Construction and Analytical Application of Internal Amplification Controls (IAC) for Detection of Food Supply Chain-Relevant Viruses by Real-Time PCR-Based Assays

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Abstract Internal amplification controls (IACs) were constructed for incorporation into real-time nucleic acid amplification assays for bovine polyomavirus, hepatitis A virus, hepatitis E virus, human adenovirus, human norovirus genogroup I, human norovirus genogroup II, murine norovirus and porcine adenovirus. The addition of optimised amounts of IAC into the assays did not affect the limits of detection for each specific target virus. A poorly performed extraction of viral nucleic acids was simulated, and the effectiveness of IACs in identifying failed assays was demonstrated. The IACs constructed in this study can be reliably used in their specific assays to provide a robust control that can be routinely applied in the analysis of foods for viruses.

Keywords Internal amplification control · False negatives · Real-time PCR · Food · Enteric virus

Introduction

Gastroenteritis produced by ingestion of food contaminated with enteric viruses is an important concern for public health. Thus, detection of the presence of enteric viruses—particularly norovirus, hepatitis A and E and adenovirus—in foods is an important issue in food safety, and a rapid

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and robust diagnostic methodology is needed (Croci et al. 2008; Greening and Hewitt 2008; Cook and Rzezutka 2006). Because at present most of these viruses cannot, or with difficulty, be cultured and integrated cell culture real-time PCR methods are useful but too time-consuming for the quick results required by the food industry, a detection approach based on nucleic acid amplification is necessary (Rodríguez-Lázaro et al. 2007; Bosch et al. 2011). However, the application of nucleic acid amplification to foodstuffs can be complicated by the presence of inhibitory substances (Rodríguez-Lázaro et al. 2004, 2006; Rodríguez-Lázaro and Hernandez 2006), which can cause false negative interpretations of the results. It is imperative therefore for the effective implementation of nucleic acid amplification in food analysis that appropriate controls are used to verify there has been no interference caused by the presence of inhibitory substances (Rodríguez-Lázaro et al. 2007; Bosch et al. 2011). The incorporation of an internal amplification control (IAC) will identify failed reactions (Bosch et al. 2011; Rodríguez-Lázaro et al. 2004, 2007). An IAC is a non-target nucleic acid sequence present in every reaction which can be co-amplified simultaneously with the target sequence (Cone et al. 1992). In a reaction without an IAC, a negative response can mean either that there is no target sequence present in the reaction or that the amplification has been inhibited. However, with the use of an IAC in each reaction, the absence of response both from the target and the IAC indicates that the reaction has failed, and the sample must be retested to avoid any false negative interpretation of its analysis.

The aim of this study was to construct IACs for nucleic acid amplification assays for viruses relevant to the analysis of foods and to define their analytical application. The viruses were human norovirus genogroups I (NoVGI) and II (NoVGII), hepatitis A virus (HAV) and hepatitis E virus (HEV), murine norovirus (MNV; which could be used to

assess the efficiency of a pre-nucleic acid amplification sample treatment), human adenovirus (HAdV; which could be used to indicate that routes of virus contamination exist from human sources), porcine adenovirus (PAdV; which could be used to indicate that routes of virus contamination exist from porcine sources) and bovine polyomavirus (BPyV; which could be used to indicate that routes of virus contamination exist from bovine sources).

Materials and Methods

Viruses and Viral Nucleic Acids

Bovine polyomavirus DNA and porcine adenovirus DNA were kindly provided by Professor Rosina Gironés of the University of Barcelona, Spain. Hepatitis A virus suspension was kindly provided by Dr. Dario de Medici of the Istituto Superiore de Sanità, Rome, Italy. Hepatitis E virus RNA was kindly provided by Dr. Malcolm Banks of the Veterinary Laboratories Agency, Weybridge, UK. Murine norovirus (MNV1) was supplied by Herbert W. Virgin IV, Washington University School of Medicine (US) and human adenovirus type 2 (HAdV2) was provided by Dr. Rosina Girones at the University of Barcelona (Spain), both viruses were replicated in the Dr. Franco Ruggeri's laboratory at the Istituto Superiore de Sanità (Rome, Italy). MNV1 was propagated in RAW264.7 cells and titrated by end-point dilution (final stock concentration, 10^7 $TCID_{50}$/ml). HAdV-2 was propagated in A549 cells and titrated by the same technique (final stock concentration, 10^7 $TCID_{50}$/ml). Human norovirus genogroup I RNA and human norovirus genogroup II RNA were kindly provided by Dr. Ana Maria de Roda Husman of RIVM, Bilthoven, The Netherlands.

Primers and Probes

The oligonucleotides used in this study are shown in Tables 1 and 2. IAC primers were designed using the software Primer Express™ version 2.0 (Applied Biosystems, Foster City, CA, USA). All oligonucleotides were purchased from MWG Biotech AG (Ebersberg, Germany), except the minor groove binder (MGB) TaqMan probes HAV150(−), MGB-ORF1/ORF2 and PrfAP that were acquired from Applied Biosystems (Warrington, UK) and NV1LCpr that was from Sigma-Aldrich (St. Louis, MO, USA).

Bovine Polyomavirus Real-Time PCR

This assay was a duplex real-time PCR using the primers and conditions described by Hundesa et al. (2010), with the inclusion of an IAC and a carryover contamination prevention system utilising uracil N-glycosylase (UNG).

The reaction contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.4 μM each primer, 0.120 μM bovine polyomavirus TaqMan probe (labelled with FAM), 50 nM IAC probe (labelled with VIC) and varying copies of bovine polyomavirus IAC. A 10-μl sample of nucleic acid extract was added to make a final reaction volume of 25 μl. The thermocycling conditions were 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C.

Hepatitis A Virus Reverse Transcription Real-Time PCR

This assay was a one-step duplex reverse transcription real-time PCR using the primers and conditions described by Costafreda et al. (2006), with the inclusion of an IAC. The reaction contained 1× RNA Ultrasense reaction mix (Invitrogen, Carlsbad, CA, USA), 0.5 μM primer HAV68, 0.9 μM primer HAV240, 0.25 μM probe HAV150(−) (labelled with FAM), 50 nM IAC probe (labelled with VIC), 1× ROX reference dye (Invitrogen), 1 μl RNA Ultrasense enzyme mix (Invitrogen) and varying copies of HAV IAC. A 10-μl sample of nucleic acid extract was added to make a final reaction volume of 20 μl. The thermocycling conditions were 15 min at 50 °C, 2 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C.

Hepatitis E Virus Reverse Transcription Real-Time PCR

This assay was a one-step duplex reverse transcription real-time PCR using the primers and conditions described by Jothikumar et al. (2006), with the inclusion of an IAC. The reaction contained 1× RNA Ultrasense reaction mix (Invitrogen), 0.25 μM each primer, 0.1 μM probe HEV-P (labelled with FAM), 50 nM IAC probe (labelled with VIC), 1× ROX reference dye (Invitrogen), 1 μl RNA Ultrasense enzyme mix (Invitrogen) and varying copies of HEV IAC. A 10-μl sample of nucleic acid extract was added to make a final reaction volume of 20 μl. The thermocycling conditions were 15 min at 50 °C, 2 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 20 s at 55 °C and 15 s at 72 °C.

Human Adenovirus Real-Time PCR

This assay was a duplex real-time PCR using the primers and conditions described by Hernroth et al. (2002), with the inclusion of an IAC and a carryover contamination prevention system utilising UNG. The reaction contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.9 μM each primer, 0.225 μM adenovirus TaqMan probe (labelled with FAM), 50 nM IAC probe (labelled with VIC) and varying copies of adenovirus IAC. A 10-μl sample of nucleic acid extract was added to

Table 1 Hybrid oligonucleotides designed in this study for the construction of the IACs and probe used for IAC detection

Target	Name	Type	Sequence (5′–3′)	IAC size (bp)
BPyV	QB-F1-1IAC	Forward primer	CTAGATCCTACCCTCAAGGGAAT**GGCTCTATTTGCGGTC**	115
	QB-R1-1IAC	Reverse primer	TTACTTGGATCTGGACACCAAC**TCTTGATGCCATCAGGA**	
HAV	HAVIACF	Forward primer	TCACCGCCGTTTGCCTAG**GGCTCTATTTGCGGTC**	107
	HAVIACR	Reverse primer	GGAGAGCCCTGGAAGAAAG**TCTTGATGCCATCAGGA**	
HEV	HEVIACF	Forward primer	GGTGGTTTCTGGGGTGAC**GGCTCTATTTGCGGTC**	106
	HEVIACR	Reverse primer	AGGGGTTGGTTGGATGAAT**CTTGATGCCATCAGGA**	
HAdV	IACAdF	Forward primer	CWTACATGCACATCKCSGG**GGCTCTATTTGCGGTCAACTT**	107
	IACAdR	Reverse primer	CRCGGGCRAAYTGCACCAG**TCTTGATGCCAT**	
NoVGI	NOR1IACF	Forward primer	CGCTGGATGCGNTTCCAT**GGCTCTATTTGCGGTC**	111
	NOR1IACR	Reverse primer	CCTTAGACGCCATCATCATTTAC**TCTTGATGCCATCAGGA**	
NoVGII	NOR2IACF	Forward primer	ATGTTCAGRTGGATGAGRTTCTCWGA**GGCTCTATTTGCGGTC**	117
	NOR2IACR	Reverse primer	TCGACGCCATCTTCATTCACA**TCTTGATGCCATCAGGA**	
MNV1	IACMuNvF	Forward primer	CACGCCACCGATCTG**TTCTGGGCTCTATTTGCGGT**	107
	IACMuNvR	Reverse primer	GCGCTGCGCCATCACTC**TCTTGATGCCATCAG**	
PAdV	IACPAdF	Forward primer	AACGGCCGCTACTGCAAG**GGCTCTATTTGCGGTC**	105
	IACPAdR	Reverse primer	AGCAGCAGGCTCTTGAGG**TCTTGATGCCATCAGGAG**	
L. monocytogenes	PrfAP	Probe	VIC-CCATACACATAGGTCAGG-MGBNFQ	

The sequences shown in bold are identical in each forward or reverse primer and correspond to a fragment of the *L. monocytogenes prfA* gene. Sequences underlined are identical to the specific primers for each target virus. Bases within the degenerated primers correspond to W=A or T; Y=C or T; K=G or T; R=A or G; S=C or G and N=A, T, C or G

make a final reaction volume of 25 μl. The thermocycling conditions were 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C.

Human Norovirus ggI Reverse Transcription Real-Time PCR

This assay was a one-step duplex reverse transcription real-time PCR using the primers and conditions described by Svraka et al. (2007), with the inclusion of an IAC. The reaction contained 1× RNA Ultrasense reaction mix (Invitrogen), 0.5 μM primer QNIF4, 0.9 μM primer NV1LCR, 0.25 μM probe NV1LCpr (labelled with FAM), 50 nM IAC probe (labelled with VIC), 1× ROX reference dye (Invitrogen), 1 μl RNA Ultrasense enzyme mix (Invitrogen) and varying copies of norovirus ggI IAC. A 10-μl sample of nucleic acid extract was added to make a final reaction volume of 20 μl. The thermocycling conditions were 15 min at 50 °C, 2 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C.

Human Norovirus ggII Reverse Transcription Real-Time PCR

This assay was a one-step duplex reverse transcription real-time PCR using the primers and conditions described by da Silva et al. (2007), with the inclusion of an IAC. The reaction contained 1× RNA Ultrasense reaction mix (Invitrogen), 0.5 μM primer QNIF2, 0.9 μM primer

COG2R, 0.25 μM probe QNIFS (labelled with FAM), 50 nM IAC probe (labelled with VIC), 1× ROX reference dye (Invitrogen), 1 μl RNA Ultrasense enzyme mix (Invitrogen) and varying copies of norovirus ggII IAC. A 10-μl sample of nucleic acid extract was added to make a final reaction volume of 20 μl. The thermocycling conditions were 15 min at 50 °C, 2 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C.

Murine Norovirus Reverse Transcription Real-Time PCR

This assay was a one-step duplex reverse transcription real-time PCR using the primers and conditions described by Baert et al. (2008), with the inclusion of an IAC. The reaction contained 1× RNA Ultrasense reaction mix (Invitrogen), 0.2 μM each primer, 0.2 μM probe MGB-ORF1/ORF2 (labelled with FAM), 50 nM IAC probe (labelled with VIC), 1× ROX reference dye (Invitrogen), 1 μl RNA Ultrasense enzyme mix (Invitrogen) and varying copies of murine norovirus IAC. A 10-μl sample of nucleic acid extract was added to make a final reaction volume of 20 μl. The thermocycling conditions were 15 min at 50 °C, 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Porcine Adenovirus Real-Time PCR

This assay was a duplex real-time PCR using the primers and conditions described by Hundesa et al. (2009), with the inclusion of an IAC and a carryover contamination

Table 2 Oligonucleotides used in this study to detect target viruses by real-time or RT real-time-PCR

Target	Name	Type	Sequence (5′–3′)	Amplicon size (bp)	Reference
BPyV	QB-F1-1	Forward primer	CTAGATCCTACCCTCAAGGGAAT	77	Hundesa et al. (2010)
	QB-R1-1	Reverse primer	TTACTTGGATCTGGACACCAAC		
	QB-P1-2	Probe	6FAM-GACAAAGATGGTGTGTATCCTGTTGA -BHQ		
HAV	HAV68	Forward primer	TCACCGCCGTTTGCCTAG	173	Costafreda et al. (2006)
	HAV240	Reverse primer	GGAGAGCCCTGGAAGAAAG		
	HAV150(−)	Probe	6FAM-CCTGAACCTGCAGGAATTAA-MGBNFQ		
HEV	JVHEVF	Forward primer	GGTGGTTTCTGGGGTGAC	70	Jothikumar et al. (2006)
	JVHEVR	Reverse primer	AGGGGTTGGTTGGATGAA		
	JVHEVP	Probe	6FAM-TGATTCTCAGCCCTTCGC-BHQ		
HAdV	AdF	Forward primer	CWTACATGCACATCKCSGG	69	Hernroth et al. (2002)
	AdR	Reverse primer	CRCGGGCRAAYTGCACCAG		
	AdP1	Probe	6FAM-CCGGGCTCAGGTACTCCGAGGCGTCCT-BHQ		
NoVGI	QNIF4	Forward primer	CGCTGGATGCGNTTCCAT	86	Svraka et al. (2007)
	NV1LCR	Reverse primer	CCTTAGACGCCATCATCATTTAC		
	NV1LCpr	Probe	6FAM-TGGACAGGAGAYCGCRATCT-BHQ		
NoVGII	QNIF2d	Forward primer	ATGTTCAGRTGGATGAGRTTCTCWGA	89	da Silva et al. (2007)
	COG2R	Reverse primer	TCGACGCCATCTTCATTCACA		
	QNIFS	Probe	6FAM-AGCACGTGGGAGGGCGATCG-BHQ		
MNV1	Fw-ORF1/ORF2	Forward primer	CACGCCACCGATCTGTTCTG	109	Baert et al. (2008)
	Rv-ORF1/ORF2	Reverse primer	GCGCTGCGCCATCACTC		
	MGB-ORF1/ORF2	Probe	6FAM-CGCTTTGGAACAATG-MGB-NFQ		
PAdV	Q-PAdV-F	Forward primer	AACGGCCGCTACTGCAAG	68	Hundesa et al. (2009)
	Q-PAdV-R	Reverse primer	AGCAGCAGGCTCTTGAGG		
	Q-PAdV-P	Probe	6FAM-CACATCCAGGTGCCGC-BHQ		

Probes were labelled with 6FAM at the 5′ end and MGB-NFQ or BHQ at the 3′ end

6FAM 6-carboxyfluorescein, *MGB-NFQ* minor groove binder non-fluorescent quencher, *BHQ* black hole quencher

prevention system utilising UNG. The reaction contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.9 μM each primer, 0.225 μM porcine adenovirus TaqMan probe (labelled with FAM), 50 nM IAC probe (labelled with VIC) and varying copies of porcine adenovirus IAC. A 10-μl sample of nucleic acid extract was added to make a final reaction volume of 25 μl. The thermocycling conditions were 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 20 s at 55 °C and 20 s at 60 °C.

IAC Construction

The principles for the construction of IACs can be explained in two different phases: First, PCR amplification of non-target DNA is performed using hybrid oligonucleotide primers. This produces a chimeric DNA molecule containing non-target sequences flanked by target sequences complementary to the virus-specific primers. This molecule is then cloned into a plasmid. If the IAC is for RNA virus detection, the plasmid should contain a T7 RNA polymerase promoter, and IAC RNA transcripts are subsequently produced by T7 RNA polymerase. The plasmid or the RNA transcript is the chimeric IAC which is co-amplified with the virus primers and detected using a fluorescent probe complementary to the internal non-target sequence (Fig. 1). When using a real-time PCR-based assay, the virus target amplicons are detected with specific hydrolysis probes, labelled with one fluorophore (e.g. FAM), and the IAC amplicons are detected with the specific IAC probe, labelled with a different fluorophore (e.g. VIC).

Each IAC in this study was designed as a DNA or RNA molecule containing sequences from the *prfA* gene from *Listeria monocytogenes* (nucleotide positions 2281–2348, AN AY512499) flanked by the sequences complementary to the primers used in the specific assays. With the exception of the *prfA* sequence, the IAC sequences did not show significant similarity to any other sequence deposited in public databases, as shown by BLAST-N searches (National Center for Biotechnology Information, Bethesda, MD, USA; www.ncbi.nlm.nih.gov). The chimeric DNA molecules were generated by PCR as previously described (Rodríguez-Lázaro et al. 2004, 2005) using as template 5 ng of *L. monocytogenes* strain CECT 935 DNA and the

specific set of construction hybrid primers for each IAC (Table 1), which contained the corresponding *prfA* target sequence plus a 5′ tail with the virus forward/reverse primer sequences. The PCR products were excised from a 2% 1× TBE agarose gel and purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), then cloned into the pCR 2.1-TOPO Vector (Invitrogen) in the case of IACs for the HAV, HEV and NoVGII assays or into the pGEM-T Easy Vector (Promega, Madison, WI, USA) in the case of IACs for the NoVGI, BPyV, HAdV, PAdV and MNV1 assays. The concentration and quality of the plasmid DNA stock solutions were determined by fluorimetry using Quant-iT PicoGreen dsDNA reagents (Invitrogen) in a NanoDrop 3300 Fluorospectrometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Production of IAC RNA

In vitro transcription was performed to obtain RNA fragments for the HAV, HEV, NoVGI, NoVGII and MNV1 IACs using the Riboprobe System—T7 (Promega). To prevent carryover contamination by DNA, RNA was treated with DNase (RQ1 RNase-free DNase, Promega) at a concentration of 1 U/μg for 15 min at 37 °C, then purified using an RNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations. The concentration and purity of the RNA stock solutions were determined by fluorimetry using Quant-iT RiboGreen RNA and Quant-iT PicoGreen dsDNA reagents (Invitrogen) in a NanoDrop 3300 Fluorospectrometer (Thermo Fisher Scientific).

Copy Number Calculation

The number of IAC copies was calculated by dividing the amount of IAC in each stock solution by the weight of one IAC molecule, as follows:

$$\text{DNA IAC copies} = \text{g in the IAC stock } /$$
$$\left(\text{bp} \times 660 \text{ DA/bp} \times 1.6 \times 10^{-27} \text{ kg/DA} \right.$$
$$\left. \times 1 \times 10^{-3} \text{ g/kg}\right)$$
$$\text{RNA IAC copies} = \text{g in the IAC stock } /$$
$$\left(\text{bp} \times 320 \text{ DA/bp} \times 1.6 \times 10^{-27} \text{ kg/DA} \right.$$
$$\left. \times 1 \times 10^{-3} \text{ g/kg}\right)$$

Optimization of IAC-Containing Nucleic Acid Amplification Assays

After construction and copy number calculation of the IACs, the following steps were performed, in this particular order:

1. Verification that the IAC could be amplified and detected in a uniplex assay

2. Verification that the IAC and the template could be simultaneously amplified and detected in the same reaction tube, i.e. a duplex assay

3. Optimization of the IAC probe concentration by performing (reverse transcription) real-time PCRs without virus nucleic acids but containing 3,000 IAC copies (or 2,000 IAC copies for RNA virus methods), 100 nM of virus target probe and increasing amounts of the IAC probe (25, 50 and 100 nM)

4. Determination of the optimal amount of IAC. First, each assay's target consistent limit of detection (LOD) was determined in the absence of an IAC by establishing the lowest number of genome equivalents (GE) that could be detected in every one of five replicate reactions. Then, decreasing numbers of IACs (down to approximately one copy) were added, and the lowest IAC number which could be consistently detected in five replicate reactions without affecting the LOD of the target was established

Demonstration of IAC Applicability in the Detection of Viruses in Food

To demonstrate the effectiveness of the IAC approach, a foodstuff artificially contaminated with two virus types was analysed. Strawberry puree (25 g) was placed into a sterile plastic bag. Approximately 10^6 TCID$_{50}$ of human adenovirus and 10^6 TCID$_{50}$ of murine norovirus were added to the puree. The sample was then processed using the method of Dubois et al. (2006). Approximately 25 g fruit was placed in a sterile beaker. Forty millilitres of Tris-glycine, pH 9.5, buffer containing 1% beef extract and 6,500 U pectinase (Pectinex™ Ultra SPL Solution, Sigma) was added to the sample, which was then agitated at room temperature for 20 min by rocking at 60 rpm. The pH was maintained at 9.0 throughout (if necessary adjusting using 4% (*w/v*) sodium hydroxide, extending the period of agitation by 10 min each time an adjustment was made. In strongly coloured berries, a change in colour of the eluate from blue/purple to red was considered indicative of acidification and was used to trigger pH adjustment). The liquid was decanted from the beaker through a strainer (e.g. a tea strainer) into one 50-ml or two smaller centrifuge tubes and centrifuged at 10,000×*g* for 30 min at 4 °C. The supernatant(s) was decanted into a single clean tube or bottle and the pH adjusted to 7.2. Volumes (0.25) of 50% (*w/v*) polyethylene glycol 8000/1.5 M NaCl were then added and mixed by shaking for 1 min. The suspension was then incubated with gentle rocking at 4 °C for 60 min before centrifugation at 10,000×*g* for 30 min at 4 °C. The supernatant was discarded and the

Fig. 1 IAC construction
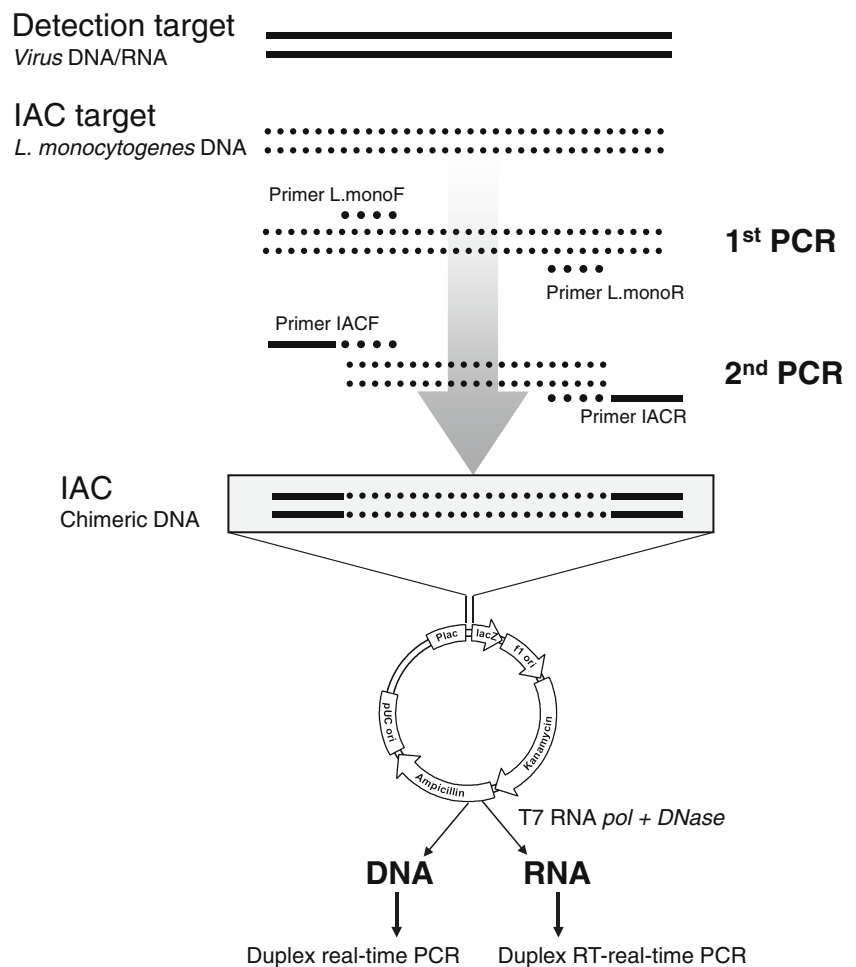
pellet compacted by centrifugation at $10,000 \times g$ for 5 min at 4 °C before resuspension in 500 μl phosphate-buffered saline. The suspension was then transferred to a chloroform-resistant tube, and 500 μl chloroform/butanol (1:1) was added and mixed by vortexing. The sample was allowed to stand for 5 min and then centrifuged at $10,000 \times g$ for 15 min at 4 °C. The aqueous phase was transferred to a clean tube and immediately used for nucleic acid extraction or stored at −20 °C. Nucleic acids were extracted using a NucliSENS miniMAG kit (bio-Mérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. The final elutions were performed with 150 μl elution buffer, resulting in a 300-μl nucleic acid extract. The nucleic acid extract was assayed immediately or stored at −70 °C.

To demonstrate how the IACs would indicate reaction failure, a situation in which nucleic acid purification had been poorly performed was modelled by adding 50 μl of non-extracted strawberry puree to 50 μl nucleic acid extract prior to nucleic acid amplification.

Results

Optimization of the IAC Probe Concentration

For each virus detection method, several experiments were performed as detailed in "Materials and Methods" in duplex (IAC and target probes) and uniplex (IAC probe) formats, and the best performance concentration chosen was that which showed the lowest C_p value with less difference between the duplex and uniplex formats and having the most similar value within the five replicates (Table 3). The results indicated that a probe concentration of 50 nM in all assays exhibits enough fluorescence intensity and that the assay performance in duplex and uniplex was satisfactory.

Determination of the Optimal Amount of IAC for Each Assay

After optimization of IAC amount per reaction as detailed in "Materials and Methods", the target LOD for each virus

was recalculated; norovirus GGI had 100 GE per reaction, and the LOD for all other target viruses was 10 GE (Table 4). These LODs were established in the amount of virus GE when the target signal was present in all five replicates, and if just one target signal was not present in all replicates, the results were not considered robust enough. It is remarkable that the LOD of the HEV system was established at 10 GE when C_p values were as high as 41.50 ± 1.80 whilst at 100 GE were 33.56 ± 0.61 (data not shown), indicating consistency of the results.

Demonstration of IAC Applicability in the Detection of Viruses in Food

The results from the analysis of the purified and inhibitor-containing nucleic acid extracts from the artificially contaminated strawberry puree are shown in Table 5. A signal was obtained for both target viruses and their IACs from the assay of the purified extract. No target virus or IAC signal was obtained from the assay of the inhibitor-containing extract.

Discussion

If monitoring of food supply chains for viruses is to be effectively performed as part of a food safety programme (Rodríguez-Lázaro et al. 2007), then it is vitally necessary that the reliability of the analytical results can be verified. Many matrices from the food supply chains which are most prone to virus contamination—salad vegetable, shellfish and soft fruit—contain substances which can inhibit nucleic acid amplification; therefore, it is essential that this verification includes the recognition of failed assays as these may mask the presence of a virus pathogen by a false negative interpretation of the results (Hoorfar et al. 2004). The use of an amplification control can provide this recognition.

There are two approaches to the use of amplification controls. The first is to run two separate reactions for each sample: One (the test) reaction contains only the sample nucleic acid, but the other (the control reaction) contains the sample nucleic acid plus the amplification control (Costafreda et al. 2006). The latter is thus termed an external amplification control (EAC). If it is successfully amplified to produce a signal, any non-production of a target signal in the test reaction is considered to signify that the sample was uncontaminated. If, however, no signal is produced in both the test and control reactions, it signifies that the nucleic acid extract contains inhibitory substances and the reaction has failed.

In contrast to an EAC, an IAC is a non-target DNA sequence present in the very same reaction as the sample nucleic acid extract (Hoorfar et al. 2004). If it is successfully amplified to produce a signal, any non-

Table 3 Optimization of the IAC probe (PrfAP) by testing three different concentrations (25, 50 and 100 nM) in uniplex and duplex formats for each of the target viruses: BPyV, HAV, HEV, HAdV, NoVGI and NoVGII, MNV1, and PAdV

PrfAP (nM)		BPyV			HAV			HEV			HAdV			NoVGI			NoVGII			MNV1			PAdV		
		25	50	100	25	50	100	25	50	100	25	50	100	25	50	100	25	50	100	25	50	100	25	50	100
Duplex	C_p	26.35	26.50	26.74	23.82	25.00	27.54	24.71	24.12	23.92	41.06	28.65	27.16	27.01	24.41	22.86	28.43	23.31	21.16	31.27	29.05	28.19	27.55	26.95	26.09
	SD	0.14	0.09	0.07	0.14	0.05	0.05	0.07	0.06	0.11	3.11	0.67	0.16	0.07	0.08	0.14	0.70	0.16	0.22	0.15	0.24	0.13	0.19	0.14	0.33
Uniplex	C_p	26.48	26.59	26.79	24.16	25.41	27.94	24.19	23.67	23.48	36.49	30.11	27.46	24.85	24.12	22.92	24.89	23.88	21.65	30.80	28.77	28.00	27.67	27.07	26.43
	SD	0.08	0.04	0.02	0.24	0.09	0.14	0.06	0.38	0.40	1.21	0.18	0.32	0.14	0.08	0.16	0.07	0.29	0.43	0.24	0.16	0.14	0.08	0.07	0.14

BPyV bovine polyomavirus, *HAV* hepatitis A virus, *HEV* hepatitis E virus, *HAdV* human adenovirus, *NoVGI* human norovirus group I, *NoVGII* human norovirus group II, *MNV1* murine norovirus, *PAdV* porcine adenovirus

Table 4 Limits of detection of BPyV, HAV, HEV, HAdV, NoVGI and NoVGII, MNV1 and PAdV assays and optimal number of IAC copies for each assay

	BPyV	HAV	HEV	HAdV	NoVGI	NoVGII	MNV1	PAdV
LOD	10	10	10	10	100	10	10	10
$C_p \pm SD$	36.37±0.59	26.28±0.61	41.50±1.80	34.62±0.60	29.78±0.21	27.54±0.09	38.74±0.56	36.54±0.56
IAC	300	300	300	100	300	300	600	100
$C_p \pm SD$	28.79±0.06	30.29±0.31	35.48±0.36	33.69±0.25	28.38±0.10	32.65±0.03	36.07±0.44	34.66±0.10

BPyV bovine polyomavirus, *HAV* hepatitis A virus, *HEV* hepatitis E virus, *HAdV* human adenovirus, *NoVGI* human norovirus group I, *NoVGII* human norovirus group II, *MNV1* murine norovirus, *PAdV* porcine adenovirus, *LOD* limit of detection of the specific target virus

production of a target signal in the reaction is considered to signify that the sample was uncontaminated. If, however, the reaction produces neither a signal from the target nor the IAC, it signifies that the reaction has failed.

Optimally, since using different primer sets may cause the amplification control to react to an inhibitory substance differently to the target, it should possess the same primer sequences as the target: This is the "competitive" strategy (Hoorfar et al. 2004). It is so called because the amplification control can compete with the target for the primers. This potential competition issue has led some workers to adopt the EAC approach, but this does contain a degree of ambiguity because one can never be completely certain that the test reaction has not individually failed, for example, through pipetting error or non-homogeneous contamination by inhibitory substances. For example, if an EAC signal is produced in the control reaction but no target signal is produced in the test reaction, one cannot be completely certain that the test reaction has not failed. Using an IAC eliminates this ambiguity since it is present in the mastermix and a signal will always appear when the reaction has not failed or high levels of competing target are not present (if they are, a target signal will be produced anyway).

The concern of the proponents of the EAC approach regards the possibility that a low level of target may be outcompeted by the IAC, leading to a false negative result.

However, a thoroughly optimised assay should not present these problems (D'Agostino et al. 2004; Rodríguez-Lázaro et al. 2010). In the current study, the amount of IAC incorporated in each assay was thoroughly optimised to ensure that it did not interfere with the analytical sensitivity of the assay. The limit of detection of the target of each assay remains the same.

Nonetheless, the IAC must be present in the reaction in sufficient quantity to perform its function, and it can only do that reliably if it consistently shows a signal in the absence of a target or in the presence of low target concentrations. The IACs developed in this study all had this capacity.

Finally, it should be demonstrated that an amplification control can identify failed reactions. In this study, using strawberries, which have been implicated in several outbreaks of viral disease (Food and Agriculture Organization of the United Nations/World Health Organization 2008) and which have often been found to contain inhibitory substances (Croci et al. 2008), the IACs showed that the performance of assays for both RNA and DNA viruses could be verified.

In conclusion, this study has produced a suite of IACs for nucleic acid amplification assays suitable for use in the analysis of food supply chain samples for viruses. The IACs constructed in this study can be reliably used in their specific assays and thus provide a robust control that can be routinely applied in the analysis of foods for viruses.

Table 5 Detection of viruses in purified and inhibitor-containing nucleic acid extracts of strawberry puree artificially contaminated with human adenovirus and murine norovirus

	Human adenovirus		Murine norovirus		Interpretation
	Target	IAC	Target	IAC	
Purified extract	20.88±0.15[b] (9/9)[c]	33.52±0.30 (9/9)	35.05±0.35 (9/9)	37.04±0.69 (9/9)	Positive
Inhibitor-containing extract[a]	Undet. (0/9)	Undet. (0/9)	Undet. (0/9)	Undet. (0/9)	False negative

[a] Non-extracted strawberry puree suspension (50 μl) added to 50 μl of nucleic acid extracted from artificially contaminated strawberry puree

[b] Mean and standard deviation of C_p values of three independent nucleic acid amplification reactions using three replicates in each

[c] Positive reactions out of nine reactions

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