

## Real-Time Reverse Transcription-Polymerase Chain Reaction for Detection and Quantitation of Turkey Coronavirus RNA in Feces and Intestine Tissues

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### Abstract

Turkey coronavirus (TCoV) infection causes acute atrophic enteritis in turkey poults, leading to significant economic loss in the turkey industry. Rapid detection, differentiation, and quantitation of TCoV are critical to the diagnosis and control of the disease. A specific one-step real-time reverse transcription-polymerase chain reaction (RT-PCR) assay using TCoV-specific primers and dual-labeled fluorescent probe for detection and quantitation of TCoV in feces and intestine tissues is described in this chapter. The fluorogenic probe labeled with a reporter dye (FAM, 6-carboxytetramethylrhodamine) and a quencher dye (Absolute Quencher™) was designed to bind to a 186 base-pair fragment flanked by the two PCR primers targeting the 3' end of spike gene (S2) of TCoV. The assay is highly specific and sensitive and can quantitate between  $10^2$  and  $10^{10}$  copies/mL of viral genome. It is useful in monitoring the progression of TCoV-induced atrophic enteritis in the turkey flocks.

**Key words** Turkey coronavirus, Real-time RT-PCR, TaqMan probe, Spike gene, Intestine, Feces

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## 1 Introduction

Turkey coronavirus (TCoV) causes atrophic enteritis in turkeys and outbreaks or cases of turkey coronaviral enteritis occurred and still occurs in the USA [1], Canada [2], Brazil [3], and Europe [4]. TCoV belongs to species *Avian coronavirus* of the genus *Gammacoronavirus* in the family *Coronaviridae*. The genome of TCoV is a linear positive-sense single-stranded RNA encoding three major structural proteins including spike (S), membrane (M), and nucleocapsid (N) protein. The amino terminal region of S protein (S1) containing receptor-binding domain and neutralizing epitopes can determine host specificity and induce the production of neutralizing antibodies [5]. The carboxyl terminal region of S protein (S2) consisting of transmembrane domain is responsible for cell fusion and virus assembly [6]. S gene is a more common target used for coronavirus (CoV) differentiation because S gene is

highly variable among different CoVs while M and N genes are more conserved. Within the S gene, the S2 gene is more conserved than S1 gene between different CoVs and between different isolates or strains of the same CoV [7]. Therefore, S2 gene is chosen as a target to detect TCoV and differentiate TCoV from other CoVs.

There is no cell culture system for TCoV; thus virus isolation is not feasible. Because the sequence information of TCoV is available, reverse transcription-polymerase chain reaction (RT-PCR)-based methods with high specificity and sensitivity have been developed [7, 8]. Real-time RT-PCR illustrated here uses a pair of TCoV-specific primers targeting a 186 base-pair fragment of TCoV S2 gene and a dual-labeled probe with a reporter dye (FAM) and a quencher dye (Absolute Quencher™) combined with the 5' to 3' exonuclease activity of *Taq* polymerase to increase the release of reporter dye fluorescence in the course of PCR amplification [9]. Quantitative data can be accessed by the standard curve established with serial dilutions of standard RNA. The procedure does not need post-PCR electrophoresis, so the processing time can be significantly reduced and the risks for carryover and cross-contamination between samples can be lessened. In this chapter, the protocol for one-step real-time RT-PCR to detect, differentiate, and quantitative TCoV RNA in the feces and intestinal tissue is presented. In **step 1**, feces or intestine tissues were collected into RNA*later* RNA stabilization reagent. In **step 2**, TCoV RNA was extracted from feces using QIAamp viral RNA mini kit or intestine tissues using RNeasy mini kit. In **step 3**, the extracted RNA was subjected to one-step real-time RT-PCR for detection and quantitation of TCoV in feces or intestine tissues. In **step 4**, a standard curve was established by serially diluted in vitro-transcribed RNA for absolute quantitation of TCoV.

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## 2 Materials

### 2.1 Sample Collection

#### 2.1.1 Reagents

1. Diethyl pyrocarbonate (DEPC) (Sigma-Aldrich, St. Louis, MO, USA): DEPC is very sensitive to moisture, so it needs to be stored at 2–8 °C to help reduce exposure to moisture.
2. Preparation of DEPC-treated water (DEPC-H<sub>2</sub>O):
  - (a) Prepare 0.1 % (v/v) DEPC in the water undergone reverse osmosis filtration and deionization. Add 0.1 mL DEPC solution to 100 mL of water. DEPC appears as globules and needs continuous stirring until the globules disappear. It takes 12 h at 37 °C in a fume hood. DEPC can dissolve plastic, so it is better to store DEPC solution in a glass bottle.

- (b) Autoclave 0.1 % DEPC solution at 121 °C for 15 min to remove any trace of DEPC. The autoclaved DEPC-H<sub>2</sub>O can be stored at any temperature.
3. *RNAlater* RNA stabilization solution (Qiagen, Valencia, CA, USA): It can be stored at room temperature (15–25 °C) for at least 1 year. Samples in *RNAlater* stabilization reagent can be kept at 37 °C for 1 day, 15–25 °C (room temperature) for 7 days, 2–8 °C (refrigerator) for 4 weeks, and –20 or –80 °C for a longer storage. Samples in *RNAlater* stabilization reagent stored at –20 or –80 °C can remain stable for 20 rounds of freeze and thaw.
4. β-Mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA): It should be stored at 2–8 °C and processed in a chemical hood.
5. Phosphate-buffered saline (PBS) is composed of 1.44 g Na<sub>2</sub>HPO<sub>4</sub>, 8 g NaCl, 0.24 g KH<sub>2</sub>PO<sub>4</sub>, and 0.2 g KCl in 1 L ddH<sub>2</sub>O. The solution is adjusted to pH 7.2 and autoclaved before use.

## 2.2 RNA Extraction

### 2.2.1 Reagents

1. RNase AWAY™ spray (VWR, Batavia, IL, USA): It is a surfactant that removes RNA and RNases from laboratory equipment and glassware.
2. Absolute ethanol (200 Proof) (Thermo Fisher Scientific, Waltham, MA, USA): ≥99.5 % (v/v), molecular biology grade, DNase-, RNase-, and protease-free.
3. QIAamp® viral RNA mini kit (Qiagen, Valencia, CA, USA): The kit contains lyophilized carrier RNA, Buffer AVL (lysis), AW1 (wash), AW2 (wash), and AVE (elute). Buffer AVL and AW1 contain guanidine thiocyanate, which is hazardous to health. Viral RNA can bind specifically to the silica membrane of QIAamp spin columns and pure viral RNA is eluted in either water or Buffer AVE.
  - (a) Prepare carrier RNA: Add 310 μL Buffer AVE to the tube containing 310 μg lyophilized carrier RNA to obtain a solution of 1 μg/μL and store it at –20 °C. Do not freeze–thaw the aliquots of carrier RNA more than three times. Incubate Buffer AVL at 80 °C if it has precipitate until it is dissolved. Add 100 volumes of Buffer AVL to 1 volume of carrier RNA-AVE solution. Mix gently by inverting tubes ten times without using a vortexer to avoid any foaming. AVL-carrier RNA solution can be stored at 4 °C for 48 h. Incubate AVL-carrier RNA solution at 80 °C for less than 5 min if there is precipitant. Do not warm the solution more than six times.
  - (b) Add 25 mL of 96–100 % ethanol into 19 mL of concentrated Buffer AW1.
  - (c) Add 30 mL of 96–100 % ethanol into 13 mL of concentrated Buffer AW2.

4. RNeasy mini kit (Qiagen, Valencia, CA, USA): The kit contains Buffer RLT (lysis), RW1 (wash), and RPE (elute). Buffer RLT and AW1 contain guanidine thiocyanate, which is harmful. RNA from intestine tissues can bind to silica membrane of RNeasy spin columns with a binding capacity of 100  $\mu\text{g}$ .
  - (a) Add 10  $\mu\text{L}$   $\beta$ -mercaptoethanol ( $\beta$ -ME) per 1 mL Buffer RLT in a fume hood. Buffer BLT with  $\beta$ -ME can be stored at room temperature for up to 1 month.
  - (b) Add 44 mL of 96–100 % ethanol to 11 mL of concentrated Buffer RPE.

### 2.2.2 Equipment

1. Bio-Gen Pro200 homogenizer (Pro Scientific, Oxford, CT, USA).
2. GeneQuant 1300 Spectrophotometer (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).
3. Eppendorf 5424 Centrifuge (Eppendorf, Hamburg, Germany).

## 2.3 Real-Time RT-PCR

### 2.3.1 Reagent

1. Primers and probe can be synthesized by Integrated DNA Technologies (IDT, San Jose, CA, USA). The primers and primer sequence information are listed in Table 1. The lyophilized powders of products prepared by company are to be dissolved in DEPC- $\text{H}_2\text{O}$  to 100  $\mu\text{M}$  as stock solution (100 $\times$ ). The working solution of primers and probe is 10  $\mu\text{M}$ . Both stock and working solution of primers and probe are stored at  $-20^\circ\text{C}$ .
2. Platinum<sup>®</sup> Quantitative RT-PCR ThermoScript<sup>™</sup> One-Step System (Invitrogen<sup>™</sup>, Life Technologies, Grand Island, NY, USA): It can be stored at  $-20^\circ\text{C}$  for at least 6 months. The kit contains ThermoScript<sup>™</sup> RT/Platinum<sup>®</sup> *Taq* mix, 2 $\times$  ThermoScript<sup>™</sup> reaction mix (a buffer containing 0.4 mM of each dNTP, 6 mM  $\text{MgSO}_4$ ), and a vial of 50 mM  $\text{MgSO}_4$ .

### 2.3.2 Equipment

1. Rotor-Gene 3000<sup>™</sup> real-time thermocycler (Corbett Research, Sydney, Australia): This model was discontinued. The alternative model is Rotor-Gene Q provided by Qiagen (Valencia, CA, USA).

**Table 1**  
**Primers and sequence information for turkey coronavirus**  
**real-time RT-PCR**

| Primer | 5' → 3' sequence                                  |
|--------|---|
| QS1F   | TCGCAATCTATGCGATATG                               |
| QS1R   | CAGTCTTGGGCATTACAC                                |
| QS1P   | Absolute<br>Quencher-TCTGTGGCAATGGTAGCCATGTTC-FAM |
| 6F     | GACCATGGGATTTGTTGAA                               |
| 6R     | TTTTTAATGGCATCTTTTGA                              |

## 2.4 Standard Curve

### 2.4.1 Reagent

1. pTriEx™-3 DNA-Novagen vector (EMD Millipore Corporation, Billerica, MA, USA): The vector was sold by Novagen (Madison, WI, USA) originally and is handled by Merck Millipore now. The availability of this vector is limited. Alternatively, any vector using T7 promotor can be used for in vitro transcription in the current protocol.
2. Restriction enzymes, *Kpn*I and *Nco*I (New England Bio Labs Inc., Ipswich, MA, USA).
3. T4 DNA ligase (Promega, Madison, WI, USA).
4. QIAprep Spin Miniprep kit (Qiagen, Valencia, CA, USA).
5. RiboMAX™ large-scale RNA production system-T7 (Promega, Madison, WI, USA): The kit contains enzyme (RNA Polymerase, Recombinant RNasin® Ribonuclease Inhibitor and Recombinant Inorganic Pyrophosphatase), transcription 5× buffer, each of 4rNTPs (100 mM), RQ1 RNase-free DNase (1 U/μL), 3 M sodium acetate (pH 5.2), and nuclease-free water.
6. RQ1 DNase (Promega, Madison, WI, USA).
7. DNA clean and concentration™-5 kit (Zymo, Irvine, CA, USA): The kit contains DNA-binding buffer, Zymo-Spin™ Column, DNA wash buffer, and DNA elution buffer.

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## 3 Methods

### 3.1 Sample Collection

#### 3.1.1 Feces from Cloaca of Turkeys

1. Use individual packed sterile cotton swab to take feces from turkey cloaca.
2. Place about 100 mg of feces on the swab into a 1.7 mL microcentrifuge tube containing 1 mL of RNAlater RNA stabilization reagent. The space occupied by 100 mg feces is about 1/3 to 1/4 of 1.7 mL microcentrifuge tube.
3. Use sterile tips to suspend fecal matters in RNAlater RNA stabilization reagent (*see Note 1*).

#### 3.1.2 Feces from Floor

1. Use sterile forceps rinsed with DEPC-H<sub>2</sub>O to collect fresh feces from floor and place about 100 mg of feces into a 1.7 mL microcentrifuge tube containing 1 mL of RNAlater RNA stabilization reagent. The space occupied by 100 mg feces is about 1/3 to 1/4 of 1.7 mL microcentrifuge tube.
2. Use sterile tips to suspend fecal matters in RNAlater RNA stabilization reagent (*see Note 1*).

#### 3.1.3 Intestine Tissue

1. Use sterile forceps and scissors to cut 0.5 cm long segment of duodenum, jejunum, ileum, or cecum.

2. Use the scissors to open the intestine segment longitudinally and rinse away intestinal content with chill sterile phosphate-buffered saline (PBS, pH=7.2).
3. Put the rinsed intestine segment into a 1.7 mL microcentrifuge tube containing 1 mL of RNA*later* RNA stabilization reagent (*see Note 2*).

### 3.2 RNA Extraction

#### 3.2.1 Fecal Samples Using QIAamp® Viral RNA Mini Kit

1. Take 1.7 mL microcentrifuge tubes containing feces and RNA*later* RNA stabilization reagent for centrifugation at 3000 rpm ( $845 \times g$ ) for 2 min at 4 °C.
2. Take 140  $\mu$ L of supernatant into 560  $\mu$ L of prepared Buffer AVL containing carrier RNA in a 1.7 mL microcentrifuge tube.
3. Incubate at room temperature for 10 min.
4. Briefly centrifuge the tube to remove drops from the inside of the lid.
5. Add 560  $\mu$ L of 96–100 % ethanol to the sample and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from the inside of the lid.
6. Apply 630  $\mu$ L of the solution from **step 5** to the QIAamp spin column in a 2 mL collection tube and centrifuge at 8000 rpm ( $6010 \times g$ ) for 1 min. Discard the old 2 mL tube containing the filtrate and transfer the QIAamp spin column to a new 2 mL collection tube.
7. Apply remaining part of the solution from **step 5** to the QIAamp spin column and centrifuge at 8000 rpm for 1 min. Discard the old 2 mL containing the filtrate and transfer the QIAamp spin column to another new 2 mL collection tube.
8. Add 500  $\mu$ L of Buffer AW1 and centrifuge at 8000 rpm for 1 min. Place the QIAamp spin column into a clean 2 mL collection tube and discard the tube containing the filtrate.
9. Add 500  $\mu$ L of Buffer AW2 and centrifuge at 14,000 rpm ( $18407 \times g$ ) for 3 min. Change the direction of tubes and centrifuge at 14,000 rpm for another 1 min.
10. Place the QIAamp spin column in a clean 1.7 mL microcentrifuge tube and add 60  $\mu$ L of Buffer AVE. Incubate at room temperature for 1 min and centrifuge at 8000 rpm for 1 min.
11. Take 4  $\mu$ L RNA into 96  $\mu$ L Buffer AVE and measure the amount of RNA by GeneQuant following the manufacturer's instruction.
12. Aliquot the RNA into 5  $\mu$ L each tube and store in  $-80$  °C freezer for use in real-time RT-PCR.

#### 3.2.2 Intestine Tissue Samples Using RNeasy Mini Kit

1. Take intestine segment out of 1.7 mL microcentrifuge tubes containing 1 mL of RNA*later* RNA stabilization reagent and put the tissue specimen on top of the plastic paper treated with RNase Away and DEPC H<sub>2</sub>O.

2. Use sterile forceps and scissors to cut the tissue into small pieces at room temperature.
3. Weight 30 mg of tissue and homogenize it in 600  $\mu\text{L}$  of Buffer RLT by using Pro200 homogenizer (*see Note 3*).
4. Centrifuge the lysate from **step 3** at 14,000 rpm for 3 min. Transfer the supernatant to a new 1.7 mL tube.
5. Add the same volume of 70 % ethanol to the supernatant and mix immediately by pipetting. Transfer 700  $\mu\text{L}$  of the mixture to the RNeasy spin column in a 2 mL collection tube.
6. Centrifuge at 10,000 rpm ( $9391\times g$ ) for 15 s. Discard the filtrate inside the 2 mL collection tube and place the same 2 mL collection tube back with the RNeasy spin column.
7. Apply the remaining part of mixture in **step 5** to the same RNeasy spin column and centrifuge at 10,000 rpm for 15 s. Discard the filtrate inside the 2 mL collection tube and place the same 2 mL collection tube back with the RNeasy spin column.
8. Add 700  $\mu\text{L}$  Buffer RW1 to the RNeasy spin column and centrifuge at 10,000 rpm for 15 s. Discard the filtrate and reuse the 2 mL collection tube.
9. Add 500  $\mu\text{L}$  Buffer RPE to the RNeasy spin column and centrifuge at 10,000 rpm for 15 s. Discard the filtrate and reuse the 2 mL collection tube.
10. Add another 500  $\mu\text{L}$  Buffer RPE to the RNeasy spin column and centrifuge at 10,000 rpm for 15 s.
11. Place the RNeasy spin column into a new 2 mL collection tube. Discard the 2 mL collection tube containing the filtrate. Centrifuge at 14,000 rpm for 1 min to get rid of the remaining ethanol.
12. Place the RNeasy spin column into a 1.7 mL microcentrifuge tube. Add 50  $\mu\text{L}$  of DEPC- $\text{H}_2\text{O}$  and centrifuge at 10,000 rpm for 1 min to elute RNA.
13. Take 4  $\mu\text{L}$  RNA into 96  $\mu\text{L}$  DEPC- $\text{H}_2\text{O}$  and measure the amount of RNA by GeneQuant 1300. If the RNA yield is higher than 30  $\mu\text{g}$  (0.6  $\mu\text{g}/\mu\text{L}$ ), repeat the elution step to recover the remaining RNA inside the RNeasy spin column.
14. Aliquot the RNA into 5  $\mu\text{L}$  tube individually and store in  $-80\text{ }^\circ\text{C}$  freezer for use in real-time RT-PCR.

### 3.3 One-Step Real-Time RT-PCR

1. Prepare a total of 25  $\mu\text{L}$  of reaction mixture (Table 2) on ice by using TCoV-specific primers, probe, reaction buffer from Platinum<sup>®</sup> Quantitative RT-PCR ThermoScript<sup>™</sup> One-Step System, and 5  $\mu\text{L}$  of RNA template. Mix gently by pipetting up and down (*see Note 4*).

**Table 2**  
**Reaction mixture for one-step turkey coronavirus real-time RT-PCR**

| Components   | Volume                      |
|--|-----------------------------|
| RNA  | 5 $\mu\text{L}$             |
| 2 $\times$ Reaction mix                                    | 12.5 $\mu\text{L}$          |
| SuperScript™ III RT/platinum <i>Taq</i> Mix forward primer | 0.5 $\mu\text{L}$           |
| QS1F 10 $\mu\text{M}$                                      | 2.25 $\mu\text{L}$ (900 nM) |
| Reverse primer QS1R 10 $\mu\text{M}$                       | 0.75 $\mu\text{L}$ (300 nM) |
| Probe QS1P 10 $\mu\text{M}$                                | 0.5 $\mu\text{L}$ (200 nM)  |
| DEPC-H <sub>2</sub> O                                      | 3.5 $\mu\text{L}$           |
| Total volume   | 25 $\mu\text{L}$            |

2. Set up the following real-time RT-PCR conditions in Rotor-Gene 3000 or Rotor-Gene Q. The temperature profile is 50 °C for 30 min, 94 °C for 5 min, and 45 cycles of 94 °C for 20 s and 61 °C for 1 min to acquire the fluorescence FAM.
3. Every run of real-time RT-PCR should contain triple reactions for 9 standard concentrations of standard pTriEx3-6F/6R in vitro transcripts, non-template control (water), and samples.
4. Signals are regarded as positive if the fluorescence intensity exceeded ten times the standard deviation of the baseline fluorescence.
5. Calculate the concentration (copies/ $\mu\text{L}$ ) of sample by blotting Ct values against the standard curve established by serial dilutions of TCoV standard transcripts from  $10^2$  to  $10^{10}$  copies/ $\mu\text{L}$  in Sect. 3.4, step 7 (see Note 5).

### 3.4 Standard Curve

1. Amplify partial S2 gene of TCoV in the region encompassing the fragment targeted by real-time RT-PCR by PCR with forward primer 6F and reverse primer 6R (Table 1) and the amplified fragment is designed as 6F/6R fragment. The fragments underlined in primers 6F and 6R are recognized by restriction enzymes *NcoI* and *KpnI*, respectively.
2. Double-digest the pTriEx-3 DNA-Novagen (1–3  $\mu\text{g}$  total) or 6F/6R fragment (1–3  $\mu\text{g}$  total) with 1  $\mu\text{L}$  of 1/10 diluted *NcoI* and 1  $\mu\text{L}$  of 1/10 diluted *KpnI* (NEB) in 50  $\mu\text{L}$  reaction containing 5  $\mu\text{L}$  of 10 $\times$  NEB® Buffer 1 and 5  $\mu\text{L}$  of bovine serum albumin (BSA, 1 mg/mL). Incubate the reaction at 37 °C for 1 h.
3. Ligate the treated pTriEx-3 and 6 F/6R fragment (638 bp) using T4 DNA ligase in 20  $\mu\text{L}$  reaction to become pTriEx3-6F/6R. The reaction solution contains total 64 ng of the



prepared 6F/6R fragment ( $x\mu\text{L}$ ), 2  $\mu\text{L}$  of the prepared pTriEx vector (50 ng/ $\mu\text{L}$ ), 2  $\mu\text{L}$  10 $\times$  ligase buffer, 2  $\mu\text{L}$  of 100 mM DTT, 1  $\mu\text{L}$  of 10 mM ATP, 1  $\mu\text{L}$  of T4 DNA ligase (0.2–0.4 Weiss units/ $\mu\text{L}$ ), and nuclease-free water (12 –  $x\mu\text{L}$ ). Incubate the reaction at 16 °C for 2 h.

4. Generate in vitro transcripts of pTriEx3-6F/6R by using RiboMAX™ large-scale RNA production system-T7. Digest pTriEx3-6F/6R with restriction enzyme *NcoI* to linearize DNA template. Clean up the linearized DNA template with QIAprep Spin Miniprep kit (Qiagen). Prepare 100  $\mu\text{L}$  reaction solution containing total 5–10  $\mu\text{g}$  of linearized pTriEx-6F/6R ( $x\mu\text{L}$ ), 20  $\mu\text{L}$  of 5 $\times$  T7 transcription buffer, 10  $\mu\text{L}$  of T7 enzyme mix, 30  $\mu\text{L}$  of rNTPs (25 mM ATP, CTP, GTP, UTP), and nuclease-free water (40 –  $x\mu\text{L}$ ). Incubate the reaction at 37 °C for 4 h to generate RNA transcript of pTriEx3-6F/6R.
5. Treat pTriEx3-6F/6R transcripts with RQ1 DNase (1 U/1  $\mu\text{g}$  RNA, Promega), purify it by RNeasy mini kit, and measure the concentration of RNA by GeneQuant at 260 nm.
6. Make tenfold serial dilutions in DEPC-H<sub>2</sub>O for real-time RT-PCR.
7. Create the standard curves by plotting the Ct value against each dilution of known concentration.
8. Calculate the copy number per micro liter by using the formula as follows:

$$\text{Copies} / \mu\text{L} = (\text{ng} / \mu\text{L} \times 10^9) / (\text{MW of transcripts}) \times (6.023 \times 10^{23}).$$

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## 4 Notes

1. RNAlater stabilization reagent is not designed for fecal samples but the previous study [10] showed a good stability of viral RNA in turkey feces by using RNAlater reagent. It is very critical to mix feces or cloacal swab with RNAlater to make feces submerged and contact RNAlater reagent completely for the stability of RNA in feces.
2. The thickness of tissue must be less than 0.5 cm. RNA in harvested intestine tissue is not protected until the tissue is completely submerged in a sufficient volume of RNAlater RNA Stabilization Reagent at about 10  $\mu\text{L}$  reagent per 1 mg tissue. The intestinal content may interfere with the reaction of RNAlater reagent, so it is critical to wash off the intestinal contents. Insufficient RNA reagent may cause RNA degradation during storage. It is not recommended to harvest tissues frozen in liquid nitrogen or dry ice and later thaw them for RNAlater storage or RNA extraction because the process would cause severe RNA degradation.

3. The heat produced by the process of hominization can damage the integrity of RNA in intestine tissues leading to low sensitivity of real-time RT-PCR. Therefore, it is recommended to homogenize the intestine tissue for 30 s and cool the tube in ice in cycles until the intestine tissue is homogenized thoroughly.
4. Real-time RT-PCR is a very sensitive assay, so it is very easy to be interfered by bubbles created by pipetting. To use pipettes and take small volume of reagents (<10  $\mu$ L) precisely, it is recommended to practice before performing the real test. In addition, the nonspecific reactions can be minimized by mixing reagents on the precooled (store at  $-20$  °C freezer) aluminum loading block (Qiagen) on ice.
5. Example of running a set of samples from the setup to the end of calculating the copy number is illustrated below. First, collect ileum and cecum samples in RNAlater solution from three turkeys at 5 days post-infection of TCoV and the same kind of samples from negative control turkeys without TCoV infection. Second, label the samples from turkeys infected with TCoV as d5-i1 to d5-i3 and d5-c1 to d5-c3, and those from negative control turkeys without TCoV infection as N-i1 to N-i3 and N-c1 to N-c3. Third, extract RNA from the samples according to Sect. 3.2.2. Next, arrange the samples in 96-well plate for one-step real-time RT-PCR (Table 3) and set up the real-time RT-PCR conditions. Prepare nuclease-free water as template for the non-template control

**Table 3**  
**Example of one-step real-time PCR: Sample arrangement in 96-well plate**

|   | 1    | 2    | 3    | 4     | 5     | 6     | 7    | 8    | 9    |
|---|------|------|------|-------|-------|-------|------|------|------|
| A | STD1 | STD1 | STD1 | STD9  | STD9  | STD9  | N-i2 | N-i2 | N-i2 |
| B | STD2 | STD2 | STD2 | d5-i1 | d5-i1 | d5-i1 | N-i3 | N-i3 | N-i3 |
| C | STD3 | STD3 | STD3 | d5-i2 | d5-i2 | d5-i2 | N-c1 | N-c1 | N-c1 |
| D | STD4 | STD4 | STD4 | d5-i3 | d5-i3 | d5-i3 | N-c2 | N-c2 | N-c2 |
| E | STD5 | STD5 | STD5 | d5-c1 | d5-c1 | d5-c1 | N-c3 | N-c3 | N-c3 |
| F | STD6 | STD6 | STD6 | d5-c2 | d5-c2 | d5-c2 | NTC  | NTC  | NTC  |
| G | STD7 | STD7 | STD7 | d5-c3 | d5-c3 | d5-c3 |      |      |      |
| H | STD8 | STD8 | STD8 | N-i1  | N-i1  | N-i1  |      |      |      |

Samples are arranged in Row A to H from Column 1 to 12 in one 96-well plate. STD1 to STD8 are tenfold serially diluted standard RNA of pTriEx3-6F/6R from  $10^2$  to  $10^9$  copies/ $\mu$ L. NTC is nuclease-free water as template for real-time RT-PCR

(NTC) and tenfold serially diluted standard RNA of pTriEx3-6F/6R from  $10^2$  to  $10^9$  copies/ $\mu\text{L}$ , as STD1 to STD8. After the reaction complete, establish the standard curve formula based on the Ct values of STD1 to STD8. Then, calculate the copy numbers of the tested samples by the standard curve formula. Example for calculating sample concentration is illustrated in Table 4.

**Table 4**  
**Example of one-step real-time PCR: Ct values and copy numbers after calculation**

| Well ID | Sample | Ave Ct value | Ave Log 10 copies/ $\mu\text{L}$ | Ave copies/ $\mu\text{L}$ |
|---------|--------|--------------|----------------------------------|---------------------------|
| A1-A3   | STD1   | 40           | 2                                | 100                       |
| B1-B3   | STD2   | 37           | 3                                | 1000                      |
| C1-C3   | STD3   | 33           | 4                                | 10,000                    |
| D1-D3   | STD4   | 30           | 5                                | 100,000                   |
| E1-E3   | STD5   | 26           | 6                                | 1,000,000                 |
| F1-E8   | STD6   | 23           | 7                                | 10,000,000                |
| G1-G3   | STD7   | 20           | 8                                | 100,000,000               |
| H1-H3   | STD8   | 16           | 9                                | 1,000,000,000             |
| A4-A6   | STD9   | 13           | 10                               | 10,000,000,000            |
| B4-B6   | d5-i1  | 25           | 6.42                             | 2,658,924                 |
| C4-C6   | d5-i2  | 24           | 6.72                             | 5,233,877                 |
| D4-D6   | d5-i3  | 22           | 7.31                             | 20,279,574                |
| E4-E6   | d5-c1  | 31           | 4.66                             | 45,709                    |
| F4-F6   | d5-c2  | 32           | 4.37                             | 23,221                    |
| G4-G6   | d5-c3  | 35           | 3.48                             | 3045                      |
| H4-H6   | N-i1   | Over 45      |                                  | UDL                       |
| A7-A9   | N-i2   | Over 45      |                                  | UDL                       |
| B7-B9   | N-i3   | Over 45      |                                  | UDL                       |
| C7-C9   | N-c1   | Over 45      |                                  | UDL                       |
| D7-D9   | N-c2   | Over 45      |                                  | UDL                       |
| E7-E9   | N-c3   | Over 45      |                                  | UDL                       |
| F7-F9   | NTC    | Over 45      |                                  | UDL                       |

The standard curve formula is established by the Ct values and the copy numbers (Log 10 copies/ $\mu\text{L}$ ) of STD1 to STD8:  $\gamma = -3.4167X + 46.917$  ( $R^2 = 0.9988$ ).  $\gamma$  is Ct value acquired by real-time RT-PCR and  $X$  is sample concentration presented by copies/ $\mu\text{L}$ . UDL is under detection limit

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