Amplisure® Adeno Virus PCR Kit

(Real Time Quantitative PCR Kit)

IVD

Product Insert

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Introduction

Adenoviruses are medium-sized (60-90 nm), non-enveloped icosahedral viruses containing double-stranded DNA. Adenoviruses represent the largest non enveloped viruses; because they are the maximum size able to be transported through the endosome. Adenoviruses have been implicated in a wide range of clinical diseases affectingly mainly the respiratory, ocular and the gastrointestinal systems of humans. Infections are common in children and can occur sporadically or in outbreaks. Approximately 5% of acute respiratory diseases in children and 10% of febrile illnesses and childhood pneumonias have been associated with adenovirus infection. Adenovirus serotypes 40 and 41 are commonly associated with viral gastroenteritis in infants and reported to be responsible for 4~15% of nosocomial infections in pediatric wards. This real time based assay is designed to quantitate the viral load of adenovirus for the diagnosis of early disseminated adenovirus infections as well as for the management of patient therapy.

Product Description

Amplisure® Adeno Virus PCR Kit is an *in-vitro* diagnostic kit for Quantitation of Adeno Virus in human plasma, throat swab or Urine. The kit contains the necessary reagents for performing quantitation by Real time PCR.

Pathogen detection by Real Time polymerase chain reaction (PCR) is based on the amplification of specific region (Hexon Protein) of the pathogen genome. The assay principle is based on Taqman probes which allow higher specificity and sensitivity.

In addition, the Amplisure® Adeno Virus PCR Kit contains a second amplification system to identify possible PCR inhibition by using an internal control (IC) without affecting the analytical sensitivity of the assay.

Recommended Work areas

Molecular Diagnostics work area includes:

a) Sample preparation area/room – for extraction of nucleic acids from clinical samples
b) Pre-PCR area/room - for setting up PCR reaction
c) PCR area/room – for performing PCR using the thermocyclers

As part of Good Laboratory Practices (GLP), it is recommended to have dedicated areas to avoid cross contamination.

General Precautions

Precautions while extracting Nucleic acid

Always wear proper attire (powder free gloves, facemask and Head cap) before starting the nucleic acid extraction procedure. During preparation of samples, compliance with good laboratory practices are essential to minimize the risk of cross-contamination between samples, and the inadvertent introduction of deoxyribonucleases (DNases) into samples during and after the extraction procedure. Proper aseptic technique should always be used when working with DNA.

The Sample Preparation Area is dedicated to processing samples. All reagents used in the Sample Preparation Area should remain in this dedicated area at all times. Laboratory coats, pipettes, pipette tips and vortex mixer used in the Sample Preparation Area must remain in this area and not be moved to the Pre-PCR/PCR area. Discard the gloves before leaving this area. Do not bring amplified product into the Sample Preparation Area. Usage of filter tips is recommended while sample preparation and should be performed in a Biosafety cabinet.

Precautions while setting up a PCR reaction

PCR assay is sensitive and any accidental introduction of product from previous amplification reactions leads to incorrect results. Hence measures to reduce the risk of contamination in the laboratory should include physically separating the activities involved in performing PCR and complying with good laboratory practices.
It is recommended to have proper cleaning procedures to minimize the risk of cross contamination and carry over contamination (e.g. DNA OUT™, RNase OUT™, 0.1% Sodium Hypochlorite, Fumigation etc.).
It is recommended that areas should be defined in Pre-PCR room for preparation of mastermix and addition of templates. Laboratory coats and equipment used in the Pre-PCR Area must remain in this area and should not be moved to the Sample Preparation Area.

Precautions for post PCR or equipment area/room

The Real time PCR instrument/s should be kept in a separate segregated area away from Sample preparation area and Pre-PCR area.

Precautions after completion of Real time PCR assay

The reaction tubes or strips should be properly discarded without opening the caps, after the completion of run to avoid carry over contamination.

Usage Limitations

1. The kit and all its components are for *in-vitro* diagnostics only.
2. The product is to be used by personnel specially trained in the *in-vitro* diagnostics procedures only.
3. Follow the product insert strictly for optimal PCR results.
4. Do not use the kit beyond the expiry date mentioned on the kit box.
5. Follow the guidelines provided in product insert for sample collection, storage and transport.
6. For ideal performance, store the kit under recommended conditions only.

Safety Precautions

1. All patient specimens should be considered as potentially infectious and handled in a BSL2 biosafety hood with BSL3 practices.
2. Wear personal protective equipment, including gloves, head cap, face mask and lab coats when handling kit reagents/sample extraction. Wash hands thoroughly using detergents before and after performing the test.
3. Do not smoke, drink or eat in areas where kit reagents and/or human specimens are being used.
4. Dispose of unused kit reagents and human specimens as per regulatory guidelines.

Storage Conditions and Product Stability

1. All the kit reagents should be stored at -20°C. Replace all the kit components immediately at -20°C after usage.
2. Repeated thawing and freezing (more than 6 x) of all kit reagents should be avoided, as it reduces assay sensitivity. If needed, make aliquots of the kit reagents according to the volume used in the protocol prior to freezing.
3. Allow reagents to be thawed completely on Ice/4°C prior to use.
4. Kit reagents are stable through the end of the expiration date indicated on the box when stored at -20°C.

Symbols

Description of Symbol	Denotation
	*in-vitro* Diagnostic medical device
	Consult Instruction manual (Product Insert) for use
	Lot Number of the kit or Kit contents
	Catalogue number of Kit
 <N>	Contains sufficient for <N> reactions (Pack Size)
	Manufacturer
	Temperature limitation (Storage Condition)
	Use by MMM-YYYY (Expiry Date)
	Biological risk (handle carefully)
	Important Note

Kit Components

Color Coding (Caps)	Contents	Description	50 rxns (QT-ADV-50)	100 rxns (QT-ADV-100) 2 x 50 rxns
Yellow	RAS qDNA PCR Mix	DNA Amplification	1 x 625 µL	2 x 625 µL
Brown	RAS ADV PPM	ADV Primer-Probe Mix	1 x 100 µL	2 x 100 µL
Lavender	RAS ADQS1 (2×10^5 copies/ µL)	ADV Quantitation Standards	1 x 60 µL	2x 60 µL
Lavender	RAS ADQS2 (2×10^4 copies / µL)		1 x 60 µL	2x 60 µL
Lavender	RAS ADQS3 (2×10^3 copies / µL)		1 x 60 µL	2x 60 µL
Lavender	RAS ADQS4 (2×10^2 copies / µL)		1 x 60 µL	2x 60 µL
Natural	RAS IC-A PCR Mix	Internal Control	1 x 50 µL	2 x 50 µL
Natural	RAS D-IC-A EX Mix		1 x 1.00 mL	2 x 1.00 mL
White	MBGW	Molecular Biology Grade water	1 x 1.00 mL	2 x 1.00 mL

Materials required but not supplied

The materials which are required but not supplied are listed below:

1. Viral DNA Extraction kit
2. Biosafety Cabinet
3. PCR Hood
4. Calibrated variable micropipettes
5. Sterile pipette filter tips (aerosol free)
6. Vortex mixer
7. Dry Bath

8. Benchtop centrifuge with rotor for 1.5 mL reaction tubes
9. Real Time PCR machine
10. Strip Tubes and Caps (0.2 mL) or PCR Tubes (0.2 mL) or 96 well plate
11. Cooling block (96 x 0.2 mL tubes)
12. 1.5 mL centrifuge tubes
13. 1.5 mL centrifuge tube stand
14. Cooling block (2 mL tubes)
15. Sterile powder free gloves
16. Facemask
17. Head cap
18. Lab coats

Quality Systems

In accordance with ISO-certified Quality Management System (9001:2008 and 13485: 2003) of RAS Lifesciences, each lot of Amplisure® Adeno Virus PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Sample Type/Collection/Storage/Transport

Sample Type

Plasma (K_2EDTA-Blood), throat swab and Urine
Heparinized Blood must not be used as they inhibit the PCR reaction

Sample Collection, Storage and Transport

3-5 mL of blood has to be drawn into a K_2EDTA vaccutainer. Cap and swirl the tubes for uniform mixing of blood and K_2EDTA. When K_2EDTA is used, whole blood can be collected in tubes with or without a gel separator. The K_2EDTA blood samples are centrifuged (20 minutes at 1000–1500 x *g)* to separate plasma from cellular material and in the case of a non gel separator tube, the plasma should be removed to a secondary sterile tube within 4 hours of phlebotomy.

Collection and storage of unstabilized whole blood is not recommended for PCR analysis, because DNA degradation occurs in blood stored *ex vivo*. The sensitivity of the assay can be reduced if whole blood samples are frozen or stored for a longer period of time. Urine and throat swab should be collected in a sterile container.

Plasma separated in a gel separator tube may be transported to the laboratory *in situ.* Plasma should be shipped at 2 to 8 °C and stored at -20°C as it is stable for up to five days at 2 to 8 °C and longer if frozen at -20°C or -70°C or lower. Do not store plasma samples in a "frost -free" freezer as the temperature is cycled several times per day on this type of freezer, causing degradation of nucleic acid targets. Urine and throat swab samples should be shipped at 2 to 8°C, stored at 4 °C and should be processed within 24 hours of collection.

Sample material should be transported in a leak proof, unbreakable transport container to avoid leakage of sample. The samples should be transported following the local and national instructions for the transport of pathogen material.

Assay Procedure

DNA Extraction

Amplisure® Adeno Virus PCR Kit has been validated using the following Viral DNA extraction kits:

1- Roche High Pure Viral Nucleic Acid kit (Cat. No. 11858874001)
2- QIAamp DNA Blood Mini Kit (Cat. No. 51104)

Follow the manufacturer's instructions mentioned in the manual for Viral DNA extraction. Different pack sizes of the above mentioned kits can be used. However the customer can also validate their own extraction process using other Viral DNA extraction Kits.

Recommended sample volume for extraction and elution are as follows:

S. No.	Name of the DNA Isolation Kit	Recommended Sample volume *(to be taken for DNA Extraction)*	Recommended Final Elution volume
1.	Roche High Pure Viral Nucleic Acid kit (Cat. No. 11858874001)	200 µL	100 µL
2.	QIAamp DNA Blood Mini Kit (Cat. No. 51104)	200 µL	100 µL

The analytical sensitivity of the assay in consideration of the purification was determined using the above defined volumes.

Use of Internal Control (IC)

Internal controls are supplied (RAS D-IC-A Ex Mix and RAS IC-A PCR Mix) along with Amplisure® Adeno Virus PCR Kit. This allows the user to control the Viral DNA isolation procedure as well as to check for possible PCR inhibition.

ⓘ Internal control should only be used once, either at the Extraction step or at the PCR step

Usage of Internal Control at the DNA extraction step

If internal control (IC-A) is required to be added at the time of DNA extraction, add 20 µL of RAS *D-IC-A Ex mix* per isolation to the lysis buffer along with other components of kit used for lysis (as per kit instructions) and vortex for 5 seconds prior to usage.

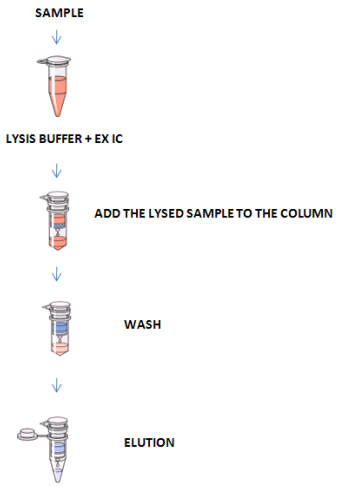

Fig1 Viral DNA Extraction Overview

Usage of Internal Control at the Real time PCR step

The internal control can optionally be used exclusively to check for possible PCR inhibition. For this application, add the internal control directly to the PCR master mix as described on Pg. No 14. Addition of Internal control should be done only once, either at the time of sample extraction or during PCR setup.

qPCR Protocol

Preparation of Reaction Mastermix

Quantitation procedure with Amplisure® Adeno Virus PCR Kit involves *one step qPCR*. It is recommended that a minimum of three standards and a negative control (MBGW should be used as negative control) are required to be included in a single run for acquiring proper results.

Set up a real time one step PCR reaction as follows:

1- qPCR reaction mix composition without Internal Control (When D-IC-A Ex Mix is added during DNA extraction)

Components	Volume per reaction (µL) (for final vol. of 25 µL)
RAS qDNA PCR Mix	12.5
RAS ADV PPM	2.0
DNA/ADQS/ MBGW	5.0
MBGW	5.5

2- qPCR reaction mix composition with Internal Control (When D-IC-A Ex Mix is not added during DNA extraction)

Components	Volume per reaction (µL) (for final vol. of 25 µL)
RAS qDNA PCR Mix	12.5
RAS ADV PPM	2.0
DNA/ADQS/ MBGW	5.0
RAS IC-A PCR Mix	1.0
MBGW	4.5

1. **Addition of IC-A PCR mix (if required) should be done at the time of preparation of master mix.**
2. **The results may be inconsistent, if the IC-A PCR mix is added individually.**

Place the PCR plate/tubes/strips in real time thermocycler.

PCR Programming

The Amplisure® Adeno Virus PCR Kit is validated on the following instruments:

- Rotor-Gene™ 6000
- Rotor-Gene™ Q 5plex
- ABI 7500 DX Real-Time PCR System
- ABI 7300 Real-Time PCR System
- Eppendorf Realplex 4
- Bio-Rad™ CFX 96

Plate Setup

1. Program the plate setup by labeling the slots as per tube/strip/plate labels. The sequence of labeling of slots should be the same way as the tube/strip/plate is kept in the machine.
2. Select the type of sample (Unknown/Standard/NTC) for each slot.
3. When Standard is selected as sample type, mention the quantitation value in copies/µL (2E4, 2E3 etc)
4. Select the channel for acquisition (FAM/Yakima Yellow)

S. No.	Name of channel	Source wavelength (nm)	Detection wavelength (nm)
1.	FAM (Pathogen target)	470	510
2.	Yakima Yellow (Internal Control)	530	555

5. For background calibration in different instruments, follow the procedure described below:

   Rotor-Gene™ 6000 - Perform 'Gain optimization'
   Rotor-Gene™ Q 5plex - Perform 'Gain optimization'
   ABI 7500 DX Real-Time PCR System - Select Passive Reference dye 'ROX'
   ABI 7300 Real-Time PCR System - Select Passive Reference dye 'ROX'
   Eppendorf Realplex 4 -Select 'ROX' for background calibration
   Bio-Rad™ CFX 96 -Select 'ROX' for background calibration

Preparation of reaction mastermix and cycling conditions are same for all the instruments listed in the product insert. For instrument specific protocols, please contact our technical support team at amplisure@raslifesciences.com

Cycling conditions

1. Configure the following program in the machine.

Steps	No. of cycles	Temperature (°C)	Time
1 (Initial denaturation)	1	95	10 min.
2 (PCR cycling)	40	95	15 sec.
		60*	60 sec
* **Plate Read/Data Acquisition in FAM and Yakima Yellow channel**			

2. Set the reaction volume as 25 µL.
3. Plate read/Data Acquisition for FAM and Yakima Yellow channel should be incorporated in the second stage of step 2 (60◦C/60 sec).
4. The ideal run time for the assay is 90 minutes. Note: *In case of Eppendorf Realplex 4, select RAMP rate as 35%.*

Preparation of reaction mastermix and cycling conditions are same for all the instruments listed in the product insert. For instrument specific protocols, please contact our technical support team at amplisure@raslifesciences.com

Data Analysis

Analyze the data after completion of the run. Check the R_n/*Cycle* amplification plot and ΔR_n/*Cycle* amplification plot to observe the amplification signal generated by different samples in the run. Compare both the plots for data analysis. Also look for noisy signals, if observed as it might not give you a proper result.

Setting the threshold for the qPCR Data analysis

The threshold should be set either automatically (by the machine itself)/ or manually just above the background signal of the negative controls and negative samples by referring to R_n/*Cycle* amplification plot. The mean threshold value calculated from these experiments will most likely work for the majority of future runs, but the user should nevertheless review the generated threshold value at regular intervals.

Result

The values for unknown samples would appear in the result column in *copies/µL* in FAM Channel. Samples showing no amplification in FAM channel should show amplification in Yakima Yellow channel, and then only results should be considered. The negative control should not show any value in the result column.

Interpretation

Interpret the values for unknown samples, only if the Slope of Standards is between -3.1 to – 3.6 and PCR efficiency is between 90%-110% (0.9 - 1.1) and there should be no amplification in negative control (FAM channel).

Observation	Interpretation	Conclusion
Amplification signal detected in ADV channel (FAM) and in Internal control channel (Yakima Yellow)	ADV DNA within quantitation range	Proceed for further Analysis
Amplification signal detected in ADV channel (FAM) but no signal in Internal Control channel (Yakima Yellow)	ADV DNA within quantitation range	
Amplification signal not detected in ADV channel (FAM) but detected in Internal control channel (Yakima Yellow)	ADV DNA below quantitation limit	
No Amplification signal detected in ADV channel (FAM) as well as Internal control channel (Yakima Yellow) in unknown samples	Possible inhibition of PCR	Dilute the DNA sample (1:100) and repeat the assay*

Troubleshoot

Observation	Possible cause	Solution
No amplification signal for Standards in FAM channel	1. One of the components may not have been added. 2. Incorrect channel selection 3. Incorrect programming of the real time machine. 4. Instrument is not working properly	1. Repeat the assay by following the correct protocol and addition of reagents 2&3. Please recheck the PCR program 4. Contact manufacturer of thermocycler for technical support.
Weak amplification signal for standards (Signal below threshold) in FAM Channel	1. Improper PCR programming. 2. Inaccurate dispensing of reagents 3. Possible deterioration of kit components due to improper storage	1. Repeat the assay by following the correct protocol 2. Minimize Pipetting errors/Check for calibration status of pipettes
Identical/Similar Ct values observed in FAM channel	1. Possible contamination of Kit reagents / Standards/Work area.	1. Use fresh aliquots of Standards/Kit Reagents (if available) 2. Clean the PCR rack/Pipettes thoroughly as per GLP 3. Clean and Fumigate the work area overnight prior to use

For any other technical query; please contact amplisure@raslifesciences.com

Assay Characteristics

Analytical Sensitivity of qPCR assay

To determine the analytical sensitivity of the Amplisure® Adeno Virus PCR Kit, a standard dilution series was set up from 1 copies/µL to 50 copies/ µL and analyzed with Amplisure® Adeno Virus PCR Kit.

Testing was carried out on 10 different days on 3 replicates. The results were analyzed by statistical analysis.

The analytical sensitivity of the Amplisure® Adeno Virus PCR Kit is 2 copies/µL and 50 copies/mL with Plasma, Throat swab and Urine samples.

Linear Range

The linear range (analytical measurement) of the Amplisure® Adeno Virus PCR Kit was determined by analyzing a dilution series of Adeno quantitation standards from 2 x 10^7 copies /µL to 2 copies /µL.

Each dilution was tested in replicates (n = 3) using the Amplisure® Adeno Virus PCR kit. The linear range of the Amplisure® Adeno Virus PCR Kit has been determined to cover concentrations from **4 copies /µL to 2 x 10^7 copies/µL.**

To convert into copies/mL use the following formula:

$$\text{Result (copies/mL)} = \frac{\text{Calculated Value (copies/µL) x Elution Volume (µL)}}{\text{Sample Volume (mL)}}$$

Specificity

The specificity of the Amplisure® Adeno Virus PCR Kit is ensured by the selection of the primers and probes, as well as the selection of stringent reaction conditions. The primers and probes were checked for possible homologies to all published sequences (Genbank) by BLAST analysis.

Cross Reactivity Data

A potential cross-reactivity of the Amplisure® Adeno Virus PCR Kit was tested using the control group listed below. None of the tested pathogens has been reactive. No cross-reactivity appeared with mixed infections.

Moreover, the specificity was validated with 50 different healthy plasma specimens/other various sample types. These did not generate any signals with the ADV specific primers and probes, which are included in the Amplisure® Adeno Virus PCR Kit.

Pathogen Tested	Cross Reactivity with the ADV Primers/Probes
Human DNA(from various sample types)	-ve
BK virus	-ve
Herpes Simplex Virus-1	-ve
Herpes Simplex Virus-2	-ve
Epstein Barr virus	-ve
Human Immunodeficiency Virus	-ve
Cytomegalo virus	-ve
Hepatitis C virus	-ve
Enterovirus	-ve
Hepatitis B Virus	-ve

Precision

The precision data of the Amplisure® Adeno Virus PCR Kit have been generated for ADV positive clinical specimens and Adeno quantitation standards.

Intra-assay variability

The precision data consists of the *Intra-assay variability* which is variability of multiple results of samples of the same concentration within one experiment.

The data obtained were used to determine the standard deviation and the coefficient of variation for the pathogen specific PCR. Precision data of the Amplisure® Adeno Virus PCR Kit have been collected using all the quantitation standards (ADQS1- ADQS4). Also included were three different samples with different viral loads. Testing was performed with 3 replicates for 10 days.

Inter-assay variability

The precision data consists of the *Inter-assay variability* which is variability of multiple results of samples of the same concentration within different experiments.

The data obtained were used to determine the standard deviation and the coefficient of variation for the pathogen specific PCR. Precision data of the Amplisure® Adeno Virus PCR Kit have been collected using all the quantitation standards (ADQS1- ADQS4). Also included were three different samples with different viral loads. Testing was performed with 3 replicates for 10 days.

The precision data was calculated on basis of the values obtained in copies/µL.

Sample Type	Mean [Log Value (copies/µL)]	Variability Testing	Standard Deviation	Coefficient of Variation (%)
ADQS1	Log 5.3	Intra-assay variability	0.070	0.004
		Inter-assay variability	0.064	0.004
ADQS2	Log 4.3	Intra-assay variability	0.120	0.007
		Inter-assay variability	0.030	0.001
ADQS3	Log 3.3	Intra-assay variability	0.010	0.001
		Inter-assay variability	0.205	0.007
ADQS4	Log 2.3	Intra-assay variability	0.195	0.008
		Inter-assay variability	0.050	0.002
Adeno Control 1	Log 3.5	Intra-assay variability	0.123	0.006
		Inter-assay variability	0.170	0.008
Adeno Control 2	Log 2.5	Intra-assay variability	0.335	0.013
		Inter-assay variability	0.405	0.014
Adeno Control 3	Log 1.5	Intra-assay variability	0.430	0.020
		Inter-assay variability	0.520	0.021

Abbreviations

Abbreviation	*Expansion*
ADV	Adeno Virus
DNA	Deoxy Ribonucleic Acid
IC	Internal Control
DNases	Deoxyribonucleases
PCR	Polymerase Chain Reaction
BSL2	Bio Safety Level 2
BSL3	Bio Safety Level 3
mL	Milliliters
µL	Microliters
K_2EDTA	Potassium Ethylene Diamine Tetra Acetate
g	Relative Centrifugal Force
qPCR Protocol	Quantitative PCR protocol
MBGW	Molecular Biology Grade Water
RT PCR	Real Time PCR
NTC	No Template Control
FAM	Carboxyfluorescein
ROX	Carboxy-X-rhodamine
IU	International Units
BLAST	Basic Local Alignment Search Tool
Rxn	Reaction

References

1. Jothikumar N, Cromeans TL, Hill VR, Lu X, Sobsey MD, Erdman DD. Quantitative real-time PCR assays for detection of human adenoviruses and identification of serotypes 40 and 41. Appl Environ Microbiol 2005; 6: 3131-36.

2. Wadell G. Adenoviruses in Principles and Practice of Clinical Virology (Zuckerman, A.J. et al. editors). John Wiley and Sons, 1990 pp. 267-287.

Ordering Information

QT-ADV-50 : 50 rxns
QT-ADV-100 : 100 rxns