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Rapid *in situ* hybridization technique for the detection of ribonucleic acids in tissues using radiolabelled and fluorescein-labelled riboprobes

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(Received 13 November 1996, Accepted 25 April 1997)

In situ hybridization (ISH) is a useful diagnostic and research tool, but is also time consuming. This study was conducted to determine if a rate enhancement hybridization (REH) buffer, developed for membrane hybridization, could be used to decrease hybridization time for ISH. Tissue from swine with an enteric disease produced by a swine coronavirus, transmissible gastroenteritis virus (TGEV), was used as a model to standardize hybridization conditions for a rapid ISH technique. Small intestinal sections from pigs experimentally and naturally infected with TGEV were hybridized for various times at 52°C and 70°C with a radiolabelled or a fluorescein-labelled RNA probe in a standard hybridization or a REH buffer. Viral RNA was detected in intestines from as early as 30 min of hybridization by using both buffers with the radiolabelled probe; however, the signal was stronger with the REH buffer. With the fluorescein-labelled probe, viral RNA was detected in virus-infected cells of the intestines after 30 min of hybridization by using the REH buffer. Signal intensity was greater with the REH buffer than with the standard hybridization buffer when compared at each hybridization time and hybridization temperature using both radiolabelled and fluorescein-labelled probes. With the REH buffer, hybridization signal intensity was greater at 70°C than at 52°C for both probes. The best results were obtained when small intestinal sections were hybridized at 70°C for 2 h using a radiolabelled or a fluorescein-labelled probe diluted in the REH buffer. The fluorescein-labelled RNA probe with REH buffer resulted in a minimal non-specific signal when compared with the radiolabelled probe. These studies demonstrated that the REH buffer can be used to decrease the time of ISH for the detection of viral RNA. This rapid ISH technique should have broad applications in the utilization of probe technology in diagnostics and research for the detection of target ribonucleic acids *in situ*. © 1997 Academic Press Limited

KEYWORDS: nucleic acid, *in situ* hybridization, coronavirus, TGEV, swine, riboprobes, rapid hybridization.

INTRODUCTION

In situ hybridization (ISH) is widely used for the detection of DNA and RNA in intact eukaryotic and prokaryotic cells by using radiolabelled or non-radiolabelled probes. Probes for ISH may be DNA, RNA

or oligonucleotides. A major disadvantage of ISH is that it is a time-consuming technique, taking about 2 days for non-isotopic ISH and 4 days or more for isotopic ISH. One of the rate limiting steps is hy-

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bridization which requires at least 16 h.¹⁻⁶ There have been two reports describing a reduction of hybridization time to 3 h using DNA probes⁷ and to 2 h using ribotypes.⁸ Recently, rate enhancement hybridization (REH) buffers have become commercially available. These buffers can decrease hybridization time for membrane-based procedures such as Southern, northern, and dot blot hybridizations.

For the development of rapid ISH, tissues from swine with experimentally induced, transmissible gastroenteritis (TGE), were used as a model. TGE is characterized by vomiting, severe diarrhoea, and high mortality in piglets during the first few weeks of life. The causative agent of TGE is a coronavirus referred to as transmissible gastroenteritis virus (TGEV). Coronavirus particles are pleomorphic and enveloped and contain a single-stranded positive-sense RNA genome.⁹ TGEV replicates in the cytoplasm of the mature absorptive enterocytes⁹⁻¹⁴ and causes villous atrophy. Here we describe a rapid ISH technique, using REH buffer, that allows decreased hybridization time for both radiolabelled and fluorescein-labelled probes.

MATERIALS AND METHODS

Virus

The virulent Miller strain of TGEV (National Veterinary Services Laboratory, Ames, Iowa) was used as a reference virus.¹⁵⁻¹⁷

Source of tissues

Four 8.5 week-old TGEV-negative pigs were used. Two pigs were inoculated orally with 10^5 plaque forming units (pfu) of virulent Miller strain of TGEV and two pigs served as non-inoculated controls. Tissues from seven pigs submitted as separate diagnostic cases to the Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Iowa State University were used to represent TGEV naturally-infected and non-infected pigs. Five samples were positive and two were negative for TGEV antigen by direct immunofluorescence of frozen intestinal sections.

Processing of tissues for *in situ* hybridization

TGEV-inoculated and non-inoculated pigs were euthanized and necropsied at 3 days post-inoculation. At the time of necropsy, 1 cm segments of the posterior end of the duodenum, and the anterior and posterior

ends of the jejunum and the ileum were collected and fixed in 10% neutral buffered formalin. A segment of the small intestine from the five pigs naturally infected with TGEV and the two TGEV negative pigs was placed in 10% neutral buffered formalin. The tissues were processed and embedded in paraffin. Consecutive sections were cut from each tissue, placed on nuclease-free silylated slides (PGC scientific), deparaffinized and hydrated as previously reported.¹⁸

Preparation of probes

RNA probes were prepared from plasmid pPSP.FP₁ containing the left half of the S gene of TGEV cloned in a phagemid vector.¹⁹ The plasmid pPSP.FP₁ contains nucleotides 1678 to 2250²⁰ of the S gene of TGEV, and was linearized with the restriction enzyme *Bam*HI (Promega). The *in vitro* transcription and labelling of the probe was performed as described previously¹⁸ by using T7 RNA polymerase, the four NTPs (ATP, CTP, TTP and GTP; Sigma Chemical Co.), DTT (dithiothreitol; Bio-Rad), and [³⁵S]-UTP α S (Amersham). The transcription reaction was stopped by adding 1 μ l (1 μ g μ l⁻¹) of DNase I (Worthington) and 1 μ l (1 μ g μ l⁻¹) of RNasin (Promega) for 20 min at 37°C. Unincorporated [³⁵S]-UTP α S was removed by passing the labelled RNA through a Sephadex G-25 spin column (Boehringer Mannheim). The fluorescein-labelled RNA probe was labelled by using a RNA colour kit (Amersham) and purified by using a Sephadex G-50 spin column (Boehringer Mannheim). The specificity of probes was determined by dot blot hybridization as previously reported.²¹

In situ hybridization

Deparaffinized tissues were treated and hybridized as reported previously.¹⁸ Briefly, tissues were treated with proteinase K (1 μ g ml⁻¹, Amersco) for 15 min at 37°C and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min at room temperature. Sections hybridized with a fluorescein-labelled RNA probe were incubated with 20% (v/v) cold acetic acid for 15 s after treatment with proteinase K to destroy endogenous alkaline phosphatase. Sections were hybridized with 50 μ l of standard hybridization buffer (50% formamide, 10% dextran sulfate, 3 \times SSC, 50 mM sodium phosphate, pH 7.4, 1 \times Denhardt's solution, 0.1 mg ml⁻¹ yeast tRNA, and 10 mM DTT) or REH buffer (Rapid-Hyb buffer, Amersham) containing 0.5 μ l of a ³⁵S-labelled RNA probe (1.43 ng μ l⁻¹ or 1.6 $\times 10^6$ cpm μ l⁻¹) or

1 μl of a fluorescein-labelled RNA probe ($1 \text{ ng } \mu\text{l}^{-1}$). Sections hybridized with a ^{35}S -labelled RNA probe were divided into 4 groups, which were incubated with the standard hybridization buffer at 52°C and 70°C , and the REH buffer at 52°C and 70°C . Sections hybridized with a fluorescein-labelled RNA probe were divided into two groups which were hybridized with the standard hybridization buffer at 52°C or the REH buffer at 70°C . Each group of sections were incubated with hybridization buffer for 20 h, 2 h, 1 h or 30 min. Sections were treated with $20 \mu\text{g ml}^{-1}$ RNase A (Worthington) at 37°C for 30 min, and washed in $2 \times \text{SSC}$ for 5 min at 52°C , $1 \times \text{SSC}$ for 5 min at 52°C , $0.5 \times \text{SSC}$ for 1 h at 52°C , and $0.5 \times \text{SSC}$ for 5 min at room temperature.

Detection of the *in situ* hybridization signal

For the ^{35}S -labelled RNA probe, sections were dehydrated and air dried. X-ray film (BIOMAX MR, Eastman Kodak Co.) was exposed to hybridization sections at -70°C for 24 h, developed and evaluated. Sections were then coated with Kodak NTB-2 emulsion gel (Eastman Kodak Co.) which was diluted 1:1 with 600 mM ammonium acetate^{1,2} and preheated to 42°C .¹⁻⁴ These sections were kept in a light-proof box at 4°C for 2 days. The slides were developed at 15°C for 3 min by using developer (Kodak D-19) diluted 1:1 with deionized water, followed by a 20 s rinse in deionized water and then fixed for 3 min in fixer (Eastman Kodak Co). Sections were then rinsed with deionized water for 5 min⁴ and counterstained with haematoxylin and eosin.

For the fluorescein-labelled RNA probe, sections were washed in Tris-buffered saline (TBS) containing 100 mM Tris-HCl, pH 7.5, and 400 mM NaCl for 5 min at room temperature. Sections were incubated for 1 h at room temperature in a moist chamber with a blocking solution consisting of 0.5% (w/v) blocking agent (Amersham) in TBS. Sections were then rinsed with TBS for 1 min and incubated with anti-fluorescein antibody conjugated with alkaline phosphatase (Amersham) diluted 1:400 in 0.5% (w/v) BSA fraction V in TBS for 1 h in a moist chamber at room temperature. Sections were washed in TBS three times for 5 min each, and in detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl_2) for 5 min at room temperature. Sections were incubated with substrate (45 μl of 4-nitroblue tetrazolium chloride and 35 μl of 5-bromo-4-chloro-3-indolyl-phosphate in 10 ml detection buffer) for 1 h in the dark, then rinsed in deionized water and counterstained with nuclear fast red for 2 min.

Controls included: tissue sections from two non-inoculated control pigs and two TGEV-free pigs submitted to the Veterinary Diagnostic Laboratory; RNase-treated tissue sections from TGEV-inoculated pigs and TGEV-naturally infected pigs; sections from TGEV-infected pigs hybridized with positive strand RNA probes; sections from TGEV-infected pigs hybridized with the excess amount of non-labelled RNA probe followed by hybridization with labelled RNA probes. For the fluorescein-labelled RNA probe, we used a hybridization buffer-free probe as an additional control.

Signal quantitation

Autoradiography results were captured on X-ray films by an AGFA Arcus II flatbed scanner and edited by using software Adobe Photoshop 3.0 on a McIntosh computer. The results were printed with a video printer (Sony Color Video Printer Up-5000). The intensity of the signal was analysed using software NIH Image 1.58 and reported as mean density and total signal area. For non-isotopic ISH, slides were scored by using semiquantitation as follows: - = negative, + = weak, ++ = moderate, +++ = strong hybridization signal.

RESULTS

In situ hybridization of sections of TGEV-infected tissues with ^{35}S -labelled RNA probe

Preliminary experiment on comparison of the effect of two hybridization buffers

Studies were first performed by using a radiolabelled riboprobe to compare the hybridization using a standard hybridization buffer with that using REH buffer. Hybridization using the standard hybridization buffer was performed at 52°C whereas hybridization with the REH buffer was performed at 70°C , both for 0.5, 1 and 2 h. Hybridization signals were detected with both hybridization buffers at all times examined; however, the signal was consistently more intense with the REH buffer than with the standard hybridization buffer. Intensity of the signal after 2 h of hybridization with the REH buffer was greater than that at 20 h using the standard hybridization buffer. This experiment suggested that the hybridization buffer and temperature may impact hybridization kinetics.

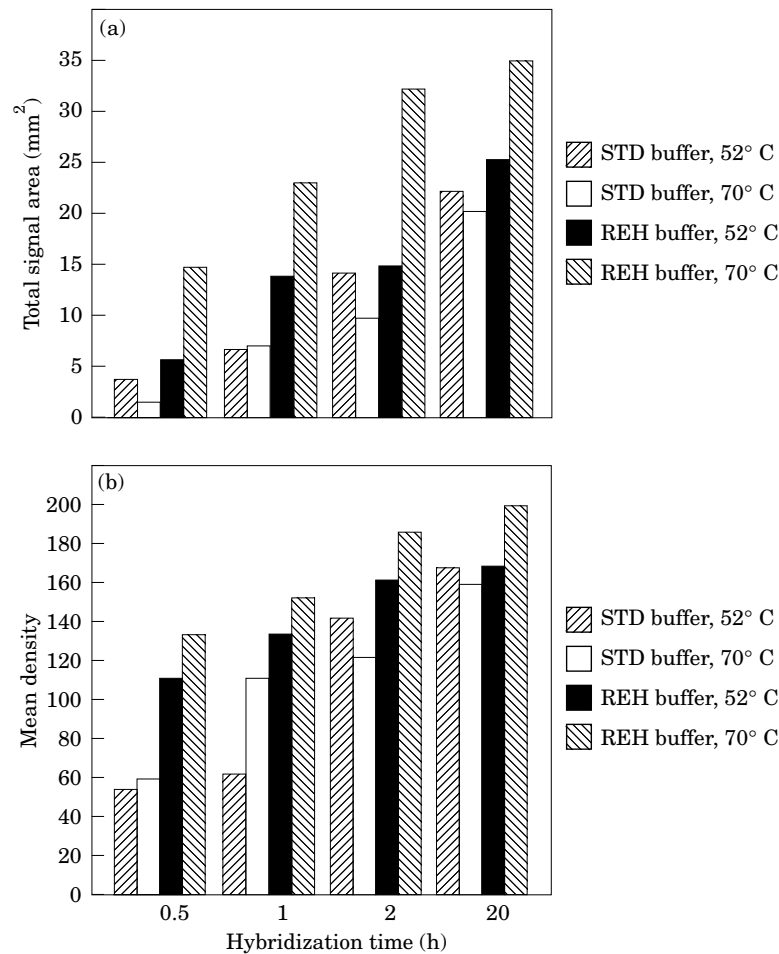


Fig. 1. Comparison of a standard hybridization (STD) buffer with the rate enhancement hybridization (REH) buffer at two different hybridization temperatures, showing the relationship between total area of signal (mm²) and hybridization times (a) and relationship between mean density of hybridization signal and hybridization times (b).

Effect of temperature on hybridization with two buffers

To further determine the effect of the temperature and the buffers, hybridization was performed using the standard hybridization and REH buffer each at two different temperatures of 52°C and 70°C for 0.5, 1, 2 and 20 h of hybridization. Hybridization with the standard hybridization buffer was best at 52°C, whereas for the REH buffer, the optimal hybridization temperature giving the best signal was 70°C (Fig. 1a & b). The hybridization signal at 70°C with the REH buffer after 2 h hybridization was equivalent to or better than that detected after 20 h of hybridization at 52°C with the standard hybridization buffer. Sections hybridized with the REH buffer for 2 h at 70°C also gave the best signal-to-noise ratio and the most intense signal when compared with the standard hybridization buffer at both 52°C and 70°C, and the

REH buffer at 52°C for 20 h (Figs 1 & 2). The intensity of the hybridization signal increased with time as the hybridization signal with the REH buffer at 70°C was less intense at 2 h compared to 20 h. However, the background was also higher after 20 h hybridization than after 2 h hybridization. Viral RNA could be detected with the REH buffer at 70°C as early as 30 min.

Microscopic examination of intestinal sections hybridized with ³⁵S-labelled RNA probe

Sections obtained from infected pigs and coated with emulsion gel had heavy concentrations of silver grains along the mucosal layer. In contrast, sections from uninoculated control animals were negative for silver grains. The silver grains were primarily concentrated over the cytoplasm of the villous enterocytes (Fig. 3),

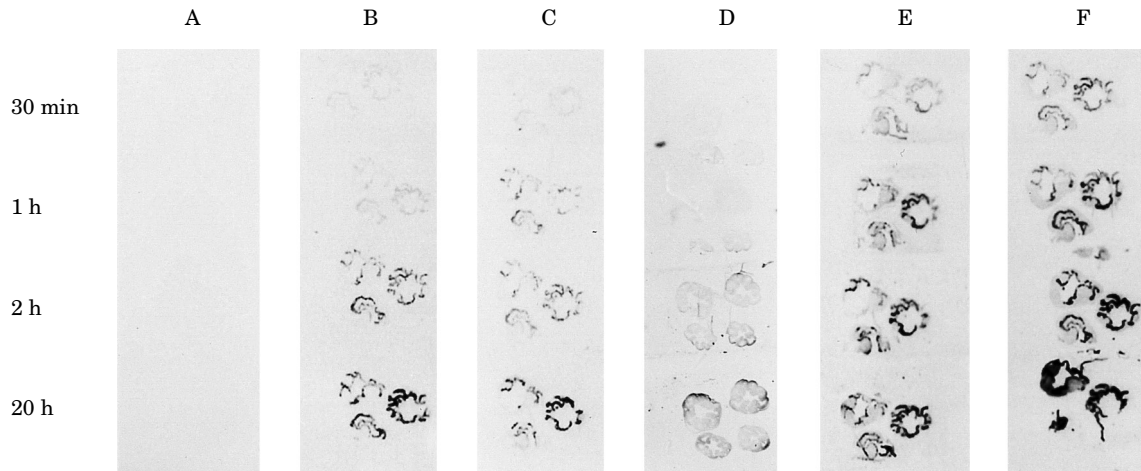


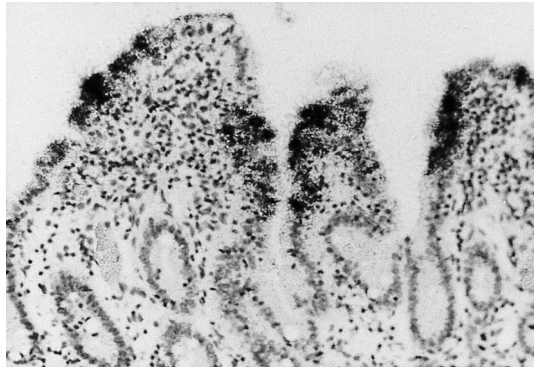
Fig. 2. Photograph of an X-ray film showing autoradiographic results of the effect of hybridization buffers and temperatures on *in situ* hybridization (ISH). Sections of formalin-fixed paraffin-embedded small intestines of TGEV-inoculated pigs (B, C, E, and F) and uninoculated control (A and D) were hybridized with ^{35}S -RNA probe with standard hybridization and rate enhancement hybridization (REH) buffer at 52°C and 70°C. Sections in panels A and B were hybridized using standard hybridization buffer at 52°C and those in panel C were hybridized using standard hybridization buffer at 70°C. Sections in panels D and F were hybridized using the REH buffer at 70°C and those in panel E were hybridized using REH buffer at 52°C. The results showed the hybridization signal as dark continuous lines along the mucosal surface of the TGEV-inoculated small intestinal sections. Such a hybridization signal was not detected in small intestines from uninoculated pigs. Best results were obtained with REH buffer after 2 h hybridization at 70°C.

and some were present in crypt epithelial cells. Signal intensity was similar to the autoradiographic results captured on X-ray films. Sections that were hybridized with the REH buffer at 70°C for 2 h had higher concentration of silver grains than those hybridized with the standard hybridization buffer at 52°C for 20 h (Fig. 3a and b). Sections that hybridized with the REH buffer had higher non-specific silver grains than standard hybridization buffer, which increased with time. The histopathologic changes observed in the small intestines from the infected animals were villous atrophy, blunted and denuded villi, and replacement of the columnar epithelial cells by flat to cuboidal cells.

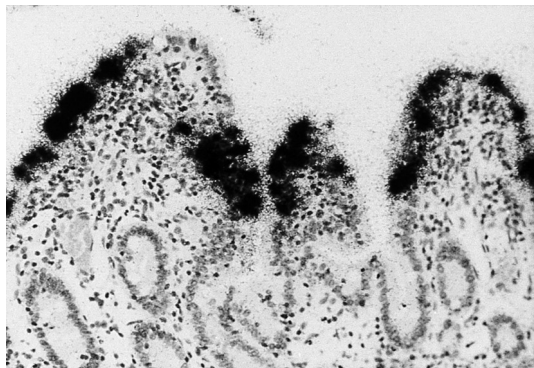
***In situ* hybridization on sections of TGEV-infected tissues using a fluorescein-labelled RNA probe**

Once the hybridization conditions were standardized with the radiolabelled probe, we compared the effect of standard hybridization buffer at 52°C with the REH buffer at 70°C by using a fluorescein-labelled RNA probe. Viral nucleic acid was detected in enterocytes of the small intestinal sections hybridized with either buffer, mainly in villous epithelial cells (Fig. 4) and some crypt epithelial cells. Dark purple colour was detected in the cytoplasm without any staining of the

nucleus. Sections hybridized with the REH buffer at 70°C clearly had a higher signal intensity and a greater number of viral infected cells than sections hybridized with standard hybridization buffer at 52°C, at each hybridization time (Table 1). Viral infected cells were detected after 2 h of hybridization using standard hybridization buffer at 52°C. In contrast, viral infected cells were detected after 30 min of hybridization with the REH buffer at 70°C. This treatment gave an equivalent or more intense signal, and a greater number of viral infected cells than sections hybridized with standard hybridization buffer at 52°C for 2 h. Sections hybridized with the REH buffer at 70°C for 20 h had the highest signal intensity, and the greatest number of viral infected cells. There was only a slight increase in signal intensity after 20 h hybridization with REH buffer at 70°C without an increase in the number of virus infected cells over those hybridized for 2 h. The signal intensity and the number of virus infected cells on sections hybridized with the REH buffer at 70°C for 2 h was equal to or higher than that with sections hybridized with standard hybridization buffer at 52°C for 20 h (Fig. 4 a & b). The signal intensity and the number of viral nucleic acid positive cells were less after 1 h and 30 min compared to 2 h hybridization. The intensity of the signals varied from purple after 30 min, to dark purple after 1 h and to very dark purple after both 2 h and 20 h hybridization. Sections hybridized with the REH



(a)



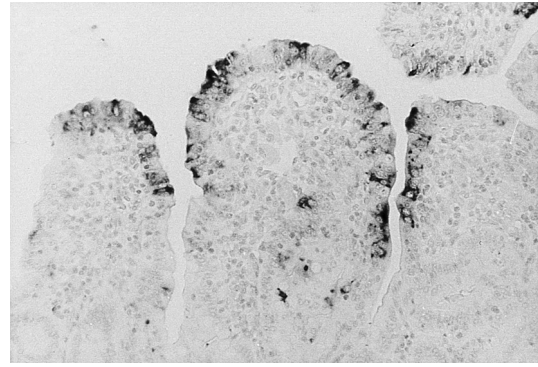
(b)

Fig. 3. Photographs of intestinal villi of small intestines from a transmissible gastroenteritis virus-inoculated pig following *in situ* hybridization with ^{35}S -RNA probe in the standard hybridization buffer at 52°C for 20 h (a), or the rate enhancement hybridization (REH) buffer at 70°C for 2 h (b).

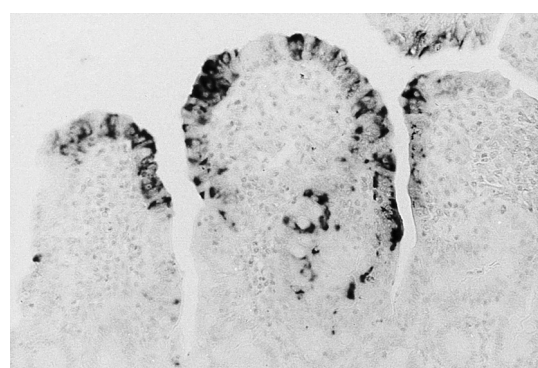
buffer containing the fluorescein-labelled RNA probe did not give a higher non-specific signal compared to the standard hybridization buffer.

DISCUSSION

In this study, we have described a rapid ISH technique for the detection of nucleic acids in formalin-fixed paraffin-embedded tissues. Hybridization of either radiolabelled and fluorescein-labelled probes with the REH buffer decreased hybridization time from 20 to 2 h without compromising the intensity or the quality of the hybridization signal. Optimal results with strong signal and signal-to-noise ratio were obtained with both radiolabelled and fluorescein-labelled probes by using the REH buffer at 70°C for 2 h. One minor disadvantage of using the REH buffer for isotopic ISH was the slight increase in non-specific



(a)



(b)

Fig. 4. Photograph of intestinal villi of small intestines from a transmissible gastroenteritis virus-inoculated pig following *in situ* hybridization with fluorescein-labelled RNA probe using the standard hybridization buffer at 52°C for 20 h (a) or the rate enhancement hybridization (REH) buffer at 70°C for 2 h (b).

signal with increased hybridization time, but this increase in non-specific signal was not a problem with 2 h hybridization and was not detected with non-isotopic ISH. Sections hybridized with the REH buffer containing a fluorescein-labelled RNA probe at 70°C detected viral infected cells as early as 30 min of hybridization whereas use of the standard hybridization buffer at 52°C only detected viral infected cells after 2 h or more of hybridization. The intensity of the signal with both types of probes decreased with shorter hybridization time of 1 h and 30 min, but the signal was still visible. Signal intensity and hybridization time in ISH are probably impacted by the copy number of the target nucleic acids which require at least 10 copies in a cell.²² It may be possible to decrease hybridization time to 1 h or even 30 min, with a higher copy number of target nucleic acid. Morphology of the cells was not impacted with either hybridization conditions

Table 1. Effect of hybridization buffer and temperature on detection of viral RNA in small intestinal sections^a by non-isotopic ISH

Buffer	Hybridization temperature (°C)	Time (h)			
		0.5	1	2	20
Standard hybridization	52	– ^b	–	+	+++
REH buffer	70	+	++	+++	+++

^aSmall intestinal sections from two inoculated pigs infected with 10⁵ pfu of virulent Miller strain of TGEV and five TGEV-positive cases by direct immunofluorescence were used in ISH.

^bHybridization results – = negative, + = weak, ++ = moderate, +++ = strong hybridization signal.

Standard ISH procedures require incubation of tissue sections with the probe for at least 16 h.^{1–4} Martinez-Montero *et al.* (1991)⁸ and Musiani *et al.* (1994)⁷ have reported methods for reducing hybridization time to 2 and 3 h, respectively, by using hybridization buffer with 50% formamide. The method of Martinez-Montero *et al.* (1991)⁸ required incubation of samples at 90°C in a hot air oven for 15 min before hybridization with a RNA probe of 600 nucleotides. Musiani *et al.* (1994)⁷ used cytospin preparations instead of formalin-fixed paraffin-embedded tissue to hybridize with a DNA probe. Both studies used higher concentrations of probes than we used in this study. We used a commercially available REH and fluorescein-labelled RNA probe with an approximate size of 600 bases. The composition of this proprietary REH buffer is not known and according to manufacturer's instructions, it was developed for membrane hybridization such as Southern blot, northern blot, and dot blot hybridization using radiolabelled DNA, RNA or oligonucleotide. The REH buffer appears to be less viscous than the standard hybridization buffer. Although the mechanism of enhancement of hybridization using REH buffer is not known, it may be due to the unfolding of viral RNA secondary structure, resulting in dissociation of protein-nucleic acid complexes so that more target molecules are accessible for hybridization with the probe. The lower viscosity of the REH buffer may play a role in the higher rate of movement of the probe to find the target RNA. We have shown that the REH buffer can be used in ISH for both radio-labelled and fluorescein-labelled RNA probes to enhance hybridization from overnight to 2 h with this buffer without compromising the intensity of the signals when compared with a standard method.¹⁸ Normally, the entire procedure could be performed within one and a half days for isotopic ISH or about 7–8 h for non-isotopic ISH. Total time could be decreased to within a day using the REH buffer with non-isotopic ISH which makes this test comparable to

immunohistochemistry and a good candidate for application in routine diagnosis. We have utilized ISH in our diagnostic laboratory for the detection of TGE virus in selected diagnostic cases. The conditions of rapid ISH technique described in this paper are for the detection of ribonucleic acids with riboprobe. It is not known whether this methodology will work for the detection of DNA or with DNA probes as the bond between RNA and RNA is stronger than that between DNA and DNA or DNA and RNA. Therefore, additional studies are needed to examine potential use of the REH buffer for detecting DNA and for use with DNA probes.

ACKNOWLEDGEMENTS

We would like to thank Drs L. D. Miller, H. W. Moon, D. C. Beitz and B. J. Thacker for review of this article. This study was supported with a grant from the Iowa Livestock Health Advisory council. Senior author T. Sirinarumitr was supported with a King's scholarship from the Anandhamahidol Foundation, Thailand. We would also like to thank Margaret Carter at ISU Image Analysis Facility for assistance with quantitation of image signal.

REFERENCES

1. Angerer L. M. & Angerer R. C. (1992). *In situ* hybridization to cellular RNA with radiolabeled RNA probe. In *In situ hybridization: A Practical Approach*. (Wilkinson, D. G., ed.) Pp. 15–32. New York: Oxford University Press.
2. Kohler C. R. & Nelsen J. A. (1990). Technique for double-labelling virus infected cells. In *Animal Virus Pathogenesis a Practical Approach*. (Oldstone, M. B. ed.) Pp. 67–86. New York: Oxford University Press.
3. Gibson S. J. & Polak J. M. (1990) Principles and applications of complementary RNA probes. In *In situ Hybridization Principles and Practice*. (Polak, J. M. & McGee, J. O., eds) Pp. 81–94. New York: Oxford University Press.
4. Wilcox J. N. (1993) Fundamental principles of *in situ*

- hybridization. *Journal of Histochemistry and Cytochemistry* **41**, 1725–33.
5. Syrjänen, S. M. (1992). Viral gene detection by *in situ* hybridization (Herrington, C. S. & McGee, J. O. eds) Pp. 103–39. New York: Oxford University Press.
 6. Morrell, J. I. (1989) Application of *in situ* hybridization with radioactive nucleotide probes to detection of mRNA in the central nervous system. (Bullock, G. R. & Petrusz, P., eds) Pp. 127–46. London: Academic Press.
 7. Musiani, M., Zerbini, M., Venturoli S. *et al.* (1994) Rapid diagnosis of cytomegalovirus encephalitis in patients with AIDS using *in situ* hybridisation. *Journal of Clinical Pathology* **47**, 886–91.
 8. Martinez-Montero J. C., Herrington, C. S., Stickland J. *et al.* (1991). Model system for optimizing mRNA non-isotopic *in situ* hybridisation: riboprobe detection of lysozyme mRNA in archival gut biopsy specimens. *Journal of Clinical Pathology* **44**, 835–9.
 9. Saif, L. J. & Wesley, R. D. (1992) Transmissible gastroenteritis. In *Diseases of Swine*. (Leman, A. D., Strauss, B. E., Mengeling, W. L., D'Allaire, S. & Taylor, D. J., eds) Pp. 362–86. Iowa: Iowa State University Press.
 10. Delmas, B., Gelfi, J., L'Haridon R. *et al.* (1992) Aminopeptidase N is a major receptor for the enteropathogenic coronavirus TGEV. *Nature* **357**, 417–20.
 11. Weingart, H. & Derbyshire, J. B. (1994). Evidence for a putative second receptor for porcine transmissible gastroenteritis virus on the villous enterocytes of newborn pigs. *Journal of Virology* **68**, 7253–9.
 12. Thake, D. C. (1968). Jejunal epithelium in transmissible gastroenteritis of swine (an electron microscope and histochemical study). *American Journal of Pathology* **53**, 149–69.
 13. Pensaert, M. B., Haelterman, E. O. & Hinsman, E. J. (1970). Transmissible gastroenteritis of swine: virus-intestinal cell interactions II. Electron microscopy of the epithelium in isolated jejunal loops. *Archiv fuer die Gesamte Virusforschung* **31**, 335–51.
 14. Wagner, J. E., Beamer, P. D. & Restic M. (1973). Electron microscopy of intestinal epithelial cells of piglets infected with a transmissible gastroenteritis virus. *Canadian Journal of Comparative Medicine* **37**, 177–88.
 15. Paul, P. S., Halbur, P. G. & Vaughn, E. M. (1994). Significance of porcine respiratory coronavirus infection. *Compendium on Continuing Education for the Practicing Veterinarian* **16**, 1223–34.
 16. Vaughn, E. M., Halbur, P. G. & Paul, P. S. (1995). Sequence comparison of porcine respiratory coronavirus isolates reveal heterogeneity in the S, 3, 3-1 genes. *Journal of Virology* **69**, 3176–84.
 17. Vaughn, E. M. & Paul, P. S. (1993). Antigenic and biological diversity among transmissible gastroenteritis virus isolates of swine. *Veterinary Microbiology* **36**, 333–47.
 18. Sirinarumitr, T., Paul, P. S., Kluge, J. P. & Halbur, P. G. (1996). *In situ* hybridization for the detection of swine enteric and respiratory coronavirus, transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV), in the formalin-fixed paraffin-embedded tissues. *Journal of Virological Methods* **56**, 149–60.
 19. Vaughn, E. M., Halbur, P. G. & Paul P. S. (1994). Three new isolates of porcine respiratory coronavirus with various pathogenicities and spike (S) gene deletions. *Journal of Clinical Microbiology* **32**, 1809–12.
 20. Britton, P. & Page, K. W. (1990). Sequence of the S gene from a virulent British field isolate of transmissible gastroenteritis virus. *Virus Research* **18**, 71–80.
 21. Vaughn, E. M., Halbur, P. G. & Paul, P. S. (1996). Use of non-radioactive cDNA probes to differentiate porcine respiratory coronavirus and transmissible gastroenteritis virus isolates. *Journal of Veterinary Diagnostic Investigation* **8**, 241–4.
 22. Nuovo, G. F. (1994). *In situ* hybridization. In *PCR in situ Hybridization: Protocols and Applications*. (Nuovo, G. F., ed.) Pp. 100–68. New York: Raven Press.