Protocol for postmortem diagnosis of Rabies in animals by direct fluorescent antibody testing (FA)

1. Place
   In a laboratory room (BSL2)

2. Equipment and Reagents
   (1) Equipment
      a. Necropsy instruments
         (1 set per sample should be sufficient quantity to prevent cross transfer of infected tissue between samples.)
      b. Microscope slides: Highest quality with coverslip, non auto-fluorescence.
      c. Specimen storage containers (large enough that reserved portions of brain stem and cerebellum): Wide mouth, screw cap, polypropylene jars or sample bottles
      d. Refrigerated storage: An explosion proof -20°C freezer for fixation of impression/smear slides and storage of acetone and other reagents; long term sample storage requires a freezer at -80°C.
      e. Acetone fixation and post-stain rinse containers
      f. Syringe: Anti-rabies conjugates are added to test slides through syringe.
      g. Incubator(37°C) and humidified staining tray or chamber: Constant humidity must be maintained during the staining process. Conjugate dried on the slides during the staining process may be mistaken for specific staining.
      h. Fluorescence microscope: The quality of the fluorescence microscope is critical to the sensitivity of the direct FA test. At a minimum, all rabies diagnostic laboratories should have a reflected light fluorescence microscope with high quality objective lenses.
      i. Autoclave and/or instrument sterilizer

   (2) Reagents
      a. Acetone (storage of -20°C) : High quality grade acetone should be employed and the acetone should not be reused.
      b. FITC-conjugated anti-rabies antibodies: Stock solutions of conjugate are stored as frozen aliquots in a non-frost-free freezer at -20°C or below.
      Reagents presently available commercially are as followings.
         (a) Fujirubio Diagnostics, Inc.
            CENTOCOR FITC-ANTI-Rabies Monoclonal Globulin Catalog # 800-090. A mixture of two IgG2a monoclonal antibodies.
         (b) Chemicon International, Inc
            Light Diagnostics Rabies DFW Reagent(Monoclonal antibody FITC-conjugate)
Catalog # 5100. A mixture of two IgG1 monoclonal antibodies and one IgG2 monoclonal antibody.
c. Conjugate diluents: phosphate buffered saline(PBS) pH7.4 to 7.6
d. Counterstains: Evans blue (1% in PBS) is added to the working dilution of the conjugate to provide contrast and lower background. An Evans blue concentration of 0.002% works is prepared by adding 2 μL of 1% stock dye solution per ml of conjugate dilution.
d. Rinse/soak buffer: A PBS formula of the same pH and molarity as the conjugate diluents is used as the rinse/soak buffer.
e. Mountant: PBS(pH8.4) with 10% glycerol
f. Immersion oil: For fluorescence applications(e.g. Cargille DF)
g. Disinfectant (Bleach, 70% ethanol)

3. Protective Personal Equipments (PPE)
   PPE is required for safe removal of brain tissue from animals submitted for rabies testing. Biosafety cabinet provide additional protection from exposure to aerosols of rabies infected material.
   a. Laboratory gown and waterproof apron
   b. Surgical masks
   c. Face shield
d. heavy rubber gloves
e. boots

4. Necropsy procedure
   (1) Remove and collect brain samples (Figure1.)
(2) Collection of brainstem (thalamus, pons, medulla), cerebellum and hippocampus

The brain stem is anterior to the cerebellum and continuous with the spinal cord. The uppermost portion of the brain stem is the thalamus; the hindbrain portion of the brain stem composed of pons and medulla oblongata.

*Shipment of samples.*

Because rabies prophylaxis is usually delayed pending a laboratory report, specimen transit time to the laboratory should be as possible, preferably within 48 hours. A fresh, unfixed brain sample is critical to a rapid and accurate diagnosis of rabies.

- Refrigeration will preserve a sample for at least 48 hours.
- Freezing at -80°C will preserve a sample over 48 hours.

5. Preparation of Impression slides/smears

(1) Sampling of cross section of brain stem (thalamus, pons, medulla), cerebellum and hippocampus

While a positive finding of rabies virus antigen in any tissue is diagnostic of rabies infection, a negative finding for rabies can be made only if the diagnostic test includes examination of at least two areas of the brain: brain stem and preferably cerebellum.

A complete cross section of the brain stem required. If brain stem is unavailable and other brain tissues are negative, the sample must be considered unsatisfactory for testing. If cerebellum is unavailable, a diagnosis may be made by examination of brain stem and hippocampus. Each brain area is tested with FITC-labeled anti-rabies reagents prepared from two different sources (i.e., two different
monoclonal antibody reagents or a monoclonal antibody reagent and a hyperimmune serum reagent).

Each sampled brain area should be of a size sufficient for examination of 40 separate views (microscopic field) at a magnification of approximately 200X. With most microscopes an impression made in a 15mm diameter well or a smear of 10mm² meets this recommendation. Over two sets of slide glasses should be prepared.

A. Brain stem

A cross (transverse) section of one of these area is necessary for rabies testing. Slides are prepared from touching the cut surface of the cross section to expose multiple ascending and descending nerve tracts.

B. Cerebellum

The cerebellum may be broadly divided into the midline structure called the vermis (“worm”) and two lateral cerebellar hemispheres. A rabies test should include examination of tissue from a cross section through the hemispheres and the vermis.

C. Hippocampus

The hippocampus is buried deep in the temporal lobe near the center of the brain and is only visible when the brain is dissected. The lateral horn-shaped protrusions of the hippocampus are the reason for its alternative name, Ammon’s horn. If used for rabies testing, a cross section areas of both horns is needed.

D. Combined areas for very small animals like bats

Slides are prepared from tissue cut through the brain at the point at which the cerebral hemispheres overlay the cerebellum. If the cut is made properly, the section will include parts of the cerebellum and midbrain, as well as both cerebral hemispheres.

E. Control slides

Control slides are prepared in the same manner as test slides, but without acetone fixation. Positive and negative control slides are fixed in acetone at the same time as test slides to control for the effect of acetone fixation on test performance. Mouse brain infected with CVS rabies virus and not are used. Slides are stored frozen at -20°C for up to one month or at -70°C for one year. Slides may be made from either brain stem or cerebellum.

Note) These area are stocked in tubes at 4°C for RT-PCR samples (Each area are stocked in two tubes).

Figure 3. Touch impression of brain stem, cerebellum and hippocampus.
• Sick impression is hard to microscopic reading, and blood become a cause of a nonspecific reaction. Slides are inverted over the absorbent paper and applied light pressure to the back of the slides to remove the excess tissue and blood.

• Prepare the two or more over impression slides per one sample.

(2) Drying and fixation

A. Impression must dry at room temperature prior to the fixation step in the biosafety cabinet. This may take 15 to 30 minutes. Do not use ovens or a hot air souse to dry slides as this may denature antigen.

B. When the tissue no longer appears wet and glistening, slides from an individual test animal are combined in one container for fixation with cold (-20°C) acetone. Do not combine slides from different animals or combine test slides with control slides in the same container.

C. Slides should be fixed for a minimum of 30 minutes to overnight at -20°C.

(3) Staining, rinsing and mounting

A. Dilution of FITC-conjugated anti-rabies antibody

A determination of a working dilution for a conjugate must be made exactly as the diagnostic reagent will be used for test samples (same diluents, tubes, syringe filters). Serial twofold dilutions (e.g., 1:10, 1:20, 1:40) are prepared for all prospective conjugates. Two or more slides are stained for each dilution. The consensus of the last dilution providing crisp +4 staining with minimal background fluorescence is the end-point dilution of the reagent. Usually The freeze-drying article of FITC is dissolved by 5mL DW. Necessary quantity is diluted with PBS (1:25), and 1% evansblue solution is added to diluted FITC-conjugate anti-rabies antibody (2µl/ml, 1:500).

B. Staining with FITC-conjugated anti-rabies antibodies

After the acetone-fixed impression control and test slides are air dried at room temperature, each anti-rabies conjugate is added by dispensing through a syringe. The slides are arranged so that the positive control slide is the first to receive the conjugate (to control for any unexpected removal of antibody by filtration) and the negative control slide is the last to receive the conjugate. The slides are then incubated for 30 minutes at 37°C in a high humidity chamber.

C. Rinsing

After staining, excess conjugate is drained from the slides or wicked onto absorbent paper and the slides are given a brief rinse under a stream of PBS for 3 to 5 minutes (control slides and slides from each test animal in a separate rinse
The PBS is discarded and replaced and the slides soaked for a second 3 to 5 minutes interval. At last slides are rinsed with distilled water 2-3 seconds.

D. Air drying

Slides are carefully blotted to remove excess liquid, the briefly air dried before mounting.

E. Mounting

Slides are mounted by dropping a small amount of 10% glycerol-PBS onto coverslips arranged on absorbent paper. Stained slides are inverted over the coverslips. Excess mountant is wicked into the absorbent paper by applying light pressure to the back of the slides. A small volume syringe fitted with a 0.45um filter and the mountant in small droplets onto the coverslips. Slides should be red within 2 hours of mounting. Rabies-specific staining should be stable for at 2 hours, and stained slides can be preserved for reference for weeks to months at refrigerator temperature or below.

6 Reading

Sample can be considered negative for rabies only when each area of the brain stained with each anti-rabies conjugate is scanned over approximately 40 fields at a magnification of approximately 200X or greater for fluorescing inclusions.

(1) Staining intensity

Rabies virus in the brains of infected animals produces intracytoplasmic inclusions of various shapes. A single microscopic field may contain dust-like particles of under 1μm in diameter and large, round to oval masses and strings 2 to 10 μm in diameter. When specifically stained with an FITC-labeled antibody, these inclusions appear smooth, with very bright margins, and a somewhat less intensely stained central area. Observations made for each test slides are recorded as staining intensity/antigen distribution (e.g., +4/+2).

Staining intensity is graded from +4 to +1. Positive control slides in all tests should always contain staining of +4 intensity. Grade +2 to +1 cannot be considered as diagnostic for a rabies infection without confirmation of specificity. Diminished staining may result from nonspecific binding of antibody to components of inflamed tissue or artifacts of tissue decomposition.

+4, a massive infiltration of large and small inclusions of varying shape in almost every area of the impression.

+3, inclusions of varying size and shape are found in almost every microscopic field, number of inclusions per field varies, but inclusions are numerous in most fields.

+2, inclusions are present in 10% to 50% of the microscopic fields and most fields
contain only a few inclusions. +1, inclusions are present in under 10% of the microscopic fields and only a few inclusions are found per field.

(2) Test interpretation
If the tissue sample submitted for testing was adequate and suitable for rabies diagnosis, results for a test animal are reported as positive or negative for rabies or non-diagnostic based on observed patterns of staining in test and control slides.

7. References
Protocol for Postmortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing, A minimum Standard for Rabies Diagnosis in the United States. From CDC website
Protocol for postmortem diagnosis of Rabies in animals by RT-PCR

1. Place
   In a laboratory room (BSL2)

2. Precautions of working with RNA
   RNases that degrade RNA are very stable and robust enzymes. Autoclaving solutions and glassware is not always sufficient to actively remove these enzymes. The first step when preparing to work with RNA is to create an RNase-free environment.
   • The RNA area should be located away from microbiological work stations.
   • Clean, disposable gloves should be worn at all times when handling reagents, samples, pipettes, disposable tubes, etc. It is recommended that gloves are changes frequently to avoid contamination.
   • There should be designated solutions, tips, tubes, lab coats, pipettes, etc. for RNA use only.
   • Clean all surfaces with commercially available RNase decontamination solutions.
   • When working with purified RNA samples, ensure that they remain on ice during downstream applications.

3. Specimen
   Brainstem (thalamus, pons, medulla), cerebellum and hippocampus (both sides) of animals

4. Equipment and Reagent
   (1) Equipments
   a. Micropipettes with an accuracy range between (0.5~10μL, 10~100μL, 100~1,000μL)
   b. Filter chips*(0.5~20μL, 2~100μL, 50~1,000μL)
   c. Microcentrifuge tube**(Eppendorf Safe-Lock Tubes™ 1.5mL)
   d. Chips**(0.5~20μL)
   e. PCR tubes 0.2ml
   f. PCR-strips 8 tubes** 0.2ml
   g. Attached lid for PCR-strips 8 tubes**
   h. Beads crusher (TOMY MS-100)
   i. Micro tube (PP) with attached screw cap (PP) 2.0 mL self-standing **
   j. Zirconia beads**
   Put 1.5g zirconia beads into 2mL screw cap micro tube, and close a cap lightly, then dry it with a dry heat sterilizers after sterilization by autoclave.
k. Homogenizer pestle** (When there is no beads crusher)
l. 20-21G hypodermic needle** (In the case that there is not any beads crusher)
m. 2~5mL syringe** (In the case that there is not any beads crusher)
n. Necropsy instruments*
op. Laboratory dishes*
q. Table top refrigerated centrifuge (KUBOTA Model 3500, TOMY MX201)
r. Refrigerator with Freezer
s. PCR bench for master mix preparation
t. PCR bench for sample extraction
u. Thermal cycler (ABI9700, Biolad I cycler)
v. Electrophoresis tub (Mupid)
w. UV gel photographic device (ATTO)
x. Thermomixer (Eppendorf)
y. Thermocycler
Notes) * PCR Grade (DNase-free, RNase-free)
** autoclaved or dry air sterilized

(2) Reagents
a. RNA extraction ready to use reagent : Ribo Pure RNA Isolation Kit (Ambion AM1924)
b. Chloroform (Molecular grade)
c. Isopropanol (2-propanol)
d. Reverse transcriptase RTase (Promega Co.ltd., AMV reverse transcriptase, No.5101)
e. Taq DNA polymerase (TaKaRa Co.ltd., TaKaRa Ex Taq, No.RR001A)
f. QIAGEN OneStep RT-PCR kit (210210 or 210212)
g. RNase decontamination solution (Promega, Recombinant RNasin Ribonuclease Inhibitor, No. N2511)
h. 100% Ethanol (ACS grade or higher quality: 500ml)
i. Distilled water: Sterilized DNAase, RNase-free distilled water
j. TE buffer (WAKO Co.ltd., pH8.0 10mM Tris·HCl, 1mM EDTA 500mL)
k. Ethidium bromide (EtBr) (WAKO Co.ltd., 10mg/mL 10mL)
l. Agarose (AMRESCO Co.ltd., Agarose 1, Biotechnology grade, No. IAMR00710)
m. DNA ladder (100bp DNA Ladder TaKaRa)
n. TBE buffer (WAKO Co.ltd., 5×TBE buffer Biotechnology grade)
o. Loading buffer (WAKO Co.ltd., 0.02%Bromophenol blue, etc.)
p. Reverse transcription primers
  10g [5' - CTA CAA TGG ATG CCG AC - 3'] or
JW12(mix) [5’-ATG TAA CAC C(C/T)C TAC AAT G-3’]
Note: JW12 is diluted to a working concentration so that C or T shown by ( ) sequences become 10 pmol/μL respectively and mixed.

q. Primer sets (2types):
(a) Product length 1468bp
  Forward primer 10g [5’-CTA CAA TGG ATG CCG AC-3’]
  Reverse primer 304 [5’-TTG ACG AAG ATC TTG CTC AT-3’]
(b) Product length 443bp
  Forward primer N1 [5’-TTT GAG ACT GCT CCT TTT G-3’]
  Reverse primer N2 [5’-CCC ATA TAG CAT CCT AC-3’]

r. Primer sets for one-step RT-PCR:
N7(mix) [5’-ATGTAA CAC C(T/C)C TAC AAT GC-3’]
Note: N7 is diluted to a working concentration so that C or T shown by ( ) sequences become 10 pmol/μL respectively and mixed.

JW6(mix)
  JW6(DPL) [5’-CAA TTC GCA CAC ATT TTG TG-3’]
  JW6(E) [5’-CAG TTG GCA CAC ATC TTG TG-3’]
  JW6(M) [5’-CAG TTA GCG CAC ATC TT-3’]
Note: JW6(DPL), JW6(E), and JW6(M) is diluted to a working concentration of 10 pmol/μL respectively and mixed.

5. RNA extraction
The handling of material should be performed within a safety cabinet or PCR bench, and wear gloves and mask certainly.
We usually use RiboPure for RNA extraction from samples. The following commercially available RNA extraction kits and procedures are useful too.
• TRIZOL(GIBCO BRL 15596)
• ISOGEN(NIPPON-GENE 317-02503)

(1) RNA extraction with RiboPure Isolation Kit(Ambion AM1924)
* Please refer to "Instruction Manual" which is attached to the kit for details.
a. Add 50 - 100 mg of tissue samples (Sample1 and Sample2) to a 2.0 mL screw cap tube with 100mg of zirconia beads.
  Sample1: The sample of 50 to 100 mg which pooled about each 20 to 30 mg of thalamus, pons, medulla oblongata.
  Sample2: The sample of 50 to 100 mg which pooled about each 20 to 30 mg of cerebellum and hippocampus(both sides).
b. Add 1mL of TRI Reagent to the tube.
c. Homogenize the sample at 4,600 rpm for 3min. (Micro Smash, TOMY)
d. Incubate the homogenate for 5 min at room temp.
e. Centrifuge at 12,000 x g for 10min at 4°C.
f. Transfer the supernatant to a new tube.
g. Add 100μL of chloroform to 1mL of homogenate.
h. Add 100μL of chloroform to 1mL of homogenate.
i. Mix by pulse-voltexting for 15 sec and incubate the homogenate for 5 min at room temp.
j. Centrifuge at 12,000 x g for 10min at 4°C.
k. Transfer the supernatant to a new tube.
l. Add 200μL of 100% ethanol and mix immediately by pulse-voltexting for 5 sec, and spin down.
m. Apply all of the sample to a Filter Cartridge.
n. Close the cap, and centrifuge at 12,000 x g for 30 sec at room temp.
o. Discard the content of the collection tube, and set Filter Cartridge to the same collection tube.
p. Add 500μL of Wash Solution, and close the cap, and centrifuge at 12,000 x g for 30 sec at room temp.
q. Repeat step 12 and 13 once again discard the the tube containing the filtrate.
r. Set Filter Cartridge to a new 1.5 mL microcentrifuge tube and add 100μL Elution buffer.
s. Incubate the homogenate for 2 min at room temp, and centrifuge at 12,000 x g for 30 sec at room temperature.

6. RT-PCR(Preparation of the master mix of reagents should be performed in the PCR bench for master mix preparation.)

(1) Two-step RT-PCR (Protocol of Rabies prevention guidelines 2001)

A. Pre-reverse transcription
   Add 10μL of RNA extraction solution to 1μL(10μM) of reverse transcription primers(10g or JW12) into a PCR tube(Table1 Pre-RT). Heat them for 1 minute at 95°C, and cool them in ice. Store them at room temperature (15-30°C).

B. Reverse transcription
   a. Add 4μL of 5x buffer, 4μL of 2.5mM dNTP, 1μL of RNasin, 1μL of AMV Reverse Transcriptase to Pre-reverse transcription solution. Total liquid volume become 21μL(Table1 RT).
   b. Heat them for 45 minutes at 42 °C, and next for 5 minutes at 95°C.

C. PCR reaction
   a. Add 1μL (10uM) of forward primer 10g and 1μL of reverse primer 304, 5μL of 10x ExTaq Buffer, 4μL of 2.5mM dNTP, 0.25uL of TaKaRa Ex Taq(5U/uL), 37.75uL of Distilled water: and 1μL of RT product to a PCR tube. Total volume becomes 50μL (Table1 PCR).
b. Add 1μL (10μM) of forward primer N1 and 1μL reverse primer N2, 5μL 10x ExTaQ Buffer, 4μL 2.5mM dNTP, 0.25μL TaKaRa Ex Taq (5U/μL), 37.75μL Distilled water, and 1μL of RT products to another PCR tube. Total volume becomes 50μL (Table 1 PCR).

**Table 1 Component of RT-PCR**

<table>
<thead>
<tr>
<th>Component</th>
<th>μL / tube</th>
<th>n=1</th>
<th>n = 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-RT</strong></td>
<td></td>
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</tr>
<tr>
<td>Template RNA</td>
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<td></td>
</tr>
<tr>
<td>Primer 10g or JW12 (10μM)</td>
<td></td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
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<td>11</td>
<td></td>
</tr>
<tr>
<td><strong>RT</strong></td>
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<td></td>
</tr>
<tr>
<td>5X buffer (Promega M5101)</td>
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<td>40</td>
</tr>
<tr>
<td>dNTP (2.5mM)</td>
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<tr>
<td>Rnase (Promega N2511)</td>
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<tr>
<td>AMV Reverse Transcriptase</td>
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<td>10</td>
</tr>
<tr>
<td>Pre-RT products</td>
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<td></td>
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<td>Total</td>
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<td><strong>PCR</strong></td>
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<tr>
<td>TaKaRa EX Taq H2O</td>
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<td>37.75</td>
<td>1887.5</td>
</tr>
<tr>
<td>10X EX Taq buffer</td>
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<td>25</td>
</tr>
<tr>
<td>dNTP (2.5mM)</td>
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<td>4</td>
<td>20</td>
</tr>
<tr>
<td>Primer 10g / N1 (10μM)</td>
<td></td>
<td>1 #10g</td>
<td>5 #N1</td>
</tr>
<tr>
<td>Primer 304 / N2 (10μM)</td>
<td></td>
<td>1 #304</td>
<td>5 #N2</td>
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<tr>
<td>EX Taq (5U/μL)</td>
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<td>Total</td>
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c. Amplification

After preparation of the reaction mixture, all samples should be transferred to the thermocycler. Amplification should be carried out according to the following protocol. Amplicons should be stored at 4°C in the fridge until analysis by agarose gel-electrophoresis.

**Table 2 Amplification protocol of RT-PCR**

<table>
<thead>
<tr>
<th>Steps</th>
<th>10g/304 primers</th>
<th>N1/N2 primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>94°C 1 minutes</td>
<td>94°C 1 minutes</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C 30 seconds</td>
<td>94°C 30 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>50°C 30 seconds</td>
<td>45°C 30 seconds</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C 90 seconds</td>
<td>72°C 90 seconds</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C 7 minutes</td>
<td>72°C 7 minutes</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>4°C</td>
</tr>
</tbody>
</table>
D. Visualization of Amplified DNA on a Agarose Gel
   a. Move to gel electrophoresis area.
   b. Make a 1%(w/v) agarose gel (1g per 100mL of 0.5X TBE) for 10g/304 primers, and make a 2%(w/v) agarose gel (2g per 100mL of 0.5X TBE) for N1/N2 primers.
   c. The congealed gel is put into the agarose gel electrophoresis chamber, which should already be filled with 0.5X TBE buffer up to the filling line.
   d. To load the gel, 6 µL of PCR amplicons is mixed well by pipette with 1 µL of Loading buffer. The mix is entered completely and carefully into the respective sample slot per line.
   e. 5 µl of the 100bp DNA ladder are loaded into both sides sample slots per line.
   f. To stain DNA amplicons 5mL of EtBr is put into the TBE buffer solution of the anode side of the electrophoresis chamber, and mix them well by pipette. Final concentration of EtBr is 0.2µg/mL.
   g. Voltage is applied and electrophoresis is carried out for 30 minutes at 100V. Time of gel electrophoresis is depended by the gel concentration.
   h. After completion of agarose gel electrophoresis amplicons are visualized using UV light in the gel documentation unit. A positive reaction produces a band of 1468 bps in length or 443 bps in length.

(2) One step RT-PCR( Protocol of Rabies diagnosis manual second edition 2013(2))

A. Reverse transcription and PCR reaction in one tube
   Enzyme Kit is QIAGEN OneStep RT-PCR kit
   Add 20µL of Distilled Water, 10µL of 5x buffer, 2µL of dNTP mixture, 3µL of forward primer JW6 and 3µL of reverse primer N7, 2µL of Enzyme mixture, and 10µL of RNA extraction solution to a PCR tube. Total volume is 50µL.

<table>
<thead>
<tr>
<th>Component of RT-PCR</th>
<th>JW6/N7 primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>QIAGEN OneStep RT-PCR kit</td>
<td>50</td>
</tr>
<tr>
<td>H₂O</td>
<td>20</td>
</tr>
<tr>
<td>5X buffer</td>
<td>10</td>
</tr>
<tr>
<td>dNTP Mixture</td>
<td>2</td>
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<tr>
<td>Primer JW6 (10µM)</td>
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</tr>
<tr>
<td>Primer N7 (10µM)</td>
<td>3</td>
</tr>
<tr>
<td>Enzyme mixture</td>
<td>2</td>
</tr>
<tr>
<td>RNA extraction solution</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
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</table>

B. Amplification
   After preparation of the reaction mixture, all samples should be transferred to the thermocycler. Amplification should be carried out according to the following
protocol. Amplicons should be stored at 4°C in the fridge until analysis by agarose gel electrophoresis.

Table 4 Amplification protocol of RT-PCR

<table>
<thead>
<tr>
<th>Steps</th>
<th>JW6/N7 primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT reaction</td>
<td>50°C 30 minutes</td>
</tr>
<tr>
<td>Initial denaturation</td>
<td>95°C 30 minutes</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C 60 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>56°C 60 seconds</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C 90 seconds</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C 10 minutes</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
</tr>
</tbody>
</table>

C. Visualization of Amplified DNA on a Agarose Gel
   a. Move to gel electrophoresis area.
   b. Make a 1.5%(w/v) agarose gel (1.5g per 100mL of 0.5X TBE).
   c. The congealed gel is put into the agarose gel electrophoresis chamber, which should already be filled with 0.5X TBE buffer up to the filling line.
   d. To load the gel, 6 µL of PCR amplicons is mixed well by pipette with 1 µL of Loading buffer. The mix is entered completely and carefully into the respective sample slot per line.
   e. 5 µL of the 100bp DNA ladder are loaded into both sides sample slots per line.
   f. To stain DNA amplicons 5mL of EtBr is put into the TBE buffer solution of the anode side of the electrophoresis chamber, and mix them well by pipette. Final concentration of EtBr is 0.2µg/mL.
   g. Voltage is applied and electrophoresis is carried out for 30 minutes at 100V. Time of gel electrophoresis is depended by the gel concentration.
   h. After completion of agarose gel electrophoresis amplicons are visualized using UV light in the gel documentation unit. A positive reaction produces a band of 606 bps in length.

7. References
(1) Rabies prevention guidelines 2001, Ministry of Health of Japan
(2) Rabies diagnosis manual second edition 2012, National institute of infectious diseases