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SeV standard and copyback genomes PCR Protocols

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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

PRINCIPLES BEHIND THE PROCEDURE MUST BE UNDERSTOOD. PLEASE CONSULT WITH EXPERIENCED LAB MEMBER THE FIRST TIME YOU USE THIS PROCEDURE. UPDATE AS A GENERAL PROCEDURE AS NECESSARY BUT DO NOT MODIFY WITH SPECIFICS TO YOUR PROJECT, INSTEAD DOWNLOAD AND PASTE A MODIFIED COPY IN YOUR NOTEBOOK.

Reverse Transcription (RT)

10m

1 ***Different RT kits and steps for cbVG and Genome**
1/3) cbVG: Use SuperScript III Kit

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 dH_2O
   - 20 µL reaction: dilute in 8 µL dH_2O
2. Pre-mix [M] 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 °C for 00:50:00 then 85 °C for 00:05:00
   Program in PCR Machine: RT-DI2
9. After program is finished keep at -20 °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 °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 °C

2 ***Different RT kits and steps for cbVG and Genome** 35m

2/3) Genome: Use Roche Kit

Procedure

*RT reaction can be completed in a 10 µL reaction or a 20 µL reaction. The 10 ∝L 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 °C for 00:10:00
   Program in PCR Machine: RT-DI1
4. Prepare the mix

REAGENT	10 ul REACTION	20 ul REACTION
Buffer (5X) contains 8mM MgCl2	2 uL	4 uL
dNTP 10mM	1 uL	2 uL
Protector Rnase Inhibitor	0.25 uL	0.5 uL
Transcriptor reverse transcriptase	0.25 uL	0.5 uL

5. Add mix to sample
   10 µL reaction: 3.5 µL
   20 µL reaction: 7 µL
6. Incubate at 50 °C for 60 minutes then 85 °C for 00:05:00 then 4 °C forever
   Program in PCR machine: RORT2SPE
7. Keep in -20 °C for at least 00:20:00 before moving on to the PCR

PCR

3 ***Same kit and steps for both cbVG and Genome***
3/3) PCR

Procedure

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 (UL)
dH_2O	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	95ºC	10 min	Hold
Denaturation	95ºC	45 sec	35 cycles total
Annealing	55ºC	30 sec	
Extension	72ºC	90 sec	
Final extension	72ºC	5 min	Hold
	4ºC	Forever	Hold

Gel Electrophoresis

4 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 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 120 volts for 30-40 minutes
7. Analyze gel bands

Sendai Virus Genomic and cbVG PCR CONCEPT

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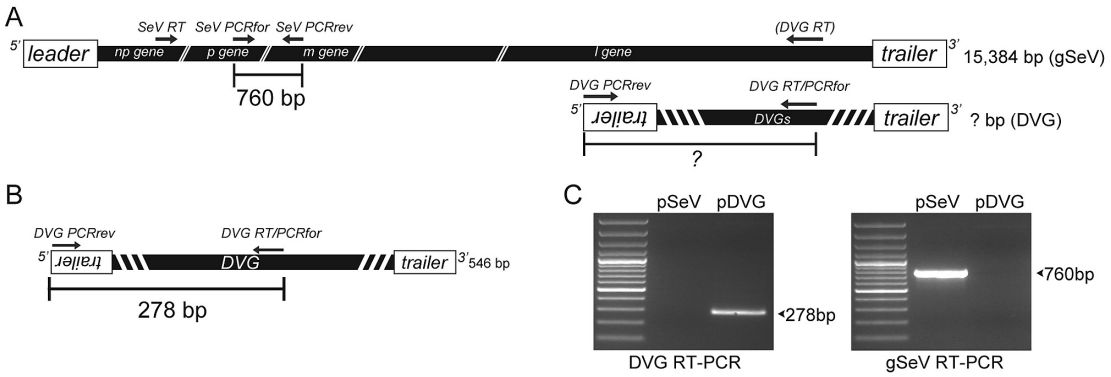

SeV copy back VG PCR strategy and validation. (**A**) Diagram of the genomic composition of the full-lengthSeV genome (gSeV) and of a representative copy-back VG of unknown length. Arrows indicate the location ofprimers used for RT and amplification (PCR). Full-length size of the genome is indicated. 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 isindicated. (**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 amplificationusing 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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