EU FP VII PROJECT "VITAL"

STANDARD OPERATING PROCEDURE

SOP VITAL 017

Detection of human adenovirus by nested PCR

CREATED:	REVISED:	APPROVED:
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— WARNING —

All samples and controls shall be handled by trained staff in a laboratory with appropriate equipment. Staff must be fully vaccinated against Hepatitis A and poliovirus. Persons using this SOP must be familiar with normal virology laboratory practice. This SOP does not presume to address fully all of the safety issues associated with its use. It is the responsibility of the user to establish appropriate health and safety practices and to ensure compliance with any national regulatory conditions.

AIM

To detect human adenovirus from food and environmental samples using a nested PCR.

PRINCIPLE

This protocol is based in the Standard Operating Procedure "Adenovirus Nested PCR" of the FP 6 Project VIROBATHE Contract 513648 (www.virobathe.org). The nested PCR is based on the method of Allard et al[1]. It incorporates an Internal Amplification Control (IAC). It also incorporates a carryover contamination prevention system which utilises uracil N-glycosylase (UNG) in the first round PCR and dUTP (replacing dTTP) in both PCRs. The reaction incorporates a hot-start polymerase (Platinum® Taq DNA polymerase, Life Technologies Inc.). The target amplicon sizes are 301 bp in the first round and 171 bp in the second round.

The IAC is plasmid-based (pGem® T-Easy vector using T/A cloning) and contains the primer sequences used in each round, flanking sequences from *Salmonella enterica*. The IAC amplicon sizes are 384 bp in the first round and 337 bp in the second round.

[1] Allard A, Albinsson B, Wadell G. (2001) Rapid typing of human adenoviruses by a general PCR combined with restriction endonuclease analysis. Journal of Clinical Microbiology 39: 498-505.

EQUIPMENT

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles.

- Laminar flow cabinet
- Pipettes
- Filter tips
- 1.5 ml microcentrifuge tubes
- Thin-walled PCR tubes (0.2 ml or 0.5 ml)
- PCR Thermocycler Machine
- Cold block for holding enzymes (eg. StrataCooler®)
- Electrophoresis gel tank and combs (eg. Bio-Rad)
- Power pack (eg. Bio-Rad)
- UV Transilluminator (eg. Ultra-Violet Products Ltd)
- Gel documentation system (eg. Bio-Rad) or Polaroid® Camera (eg. Polaroid® GelCam® Camera System with Electrophoresis Filter Kit)

REAGENTS

- Molecular biology grade water
- Platinum® Taq DNA polymerase (5U/µl) with Platinum® Taq Buffer (10 ×; in 50 µl aliquots)
- Magnesium Chloride (25 mM; in 50 µl aliquots)
- dNTP mix (2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 5.0 mM dUTP; in 50 µl aliquots)
- HK™ UNG (1U/µl; Epicentre®, Madison, Wisconsin)
- IAC
- Agarose (any supplier will be suitable)
- 100 bp ladder (eg. Fermentas)
- Loading Dye (6 x loading dye eg. Fermentas)
- 1 × Tris Borate EDTA (TBE) Buffer (eg. Sigma, supplied as 10 x concentrate)
- Ethidium Bromide / GelRed™ (recommended - supplier Insight Biotechnology Ltd.)

PRIMERS

1st round

Forward Primer: Hex1deg 5'-GCC SCA RTG GKC WTA CAT GCA CAT C-3'
Reverse Primer: Hex2deg 5'-CAG CAC SCC ICG RAT GTC AAA-3'

2nd Round

Forward Primer: nehex3deg 5'-GCC CGY GCM ACI GAI ACS TAC TTC-3'
Reverse Primer: nehex4deg 5'-CCY ACR GCC AGI GTR WAI CGM RCY TTG TA-3'

CONTROLS

A positive reaction control consisting of 10 µl of purified virus suspension or purified adenovirus DNA (20 ng μl^{-1}), and a negative reaction control (consisting of 10 µl ultrapure H_2O) should be included with each series of tests.

Internal amplification control (IAC)

The IAC must be added to every well, to verify that the reaction has worked and has not failed, e.g. through inhibition or instrument failure or operator error. Please see SOP no. 023 for details on the preparation of the IAC.

PROCEDURE

1st Round PCR

1. Set up the reaction in a PCR clean room cabinet. Wear a PCR clean coat and use a new pair of gloves.
2. Defrost the buffer, primers hex1deg and hex2deg, magnesium chloride, nucleotide mix and IAC.
3. Label sufficient thin-walled PCR tubes (0.2 ml or 0.5 ml depending on thermocycler) and place in a suitable rack.
4. Prepare the adenovirus first round PCR mix as outlined in Table 1 in a 1.5ml microcentrifuge tube. Use filter tips. Remove the Platinum® Taq polymerase from the freezer when needed, keeping it in the cold block.

Table 1: Mastermix for the Adenovirus 1st round PCR

Reagent	Working concentration	Final concentration	Volume in 1 reaction (µl)
Ultrapure H_2O			19.8
Platinum® Taq Buffer	10 ×	1 ×	5.0
$MgCl_2$	25 mM	1.5 mM	3.0
dNTP mix:	2.5mM dATP, dCTP, dGT and 5.0mM dUTP	250 µM dATP, dCTP, dGTP and 500 µM dUTP	5.0
Primer Hex1deg	50 µM	0.5 µM	0.5
Primer Hex2deg	50 µM	0.5 µM	0.5
IAC	*	*	5.0
Platinum® Taq	5 U μl^{-1}	1 U	0.2
UNG	1 U μl^{-1}	1 U	1.0

*see SOP 023 for details on IAC preparation

5. Aliquot 40 µl of the PCR mix to each tube.
6. Add 10 µl of the sample to the appropriate tube. Use a fresh filter tip for each sample and mix by pipetting the liquid up and down.
7. Load the samples in the PCR machine.
8. Program the PCR machine for the **1st round** thermocycling program:

	50 °C 10 minutes	UNG incubation
	95 °C 10 minutes	UNG inactivation and initial denaturation
followed by **45 cycles** of	94 °C 30 s	denaturation
	55 °C 30 s	annealing
	72 °C 1 min	extension
followed by **1 cycle** of	72 °C 5 min	final extension
	and a hold at 20 °C	

9. Now perform the second round PCR.

2nd Round PCR

1. Set up the reaction in the PCR clean room cabinet. Wear a PCR clean coat and use a new pair of gloves.
2. Defrost the buffer, primers nehex3deg and nehex4deg, magnesium chloride and nucleotide mix
3. Label sufficient thin-walled tubes (0.2 ml or 0.5 ml depending on thermocycler) and place in a suitable rack.
4. Prepare the adenovirus second round PCR mix as outlined in Table 2 in a 1.5ml eppendorf. Use filter tips. Remove the Platinum® Taq from the freezer when needed, keeping it in the cold block.

Table 2: Mastermix for the Adenovirus 2nd round PCR

Reagent	Working concentration	Final concentration	Volume in 1 reaction (µl)
Ultrapure H_2O			36.8
Platinum® Taq Buffer	10 ×	1 X	5.0
$MgCl_2$	25 mM	1.5 mM	3.0
dNTP mix:	2.5mM dATP, dCTP, dGT and 5.0mM dUTP	100 µM dATP, dCTP, dGTP and 200 µM dUTP	2.0
Primer nehex3deg	50 µM	0.5 µM	0.5
Primer nehex4deg	50 µM	0.5 µM	0.5
Platinum® Taq	5U μl^{-1}	1U	0.2

5. Aliquot 48µl of the PCR mix to each tube.
6. Add 2µl of the first round mix to the appropriate second round tube. Use a fresh filter tip for each sample. Mix each sample, by gently pipetting up and down.
7. Load the second round samples into the PCR machine. Program the PCR machine for the **2nd round** thermocycling program:

	94 °C 3 minutes	initial denaturation
followed by **45 cycles** of	94 °C 30 s	denaturation
	55 °C 30 s	annealing
	72 °C 1 min	extension
this is then followed by **1 cycle** of	72 °C 5 min	final extension
	and a hold at 20 °C	

Gel Electrophoresis

1. Prepare a 2 % gel by dissolving agarose in 1x TBE buffer (volume is variable depending on size of gel tank used). Completely dissolve by boiling eg. In a microwave oven.
2. Add the ethidium bromide* (0.5 µg ml^{-1}) or GelRed™* (0.1µl ml^{-1}) to the molten gel (please note, when using GelRed™ it is not necessary to cool the gel to 50ºC as with ethidium bromide). ***Note:** Discard all used gels, tips etc which have been contaminated with either ethidium bromide or GelRed™ in a suitable container i.e. one which is sealable, and dispose of according to local H&S rules.
3. Allow gel to set completely then remove comb(s) etc.
4. Add 3 µl loading dye to 10 µl reaction, then add the mixture to the wells.
5. Run at 70 V for 1 hour (variable)
6. If no IAC signal is present after the 2nd round PCR, the reaction has failed and must be repeated. If after repeating there is still no signal from the IAC, this may indicate the presence of inhibitors.

Figure 1: *Figure 1* shows a gel containing the **first round** target and IAC

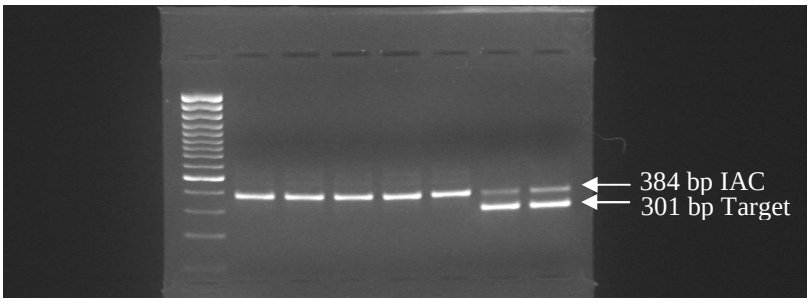

Figure 2: *Figure 2* shows a gel containing the **second round** target and IAC