



## LETTER

# A SYBR-Green I quantitative Real-time Reverse Transcription-PCR assay for rabies viruses with different virulence

Dear Editor,

To establish a more specific diagnostic assay than the current methods for rabies virus (RABV) infection in canines and wildlife species, we developed a SYBR-Green I quantitative real-time reverse transcription-PCR (RT-PCR). Three primers were designed and synthesized: one of which, located within the leader sequences and targeted for the whole viral genome amplification to obtain its cDNA, is designated as reverse transcription primer; the other two (as a pair), located within the conserved region of the nucleoprotein gene and aimed at amplifying the nucleoprotein gene, are designated as amplification primers. This real-time RT-PCR can be used for rapid qualitative analysis on dog brain samples and quantitative analysis in fundamental research.

Rabies virus is the prototype of *lyssavirus*, *Rhabdoviridae*. It is the causative agent of rabies and has caused an annual death of 2000-3000 people in recent years in China (Yang D-K, et al., 2012; Yin C P, et al., 2012; Zhang S, et al., 2009). The diagnostic methods currently for rabies include direct viral observation, assay of virus-related proteins, and detection of the viral genome (Hoffmann B, et al., 2009; Yang D-K, et al., 2012). Assay of the viral genome by real-time RT-PCR possesses the merits of specificity, sensitivity, rapidity, low cost, and simplicity (David D, 2012).

The RABV genome consists of a single, negative-stranded RNA molecule, 12 kb long, comprising five structural protein genes, Nucleoprotein gene (N gene, 1.4 kb), Phosphoprotein gene (0.9 kb), Matrix protein gene (0.8 kb), Glycoprotein gene (1.6 kb) and RNA-dependent RNA-polymerase gene (6.5 kb), in 5' to 3' orientation (Jackson A C, 2011). The N gene, the most conserved of the five structural protein genes, yields much more transcriptional mRNA than the other four (Jackson A C, 2011; Yang D-K, et al., 2012). It has been extensively used as the target gene for rabies molecular diagnosis in most laboratories (Nagaraj T, et al., 2006; Wang X, et al., 2010).

By analyzing the sequences of 200 RABV strains in GenBank database using the Bioedit Sequence Alignment Editor, we found that the leader sequences (1-18nt) of the genomes are highly specific and conserved. A reverse transcription primer (RV-Q-RT-P, shown in Table 1;

the degeneracy bases are indicated in bold letters) located in this region was therefore designed and used in this experiment to obtain the cDNA of the viral genome. A pair of primers (RV-NP-Q-Forward, RV-NP-Q-Reverse, Table 1) specific for N gene was designed and used for amplification, as described previously (Meslin FX, et al., 1996).

We tested a range of RABV strains and samples, using commercially available reagents (purchased from TaKaRa Biotechnology Co., Ltd, Dalian, China, except where noted) for RNA extraction and real-time quantitative RT-PCR to simplify the manipulation process. The RABV strains and samples comprised: BD06 (GenBank Accession No. EU549783); CVS-24 (HM535790.1); SRV9 (AF499686.2); four rabies dFA-positive brain samples (dFA, direct immunofluorescence assay) of dead Chinese ferret badgers from Jiangxi province; two rabies dFA-negative Chinese ferret badgers brain samples; Canine Parvovirus (CPV) JL09-11; Canine Distemper Virus (CDV), vaccine strain of Nobivac<sup>®</sup> Canine 3-DAPv (MERCK Animal Health Corp., New Jersey, USA) (Liu Y, et al., 2010; Zhang S, et al., 2009). All samples and strains were prepared by homogenization in 20% (wt/vol) phosphate-buffered saline (PBS) on ice in a Bio-safety Level 2 containment cabinet. After total RNA extracting, 1  $\mu$ L was moved to an Eppendorf tube. Reverse primer (RV-Q-RT-P) was added. The mixture was heated at 70  $^{\circ}$ C for 10 min and then kept on ice for 5 min. 0.5  $\mu$ L RNase inhibitor, 1  $\mu$ L dNTPs, 0.5  $\mu$ L reverse transcriptase (M-MLV), and 5  $\mu$ L 5 $\times$ M-MLV buffer were added. The final mixture was incubated at 42  $^{\circ}$ C for 1h to obtain cDNA.

Standard plasmid (pMD18T-BD06-N, 4,443 bp) containing the full-length cDNA of the BD06 N gene was used for establishing the standard curve. The copy number of the standard plasmids was calculated by the total mass of the plasmids in the solution divided by the mass of a plasmid (i.e., 1 plasmid copy=4,443 bp, 1 bp=618 g/mol,

Table 1. Primer sequences and locations

| Primer name     | Sequences (5' to 3')   | Nucleotide position |
|-----------------|------------------------|---------------------|
| RV-Q-RT-P       | ACGCTTAACAACMARAYC     | 1-18                |
| RV-NP-Q-Forward | CAAGATGTGTGCYAAAYTGGAG | 644-663             |
| RV-NP-Q-Reverse | AGCCCTGGTTCGAACATTCT   | 881-900             |

1 plasmid copy =  $4,443 \text{ bp} \times 618 \text{ g/mol/bp} = 4.5 \times 10^{-17} \text{ g}$ . The concentration of the pMD18T-BD06-N solution was determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Shanghai, China).  $1.3 \times 10^8$  copy standard plasmids were contained in 2  $\mu\text{L}$  solution. Serial dilutions were made in TE buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0) to generate six standard concentrations containing,  $1.3 \times 10^8$ ,  $1.3 \times 10^7$ ,  $1.3 \times 10^6$ ,  $1.3 \times 10^5$ ,  $1.3 \times 10^4$  and  $1.3 \times 10^3$  copies, respectively (Puglia A L, et al., 2013). The standard curve was defined as the regression line of the logarithm of copy numbers of standard plasmid *versus* cycle threshold (Ct).

The PCR mixture consists of 0.2  $\mu\text{L}$  forward primer (RV-NP-Q-Forward, 10  $\mu\text{mol/L}$ ), 0.2  $\mu\text{L}$  reverse primer (RV-NP-Q-Reverse, 10  $\mu\text{mol/L}$ ), 10  $\mu\text{L}$  2 $\times$  SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup>, 0.4  $\mu\text{L}$  passive reference ROX (50 $\times$ ), 2  $\mu\text{L}$  reverse transcribed cDNA, and 7.2  $\mu\text{L}$  sterile distilled water. For each template, quadruplicate was set. The PCR was performed at 94  $^\circ\text{C}$  for 30 s,  $40 \times (94 \text{ }^\circ\text{C} / 5 \text{ s}, 60 \text{ }^\circ\text{C} / 30 \text{ s})$ , according to the instructions of SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> and the protocol of the Eco Real-Time PCR System (Illumina Inc., San Diego, California, USA).

Single prominent peak of melting curve could be observed for BD06, SRV9, CVS-24 and the dFA-positive samples of Chinese ferret-badgers; in contrast, no peak were observed for CPV, CDV and the dFA-negative samples of Chinese ferret badgers, respectively. This indicated that the real-time RT-PCR is specific. Among all the RABV samples, the peaks of melting curves of CVS-24 appeared later than BD06, SRV9 and other rabies samples. The reason is probably that CVS-24 possesses a higher G+C% (44.19%) content in the nucleoprotein sequence than BD06 (41.47%), SRV9 (40.31%) and others. The Ct  $\pm$  standard deviation (SD) of the serially diluted standard plasmids were  $7.10 \pm 0.04$ ,  $10.67 \pm 0.06$ ,  $14.30 \pm 0.07$ ,  $17.29 \pm 0.03$ ,  $20.97 \pm 0.14$ ,  $24.51 \pm 0.09$ , respectively. The coefficient of variation (CV) were 0.56%, 0.56%, 0.48%, 0.17%, 0.60%, 0.36%, correspondingly, indicating that the real-time RT-PCR were steady with excellent repeatability. According to the standard curve ( $Y = -3.46X + 27.95$ , Efficiency=94.5%,  $R^2=0.99$ ) and the Ct of sample, the copy numbers of BD06, SRV9, CVS-24 and four rabies dFA-positive brain samples of dead Chinese ferret badgers were determined to be 492654, 244531, 1425081, 777, 2754, 2290 and 794, respectively.

Several previously reported real-time Taqman RT-PCR assays (Hoffmann B, et al., 2010; Wacharapluesadee S, et al., 2012) for RABV have been tested in our lab, but seldom showed good specificity to some Chinese RABV isolates. This probably is due to incompletely unpairing between the probe and the targeted sequence. Therefore, a SYBR-Green I quantitative real-time reverse transcription-PCR assay was established, in which the primer for reverse transcription is located in the leader sequence.

The reverse primer can only transcribe the viral genome rather than the transcribed RNA, in which leader sequence is processed and absent. This further increases the specificity.

The titers of different RABV strains are usually compared by either the median lethal dose (LD<sub>50</sub>) in mice or the median tissue culture infective dose (TCID<sub>50</sub>) in a cell line. However, it is difficult to compare the titers of a street virus or a fixed virus with an attenuated virus accurately at the same time, as street virus are less adapted to a cell lines and an attenuated virus is usually not lethal to adult mice. The quantitative real-time SYBR-green I RT-PCR assay developed in this study is versatile for all the strains with different virulence.

## FOOTNOTES

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All the authors declare that they have no competing interest. All institutional and national guidelines for the care and use of laboratory animals were followed.

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