



ADIAVET™ SCHMALLENBERG REAL TIME

TEST FOR THE DETECTION OF SCHMALLENBERG VIRUS BY REAL-TIME ENZYMATIC AMPLIFICATION (RT-PCR TEST)

References:

ADI491-50 (50 reactions)
ADI491-100 (100 reactions)



NOTE

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English version

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I.	REVISION HISTORY	3
II.	GENERAL INFORMATION.....	4
1.	Purpose of the test	4
2.	Pathogen.....	4
3.	Description and purpose of the test	4
III.	MATERIAL AND REAGENTS.....	5
1.	Reagents provided with the kit.....	5
2.	Validity and storage	5
3.	Use of SBV CTL+.....	5
4.	Equipment required but not supplied	5
IV.	RECOMMENDATION BEFORE THE ANALYSIS OF SAMPLES	6
1.	Precautions.....	6
2.	Storage of samples and RNA extracts	6
3.	Samples preparation	6
	A. <i>Tissue</i>	6
	B. <i>Blood</i>	6
	C. <i>Serum</i>	6
4.	Controls to include	7
V.	EXTRACTION AND PURIFICATION	8
1.	Using QIAamp® Viral RNA kit.....	8
2.	Using NucleoSpin® RNA Virus kit.....	9
3.	Using Nucleospin® 96 Virus.....	10
4.	Using DNA/RNA magnetic beads kit	11
VI.	AMPLIFICATION.....	12
VII.	INTERPRETATION OF RESULTS.....	13
1.	Definitions	13
2.	Validation and interpretation of results.....	13
	A. <i>Validation of the run</i>	13
	B. <i>Result interpretation</i>	14
VIII.	INDEX OF SYMBOLS	15

I. Revision history

N/A	Not Applicable (first publication)
Correction	Correction of document anomalies
Technical change	Addition, revision and/or removal of information related to the product
Administrative	Implementation of non-technical changes noticeable to the user

Note: minor typographical, grammar and formatting changes are not included in the revision history.

Release Date	Part Number	Change type	Change summary
2012/05	NE491-05	Technical change	Addition of "Extraction and Purification of RNA using Nucleospin® 96 Virus" paragraph, in page 10, § V-3.
2012/05	NE491-05	Technical change	Addition of "Extraction and Purification of RNA using DNA/RNA magnetic beads kit" paragraph, in page 11, § V-4.
2014/12	NE491-06	Technical change	Addition of "Index of symbols" section, in page 15.
2014/12	NE491-06	Technical change	Removal of reference ADI491-50 (50 reactions)
2016/07	NE491-07	Administratif	Changing logos
2016/07	NE491-07	Administratif	Add legal requirements Biosearch
2016/07	NE491-07	Administratif	Addition of table "Analysis options according to the specimen" §I.3
2016/07	NE491-07	Technical change	Addition of reference ADI491-50 (50 reactions)

II. General information

1. Purpose of the test

ADIAVET™ SCHMALLENBERG REAL TIME kit is intended to detect Schmallenberg Virus (SBV) using real-time Polymerase Chain Reaction (PCR) technology from tissue, brain, whole blood and serum specimens of bovine, ovine and caprine.

2. Pathogen

The Schmallenberg virus was isolated for the first time in Germany in 2011 by the FLI from blood of infected cows. The name is based on the geographic origin of the virus (village of the North Rhine-Westphalia). First phylogenetic analyses show that the viral genome presents the most similar sequences with Shamonda viruses within the Simbu serogroup. These suggest that the novel virus is a Shamonda-like virus within the genus Orthobornavirus.

Clinical signs of Schmallenberg virus infection in adult ruminants are mainly mild or non-existent but transient fever, loss of appetite, a reduction in milk yield and diarrhoea have been observed. The main clinical signs of Schmallenberg virus are congenital malformations (severe arthrogryposis, torticollis, brachygnathia, hydrocephalus and other severe brain malformations) in newborn animals similar to those observed in infections by the Akabane virus, the main known virus of the genus.

If infection occurs prior to pregnancy a normal pregnancy is expected to occur. Malformations in fetuses would be observed when the infection occurs during a vulnerable stage of the pregnancy. In analogy to Akabane virus, the vulnerable stage of pregnancy may be between days 28 and 36 in sheep and between days 75 and 110 in cattle.

The viruses of Simbu serogroup are transmitted by insects (Culicoides midges and mosquitoes). It is likely that Schmallenberg virus is also transmitted by these insects but this has not been confirmed yet. This way of transmission is reinforced by the clinical signs of Schmallenberg virus infection in adults that were observed from August onwards, coinciding with the density peak of the putative vectors. Considering a gestation period of 5 and 9 months for respectively sheep/goats and cows it could be expected that the majority of the deformed lambs/kids would be born from December to February and the majority of deformed calves between March and May.

Today, neither vaccine nor treatment exists against the viral infection and no serological test is available. Viral culture and PCR amplification are the only ways to detect the virus.

Current knowledge suggests that it is unlikely that Schmallenberg virus can cause disease in humans.

3. Description and purpose of the test

This test is based first on the reverse transcription (RT) of RNA into complementary DNA. Then, cDNA is amplified (PCR) by a DNA polymerase using specific primers. Both enzymatic reactions occur in the same tube (One-step RT-PCR).

Amplified products are detected in real-time thanks to a specific labelled hydrolysis probe (5'-exonuclease technology).

The ADIAVET™ SCHMALLENBERG REAL TIME kit enables the simultaneous detection of:

- Schmallenberg virus (probe labelled in FAM),
- GAPDH, an internal control of extraction and amplification steps specific from an endogenous RNA (probe labelled with a fluorochrome read in the same spectra as VIC and HEX).

ADIAGENE has validated the test using RNA purification kits (Qiagen, Macherey-Nagel). Other purification kits can be used if they have been validated by the user.

Analysis options according to the specimen:

Specimen	Individual analysis
Tissue (spleen, tonsil, ganglion...)	☒
Brain	☒
Whole blood	☒
Serum	☒

III. Material and reagents

1. Reagents provided with the kit

Designation	Reagent	ADI491-50	ADI491-100
A5	Amplification solution	1 x 1000 µl green tube	2 x 1000 µl green tubes
SBV CTL+	Positive control Schmallenberg Virus	1 purple tube	1 purple tube

2. Validity and storage

On receipt, the kit should be stored at <-15°C.

It is recommended to make fractions of A5 solution if it should be defrosted more than 3 times.

Do not defrost reagents more than 3 times.

Realtime reagents are susceptible to light: store them in the darkness.

The A5 reagent is ready to use for PCR reaction.

Do not mix reagents of two different batches.

3. Use of SBV CTL+

Add **200 µl** of Nuclease-free water to the SBV CTL+ tube included in the kit. Homogenize tube contents using a mixer such as vortex, at least 20 seconds. Aliquot this solution by 6 or 12 µl and store them to <-15°C.

For each analysis, we recommend to use 5 µl of SBV CTL+ in a well.

4. Equipment required but not supplied

Material should be Nuclease-free (e.g. autoclaved 25 minutes twice at +120°C or once 60 minutes at +121°C)

- Thermal cycler with consumables for real-time PCR: 0.2 ml PCR tubes or closed 96-wells PCR plates with optical quality.
- Class II Microbiological Safety Cabinet
- Centrifuge for microtubes
- Grinder (Mixer Mill or Fast Prep)
- Instrument for homogenous mixing of tubes
- 1 - 10 µl pipette, 20 - 200 µl pipette and 200 - 1000 µl pipette
- Nuclease-free filter tips
- Nuclease-free microtubes: 1.5 ml and 2 ml
- Tubes Lysing Matrix D (MP Biomedicals, 100 extractions: ref. 116913.100) only for grinder Fast Prep
- Powder-free latex gloves
- Metal beads 3 mm (e.g. Qiagen, 200 extractions: ref. 69997) only for grinder Mixer Mill
- Scalpel blades
- 96-100% ethanol solution
- Sterile distilled water
- PBS buffer 1X pH 7.4

- Manual RNA extraction kit (individual columns)

- QIAamp® Viral RNA (Qiagen, 50 extractions: ref. 52904 or 250 extractions: ref. 52906)
- NucleoSpin® RNA Virus (Macherey-Nagel, 50 extractions: ref. 740956.50 or 250 extractions: ref. 740956.250)

or

- 96-wells plates extraction

- Nucleospin® 96 Virus kit (Macherey-Nagel, 2x96 extractions: ref. 740691.2; 4x96 extractions: 740691.4)

or

- Automated DNA/RNA extraction kit (magnetic beads)

See the NEKF user manual available on the web site mentioned on the certificate of analysis included in the used ADIAVET™ kit

IV. Recommendation before the analysis of samples

1. Precautions

Adiogene has elaborated this PCR test with the use of Qiagen and Macherey-Nagel extraction kits. Other extraction kits can be used with a previous validation.

Follow the supplier's instructions for the storage, the preparation and the use of the extraction reagents.

We strongly recommend that only appropriately trained personnel perform this test. Ensure the accuracy and precision of the micropipettes used. The quality of the obtained results depends upon rigorous respect of good laboratory practices.

The PCR generates large amount of amplified DNA. A few molecules of amplified products are sufficient to generate a positive result. It is important to reserve 2 rooms, one for manipulation of samples to be tested, and another one for amplified products analysis. Do not open the PCR tubes after amplification.

Samples for analysis should be handled and disposed of as biological waste. **Take all measures of security and confinement required for the manipulation of the concerned biological agents.**

Before starting the test, read the entire protocol and scrupulously respect it.

2. Storage of samples and RNA extracts

Samples can be stored a couple of days at +2/8°C. After 2 days, we recommend to store them at <-15°C. Samples of blood with anticoagulant reagent must not be frozen.

Extracted RNAs are quite sensitive molecules. Extraction is made at room temperature and should be performed as fast as possible to avoid degradations. We then recommend to read the entire protocol before starting the test and to rigorously respect it. Crude extracts should be stored at the end of extraction on melting ice or at +2/8°C for few hours, then at <-15°C.

3. Samples preparation

A. Tissue

The analysis is carried out from 0.1 g of tissue (brain, spleen). The rest of the sample can be frozen for other analyses.

a) Using a grinder such as Mixer Mill

Place **0.1 g** of sample in a 2 ml microtube.

Add **1 metal bead** and **1 ml** of **buffer PBS 1X**.

Grind **2 minutes** at **30 Hz**.

Centrifuge 2 minutes at 6000 g.

The RNA extraction is performed from the supernatant (see § V).

b) Using a grinder such as Fast Prep or Ribolyser

Place **0.1 g** of sample in a Tube Lysing Matrix D.

Add **1 ml** of **buffer PBS 1X**.

Grind twice 20 seconds at 6m/sec with 5 minutes waiting on ice between both.

Centrifuge 3 minutes at 2000 g.

The RNA extraction is performed from the supernatant (see § V).

B. Blood

The RNA extraction is performed from the sample without previous preparation (see § V).

C. Serum

The RNA extraction is performed from the sample without previous preparation (see § V).

4. Controls to include

Several controls should be included per trial of analysis.

The internal endogenous control (GAPDH) naturally found in the samples allows verifying the extraction and amplification steps of each sample. The SBV CTL+ allows validating the amplification of the Schmallenberg virus target.

The mix of the different controls allows validating all the steps (extraction and amplification) of the analysis process for all the samples.

Other controls should or must be added:

- **Negative control of extraction (required)**

To verify the absence of cross-contamination, at least one negative control must be included per trial (e.g. AFNOR NF U47-600 guidelines suggest to include a negative control per 24 columns centrifuged). The control is a negative sample, for example a buffer used for dilution.

- **Positive control of extraction (recommended)**

A positive control could be added in each trial. The control is a sample including Schmallenberg virus.

It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of Schmallenberg virus. This positive control will be closed to the limit of detection of the method. It will inform about the fidelity of the obtained results between different trials.

V. Extraction and purification

1. Using QIAamp® Viral RNA kit

All the centrifugations are performed at room temperature.

	Tissues (brain/spleen)	Blood	Serum Viral culture
Lysis	Take 140 µl of sample , prepared as previously descibed.	Place 100 µl of sample in a microtube.	Place 140 µl of sample in a microtube.
	Add 560 µl of AVL buffer + RNA Carrier . Homogenize ~15 seconds and incubate 10 minutes at room temperature.		
Binding preparation	Add 560 µl of ethanol 100% . Homogenize by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).		
Transfer to columns and binding to the membrane	Identify columns, apply 630 µl of the obtained solution to the corresponding column and centrifuge 1 minute at 10 000 g. Put the rest of the mix on the column and centrifuge 1 minute at 10 000 g.		
1 st wash	Change the collection tube and add 500 µl of AW1 buffer to the column. Centrifuge 1 minute at 10 000 g.		
2 nd wash	Change the collection tube and add 500 µl of AW2 buffer to the column. Centrifuge 1 minute at 10 000 g.		
Column dry step	Change the collection tube. Centrifuge 3 minutes at 10 000 g.		
Elution	Transfer the column to a microtube. Add 60 µl of AVE buffer . Incubate ~1 minute at room temperature and centrifuge 1 minute at 10 000 g.		
Storage	Close the tubes, identify and store on ice if using immediately or at <-15°C.		

2. Using NucleoSpin® RNA Virus kit

All the centrifugations are performed at room temperature.

	Tissues (brain/spleen)	Blood	Serum Viral culture
Lysis	Take 140 µl of sample , prepared as previously descibed.	Place 100 µl of sample in a microtube.	Place 140 µl of sample in a microtube.
	Add 560 µl of RAV1 buffer + RNA Carrier . Homogenize ~15 seconds and incubate 10 minutes at room temperature.		
Binding preparation	Add 560 µl of ethanol 100% . Homogenize by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).		
Transfer to columns and binding to the membrane	Identify columns, apply 630 µl of the obtained solution to the corresponding column and centrifuge 1 minute at 10 000 g. Put the rest of the mix on the column and centrifuge 1 minute at 10 000 g.		
1 st wash	Change the collection tube and add 500 µl of RAW buffer to the column. Centrifuge 1 minute at 10 000 g.		
2 nd wash	Change the collection tube and add 500 µl of RAV3 buffer to the column. Centrifuge 1 minute at 10 000 g.		
Column dry step	Change the collection tube. Centrifuge 3 minutes at 10 000 g.		
Elution	Transfer the column to a microtube. Add 60 µl of Nuclease-free water . Incubate ~1 minute at room temperature and centrifuge 1 minute at 10 000 g.		
Storage	Close the tubes, identify and store on ice if using immediately or at <-15°C.		

3. Using Nucleospin® 96 Virus

Three MN Square well block plates are included in each kit. These plates are either mix plates or recovery plates. Once they have been used, they can be emptied, decontaminated with HCl 0.4 M during 1 minute, washed with distilled water and autoclaved.

All centrifugations are achieved at 5900 tr/min (5600 to 5800g) and at room temperature.

Before starting the extraction, place the RAV1+RNA carrier buffer and the Nuclease-free water at +70°C in a water bath or a heating block.

	Tissues (brain/spleen) – Blood – Serum - Viral culture
Lysis	Place 100 µl of sample (prepared as previously described) and in each well of a Round-well Block plate. For extraction negative controls, use 100 µl of PBS 1X .
	Add 400 µl of pre-warmed RAV1 buffer + RNA carrier + 20 µl of proteinase K in each well of the Round-well Block plate. Close the plate with an adhesive seal Self-adhering PE Foil. Mix gently during 15 seconds with a plate agitator. Incubate 10 minutes at +70°C.
Binding preparation	Add 400 µl of ethanol 100 % in an MN Square well Block plate. Carefully remove the adhesive seal of the Round-well Block plate and transfer the whole content of each well in the MN Square well Block plate containing ethanol. Homogenize the mix 5-fold (very important) with a multichannel pipette P1000.
Transfer to columns and binding to the membrane	Place a Nucleospin® Virus Binding plate (blue) on a new MN Square well Block plate. Place the whole mix with a multi pipette P1000 on the Nucleospin® Virus Binding plate. Place a new adhesive seal Self adhering PE Foil on the plate. Centrifuge 2 minutes. If the whole mix has not filtered, centrifuge one more time 3 minutes.
1st wash	Place the Nucleospin® Virus Binding plate on a new MN Square well Block plate. Remove the adhesive seal from the Nucleospin® Virus Binding plate. Add 500 µl of RAW buffer in each well. Place a new adhesive seal Self adhering PE foil on the plate. Centrifuge 2 minutes.
2nd wash	Remove the adhesive seal of the Nucleospin® Virus Binding Plate. Add 900 µl of RAV3 buffer in each well. Place a new adhesive seal Self adhering PE Foil on the plate. Centrifuge 5 minutes.
Column dry step	Place the Nucleospin® Virus Binding Plate on an empty and dry 96 well plate. Centrifuge 10 minutes.
Elution	Place the Nucleospin® Virus Binding Plate on the Rack plate with MN tube strips. Remove the adhesive seal from the plate. Add 100 µl of pre-warmed Nuclease-free water in each well of the Nucleospin® Virus Binding plate. <u>Do not use the RE buffer.</u> Centrifuge 2 minutes.
Storage	Discard the Nucleospin® Virus Binding plate. Close the Rack plate with MN tube strips with Caps for strips. Store it on melting ice if analysis is immediately achieved, then at <-15°C.

4. Using DNA/RNA magnetic beads kit

See the NEKF user manual available on the web site mentioned on the certificate of analysis included in the used ADIAVET™ kit.

VI. Amplification

a- Determine the number samples analysed including the control (e.g. positive and negative extraction control, positive control of amplification (SBV CTL+) and PCR reagent control (NTC)).

b- Defrost the A5 solution at room temperature. Homogenize. Dispense **20 µl** of A5 solution in each PCR tubes or PCR plate wells with a micropipette with a Nuclease-free tip.

c- **Immediately replace the A5 solution tube at <-15°C and in darkness.**

d- For each sample, the extraction negative control (obligatory) and the extraction positive control (recommended) add **5 µl** of purified extract to the 20 µl of A5 solution.

For the positive control, add **5 µl** of the solution obtained in § II-3 to the 20 µl of A5 solution.

For the PCR reagent control (NTC), nothing is added to the A5 solution.

Immediately replace purified RNA extracts on melting ice or at <-15°C. Take care to have no bubbles in the bottom of the wells.

e- Store the plate or the tubes on melting ice or at +2/8°C until the cycler is programmed and start quickly the run after you have placed the plate or the tubes in the cycler.

The Schmallenberg virus target is read in FAM. The Internal Control is read in VIC or HEX. The Quencher is non fluorescent. The solution contains a passive reference ROX for the ABI machines. Fluorescence is read during the elongation step (1 minute at 60°C).

The following program is defined for **ABI Prism** thermalcyclers (like 7500, StepOne...) from **Applied Biosystems** (check the "emulation 9600" option if available), for the **Rotorgene** from **Qiagen** and for the **Chromo 4** from **Biorad**:

10 minutes 45°C

10 minutes 95°C

15 seconds at 95°C and 1 minute at 60°C during 45 cycles

This program is concerning the **MX3000P** and **MX3005P** of **Stratagene**:

10 minutes 45°C

10 minutes 95°C

30 seconds at 95°C and 1 minute at 60°C during 45 cycles

Roche diagnostic: LightCycler 2*, LightCycler 480*

*** NOTE:** *The use of LightCycler thermalcyclers requires a calibration manipulation. AdiaGene will furnish process chart and reagents required for this calibration.*

Contact us if you wish to use other thermalcyclers.

VII. Interpretation of results

1. Definitions

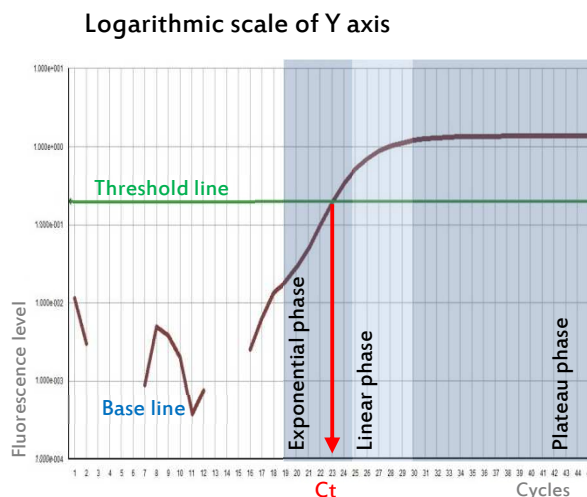
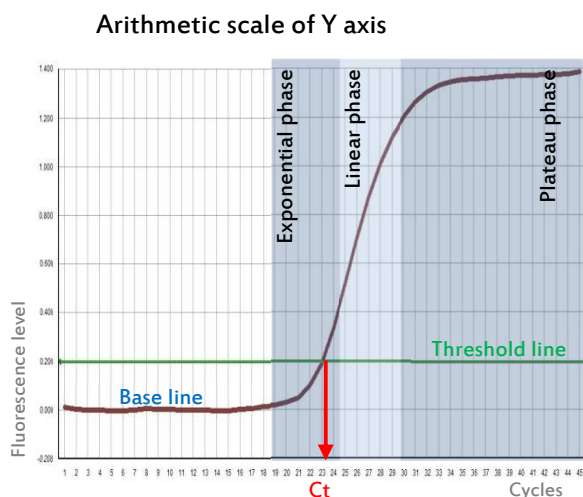
The « **base line** » corresponds to the background of fluorescence and qualifies the non-characteristic part of the curve observed during the first cycles.

The « **Characteristic amplification curve** » qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

The « **threshold line** » has to be placed over the background in the exponential phase of a characteristic amplification curve or a group of characteristic amplification curves.

The « **threshold cycle** » (**Ct**) of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present in the PCR well.

Example of characteristic amplification curve



2. Validation and interpretation of results

Display the FAM curves from the plate and set the threshold value as indicated above.
Proceed in the same mean for the VIC or HEX curves.

A. Validation of the run

Amplification is considered to be **valid** if the following results are obtained for the controls:

Controls	Reagent control (NTC)	SBV CTL+	Extraction negative control	Extraction positive control *
FAM amplification	no	yes	no	yes
VIC/HEX amplification	no	no/yes	no	no/yes
Validation of	Absence of contamination for amplification	Amplification of the target	Absence of contamination for extraction	Extraction and amplification steps

* Optional

The indicative Ct values (FAM and VIC/HEX dyes) of the SBV CTL+ were indicated in the certificate of analysis of the kit.

B. Result interpretation

RNA extraction and amplification for each sample are considered to be **valid** if at least a characteristic amplification curve is observed for target (FAM) or for the internal control (VIC or HEX).

Example	A	B	C	D
FAM amplification	no	yes	yes	no
VIC/HEX amplification	yes	no	yes	no
Result	negative	positive	positive	undetermined

The sample is considered as **negative** if a characteristic amplification curve is observed in VIC or HEX without any amplification in FAM (A example).

The sample is considered as **positive** if a characteristic amplification curve is observed in FAM (B example). Internal control can be co-amplified (C example).

A total absence of characteristic amplification curve for a sample (example D) shows a defective RNA extraction (lost or destruction of RNA) or a deficient real-time PCR (inhibitors in the sample, program error or no template added). In this case, we recommend first to repeat the test with pure and tenfold diluted RNA in sterile nuclease-free water. Then, if the test is still not valid, a new extraction is recommended.

VIII. Index of symbols

Symbol	Meaning
	Catalogue number
	Manufacturer
	Upper temperature limit
	Use by date
	Batch code
	Consult Instructions for Use
	Contains sufficient for <n> tests
	Keep away from sunlight
	For veterinary in vitro use only – For animal use only

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