-PCR are used to identify how much amplifiable STO DNA template remains in the RNA preparation and to crudely estimate the amplifiable RNA content of the preparation, respectively. The procedure is as follows:

- Thaw one vial, label as having been thawed
- Pre-label 10X 2ml screw-capped tubes
- Add 495µl RNase-free water to the first tube and 450µl to the other nine tubes
- Prepare a 10-fold serial dilution from 10^{-2} to 10^{-11} in the prepared tubes keeping tubes on ice between transfer and vortexing steps
- In a purpose-specific BSCII, transfer 5µl of undiluted stock *in vitro* transcribed STO RNA into the tube preloaded with 495µl water – this is the 10^{-2} dilution. We start at this dilution to save stock and avoid too much RNA being brought into the template addition area.

- Using a vortex in BSCII, subject the re-capped tube to three pulses of 2-5 seconds each. Return the stock to the freezer.
- Transfer 50µl to the next tube, this one preloaded with 450µl RNase-free water and repeat this process, with vortexing as described after each transfer until complete, discarding the final 50µl

The synPri or synPrb RNA STO control is added as if it was the biospecimen extract sample. The only difference is that mixes must also incorporate the required UBE2D2 primers or probe to "complete" the missing pieces needed to amplify the hybrid templates.

Sufficient PCR mix is made to test the entire dilution series in duplicate + NTC + a small excess. See an example in the **Table** below, and scale up as required. Add STO RNA/DNA to rPCR and RT-rPCR mixes in duplicate and aliquot into tubes, plate or ring appropriate to the machine being used and space required.

We initially test the dilution series using both RT-rPCR and rPCR (specific PCR mixes, not an RT-rtPCR without the RT step) to identify the amount of amplifiable DNA remaining in the RNA preparation. There will always be some, but it's important to gauge how much. When using an RNA STO, if the rtPCR comes up positive it is still possible to create a stock of useful RNA STO so long as the last RT-PCR positive dilution is $\geq$1 $\log_{10}$ dilution greater (more dilute) than the last PCR positive dilution. This is an arbitrary value.

- If the final dilution to amplify by rPCR is <1 dilution greater (more dilute) than the last RT-rPCR-positive dilution, the remaining original RNA preparation can be thawed, pooled and subjected to another round of DNase treatment and column purification. There will be some loss of RNA.

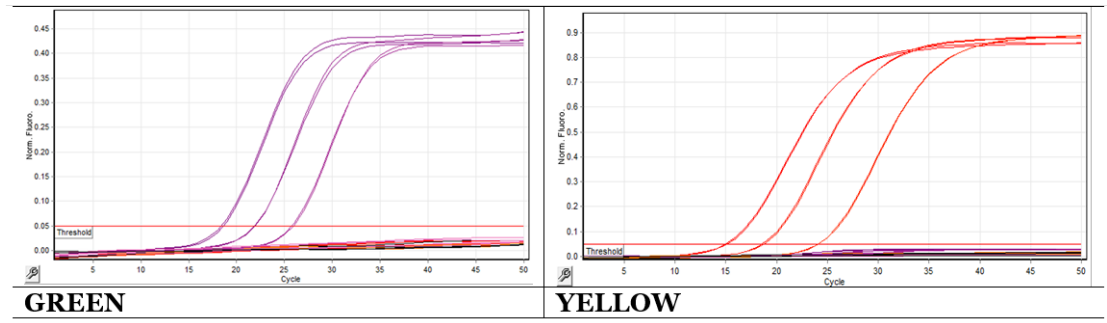
Volumes (µl)	**PRIMER control (µl)**	**PROBE control (µl)**
rPCR/RT-rPCR Mastermix (incl water & virus-specific primers & probe)	14.0	14.0
UBE2D2 Fwd & Rev primer working stock (6µM & 18µM)	-	1.0
UBE2D2 probe working stock (5µM)	1.0	-
synPrb STO at chosen dilution	-	5.0
synPri STO at chosen dilution	5.0	-
Total volume	***20***	***20***

Oligonucleotide stocks are stored at -20°C at 200µM (primer) and 100µM (oligoprobe) concentrations.

An example result for the EV-D68 is shown below.

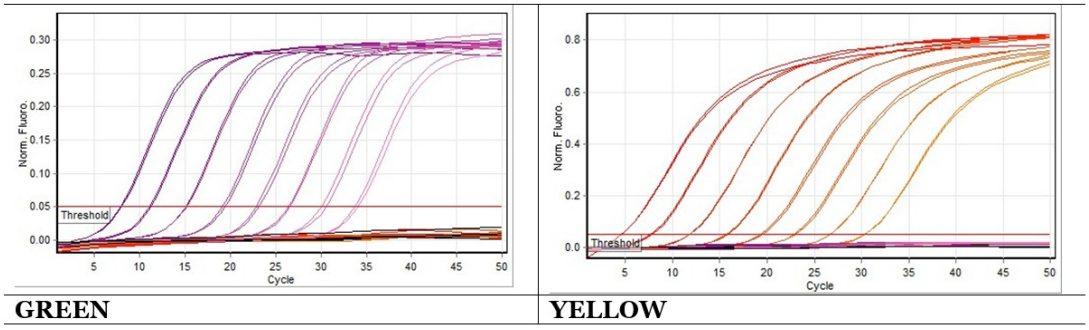
The RNA tested was the follwing dilution series of synPri and synPrb STO RNA: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11} The highest conentraiton generates a fluorescnet signal first (lowest $C_T \equiv$highest template load), followed by the next dilution, then the next, and so on. Sigmoidal curves are ideally separated by ~3.3 cycles.

rPCR TESTING FOR RESIDUAL DNA CONTENT



DNA has amplified from 2/2 duplicate reactions of the PROBE control (green channel for the FAM-labelled target virus probe) for the dilutions from 10^{-2} to 10^{-4}.
DNA has amplified from 2/2 duplicate reactions of the PRIMER control (yellow channel for the CALFLUOR560-labelled UBE2D2 probe) for the dilutions from 10^{-2} to 10^{-4}.

RT-rPCR TESTING FOR RESIDUAL RNA CONTENT



GREEN | **YELLOW**

RNA has been reverse-transcribed and amplified from 2/2 duplicate reactions of the PROBE control (green channel for the FAM-labelled target virus probe) for dilutions from 10^{-2} to 10^{-9}.
RNA has been reverse-transcribed and amplified from 2/2 duplicate reactions of the PRIMER control (yellow channel for the CALFLUOR560-labelled UBE2D2 probe) for dilutions from 10^{-2} to 10^{-9}.

For routine use, I'd recommend 10^{-6} as the usual dilution for routine RT-rPCR control use, in this example. This dilution produced a good sigmoidal shaped curve of suitable height that fell between a C_T of 20-25 in this test in our hands.

The repeatability and reproducibility of this can be used in the subsequent assay validation process and for ongoing monitoring of the test's and control's behaviour over time.

Regular use

10 Once testing is complete, the NEAT stocks are returned to long-term storage.

Working dilutions that are of use (10^{-5} to 10^{-9} in the example above) can also be stored until needed.

For regular use, stocks of synPri+UBE2D2 probe+water (PRIMER controls) and synPrb+UBE2D2 primers+water (PROBE controls) can be made and frozen. Batches of multi-use tubes and single-use tubes can be made depending on your laboratory's throughput.

Each diagnostic rPCR or RT-rPCR run muct include one of each control mix tube.

	Premade PRIMER Control mix	
	1X	100X
Hb5c2 Prb (100µM)	0.05µl	5µl
H_2O	4.95µl	495µl
TOTAL	5.0µl	500µl

A single 5µl volume can be added to a PCR/RT-rPCR tube already containing all the virus primers and probes and this tube is included in each run of samples that test for that virus.

	Premade PROBE Control mix	
	1X	100X
UBE2D2_01.2 (200µM)	0.03µl	3µl
UBE2D2_02.2 (200µM)	0.09µl	9µl
H2O	4.88µl	488µl
TOTAL	5.0µl	500µl

A further variation can be adopted for eh high-throughput PCR laboratory. The mixes above are adjusted to 2.5ul final volumes. Separate virus oligonucleotide mixes (primers, probes and water) are prepared to a final 2.5ul volume. Bulk numbers of mastermix aliquots are made and stored. Then mastermix can be thawed and a range of different virus-specific RT-rPCR/rPCR can be run on the same cycler by efficiently combining appropriate oligonucleotide and control mixes with the generic RT-rPCR or rPCR mastermix, as required.

References

11
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