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RSV standard and copyback genomes PCR Protocols V.2

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We use this protocol and it's working

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Abstract

Protocol for the detection of the Respiratory Syncytial Virus (RSV) standard and copyback genomes by PCR

Materials

Reverse Transcription (RT)

REAGENT	SOURCE	IDENTIFIER
2 µM Primer (need to make dilution from the 100 µM lab stock)		
Small DVGs: RT/anchorA-gRSV rev	IDT	5' **ggcagtatcgtgaattcgatgc**CCTCCAAGATTAAAATGATAACTTTAGG3'
Big DVGs: RT/anchorB-RSV DI 1	IDT	5' **ggccgtcatggtggcgaataa**CTTAGGTAAGGATATGTAGATTCTACC3'
Genome: RT/anchorC-RSVgenom1	IDT	5' **gaccatctagcgacctccac**GGAGGTTATATATGGGAAATGATGG 3'
dNTPs (10mM)	Thermo	Cat # R0194
SuperScript® III First-Strand Synthesis System (comes with everything) or SuperScript™ III Reverse Transcriptase	Thermo	Cat # 18080051 Cat # 18080085
RNaseOUT Recombinant Ribonuclease Inhibitor	Thermo	Cat # 10777019
Ribonuclease H	Thermo	Cat # 18021071

PCR

REAGENT	SOURCE	IDENTIFIER
Platinum Taq polymerase kit	Thermo	Cat # 10966018
$MgSO4_4$(35 mM)	Sigma	Cat # 83266100ML-F
dNTPs (10 mM)	Thermo	Cat # R0194
Forward Primer (10 µM) (need to make dilution from the 100 µM lab stock)		
Small DVGs: anchorA-Fwd	IDT	5' GGCAGTATCGTGAATTCGATGC 3'
Big DVGs: anchorB-Fwd	IDT	5' GGCCGTCATGGTGGCGAATAA 3'
Genome: anchorC-Fwd	IDT	5' GACCATCTAGCGACCTCCAC 3'
Reverse Primer (10 µM) (need to make dilution from the 100 µM lab stock)		
Small DVGs: RSV DVG rev small	IDT	5' CGAGAAAAAAAGTGTCAAAAACTAATATC 3'
Big DVGs: gRSV rev	IDT	5' CCTCCAAGATTAAAATGATAACTTTAGG 3'
Genome: RSV genom1 Rev	IDT	5' GTGCTTCCTACTTTGTGTAATAG 3'

Before start

READ ME FIRST!

What is a PCR hood/cabinet?
Our PCR hood is a dead air box that prevents contamination between samples by minimizing air circulation in the environment. The UV light helps decontaminate the workspace between uses.

When should you use the PCR hood?
cbVGs are highly stable in their RNA form. In addition, some species in highly abundant and once they are reverse transcribed into cDNA, you have a lot of very stable cDNA in the environment. Therefore, to avoid contaminations in PCRs and sequencing cbVG cDNA should be handled in the PCR hood to minimize contamination. Specifically,
After performing cbVG-specific RT reactions, RNaseH should be added in the PCR hood after RT. The aerosol that occurs when opening the cDNA-containing PCR tubes is a major source of contamination.
After PCR master mix is aliquoted at the bench, cDNA should be added to each reaction in the PCR hood.

Note, PCR hoods are a dedicated workspace designed to minimize DNA contamination; they are not RNase-free environments, so they are not the best place to handle RNA. RNases are ubiquitous in the environment and even miniscule amounts can quickly degrade an RNA sample. PCR amplifies huge numbers of the target DNA, and these amplicons can easily contaminate subsequent reactions if proper spatial separation and clean practices are not maintained.

Tips for using the PCR hood:
The PCR hood is not a Biosafety Cabinet. PCR cabinets do not protect the operator; they only protect samples inside the work zone.
Do not store unnecessary things in the PCR hood, as this can reduce containment.
The workstation should be wiped down with disinfectant prior to and after use, and all external items should be disinfected before they enter the PCR hood (things like tube racks).
Pipettes should be disinfected before and after use: spray disinfectant (10% bleach) on a paper towel and wipe them down (do not spray the pipettes directly).
When applicable, minimize contamination risk by processing LD samples first, followed by HD or higher concentration samples.
Do not reach over your samples. This can contaminate your product.
When finished, turn on the UV light to decontaminate the workspace between uses and eliminate any airborne contaminants (for our hood, the knob is broken so you need to use the tool that is usually sitting on top to turn it on).
Change gloves immediately when finished to reduce contamination to other areas in the lab.

RSV standard and copyback genomes PCR Protocols

1 2h 5m

*The “Big” DVG design can detect cbVGs with break before 14882 and rejoin before 15112 (RSVA2). The “Small” DVG design can detect cbVGs with break before 15112 and rejoin before 15194 (RSVA2). The “Small” DVG design should theoretically also pick up big DVGs, but bigger amplicons do not get amplified as well. “gRSV rev” has been tested as an RT primer for both “Big” and “Small” DVG design, however, gave an unspecific amplicon with the “Big” DVG design at around 500bp. As a consequence, **two separate RT and PCR reactions must be run when wanting to look for both big DVGs and small DVGs**. For most experiments, it is enough to only use the “Big” DVG design. RSV Genome can also be detected using this protocol when using the Genome primers. Primer locations are 387-410 and 894-918 (RSVA2), however not an optimal match (seems to have been designed for RSV B?).

Procedure

Reverse Transcription (RT): *RT reaction can be completed in a 10 µL reaction or a 20 µL reaction. The 10 µL reaction is preferred, if RNA concentrations allow, to help conserve reagents.

1. Start with 500 ng of RNA diluted in dH_2O (range 200-2000ng (background increases with more RNA))
   - 10 µL reaction: dilute in 4 µL dH_2O
   - 20 µL reaction: dilute in 8 µL dH_2O
2. Add primer ([M] 2 micromolar (µM))
   - 10 µL reaction: 0.5 µL
   - 20 µL reaction: 1 µL
3. Add dNTPs
   - 10 µL reaction: 0.5 µL
   - 20 µL reaction: 1 µL
4. Incubate at 65 °C for 00:10:00
   Program in PCR Machine: RT-DI1
5. Prepare the mix

REAGENT	10 uL REACTION	20 µL REACTION
Buffer	1 µL	2 µL
$MgCL_2$(25mM)	2 uL	4 uL
0.1 DTT	1 uL	2 uL
RNase Out	0.5 uL	1uL
SS III	0.5 uL	1uL

6. Add mix to sample
   10 µL reaction: 5 µL
   20 µL reaction: 10 µL
7. Incubate at 50 °C for 00:50:00 then 85 °C for 00:05:00
   Program in PCR Machine: RT-DI2
8. After program is finished keep at -20 °C for at least 00:20:00
9. Spin down tubes in mini microcentrifuge
10. Add RNase H (add in the PCR hood! DVGs are very stable!)
    10 µL reaction: 0.5 µL
    20 µL reaction: 1 µL
11. Incubate at 37 °C for 00:20:00
    Program in PCR Machine: RNaseH
12. Keep in -20 °C for at least 00:20:00 before moving on to the PCR

PCR

2 1. Prepare master mix (can be done on bench)
*Volumes below are for when 1 µL of cDNA is used. Adjust dH_2O volume accordingly if more cDNA is added. Up to 4 µL cDNA can be added but increases background. Total reaction volume should equal 25 µL.

REAGENT	VOLUME (µL)
dH_2O	14.75
Buffer (10X)	2.5
$MgSO4_4$(35 mM)	2.5
dNTPs (10 mM)	2.0
Forward primer (10 µM)	1
Small DVGs: anchorA-Fwd	
Big DVGs: anchorB-Fwd	
Genome: anchorC-Fwd	
Reverse primer (10 µM)	1
Small DVGs: RSV DVG rev small	
Big DVGs: gRSV rev	
Genome: RSV genom1 Rev	
Taq polymerase (5 units/µL)	0.25

2. Add 24 µL of master mix to PCR tubes (can be done on bench)
3. Thaw cDNA samples and spin down in mini microcentrifuge
4. Add 1 µL of cDNA sample to master mix (add in PCR hood! DVGs are very stable!)
5. Run PCR

Program in PCR Macine: RSVDI233

STEP	TEMPERATURE	TIME	
Denaturation	95ºC	10 min	Hold
Denaturation	95ºC	30 sec	33 cycles total (up to 35 cycles but background increases with more cycles)
Annealing	55ºC	30 sec	
Extension	72ºC	90 sec	
Final extension	72ºC	5 min	Hold
	4ºC	Forever	Hold

Gel Electrophoresis:

30m

3

1h 2m

1. Prepare 1% agarose gel

1 g pure agarose in 100 mL of 1X TAE buffer

Microwave until agarose solution dissolves completely (00:02:00)

Let agarose solution cool before adding Ethidium bromide (if you can keep your fingers on the flask without burning, then it is at an appropriate temperature)

Add 1-5 µL of Ethidium bromide and mix by swirling flask

2. Pour agarose solution into gel cast (remember to put in the well comb)

3. Let the agarose solidify (wait at least 00:30:00)

4. Place gel in electrophoresis chamber containing 1X TAE buffer (make sure buffer covers the gel)

5. Load ladder and samples to wells

6 µL of Ladder

Ladder stock recipe:

100 µL GeneRuler 100 bp Plus DNA ladder from Thermo Scientific: SM0321

100 µL DNA Gel Loading Dye (6X) from Thermo Scientific: R0611

400 µL dH_2O

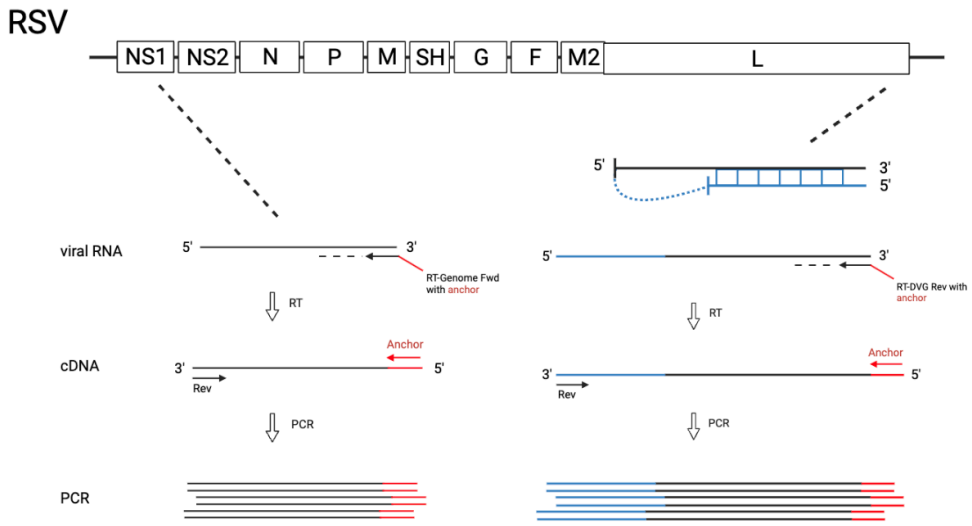
30 µL of Sample + Loading dye mix (dilutes from 6X to 1X)

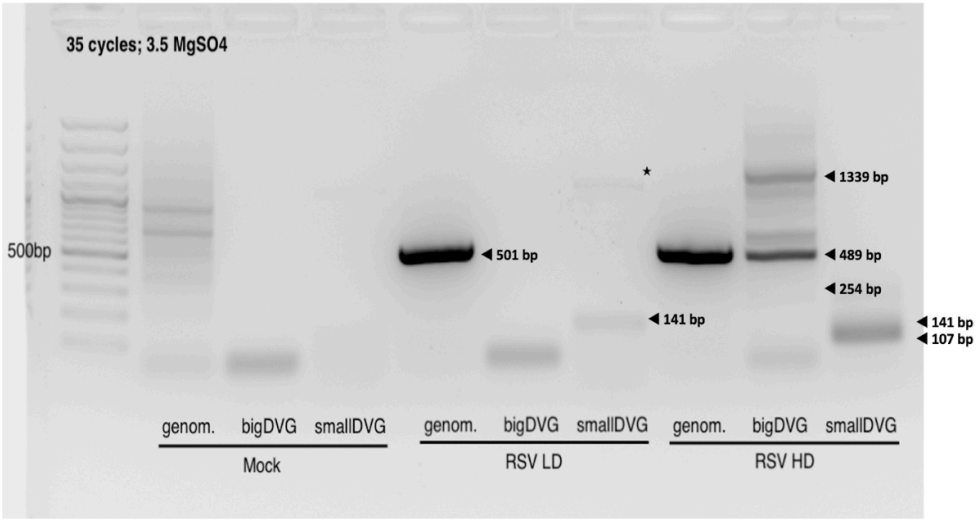
5 µL of DNA Gel Loading Dye (6X) from Thermo Scientific: R0611

25 µL of sample

6. Run at 110 volts for 00:30:00

7. Analyze gel bands





Band sizes and sequences were confirmed with Sanger sequencing.