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SeV standard and copyback genomes PCR Protocols V.2

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Protocol status: Working

We use this protocol and it's working

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Abstract

Protocols for the detection of Sendai Virus (SeV) standard and copy-back genomes by PCR

Materials

Reverse Transcription (RT) **Different RT kits and steps for cbVG and Genome** cbVG

REAGENT	SOURCE	IDENTIFIER
10 uM Primer (need to make dilution from the 100-uM lab stock)		
cbVGs: DI	IDT	5' GGTGAGGAATCTATACGTTATAC 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

Genome



REAGENT	SOURCE	IDENTIFIER
2.5 uM Primer (need to make dilution from the 100-uM lab stock)		
Genome: SEV (SC 2 (+) 3)	IDT	5' TGTTCTTACTAGGACAAG 3'
dNTPs (10mM)	Roche	Cat # 04379012001
Transcriptor first strand cDNA Synthesis kit Roche	Roche	Cat # 04896866001 Cat # 04897030001
Protector Rnase Inhibitor,40U/ul	Roche	Cat #04897030001

PCR *Same kit and steps for both cbVG and Genome*

REAGENT	SOURCE	IDENTIFIER
Platinum Taq polymerase kit	Thermo	Cat # 10966018
MgSO ₄ (50 mM)	Sigma	Cat # 83266100ML-F
dNTPs (10 mM)	Thermo	Cat # R0194
Forward Primer (10 uM) (need to make dilution from the 100 uM lab stock)		
cbVG: DI1	IDT	5' GGTGAGGAATCTATACGTTATAC 3'
Genome: SC 4.1 (-) 4	IDT	5' AATCTAGGTATCTCACTCCATG 3'
Reverse Primer (10 uM) (need to make dilution from the 100 uM lab stock)		
cbVG: DI/gSeV	IDT	5' ACCAGACAAGAGTTTAAGAGATATGTATT 3'
Genome: SC 2 (+) 6	IDT	5' AAGAGATTCTCTGAGTATCAGAA 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 are 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,

1. 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.
2. 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.

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Tips for using the PCR hood:

1. The PCR hood is not a Biosafety Cabinet. PCR cabinets do not protect the operator; they only protect samples inside the work zone.
2. Do not store unnecessary things in the PCR hood, as this can reduce containment.
3. 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).
4. 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).
5. When applicable, minimize contamination risk by processing LD samples first, followed by HD or higher concentration samples.
6. Do not reach over your samples. This can contaminate your product.
7. 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).
8. Change gloves immediately when finished to reduce contamination to other areas in the lab.

Reverse Transcription (RT)_Several options depending on the application

10m

1 For cbVG cDNA: SuperScript III

1h 35m

Procedure:

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 1 µg of RNA
 - 10 µL reaction: dilute in 4 µL dH₂O
 - 20 µL reaction: dilute in 8 µL dH₂O
2. Pre-mix 10 micromolar (µM) primer and dNTPs
 - 10 µL reaction: 0.5 µL of each, per sample
 - 20 µL reaction: 1 µL of each, per sample
3. Add Primer/dNTP mix to the RNA sample
 - 10 µL reaction: 1 µL
 - 20 µL reaction: 2 µL
4. Mix and spin down
5. Incubate at 65 °C for 00:10:00
Program in PCR Machine: RT-DI1
6. Prepare the mix (see TABLE below)

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



- Add the SIII enzyme separately to each sample, don't include it in the mix.

7. Add mix to sample, vortex briefly, and spin down

10 μL reaction: 5 μL

20 μL reaction: 10 μL

8. Incubate at 50 $^{\circ}\text{C}$ for 00:50:00 then 85 $^{\circ}\text{C}$ for 00:05:00

Program in PCR Machine: RT-DI2

9. After program is finished keep at -20 $^{\circ}\text{C}$ or on ice for at least 00:05:00

_____ Move to PCR Hood (for all later steps)

_____ (Clean PCR hood with 10% bleach. Wipe down pipette

with bleach in between samples.)

10. Add RNase H

10 μL reaction: 0.5 μL

20 μL reaction: 1 μL

11. Incubate at 37 $^{\circ}\text{C}$ for 00:20:00

Program in PCR Machine: RNaseH

12. Keep on ice for at least 00:05:00 then you can do the PCR or store at

-20 $^{\circ}\text{C}$

2 For viral genome cDNA: OPTION A: SuperScript III

Procedure:

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 1 μg of RNA

10 μL reaction: dilute in 4 μL dH₂O

20 μL reaction: dilute in 8 μL dH₂O

2. Pre-mix [M] 2.5 micromolar (μM) primer (**SEV (SC2 (+)3**) and dNTPs

10 μL reaction: 0.5 μL of each, per sample

20 μL reaction: 1 μL of each, per sample

3. Add Primer/dNTP mix to the RNA sample

10 μL reaction: 1 μL

20 μL reaction: 2 μL

4. Mix and spin down

5. Incubate at 65 $^{\circ}\text{C}$ for 00:10:00

Program in PCR Machine: RT-DI1



6. Prepare the mix (see TABLE below)

REAGENT	10 μ L REACTION	20 μ L REACTION
Buffer	1 μ L	2 μ L
MgCL*(25mM)	2 μ L	4 μ L
0.1 DTT	1 μ L	2 μ L
RNase Out	0.5 μ L	1 μ L
SS III	0.5 μ L	1 μ L

7. Add mix to sample, vortex briefly, and spin down

10 μ L reaction: 5 μ L

20 μ L reaction: 10 μ L

8. Incubate at 50 $^{\circ}$ C for 00:50:00 then 85 $^{\circ}$ C for 00:05:00

Program in PCR Machine: RT-DI2

9. After program is finished keep at -20 $^{\circ}$ C or on ice for at least 00:05:00

_____ Move to PCR Hood (for all later steps)

_____ (Clean PCR hood with 10% bleach. Wipe down pipette

with bleach in between samples.)

10. Add RNase H

10 μ L reaction: 0.5 μ L

20 μ L reaction: 1 μ L

11. Incubate at 37 $^{\circ}$ C for 00:20:00

Program in PCR Machine: RNaseH

12. Keep on ice for at least 00:05:00 then you can do the PCR or store at

-20 $^{\circ}$ C

3 For viral genome cDNA: OPTION B: ABRT Roche Kit

35m

Procedure:



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  1000 ng of RNA diluted in dH_2O

 10 μL reaction: dilute in  3 μL dH_2O

 20 μL reaction: dilute in  6 μL dH_2O

2. Add primer:

 10 μL reaction:  3.5 μL

 20 μL reaction:  7 μL

3. Incubate at  65 $^{\circ}\text{C}$ for  00:10:00

Program in PCR Machine: RT-DI1

4. Prepare the mix

REAGENT	10 μL REACTION	20 μL REACTION
Buffer (5X) contains 8mM MgCl_2	2 μL	4 μL
dNTP 10mM	1 μL	2 μL
Protector Rnase Inhibitor	0.25 μL	0.5 μL
Transcriptor reverse transcriptase	0.25 μL	0.5 μL

5. Add mix to sample

 10 μL reaction:  3.5 μL

 20 μL reaction:  7 μL

6. Incubate at  50 $^{\circ}\text{C}$ for 60 minutes then  85 $^{\circ}\text{C}$ for  00:05:00 then

 4 $^{\circ}\text{C}$ forever

Program in PCR machine: RORT2SPE

7. Keep in  -20 $^{\circ}\text{C}$ for at least  00:20:00 before moving on to the PCR

PCR

4 *Same kit and steps for both cbVG and Genome*

Procedure:



1. Prepare master mix (can be done on bench) * Volumes below are for when  1 μL of cDNA is used. Adjust dH₂O 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 (UL)
dH ₂ O	14.75
Buffer (10X)	2.5
MgSO ₄ (50 mM)	2.5

dNTPs (10 mM)	2.0
Forward primer (10 uM)	1
cbVG: DI1	
Genome: SC 4.1 (-) 4	
Reverse primer (10 uM)	1
cbVG: DI/gSeV	
Genome: SC 2 (+) 6	
Taq polymerase (5 units/uL)	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! cbVG are very stable!)
5. Run PCR

Program in PCR Machine: DIPCR



STEP	TEMPERATURE	TIME	
Denaturation	95°C	10 min	Hold
Denaturation	95°C	45 sec	35 cycles total
Annealing	55°C	30 sec	
Extension	72°C	90 sec	
Final extension	72°C	5 min	Hold
	4°C	Forever	Hold

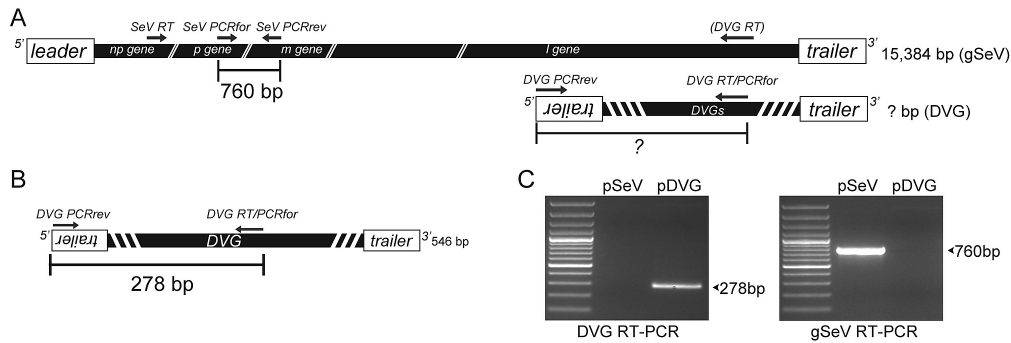
Gel Electrophoresis

- 5 1. Prepare 1% agarose gel 30m
- 1 g pure agarose in  100 mL of 1X TAE buffer
- Microwave until agarose solution dissolves completely (~3 min)
- 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 an electrophoresis chamber containing 1X TAE buffer (make sure buffer covers gel)
5. Load ladder and samples to wells
-  6 µL of Ladder
- Ladder stock recipe:
-  100 µL Gene Ruler 100 bp Plus DNA ladder from Thermo
- Scientific: SM0321
-  100 µL DNA Gel Loading Dye (6X) from Thermo Scientific: R0611
-  400 µL dH₂O
-  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 120 volts for 30-40 minutes
7. Analyze gel bands

Sendai Virus Genomic and cbVG PCR CONCEPT

6



SeV copy back VG PCR strategy and validation. (A) Diagram of the genomic composition of the full-length SeV genome (gSeV) and of a representative copy-back VG of unknown length. Arrows indicate the location of primers used for RT and amplification (PCR). Full-length size of the genome is indicated. Expected amplicon size of 760 nt of the gSeV to be detected through our PCR assay is indicated. This strategy allows detection of most copy-back VGs replicating in an infected cell. **(B)** Schematics of SeV strain Cantell's predominant 546 nt long cbVG. Expected amplicon size of 278 nt of this particular cbVG to be detected through our PCR assay is indicated. **(C)** Validation of the cbVG PCR assay. cbVG and gSeV amplicons from plasmids encoding the full-length SeV strain Cantell genome (lane 1) or the SeV strain Cantell dominant cbVG (lane 2) after amplification using the primers depicted in (A). <https://doi.org/10.1371/journal.ppat.1003703>.



Note

The primers used to detect the Sendai virus genome, are designed between intergenomic regions, that are not transcribed which ensures that the genome pcr product is totally from genomic RNA and not mRNA.

The cbVG product shown is not the only cbVG that is presented in your samples, it's just the one that was detected using these primers and this protocol sensitivity.

Same primers that are used in this protocol are the ones currently applied for Sev52 and SevC, SevCB as well.

Using random primers is not preferable as nonspecific products might be detected.

Protocol references

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