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Development and validation of RT-PCR and nested PCR assays for detection of Sabiá virus (SABV)

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We are still developing and optimizing this protocol

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Abstract

Note

This protocol describes the design, validation, and application of conventional RT-PCR and nested PCR assays for detection of Sabiá virus (SABV). Primer sets were designed based on the 1990 reference genome and newly generated SABV genomes, targeting conserved regions of the nucleoprotein gene. The protocol includes RT-PCR screening and nested PCR amplification optimized for low-viremia samples.

Materials

1 **Reagents**

2 Q5 DNA Polymerase (New England Biolabs)

3 5× Q5 Reaction Buffer

4 dNTPs (10 mM)

5 ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs)

6 Random Hexamers (Thermo Fisher Scientific)

7 Nuclease-free water

8 E-Gel EX Agarose 2% (Thermo Fisher Scientific)

9 E-Gel DNA Sizing Ladder

10 RNAse P primers and probe (optional internal control)

11 **Equipment**

12 Thermal cycler

13 Gel electrophoresis system (E-Gel)

14 UV or blue-light transilluminator

Primer tables

15 **RT-PCR**

	A	B	C	D	E
	Name	Sequence 5′ - 3′	Numer of bases	Amplicon lenght	Tm
	Pos_29F	GTCACGCTTAAATCTTTGATTGC	23	350 bp	55° C
	Pos_381R	ACAGACACCTCAAGACACCA	20		
	All_3F	TCACGCTTAAATCTTTGATTGCG	23	121 bp	65 °C
	All_3R	GCAGCAATYAAGCTCACTGC	20		

f = forward primer, r = reverse primer, bp = base pairs, Tm = melting temperature.

Nested-cPCR

	A	B	C	D	E
	Name	Sequence 5′ - 3′	Numer of bases	Amplicon lenght	Tm
	Pos_29F_outer	GTC ACG CTT AAA TCT TTG ATT GC	23	350 bp	55 oC *
	Pos_381R_outer	ACA GAC ACC TCA AGA CAC CA	20		
	Pos_171F_inner	AAC CTG TGG AAG AGT GGC CT	20	200 bp	52 oC *

	A	B	C	D	E
	Pos_381R_inner	ACA GAC ACC TCA AGA CAC CA	20		
	S_outer_1_f	TCA GTG CAG GGA CAG ATC CA	23	494 bp	69 oC *
	S_outer_1_r	TCC CTG AGA AGA GGG CTC AG	20		
	S_inner_1_f	TAC AAC CCC TGG AGA CCT CA	20	112 bp	68 oC *
	S_inner_1_r	TCA GGA GGT GTG TAC CTG GG	20		
	RNAse P-f	AGA TTT GGA CCT GCG AGC G	19		65.5 oC
	RNAse P-r	GAG CGG CTG TCT CCA CAA GT	20		67.4 oC
	RNAse P-p	HEX-TTC TGA CCT /ZEN/ GAA GGC TCT GCG CG	22		71.6 oC

f = forward primer, r = reverse primer, p = probe, bp = base pairs, Tm = melting temperature.

Step-by-step - cDNA synthesis

16 Per reaction, combine and mix thoroughly the following in a 0.2 mL PCR tube:

	A	B
	Component	Volume
	Random Hexamers (50 µM)	1 µL
	RNA	7 µL
	Total	8 µL

Place on the thermocycler and start the program – **65 ° C for 5 min** – when finished, place on ice. Add the following to each tube:

	A	B
	Component	Volume
	Protoscript II Reaction Mix (2X)	10 µL
	Protoscript II Enzyme Mix (10X)	2 µL
	Total	12 µL

Incubate on thermocycler as follows:

	A	B
	Temperature	Time
	25°C	5 minutes
	48°C	15 minutes
	80°C	5 minutes
	Hold	Hold

Step-by-step - RT-PCR protocol

17 Combine the following per reaction to create the amplification master mix:

	A	B
	Component	**Volume**
	Q5 Reaction Buffer 5x	5 µL
	dNTPs (10 mM)	0.5 µL
	Q5 DNA Polymerase	0.25 µL
	Forward primer (10 µM)	1.25 µL
	Reverse primer (10 µM)	1.25 µL
	cDNA	2.5 µL
	Nuclease-free water	25 µL
	Total	25 µL

Incubate on thermocycler as follows:

	A	B	C
	Temperature	**Time**	**Cycles**
	98 °C	1 minute	1
	98 °C	30 seconds	35
	Annealing at primer-specific Tm	30 seconds	35
	72 °C	1 minute	35
	72 °C	2 minutes	1
	4 °C	Hold	1

Nested-cPCR protocol

18 **Outer PCR**

Combine the following per reaction to create the amplification master mix:

	A	B
	Component	**Volume**
	Q5 Reaction Buffer 5x	5 µL
	dNTPs (10 mM)	0.5 µL
	Q5 DNA Polymerase	0.25 µL
	Forward primer (10 µM)	1 µL
	Reverse primer (10 µM)	1 µL
	cDNA	2.5 µL
	Nuclease-free water	25 µL
	Total	25 µL

Incubate on thermocycler as follows:

	A	B	C
	Temperature	Time	Cycles
	98 °C	1 minute	1
	98 °C	30 seconds	35
	Annealing at primer-specific Tm	30 seconds	35
	72 °C	1 minute	35
	72 °C	2 minutes	1
	4 °C	Hold	1

Inner PCR

Note

Use 2.5 µL of the outer PCR product as template and repeat the reaction using inner primers under the same cycling conditions.

Visualization

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Gel electrophoresis

Note

Load 5 µL of PCR product mixed with 15 µL nuclease-free water on a 2% E-Gel EX agarose gel. Run according to manufacturer's instructions until bands are clearly visible by transillumination.

Notes

20 Degenerate bases were incorporated in selected primers to accommodate sequence variability.

21 Shorter amplicons were prioritized for degraded RNA.

22 Nested PCR should be handled carefully to avoid cross-contamination.