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Journal of Virological Methods 119 (2004) 57–64

Journal of Virological Methods
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Protocol

Immunopurification applied to the study of virus protein composition and encapsidation

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Received 22 January 2004; received in revised form 4 March 2004; accepted 8 March 2004

Abstract

A protocol for obtaining small amounts of highly pure virus preparations starting from reduced volumes (<5 ml) of infected tissue culture supernatants is described. This procedure was adapted to the study of transmissible gastroenteritis coronavirus (TGEV) protein composition and RNA encapsidation. This protocol relies on virion capture by monoclonal antibodies specific for a virion membrane protein. These antibodies were bound to protein A-coated ELISA wells armed with rabbit anti-mouse IgG antibodies. As an example, the purification of ^{35}S-labelled TGEV virions was performed. The quality of virus preparations was assessed by quantifying common contaminating RNAs by real-time RT-PCR. The most critical factors influencing the purity degree are analysed and discussed.

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Keywords: Coronavirus structure; Composition and encapsidation; Packaging signal; Virus immunopurification

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1. Type of research

Purification of virus particles is a standard protocol in virus basic research. The study of virus composition relies on high quality virus preparations free of cellular contaminants. The most widely used protocol for virus purification is centrifugation through continuous sucrose gradients (Fields et al., 1996). This method usually requires large amounts of infected-cell culture supernatants as a starting point, frequently harvested after extensive cytopathic effect. Therefore, contamination of virus preparations with proteins and nucleic acids stuck to the surface of virion particles is common. The purpose of this study is to provide a specific and rapid protocol for obtaining highly pure virus preparations, starting from low amounts (usually less than 5 ml) of infected-cell culture supernatants.

TGEV belongs to the family Coronaviridae that groups important pathogens, causing a variety of diseases including the recently described coronavirus causing the severe and acute respiratory syndrome in humans (SARS-CoV) (Enjuanes et al., 2000a; Marra et al., 2003; Rota et al., 2003). TGEV virions present three structural levels (Fig. 1) (Escors et al., 2001b): (i) the envelope, in which the spike (S), envelope (E) and membrane (M) proteins are embedded; (ii) the internal core made of the nucleocapsid and the carboxy-terminus of the M protein; and (iii) the nucleocapsid, consisting of the positive-sense, single-stranded RNA genome and the nucleoprotein (N) (Almazán et al., 2000; Escors et al., 2001a; Penzes et al., 2001). Virus composition and RNA encapsidation have been subjects of interest in our laboratory and virion immunocapture (immunopurification), frequently used as a diagnostic technique (Le Gall-Reculé et al., 2001; Nolasco et al., 1993; Plakokefalos et al., 2000; Ptacek et al., 2002; Rimhanen-Finne et al., 2002; Sefc et al., 2000; Sharman et al., 2000; Wetzel et al., 1992), has been a valuable tool. Accordingly, two topologies for the M protein in the envelope of TGEV virions have been characterized using the technique described in this protocol (Escors et al., 2001b). Additionally, by the powerful combination of the specificity of immunopurification with the sensitivity of quantitative real-time RT-PCR, it has been demonstrated that TGEV mRNAs are not encapsidated into virus particles (Escors et al., 2003), in contrast to previous publications using non-homogeneous virus preparations

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doi:10.1016/j.jviromet.2004.03.004

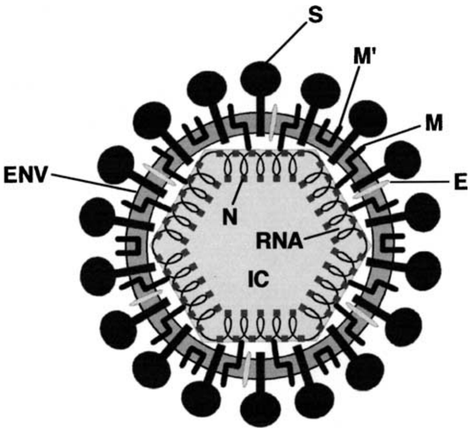

Fig. 1. Transmissible gastroenteritis coronavirus structure and composition. A scheme of the TGEV virion structure is shown. The TGEV particle is represented as a spherical particle made of a virus envelope (ENV) presenting a corona of peplomers made of the S protein (S). The envelope surrounds the internal core (IC) made of the virus RNA genome (RNA), the nucleoprotein (N) and the carboxy-terminus of the M protein in a Nexo-Cendo topology (M). M′, membrane protein molecules adopting a Nexo-Cexo topology (Escors et al., 2001b). E, small envelope protein.

that reported the encapsidation of viral mRNAs within virus particles (Hofmann et al., 1990; Sethna et al., 1989, 1991; Zhao et al., 1993). Using the same technique the TGEV packaging signal has been located (Escors et al., 2003).

2. Time required

Preparing ELISA well plates for immunopurification takes 1 day. Plates can then be stored at −20 °C for several months (up to 4). For optimal results, virus samples are incubated in ELISA plate wells overnight. However, this incubation step can be reduced to 1 h at 37 °C. A high number of different virus purifications (theoretically up to 96) can be performed within two working days in a single ELISA plate, by a single operator.

3. Materials

3.1. Cells and viruses

TGEV strain PUR46-MAD (Almazán et al., 2000; Jiménez et al., 1986; Penzes et al., 2001) was used in this study, and swine testicle cells (ST) (ATCC number CRL-1746) were used to grow TGEV virus.

3.2. Monoclonal antibodies

Monoclonal antibodies 25.22 and 3D.C10, specific for the TGEV M protein and for the N protein, respectively, have been described before (Charley et al., 1988; Escors et al., 2001b; Laude et al., 1992; Martín-Alonso et al., 1992). MAb 25.22 was used to immunopurify TGEV virions. MAb 3D.C10 was used as a control. An unrelated antibody specific for beta-glucuronidase (GUS) was used as a control (Escors et al., 2001a). Purified antibodies from hybridoma supernatants, or hybridoma supernatants directly, were used for the assays.

3.3. Primers

Primers for RT-PCR were synthesized by Sigma-Genosys Ltd. (Pampisford, UK), and aliquots of 50 ng/μl were performed in DEPC (diethyl pyrocarbonate)-treated water. For RT-PCR amplification of the TGEV genome two primers were used, a reverse primer (5′-TGCAAGGCATGCTGGCATTTTATAC-3′) and a forward primer (5′-ACTCATTGAATTTAGGCAGCAAAGC-3′). Amplification of the virus mRNA coding for the TGEV N protein (mRNA-N) was carried out using the reverse primer (5′-CCTGGTTGGCCATTTAGAAGTTTAG-3′) and the forward primer (5′-AGATTTTGTCTTCGGACACCAACTCG-3′). Amplification of β-actin mRNA was performed using the reverse primer (5′-CAGAGTCCATGACAATGCCAGTGGT-3′) and forward primer (5′-ATGTTTGAGACCTTCAACACGCC-3′).

3.4. Special equipment

Quantitative PCR materials: Optical Adhesive Cover Starter Kit and 96-well Optical Reaction Plate (Applied Biosystems, Warrington, UK). Pipette tips with filters (Sorenson Bioscience Inc., Salt Lake City, United States). BioRad mini-protean II electrophoresis apparatus (Bio-Rad, Richmond, USA); ELISA 96-well plates (NUNC, Rochester, USA); BECKMANN XL-90 Ultracentrifuge and SW60.Ti Beckmann rotor and tubes (Beckmann Instruments Inc., Palo Alto, United States); Konica medical film 18 cm × 24 cm (Konica Corporation, Tokyo, Japan); cell culture plates (10 mm diameter) (NUNC, Rochester, USA); slab gel dryer (E-C Apparatus Corporation, Florida, United States); 3 MM chromatography paper (Whatman International Ltd., Maidstone, England).

3.5. Optional equipment

To avoid contamination of samples for quantitative RT-PCR, a laminar flow cabinet could be useful. An ABI PRISM 7000 PCR detection system, and data processing software (ABI PRISM 7000 SDS program, Applied Biosystems, Warrington, UK) for real-time RT-PCR analyses. This is optional since semi-quantitative RT-PCR can also be performed.

3.6. Reagents

Most of the reagents used in the protocol are commonly found in molecular virology laboratories. Only the special reagents are listed below:

- Acrylamide/bisacrylamide 37.5:1 solution (Amresco, Ohio, USA).
- Bovine Serum Albumin (BSA, fraction V, SIGMA Chemical Co., St. Louis, USA).
- Complete Protease Inhibitor Cocktail tablets (Boehringer Mannheim GmbH, Germany).
- Diethyl pyrocarbonate (DEPC, SIGMA Chemical Co., St. Louis, USA).
- Glycogen (SIGMA Chemical Co., St. Louis, USA).
- HPLC-purified rabbit anti-mouse IgG antibodies (Cappel, Turnhout, Belgium).
- Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega, USA).
- Pro-mix L-^{35}S in vitro cell labelling mix (Amersham Pharmacia Biotech, Little Chalfont, UK).
- Protein A (Amersham Pharmacia Biotech, Little Chalfont, UK).
- RNAsin (Promega, Madison, USA).
- Sodium dodecyl sulphate (SDS, SIGMA Chemical Co., St. Louis, USA).
- Sodium salicylate (Merck, Darmstadt, Germany).
- SybrGreen PCR master mix (Applied Biosystems, Warrington, UK).
- ULTRASPEC RNA extraction reagent (Biotecx laboratories Inc., Houston, USA).

4. Detailed procedure

4.1. General considerations

Basic rules of good laboratory practice are the only requirements for this protocol. Special care has to be taken when real-time RT-PCR is performed to avoid cross-contaminations. For this, all reagents have to be kept in separate aliquots. Use of pipette tips with filters is highly recommended. Specific measures have to be taken for manipulation of radioactive materials.

A purification of TGEV virions in continuous density sucrose gradient was also performed. The specific protocol for purification of TGEV virions by sucrose gradients is described elsewhere (Jiménez et al., 1986).

4.2. Controls

For enveloped viruses, such as coronaviruses, a monoclonal antibody specific for an internal virion protein must be used in order to assess virus integrity (Fig. 1). In the example provided within the protocol, MAb 3D.C10, specific for TGEV N protein (Martín-Alonso et al., 1992), is used as a control. A purification using non-specific MAbs has to be performed as a control for antibody specificity.

4.3. Cell culture

Basic cell culture facilities are the only specific requirements.

(1) Swine testicle cells were grown in 10 ml of Dulbecco Modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) in a l0-mm diameter cell culture plate. Cells were grown at 37 °C to confluence.

4.4. Infection of ST cells with TGEV virus

(1) Medium from confluent ST cells was removed and 5 ml of DMEM containing 2% FCS was added to the cell culture.
(2) From a TGEV virus stock (title 8×10^8 pfu/ml) 50 μl were added to the cell culture plate so that cells were infected with a multiplicity of infection of 5.
(3) Infected cells were either metabolically labelled as described below or incubated at 37 °C for 14 or 48 h.

4.5. Metabolic labelling of infected cells

Metabolic labelling with ^{35}S-methionine/cysteine is performed only when it is required for research. Radioactive labelling is not necessary for detection of encapsidated RNA by RT-PCR.

(1) Medium from infected cells was removed and cells were washed three times with 2 ml of DMEM lacking methionine/cysteine. The washing medium was removed by vacuum.
(2) Cells were starved by incubation for 1 h at 37 °C in 5 ml of DMEM without methionine/cysteine.
(3) Hundred microcurie of ^{35}S-labelled methionine/cysteine were added to the infected-cell culture (about 10 μl of cell labelling mix). Cells were placed inside a protective methacrylate box and incubated at 37 °C for 18 h.

4.6. Sedimentation of TGEV virions

(1) Previous to virus sedimentation, a solution of 20% sucrose in phosphate buffered saline (PBS) was prepared. One millilitre of this solution (sucrose cushion) was added to a centrifuge tube for a SW60.Ti Beckmann rotor.
(2) Supernatants from infected ST cells (about 5 ml) were recovered at 14 and 48 h post-infection (hpi) and clarified by slow speed centrifugation in a Beckmann centrifuge. Cell debris was discarded and clarified supernatants recovered.
(3) Clarified supernatants were added on top of sucrose cushions, avoiding mixing.
(4) Virions were sedimented by ultracentrifugation at 27 000 rpm for 50 min at 4 °C in a SW60Ti Beckman rotor.
(5) Sedimented virions were recovered by gentle pipetting a few times in 200 μl of PBS buffer containing protease inhibitors and were kept on ice (partial purifications).

4.7. Coating of ELISA well plates with specific antibodies

(1) NUNC ELISA 96-well plates were coated with 100 μl of protein A in PBS buffer (50 μg/ml). Plates were incubated overnight at 37 °C, covered with a plastic sheet to avoid evaporation.
(2) Excess of protein A was removed by a flick of the plate over adsorbent paper. Wells were blocked with 150 μl per well of 5% BSA in PBS. Blocking was carried out by incubation at 37 °C for 2 h.
(3) Excess of BSA was removed and 150 μl per well of 0.1% BSA in PBS (washing solution) was added. This washing step was repeated three times.
(4) Washing solution was removed and 60 μl of HPLC-purified rabbit anti-mouse IgG antibodies (90 μg/ml) were added to each ELISA well. Binding of rabbit antibodies to protein A was carried out for 1 h at 37 °C.
(5) Wells were washed three times as described above and specific monoclonal antibodies for the M protein (25.22), the internal nucleoprotein (3D.C10) and GUS-specific Abs were added to the corresponding wells. As a control, two wells were left without MAbs. Binding of MAbs was carried out for 1 h at 37 °C (Fig. 2A).

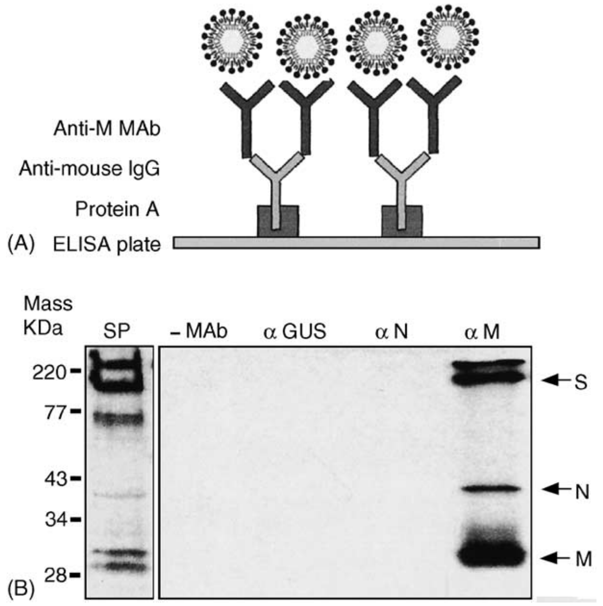

Fig. 2. Immunopurification of TGEV particles. (A) A scheme showing the immunopurification setup is shown. Protein A is bound to ELISA wells. Anti-mouse IgG is then bound to protein A, and in turn, the membrane protein-specific MAb is bound to the anti-mouse antibody. Virion particles binding the M protein-specific MAb are represented as spheres. (B) Representative SDS-PAGE electrophoresis and fluorography of immunopurified radioactively labelled TGEV virions using the indicated antibodies above the gel lanes. αGUS, GUS-specific antibody; αN, MAb 3D.ClO; αM, MAb 25.22; SP, infected-cell supernatant; arrows indicate the positions of TGEV structural proteins. S, spike protein; N, nucleoprotein; M, membrane protein.

(6) Wells were washed twice and 3 μg (total protein content) of partially purified or sucrose gradient-purified virions were added to the ELISA wells. Washing solution was added if required to reach a final volume of 60 μl per ELISA well.
(7) Wells were washed 10 times as described above. For protein analyses, viruses were directly recovered from the wells by adding 30 μl of SDS-PAGE loading buffer (50 mM Tris–HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol) heated at 60 °C to dissociate virus proteins from antibodies.

4.8. Protein detection

Radioactively labelled virions were used to detect virion proteins.

(1) SDS-polyacrylamide gels were prepared for a Bio-Rad mini-protean electrophoresis apparatus. Samples of immunopurifications (15 μ1) were separated by SDS-PAGE by applying 100 V at room temperature for 2 h.
(2) Gels were placed in 50 ml of distilled water for 10 min at room temperature.
(3) Gels were incubated for 1 h at room temperature in 25 ml of a solution containing 15% sodium salicylate in distilled water.
(4) Gels were placed on a piece of 3 MM chromatography paper, covered with a transparent plastic film and dried at 80 °C for 1 h in a slab gel dryer.
(5) Dried gels were exposed to a Konica medical film from 1 to 5 days at −80 °C inside a film cassette, and labelled proteins were developed in an X-OMAT Processor (Kodak, Rochester, United States).

4.9. RNA extraction from immunocaptured virions and detection by RT-PCR

(1) After washing immunocaptured virions, 100 μl of ULTRASPEC RNA extraction reagent was added in each ELISA well and pipetted up and down three times.
(2) Extracted RNA in ULTRASPEC reagent was placed in ice-cold eppendorf tubes and 20 μl of chloroform (Merck, Darmstadt, Germany) were added per sample. The mix was vortexed and left in ice for 15 min.
(3) The mix was centrifuged for 15 min at 4 °C in a bench centrifuge at maximum speed (10 000 rpm). Three quarters of the aqueous supernatant were carefully retrieved and transferred to new cold eppendorf tubes.
(4) For RNA precipitation, 2 μl of a glycogen solution in DEPC-treated water (20 mg/ml) were added per sample, vortexed and 50 μl of isopropanol (Merck, Darmstadt, Germany) were added. The mix was vortexed and incubated in ice for 15 min.
(5) RNA was pelleted by centrifugation in a bench microcentrifuge for 15 min at 4 °C and the supernatant was

removed. The pellet was washed twice with 200 μl of 70% ethanol in DEPC-treated water.

(6) RNA pellets were dried at room temperature for 10 min and dissolved in 50 μl of DEPC-treated water containing 40 units of RNAsin.

(7) Purified RNAs were either stored at −80 °C or quantified by real-time RT-PCR. The protocol followed for real-time RT-PCR analyses is explained elsewhere (Escors et al., 2003).

4.10. Estimation of encapsidation efficiencies

Conditions and experimental design for real-time RT-PCR analyses are described elsewhere (Escors et al., 2003). Briefly, calculations were performed as follows:

(1) Relative quantification of genome RNA from immunopurified virions was calculated dividing the number of virus genome molecules in each ELISA well coated with antibodies by the number of genome molecules detected using a non-specific MAb. Three different estimations were performed and means with standard deviations were obtained.

(2) Relative apparent encapsidation efficiencies for mRNA-N and β-actin mRNA in relation to TGEV genome encapsidation were calculated for purified virions by dividing the molar ratios between each mRNA-to-genome RNA in virions by the ratio in infected cells, as described (Escors et al., 2003).

5. Results

To assess the specificity of the technique, immunopurification of ^{35}S-labelled virions was carried out as described in the protocol. Previously, the infected-cell supernatant was analysed by SDS-PAGE and fluorography, containing virus and cellular proteins (Fig. 2B). Three different antibodies were used in immunopurifications, MAb 25.22 specific for the M protein, MAb 3D.Cl0, specific for the N protein and an MAb specific for the GUS protein. A well without antibodies was left as a negative control. Immunopurified virions were analysed by SDS-PAGE and fluorography. TGEV structural proteins S, N and M were detected when the M protein-specific MAb 25.22 was used (Fig. 2B). In contrast, virus proteins were not detected using the N protein-specific MAb 3D.C10, confirming the integrity of TGEV virions, since the N protein is exclusively present inside the virus particle (Figs. 1 and 2B). As expected, no virion proteins were detected when the GUS-specific Ab was used, or in the absence of antibodies (Fig. 2B). To confirm the specificity of the assay, genome RNA levels were quantified by real-time RT-PCR. Genome RNA was detected at high levels when the M-specific MAb was used (around 7 × 10^9 genome molecules per ELISA well), as expected, compared to those using the N-specific MAb or GUS antibody (about a 1000-fold less).

Table 1
Percentage of relative mRNA-to-genome encapsidation efficiencies in virions purified by standard techniques

	Partial purification 14 hpi	Partial purification 48 hpi	Sucrose gradient purification
Genome	100	100	100
mRNA-N	30 ± 0.6	20 ± 0.3	20 ± 0.01
β-Actin mRNA	3 ± 0.06	1 ± 0.06	8 ± 0.4

Table 2
Percentage of relative mRNA-to-genome encapsidation efficiencies in virions purified by immunopurification from the indicated virus sources

	Partial purification 14 hpi	Partial purification 48 hpi	Sucrose gradient
Genome	100	100	100
mRNA-N	0.8 ± 0.02	2 ± 0.03	ND
β-Actin mRNA	ND	ND	ND

ND: not detected.

To evaluate the purity degree of TGEV virus preparations, relative apparent encapsidation efficiencies were estimated for usual contaminating RNAs, virus mRNA-N and cellular β-actin mRNA. First, the source of TGEV virions used for immunopurifications (partially purified preparations or sucrose gradient purifications) was evaluated. In this example, the apparent mRNA-N encapsidation efficiencies were high, ranging from 20 to 30% compared to TGEV genome encapsidation in all cases (Table 1). The levels of cellular β-actin mRNA varied from 1 to 8%, indicating that even after sucrose density gradients, all the preparations were contaminated with cellular and viral mRNAs (Table 1). In contrast, after immunopurification very low levels of mRNA-N were detected and β-actin mRNA was not detected at all (Table 2). TGEV virion preparations virtually free of mRNA-N and β-actin mRNA were obtained when sucrose-gradient-purified virions were used in immunopurifications (Table 2). When partially purified virions recovered at 14 hpi were used for immunopurification, very low levels of contaminating virus mRNA-N (0.8%) were detected (Table 2). These results show that a combination of sucrose gradients and immunopurification provides a high degree of purity. Virus preparations with the highest purification degree are obtained using previously purified virions, or partially purified virions harvested early in infection.

6. Discussion

6.1. Trouble-shooting

6.1.1. Cell culture and virus infection

To adapt this protocol to other viral systems, cell culture and virus growth have to be determined.

6.1.2. *Metabolic labelling of infected cells*

Cells should be starved in methionine/cysteine-free medium for one hour at 37 °C in order to ensure a good incorporation of radioactive aminoacids. Incubation of metabolically labelled infected cells should be carried out inside a protective methacrylate box. The use of radioactively labelled virions, although not essential, it is recommended since a great increase in sensitivity is obtained and only proteins incorporated in the virion are detected. Unlabelled virions can also be used when other analytical techniques are used, such as mass spectrometry.

6.1.3. *Recovery of supernatants from infected cells*

As it has been shown in the results provided within the protocol, in order to obtain highly pure virus preparations it is recommended to recover supernatants from infected cells early during infection, when the cytopathic effect is low.

6.1.4. *Sedimentation of TGEV virions*

Although virions released to the cell culture supernatant can be directly captured, a previous concentration of virions though a sucrose cushion (partial purification) considerably increases the yield and purity of the virus preparation. The use of sucrose cushions at appropriate concentrations is also highly recommended to avoid damage of virion structure.

6.1.5. *Coating of ELISA well plates with antibodies*

It is extremely important to avoid drying of ELISA wells during washing or addition of any of the components. This will result in low yield of virions and contaminated preparations. Washing stringency can be increased by adding ionic salts (sodium and potassium chloride) or detergents (Tween-20) to the washing solution.

6.1.6. *Protein detection*

Detection of virion proteins using radioactively labelled virions is straightforward. When ^{35}S-methionine/cysteine is used, proteins can be detected either by autoradiography or fluorography. However, since small amounts of virions are purified, it is recommended to detect proteins by fluorography using sodium salicylate, as described in the protocol. Using this protocol, radioactive signals are amplified four-fold. Additionally, the use of low amounts of labelled immunopurified virions greatly reduces irradiation risks and contaminations.

6.1.7. *RNA extraction and detection*

It is important to avoid RNA degradation using DEPC-treated solutions and working with gloves. It is extremely important to avoid overdrying of pelleted RNA, since it will be difficult to dissolve it in DEPC-treated water.

6.2. *Alternative and support protocols*

6.2.1. *Detection of virion protein modifications*

Phosphorylated virion proteins or encapsidated RNA can also be detected when cells are metabolically labelled with ^{32}P. Other protein modifications can potentially be detected using the appropriate radioactive isotope.

6.2.2. *Variations in immunocapture protocol*

In most immunocapture protocols pathogen-specific antibodies are directly bound to a solid phase. The major advantage of the protocol described in this article is the use of two steps that increase the effective binding surface. The first step is coating of ELISA well plates with protein A. The second step is binding rabbit anti-mouse antibodies to protein A, which will bind virus-specific mouse MAbs by the Fc (Fig. 2A). Specific MAbs can be directly bound to protein A-coated ELISA wells, although some mouse IgG isotypes do not bind protein A efficiently, therefore the capture of specific mouse IgGs by rabbit anti-mouse MAb also avoids this problem.

6.2.3. *Detection of encapsidated RNA*

To study RNA encapsidation, semi-quantitative RT-PCR can also be used instead of quantitative RT-PCR.

Acknowledgements

We thank Hubert Laude for providing M protein-specific MAb 25.22. This work was supported by grants from the Comisión Interministerial de Ciencia y Tecnología (CICYT), La Consejería de Educatión y Cultura de la Comunidad de Madrid, Fort Dodge Veterinaria and the European Communities (Frame V, Key Action 2, Control of Infectious Disease Projects QLRT-1999-00002, QLRT-1999-30739, and QLRT-2000-00874). DE received a postdoctoral fellowship from the European Union (Frame V, Key Action 2, Control of Infectious Disease Projects). CC received a predoctoral fellowship from the Community of Madrid.

Appendix A. Quick procedure

1. Cell culture
   - ST cells were grown to confluence.

2. Infection of ST cells with TGEV virus
   - ST cells were infected with a TGEV virus stock with an moi of 5.
   - Infected cells were metabolically labelled or incubated for 14 or 48 h.

3. Metabolic labelling of infected cells
   - Infected cells were starved in DMEM lacking methionine/cysteine.
   - Hundred microcurie of ^{35}S-labelled methionine/cysteine was added to infected cells.
   - Labelled infected cells were incubated for 18 h at 37 °C.

4. Sedimentation of TGEV virions
    - Supernatant from infected cells was clarified by slow-speed centrifugation.
    - TGEV virions from supernatants were sedimented through a 20% sucrose cushion by ultracentrifugation. Sedimented virions were recovered in 200 μl PBS.

5. Coating of ELISA well plates with specific antibodies
    - Protein A (5 μg/well) was fixed to ELISA well plates overnight.
    - Protein A was removed by washing with 0.1% BSA in PBS (washing buffer).
    - Wells were blocked with 5% BSA-PBS by 2 h incubation at 37 °C.
    - Rabbit anti-mouse MAb (5 μg/well) was added to each protein A-coated ELISA wells and incubated for 1 h at 37 °C.
    - Wells were washed three times in washing buffer.
    - Virion protein-specific MAbs were added to each well and incubated for 1 h at 37 °C.
    - Wells were washed three times in washing buffer.
    - About 3 μg of total virus protein were added to each antibody-coated well and samples were incubated overnight at 4 °C.

6. Protein detection
7.
    - Virion proteins were recovered in 30 μl of SDS-PAGE loading buffer and separated in 10% SDS-PAGE gels.
    - Gels were incubated in 15% sodium salicylate for 1 h at room temperature.
    - Gels were dried and exposed to a medical film at −80 °C for a variable time.

8. RNA extraction from immunopurified virions and detection by RT-PCR
    - ULTRASPEC RNA extraction reagent (100 μl) was added to each ELISA well containing viruses, and transferred to eppendorf tubes.
    - Chloroform (20 μl) was added to each sample, vortexed, and incubated in ice for 15 min.
    - Samples were centrifuged in a bench microcentrifuge at 4 °C for 15 min.
    - The aqueous supernatant was recovered and 20 μg of glycogen added to each sample.
    - The same volume of isopropanol was added to each sample, vortexed and incubated in ice for 15 min.
    - RNA was pelleted by centrifugation at 4 °C in a bench microcentrifuge.
    - RNA pellet was washed in 70% ethanol twice, dried at room temperature and resuspended in 50 μl of DEPC-treated water.
    - Samples were analysed by quantitative or standard RT-PCR.

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