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ScienceDirect
Journal of Virological Methods 146 (2007) 401–404
Journal of Virological Methods
www.elsevier.com/locate/jviromet

Short communication

# Inactivation of orthopoxvirus for diagnostic PCR analysis

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Received 10 November 2006; received in revised form 29 July 2007; accepted 30 July 2007
Available online 11 September 2007

**Abstract**

Diagnoses of ongoing viral infections commonly rely on PCR methodology. Sample material that may contain hazardous virus should be efficiently inactivated in biological containment or bed-side before diagnostic PCR analysis. Surprisingly little documentation is available for inactivation of human viral pathogens by inactivation reagents that allow for subsequent PCR diagnostics. It is now shown that pathogenic DNA viruses (orthopoxvirus) are completely inactivated by a commercially available Roche MagNA Pure lysis/binding buffer as evaluated by subsequent cell culture. However, inactivation reagents are typically toxic and therefore problematic in cell culture. Using the relatively large orthopoxvirus, a method was developed in which virus is precipitated by high-speed centrifugation after inactivation but prior to application onto the target cells, thereby eliminating the cytotoxic effect of the lysis buffer. The results from quantitative PCR analysis indicate that the viral DNA from the completely inactivated virus particles, remain associated to macromolecules and aggregates. The use of inactivation buffers for bed-side inactivation of special patient samples taken for PCR diagnostics should be considered in cases where high containment would otherwise be required.


*Keywords:* Inactivation; *Orthopoxvirus*; Diagnostic PCR; Bioterror

## 1. Introduction

Accurate and timely diagnostics of viral infections include a variety of PCR and genotyping methods. Many diagnostic laboratories now utilize automated sample extraction systems such as MagNA Pure (Roche) or other. A number of human virus pathogens require high-biological containment for targeted research and virus cultivation, whereas equally high containment is generally not required for untargeted laboratory work, such as common diagnostic procedures. However, inactivation of possible hazardous virus in patient samples is preferred before performing diagnostic PCR; for example if an infection is suspected to include a potential bioterror agent. The majority of such virus includes enveloped RNA virus (e.g. members of the *Flavivirus*, *Filovirus*, *Bunyavirus* and *Alphavirus* genera) but also a few enveloped DNA virus (e.g. *Orthopoxvirus*) (Mahy, 2003; Whitley, 2003). Among the *Orthopoxvirus* genus the major human pathogens include *Variola virus*, *Vaccinia virus*, *Monkeypox virus* and *Cowpox virus* (Esposito and Fenner, 1996). In cases where inactivation is desirable this should be completed at earliest possible time, preferably at the time of sample collection (bed-side).

Although it is assumed that nucleic acid extraction inactivates the viral pathogens, surprisingly little documentation is available. Only one study has been published demonstrating inactivation of pathogenic RNA virus using two commercially available lysis buffers; AVL (Qiagen) and TRIzol® (Invitrogen) (Blow et al., 2004). Here, it is shown that two species of *Orthopoxvirus* (*Vaccinia virus* and *Cowpox virus*) are efficiently inactivated by a commercially available Roche MagNA Pure lysis/binding buffer. This buffer contains both strong detergent (20% Triton X-100) and chaotropic salt (>25% guanidine isothiocyanate).

Inactivation reagents containing detergent and chaotropic salt are toxic and thereby problematic in cell culture experiments. Their cytotoxic effect on target cells often appears visually indistinguishable from virus-mediated cytopathic effect (CPE) in dilutions less than $10^2$ to $10^4$. As a result the presence of residual infectious activity cannot be excluded in high-tittered samples. Using the relatively large orthopoxvirus as model for variola, a method was developed in which the cytotoxic effect of the lysis buffer is eliminated before applying onto the CPE assay target cells.

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doi:10.1016/j.jviromet.2007.07.025

The results support the finding of Blow et al. (2004) that demonstrated inactivation of enveloped RNA virus, and provides documentation for inactivation of orthopoxvirus. The use of inactivation buffers for safe bed-side inactivation of patient samples should be considered in extraordinary cases where high containment would otherwise be required.

## 2. Materials and methods

### 2.1. The virus isolates

The following human pathogens was included belonging to the *Orthopoxvirus* genus. Vaccinia virus was selected as a model for variola. The Western Reserve strain was expanded to titers greater than $1 \times 10^9$ PFU/ml. The cowpox virus (CoPV/Den98) was isolated from a Danish patient (Christensen et al., 2005) and was cultivated to titers of $>1 \times 10^5$ PFU/ml.

### 2.2. Assay for cytopathic effect (CPE)

Vaccinia and cowpox assays were performed in Vero cells cultivated in MEM with Hanks' salts, 10% FCS, 100 U/ml pen. strep. and 10 mM Hepes. In some vaccinia virus experiments target cells were CV-1 cells cultured in Eagles' minimal essential media (EMEM) (Gibco 42360-024) supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyrovate, 0.1 mM non-essential amino acids and 100 U/ml pen. strep. (media).

Four sample materials were investigated: (1) cell-free virus supernatant, (2) human plasma or serum from sero-negative donor spiked with 10% cell-free vaccinia virus supernatant, (3) EDTA blood spiked with vaccinia virus-infected VERO cells and (4) media with vaccinia virus-infected VERO cells. To obtain vaccinia virus-infected cells, 90% confluent Vero cells were inoculated with approximately 1 MOI and grown with for 3 days. At the time of harvested abundant CPE was visible. A total of $8–9 \times 10^5$ infected cells were used for each spiked sample.

Equal volumes (50 μl) of sample material and MagNA Pure lysis/binding buffer (Roche Cat no.: 03246779001) were mixed (mix 1). In parallel, mixtures of sample and media (mix 2) and media with lysis buffer (mix 3) were prepared. Mixes 1–3 incubated for 15 min at room temperature. After addition of 1200 μl media, mixtures were centrifuged at $20{,}000 \times g$ for 60 min at 4 °C to precipitate any infectious material. The supernatant was carefully removed and visual pellets resuspended in 1000 μl media (dilution $10^1$) from which serial 10-fold dilutions were made ($10^6$ to $10^{12}$). Eight replicates of 90% confluent CV-1 cells or Vero cells in 96-well plates were infected with 50 μl of each dilution of mixes 1–3, respectively. For the initial experiments infection was continued for 2 h at 37 °C, after which the supernatants were replaced in all wells with 150 μl fresh media. For the simulated patient material, infection was continued until the evaluation of CPE (plaque formation) on plates 1 and 2 (mixes 1 and 2) after 3 days. Media was replaced by 100 μl crystal violet solution (0.1% (w/v) in 20% (v/v) ethanol in $H_2O$). Cells were stained for 5 min and washed twice with 200 μl $H_2O$. Viral titers were calculated according to Reed and Muench (1938).

### 2.3. Assay for cytotoxic effects

Cytotoxic effect of mix 3 was measured by a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay the day after infection by addition of 30 μl of MTT reagent (5 mg/ml in PBS) to all wells. Plates were incubated for 4 h at 37 °C and 150 μl supernatant was replaced by 125 μl stop solution (isopropanol containing 10% Triton X-100 and 40 mM HCl). Absorbance was measured at 540–620 nm.

### 2.4. Quantitative PCR

The serial dilutions of mixes 1 and 2 were extracted by MagNA Pure Total NA kit (Roche) according to manufacturer's instructions. The concentration of orthopoxvirus DNA was examined by quantitative PCR using a commercially available orthopoxvirus Lightcycler PCR kit (Artus) according to the kit instructions (Olson et al., 2004).

## 3. Results

### 3.1. Inactivation of vaccinia virus and cowpox virus by commercially available lysis/binding buffer

The results of the orthopoxvirus inactivation experiments are shown in Table 1. Initial experiments showed an apparent inactivation of vaccinia virus between $4 \times 10^8$ and $2.1 \times 10^9$ pfu/ml. However, cytotoxic effect of the inactivation reagent was observed in dilutions $\leq 10^4$ (Fig. 1A) that is difficult to distinguish from virus-mediated cytopathic effects. To eliminate the cytotoxic effect a 1-h high-speed ($20{,}000 \times g$) centrifugation step was included into the protocol to pellet any infectious material upon inactivation. This centrifugation did not reduce the viral titer of non-inactivated vaccinia virus. Again, complete inactivation of vaccinia virus and cowpox virus corresponding to an 8–9 and 5 log reduction in viral titer, respectively (Table 1). The centrifugation procedure reduced the cytotoxic effect of the inactivation below detection level, as measured by MTT assays (Fig. 1B). Thus, using the centrifugation method enables detection of residual non-inactivated infectious units.

Quantitative PCR was used as a second measure of the virus content in the cell-free virus supernatants of the CPE assay. As expected, orthopoxvirus DNA was measured in the non-inactivated virus dilutions that decreased with increasing dilutions, e.g. $10^1$ to $10^5$ in the cowpox virus experiments (Table 2). As for the inactivated virus, orthopoxvirus DNA was also readily detectable, and decreasing, in dilutions $10^1$ to $10^4$ of cowpox virus. Similar results were obtained for the vaccinia virus supernatant (Table 2). Comparing equal dilutions of inactivated and non-inactivated virus after the centrifugation procedure, the DNA content was consistently lower (1.6–8.3-fold) in the inactivated virus, suggesting some loss of viral DNA and/or viral particles during the centrifugation protocol. In contrast, this relatively modest loss of viral DNA indicates that although completely inactivated, the viral particles are not entirely disintegrated by the lysis buffer and likely the viral DNA remain associated to macro-molecular cell debris or aggregates.