RNA *IN SITU* HYBRIDIZATION FOR THE DETECTION OF FELINE IMMUNODEFICIENCY VIRUS IN INFECTED CELLS

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Abstract. In the present study, we have applied a novel approach to generate specific digoxigenin- and biotin-labeled RNA probes to detect Feline Immunodeficiency Virus (FIV) *gag* gene in the FIV-infected feline T-lymphoblastoid cell line (MYA-1). This involved cloning of the FIV *gag* gene into a PCR Script vector containing both T3 and T7 promoters, followed by amplification of the insert and the two promoter sequences, using specific primer sequences. The FIV RNA probes were more sensitive than FIV DNA probes. This approach should make RNA *in situ* hybridization more accessible for use in routine diagnosis.

INTRODUCTION

Feline Immunodeficiency Virus (FIV) is an RNA virus and a member of the lentivirus group (Angerer *et al*, 1992). FIV infects the central nervous system shortly after infection, and is associated with neuropathology in natural and experimental infections (Johnson *et al*, 1988; Dow *et al*, 1990; Hurtel *et al*, 1992; Boche *et al*, 1996).

In situ techniques such as RNA *in situ* hybridization (ISH) allow visualization of gene expression in individual cells within their histological context (Angerer *et al*, 1992; Gibson and Polak, 1992; Looi and Cheah, 1992). The type of probe used and how it is labeled is very important for the outcome of ISH (Wahl *et al*, 1979; Gibson and Polak, 1992). DNA probes are generally easy to generate, but they are less sensitive than their RNA counterparts (Gibson and Polak, 1992). However, the preparation of specific and sensitive RNA probes can be problematic. In order to generate RNA probes, the fragment of interest must be sub-cloned into a suitable vector with T3, T7 and SP6 phage promoters (Sunday *et al*, 1991). This allows for the generation of both control sense and anti-sense RNA probes from the same subclone. Then this cDNA template must be linearized at one end of the insert to limit the length of the probe to the insert size or smaller (Gibson *et al*, 1992).

Previously, we have generated RNA probes using a novel approach to detect the expression of RNA component of telomerase (hTR), in oral lichen planus (O'Flatharta *et al*, 2002). This approach is based on designing specific primers to amplify by PCR both T3 and T7 promoters and the insert (gene of interest) located between these promoters within the PCR script vector. This PCR product contains the gene of interest insert (FIV *gag* gene) flanked by T3 and T7 promoters (see Fig 1 for further details). This approach does not require the presence of promoter sequence at the 5′ end of oligonucleotide used to prime the PCR as described previously (Wu *et al*, 1995). In addition, this method does not require the linearization of the cDNA template and, PCR amplification results in a high copy of the insert containing both the T3 and T7 promoters, which ultimately leads to the generation of specific and sensitive digoxigenin or biotin-labeled RNA probes for ISH. In the present study, we have utilized the same approach to generate a specific RNA probe to detect FIV RNA in FIV infected feline T-lymphoblastoid cell line (MYA-1).

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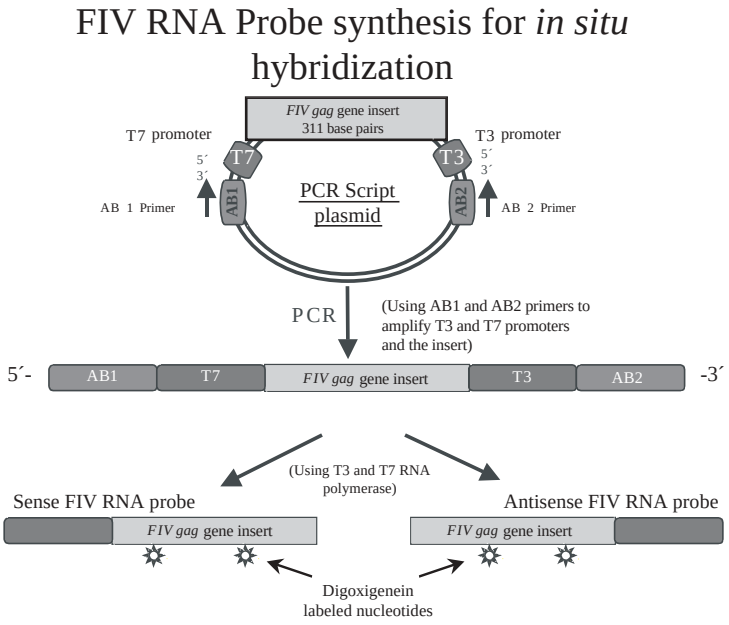


Fig 1–Flow diagram of probe generation for *in situ* hybridization. The 311bp FIV *gag* gene fragment was transformed into a PCR script plasmid. Using AB1 and AB2 primer sites, the *gag* insert and T3 and T7 promoters were amplified by PCR. Using T3 and T7 RNA polymerase, sense and antisense RNA strands were generated, which were then labeled either with digoxigenin or biotin.

MATERIALS AND METHODS

All chemicals used in these methods were obtained from Sigma Chemical, St Louis, USA unless otherwise stated. The FIV infected feline T-lymphoblastoid cell line (MYA-1), was previously established by Miyazawa *et al* (1989). The cell lines were formalin-fixed and paraffin-embedded.

Probe preparation

The FIV DNA clone was a gift from Dr Margaret Hosie, University of Glasgow. This clone contained the entire 9467 bp FIV_{GL-8} genome. A 311bp, a 618bp and a 1064bp fragment from the *gag* gene were amplified by PCR using specific ologonucleotide primers (Table 1) and pfu polymerase.

PCR was conducted in a 50 µl reaction volume. Each reaction contained 1X PCR buffer (10mM Tris-HCl, pH 9, 50mM KCl and 0.1% Triton X-100) 1mM $MgCl_2$, 0.2 mM of each dNTP and 5U of *pfu* DNA polymerase (Promega, USA). The PCR was carried out under the following conditions: initial denaturation step at 94ºC for 2 minutes; cycling step at 94ºC for 30seconds, 52ºC for 1 minute and 72ºC for 1 minute for 25 cycles. After thermal cycling, the products were separated on a 1% agarose gel and stained with ethidium bromide (Fig 3.1). Two fragments, 311 bp and 618 bp were excised from the gel, and purified using a QIAquick gel extraction kit (Qiagen, UK).

All purified fragments were ligated into PCR Script AMP SK(+) at *saf I* site and then transformed into Epicurian Coli XL-10 Blue MRF' Kan ultracompetent cells (Stratagene, UK). White colonies were then chosen and grown in Lemco broth overnight at 37ºC with agitation in an orbital incubator.

Plasmid DNA was recovered using the Genelute™ Plasmid Miniprep Kit (Sigma, UK) as described by the manufacturers. The 311 bp PCR product was sequenced using Visible Genetic DNA sequencer (Visable Genetics, USA). The 618 bp fragment was sent to MWG, UK for sequencing.

PCR amplification of gag gene from PCR Script plasmid was performed with (sense) primer AB2 (5´-GTT TTC CCA GTC ACG ACG-3´) and (antisense) primer AB1 (5´-GAA TTG TGA GCG GAT AAC-3´). These primers were designed to amplify a region containing the FIV *gag* insert flanked by the T3 and T7 promoters. PCR was conducted in a 50 ml reaction volume. Each reaction contained 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl and 0.1% Triton X-100, pH 9) 1mM $MgCl_2$, 0.2 mM of each dNTP and 5U of Taq DNA polymerase (Promega, Germany). The reaction was performed for an initial denaturation cycle of 95ºC for 5 minutes followed by 30 cycles of denaturation at 94ºC for 45 seconds, annealing at 37ºC for 45 seconds and extension at 72ºC

Table 1
FIV primer sequences used for amplifying the *gag* gene.

Primer	Sequence 5′ - 3′
Forward primer	
FIV 771f	*Aga acc tgg tga tat acc aga gac*
Reverse primer	
FIV 1081r	Ttg ggt caa *gag* cta cat att g
Forward primer	
M21s (FIV 1083)	*Atg gtg tcc att ttt atg g**
Reverse primer	
296as (FIV 1699)	Aag agt tgc att tta tat cc
Forward primer	
FIV 566f	Acc ttc aag cca gga gat tc
Reverse primer	
FIV 2167r	Cct cct cct act cca atc at

* This primer sequence is for FIV Petaluma strain and differs from FIV_{GL-8} by 2 nucleotides. The FIV_{GL-8} sequence is as follows ATG GTG TCT ATT TTTC.

for 1 minute. Reactions were performed in a programmable DNA thermal cycler (Hybaid, UK). A 5-minute additional extension step was carried out after the last cycle to ensure complete polymerization. For the electrphoretic analysis of amplified DNA, a 5 µl aliquot of PCR product was electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining.

Digoxigenin and biotin labeling of FIV RNA probes

Probe labeling was performed using T3 RNA polymerase and T7 RNA polymerase (Boehringer Mannheim/ Roche Molecular Biochemicals, UK). Labeling was conducted in a 20 µl reaction volume. Each reaction volume contained 10X transcription buffer, digoxigenin or biotin labeling mix, either T3 or T7 RNA polymerase (all from Boehringer Mannheim/ Roche Molecular Biochemicals), Rnasin (RNAse inhibitor) (Promega, Germany), sterile RNase-free water and DNA template (PCR product). The labeling procedures were carried out as described previously by O'Flatharta *et al* (2002).

Digoxigenin and biotin labeling of FIV DNA probes

Three different approaches were used for generating FIV DNA probes. The 311 bp, 617 bp and 1,084 bp DNA fragments of FIV were labeled by nick translation using Bionick™ kit (Lifetechnologies, USA), as instructed by the manufacturer. The 311 bp and 617 bp fragments were also labeled with digoxygenin by Dig-High prime (Boehringer Mannheim/Roche Molecular Biochemicals, UK), which labels by random priming. Two fragments, 617 bp and 1084 bp were labeled using chemlink (Boehringer Mannheim/ Roche Molecular Biochemicals, UK), according to manufacturer's instructions. Briefly, the chemlink and template were incubated at 85ºC for 30 minutes. Chemlink labels guanosine and adenosine bases with biotin.

In situ hybridization

Pre-hybridization and post-hybridization treatment using FIV RNA probes protocols were carried out as described in our previous report (O'Flatharta *et al*, 2002). Briefly the pre- and post-hybridization procedures were carried as the following:-

Pre-hybridization treatment. The sections were treated with 50 µg/ml Proteinase K in Proteinase K buffer (50 mM Tris-HCl pH 7.5, 5mM EDTA) for 7 minutes and immediately washed in PBS for 5 minutes. Once the tissue sections were dehydrated fully the probe was then added.

The concentration of probe added to each section was 5 µg probe/130 µl hybridization buffer (Dako mRNA *in-situ* hybridization solution, DAKO Corporation, Carpinteria, CA 93013, USA). The slides were coverslipped and sealed in a humid reaction chamber, to prevent the sections from drying out. The probe was allowed to hybridize for 12-16 hours at 52ºC.

Post-hybridization treatment. Two 15-minutes 2X SSC stringency washes were performed to remove unhybridized probe from sections. A 20-minutes 50% formamide wash (made up using 2X SSC) was carried out at this point. All these washes were carried out at room temperature.

The slides were transferred into a jar containing Ribonuclease A buffer (10mM Tris-HCl pH8.0, 5mM EDTA, 0.5M NaCl) at 37ºC for 10 minutes, then treated with RNase A at a concentration of 10 µg/µl for 20 minutes and finally rinsed in large volumes of RNase A buffer at 37ºC

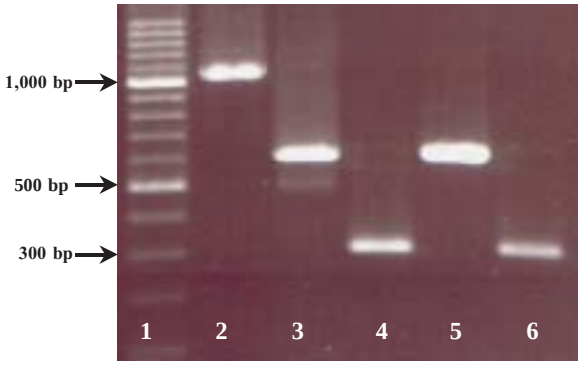


Fig 2–PCR products amplified from FIV *gag* gene. (Lane 1) molecular weight marker; (lane 2) PCR product from the primers FIV 1082f and FIV 2167r (1084bp); (lanes 3 and 5) PCR product from the primers 1082f and 1700r (618bp) and (lanes 4 and 6) PCR product from the primers FIV 771f and 1081r (311bp).

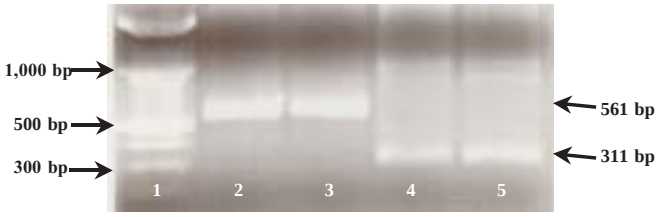


Fig 3–PCR product amplified from the 561 bp AB1/ AB2 primed PCR product. Lane1: 100 bp marker. Lanes 2, 3: PCR Script containing 311 bp insert amplified as in Fig 3; lanes 4,5: PCR Script containing 311 bp insert amplified using *gag* primers

(5 X five minutes washes).

Following this, the slides were washed in 4 changes of 2X SSC with a further treatment for 15 minutes in 0.1X SSC, all of these washes were carried out at 52ºC, with the 2X SSC washes needing gentle agitation.

The protocol used for DNA probes was as described by Mabruk *et al* (1994).

Visualization of bound DNA and RNA digoxigenin-labeled probe were carried out as described by the manufacturer using the digoxigenin nucleic acid detection kit (Boehringer Mannheim/ Roche Molecular Biochemicals), with the following modification: (1) Two cycles of the anti-digoxigenin antibody were applied (Roche);

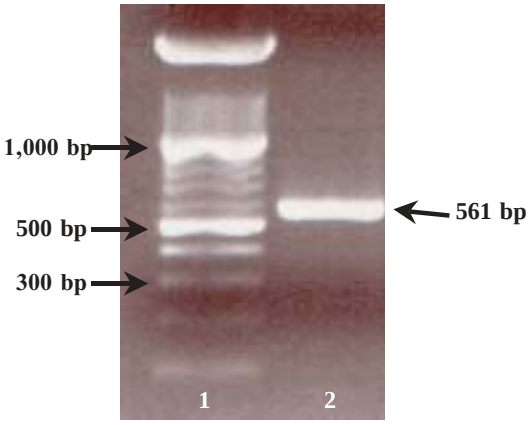


Fig 4–PCR product amplified using AB1 and AB2 primers in the PCR Script vector containing 311 bp *gag* insert. The AB primers, together with the T3 and T7 promoters, add approximately 250 base pairs to the fragment it amplifies. Lane 1: 100 bp marker. Lane 2: PCR Script vector containing 311 bp *gag* insert.

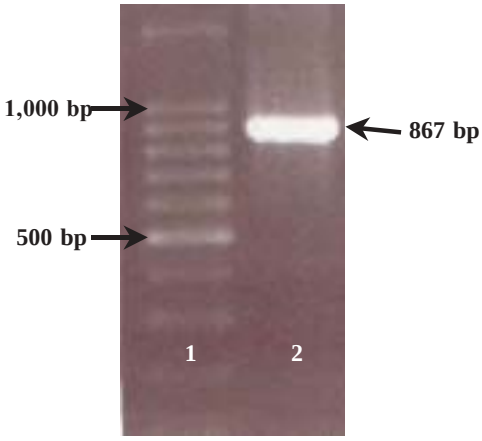


Fig 5–PCR product amplified using AB1 and AB2 primers in the PCR Script vector containing 617 bp *gag* insert. The AB primers, together with the T3 and T7 promoters add an extra 250 base pairs to the fragment it amplifies. Lane 1; 100 bp marker. Lane 2: PCR Script vector containing 617 bp *gag* insert.

(2) after visualization of the signals, the slides were placed in T.E. Buffer (stop buffer) for approximately 36 hours to ensure that further signal development was completely halted. G.V.A mounting solution (Zymed laboratories, USA), was used as mounting medium. The biotin-labeled probes were visualized using a Genpoint™ System (Dako, CA).

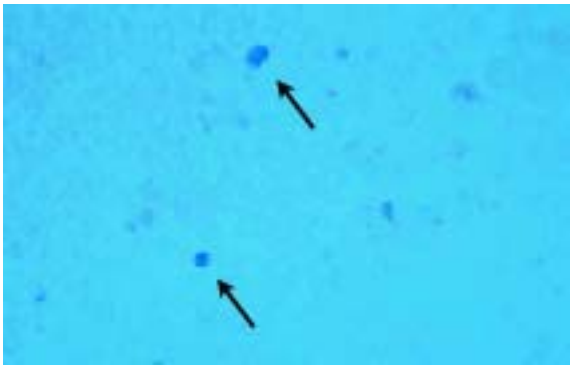
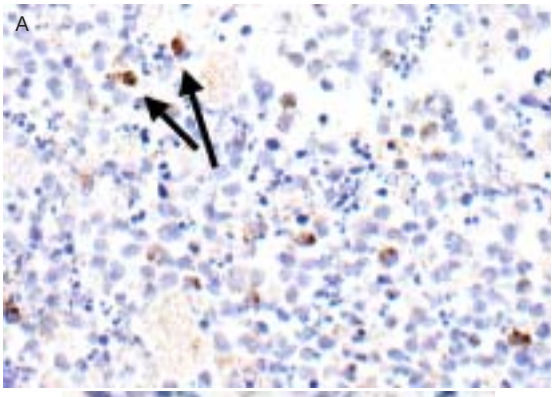


Fig 6–FIV RNA detection in FIV infected MYA-1 cell line using an antisense probe. A digoxigenin labeled FIV RNA, 311bp antisense probe was used to detect FIV RNA in MYA-1 infected cells. Positive signals for FIV RNA appeared as a dark purple stain (arrow head); (Magnification x400).

Fig 7–FIV RNA detection in FIV infected MYA-1 cell line using a sense probe. A digoxigenin labeled FIV RNA, 311bp sense (control) probe was applied to this section. The sense probe was used as a negative control. The lack of staining indicates that the sense probe does not non-specifically bind to the target nucleic acid in FIV infected MYA-1 cell line (Magnification x400).

RESULTS

Fragments of length 311bp, 617bp and 1,084 bp were successfully amplified from the PCR Script plasmid by PCR (Fig 2). The 311 bp and 618 bp fragments were then cloned into PCR Script plasmid as demonstrated by amplifying the two fragments of correct size using the primers AB1 and AB2 contained within the PCR Script plasmid. These two fragments were se-



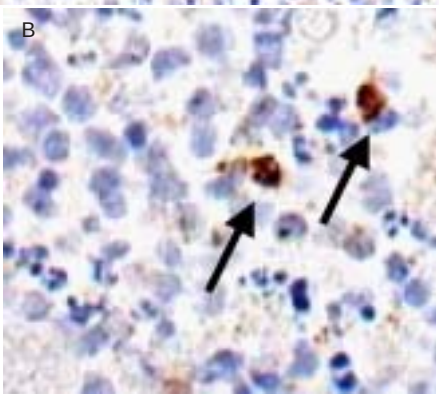


Fig 8–(A). FIV RNA detection in FIV infected MYA-1 cell line. A biotin labeled FIV RNA, 311bp T3 antisense probe was used to detect FIV RNA. After color development, biotin labeled probes produce a brown color staining in FIV infected cells (arrow head); (Magnification x400). (B) Clearer illustration of Fig 8 (A).

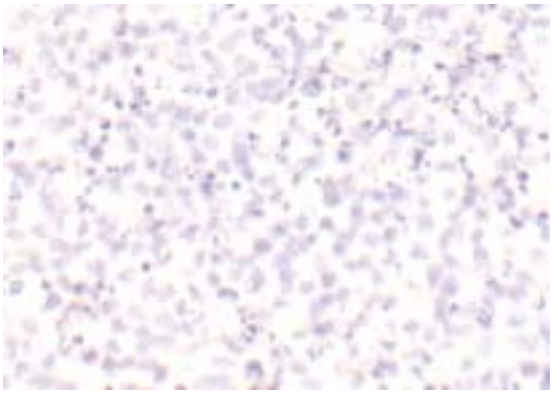


Fig 9–FIV RNA detection in FIV infected MYA-1 cell line. A biotin labeled FIV RNA, 311bp T7 sense (control) probe was applied to FIV infected MYA-1cell line. The lack of staining indicates that the sense probe does not non-specifically bind to section. (Magnification x400).

quenced and found to have the expected FIV *gag* sequences. To confirm that the 311 bp *gag* insert was included in the AB1/AB2 PCR product, a PCR was conducted on the AB1/AB2 PCR product using *gag* primers (771-1081) for the *gag* insert producing the expected 311 bp insert (Fig 3).

The T3, T7 promoters and the gene of interest (FIV *gag*) were successfully amplified by PCR using the specifically designed AB1 and AB2 primer sequences. There were two PCR products, one from amplifying the 311 bp *gag* gene insert and the other from amplifying the 617 bp fragment. The AB primers, together with the T3 and T7 promoters, add approximately 250 bps to each fragment resulting in PCR products of 561 bp (Fig 4) and 867 bp (Fig 5).

The FIV antisense digoxigenin-labeled RNA probe gave clear blue/purple staining in the FIV-infected MYA-1 cell culture (Fig 6). Using the FIV sense RNA probe, no staining was seen in FIV infected MYA-1cell culture (Fig 7). When both probes were applied to FIV non-infected MYA-1 cell culture, no digoxigenin staining was observed thus indicating there was an absence of non-specific staining. Biotin labeled antisense RNA probe gave clear brown staining in the FIV infected MYA-1 cell culture (Fig 8). Using sense biotin-labeled RNA FIV probe no staining was seen in FIV infected MYA-1cell culture (Fig 9).

No signals for FIV were detected in MYA-1 infected cells using the DNA probes labeled with Bionick, Chemlink or Dig-High prime, indicating that the DNA probes were not sensitive enough to detect FIV RNA in MYA-1 infected cells.

DISCUSSION

In situ hybridization (ISH) is a technique that enables detection of specific nucleic acid sequences (DNA, RNA) at a single cell level with respect to distribution as well as quantification (Shin and Koji, 1998; Speel *et al*, 1999). Although, ISH is a powerful tool, optimization of the technique has proven to be a difficult and wearisome task (Erdtmann-Vourliotis *et al*, 1999). The probe generated has to be sensitive and specific enough to detect the target nucleic acid sequence.

Investigators have conducted many approaches in order to overcome these problems with the use of fluorescent labels (Nederlof *et al*, 1992; Durrant *et al*, 1995) and the use of signal amplification buffers (Huang *et al*, 1998). In the present study, the capability of using a novel approach to generate a specific and sensitive RNA probe for the detection of FIV RNA in MYA-1 cells infected experimentally with FIV was demonstrated. This approach involved the use of PCR with specifically designed AB1/AB2 primers. These primers amplify T3, T7 promoters and the insert (gene of interest) located between these promoters within the PCR Script vector. This was followed by *in vitro* transcription, which incorporates a digoxigenin-labeled nucleotide, in order to produce control sense and anti-sense RNA probes. (O'Flatharta *et al*, 2002). The RNA probe label was also changed from digoxigenin to biotin, which also detected FIV RNA in cell culture.

In one study, the use of *in situ* hybridization and PCR (PCR-ISH) was employed to detect FIV nucleic acid, which although provides far greater sensitivity, it is extremely difficult to optimize and not broadly accepted as a reliable technique (Elder *et al*, 1993).

In addition and within the present study, FIV specific DNA probes were then generated from the same two regions of the *gag* gene (771-1081 and 1082-1700) and labeled with biotin and digoxigenin. No signal were detected in FIV infected MYA-1 cell culture. This finding is in agreement with previous findings that DNA probes are less sensitive than RNA probes (Gibson and Polak, 1992).

In summary, the detection of FIV RNA in cell culture and telomerase in oral lichen planus (O'Flatharta *et al*, 2002) has validated this probe labeling technique. Also, this study clearly indicated that RNA probes, generated using our approach, were more sensitive than DNA probes. This technique will help to simplify the *in situ* hybridization procedures in diagnostic molecular virology and pathology laboratories.

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