DNA extraction from liquid and tissue samples

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1. Purpose and scope

This procedure describes the preparation of liquid or tissue samples, the non-automated extraction of genomic DNA and the storage of the obtained DNA.

2. Definitions and abbreviations

DEPC-H_2O	diethylpyrocarbonate-treated water
EC_CAPV	External control DNA
NEC	Negative extraction control
PCR	Poymerase chain reaction
SDS	Sodium dodecyl sulfate

3. Principle of the method

3.1. Liquid samples

DNA is extracted from liquid samples (such as blood, serum/plasma or cell culture) by silica membrane binding using the Nucleospin Blood colums (Macherey Nagel). Following lysis of cells and virus by addition of B3 buffer (containing large amounts of chaotropic ions) and proteinase K to the sample, conditions are optimized for the DNA to bind to the silica membrane by addition of ethanol to the lysate. Contaminations are then removed by a washing step. Total DNA is finally eluted from the column under low ionic strength in a slightly alkaline elution buffer.

3.2. Tissue samples

DNA is extracted from tissue samples by silica membrane binding using the Nucleospin Tissue colums (Macherey Nagel). Samples are homogenized, in the presence of SDS and Proteinase K, using silicon carbide beads. Following lysis of cells and virus by addition of B3 buffer to the sample, conditions are optimized for the DNA to bind to the silica membrane by addition of ethanol to the lysate. Contaminations are then removed by washing steps with 2 different buffers. Total DNA is finally eluted from the column under low ionic strength in a slightly alkaline elution buffer.

3.3. Monitoring assay quality

The efficiency of the extraction is controlled by adding a synthetic DNA fragment (EC_CAPV) to each sample at the time of cell/virus lysis. EC_CAPV will be extacted together with the viral and celluar DNA. In the subsequent real-time PCR analysis, the EC_CAPV will be detected. The EC_CAPV serves as a positive control for the extraction and subsequent real-time PCR for each individual sample.

3.4. Monitoring sample quality

The quality of the received samples can vary greatly depending on the transport duration, status of the animal, sample type, temperature,… Low quality samples can impact extraction efficiency and increase the risk of false negative results. To monitor sample quality cellular DNA, extracted together with the viral DNA is also detected in the subsequent real-time PCR analysis. This internal control (IC-EXTR) indicates high sample quality when positive in PCR and low sample quality when negative in PCR.

4. Safety, biosafety and environment

Buffer B3 contains guanidine hydrochloride (36-50%) which is harmful when swallowed and causes serious eye irritation.

Buffer BQ1 contains guanidine hydrochloride (50-66%) which is harmful when swallowed and causes skin irritation and serious eye irritation.
Buffer BW contains guanidine hydrochloride (36-50%) and 2-propanol (20-50%) which is a flammable liquid and vapour which is harmful when swallowed and causes serious eye irritation and May cause drowsiness or dizziness.
Liquid proteinase K (90-100%) may cause an allergic skin reaction as well as allergy or asthma symptoms or breathing difficulties if inhaled.

- The test is performed under BSL-3 conditions. Specific biosafety procedures are written down in SOP/BIO/ADM/05 (Specific biosafety measures in the L3 containment zone of Building D)
- The entire extraction procedure occurs in a Bio-Safety Cabinet II and/or chemical flow cabinet.
- Gloves are worn throughout the procedure.
- Liquid waste is discarded according to SOP/BIO/ADM/13 (Inactivation of liquid laboratory waste).
- Disposable material is discarded in autoclavable trash bags and discarded according to PRO/5.1/04.

5. Experimental

5.1. Consumables

5.1.1. Reagents

- DNAExitusPlus (PanReac AppliChem) or DNA Remover (Minerva Biolabs)
- Ethanol (100%) stored at 20 ± 5°C
- DEPC-H2O stored at 20 ± 5°C
- EC_CAPV stored at <-72°C
- Nucleospin Blood Genomic DNA frm blood 250 preps, Macherey-Nagel, stored at 20 ± 5°C
    - Buffer B3: 60 ml
    - Wash buffer BW: 150 ml
    - Wash buffer B5 concentrate: 50 ml
    - Elution buffer BE: 60 ml
    - Proteinase K (lyophilized): 2x 75mg
    - Proteinase Buffer PB: 8 ml
- Nucleospin Blood Colums (red rings): 250
    - Collection tubes (2 ml): 500
    - Nucleospin Tissue Genomic DNA from tissue 250 preps, Macherey-Nagel, stored at 20 ± 5°C
    - Lysis buffer T1: 100 ml
    - Lysis buffer B3: 75 ml
    - Wash buffer BW: 150 ml
    - Wash buffer B5 concentrate: 50 ml
    - Elution buffer BE: 60 ml
    - Proteinase K (lyophilized): 2 x 75 mg
    - Proteinase buffer PB: 8 ml
    - Nucleospin Tissue colums (green rings): 250
    - Collection tubes (2ml): 500

When a new extraction kit is opened, the date of opening and the initials of the technician who opened it have to be noted on the box. Do not mix buffers with different batch numbers.
For each test kit the Quality Control Data Sheet together with the batch number has to be archived

5.1.2. Lab equipment

- Petri dishes
- Scalpels
- Silicon carbide beads (1,0 mm)
- 2 ml tubes
- Vortex

- Disposabel micropipette tips
- Latex/nitril exam gloves
- Biosafety cabinet II
- Chemical cabinet
- Centrifuge for microtubes
- Tissuelyser
- Refrigerator (5°C ± 3°C)
- < -72°C freezer
- < -20°C freezer
- Pipettes
- Precision balance
- Heating block for (37°C ± 5°C), (56°C ± 5°C) and (70°C ± 5°C)

5.2. Environmental conditions

The test is performed at room temperature (21°C ± 5°C)

5.3. Method

5.3.1. Preparation of internal controls

- Internal controls
  - Negative exctraction control (NEC)

    The NEC is DEPC-treated water, run in parallel to the samples (simultaneously and using the same protocol).

  - Positive extraction control (EC_CAPV)

    The positive extraction control is a synthetic DNA with a random sequence unrelated to the tested pathogens)

5.3.2. Preparation of solutions

- Lysis buffer B3+ EC_CAPV
  Dilute EC_CAPV 1/100 in B3 buffer and mix manually. B3+EC_CAPV can be stored for 3 days at 5°C ± 3°C.

- Wash buffer B5
  Add 200 ml of ethanol (96-100%) to the wash buffer B5 concentrate. Mark the label of the bottle to indicate that ethanol was added. B3 can be stored at 20 ± 5°C for 1 year.

- Proteinase K
  Add the indicated volume (3.35 ml per vial) of Proteinase buffer PB to dissolve the lyophilized proteinase K and prepare aliquots of 100 µl. The proteinase K solution is stable at -20°C for up to 6 months. Do not mix proteinase K and Buffer T1 more than 10 min before adding them to a tissue sample. Proteinase K will self-digest in buffer T without substrate.

5.3.3. Precaution of avoiding contamination

In order to avoid cross-contamination, the preparation of the positive amplification controls, the DNA extraction, the preparation of primer, probe/enzyme mixes and the real-time PCR amplification must always be performed in 4 separate rooms. Each room must have gloves, pipettes, microtube holders etc that should never be exchanged between rooms. Gloves must be worn during all manipulation and removed whenever leaving a room. Prior to each manipulation, the work surface of the laminar flow cabinets, pipette shafts and interior of the centrifuges must be cleaned with DNA ExitusPlus or DNA Remover. Microtubes/-plates must be kept closed whenever possible and always centrifuged briefly before opening. Only sterile RNase- and DNase-free plastic disposables may be used.

5.3.4. Preparation of samples, lysis of cells and virus and optimizing binding conditions

- Liquid samples
  - Preparation
    Check if buffer B5 and Proteinase K were prepared. Set the heating block to 70°C and preheat the Elution buffer BE to 70°C. Prepare Lysis buffer B3+EC (1/100).

  - Lysis of sample
    Thoroughly mix the sample by inverting and pipet 200µl sample into a 2ml tube. Add 25µl Proteinase K solution. Add 200 µl of Lysis buffer B3+EC to the sample and vortex vigorously (10-20 sec). Vigorously mixing is important to obtain high yield and purity of the extracted DNA. Incubate at 70°C ± 5°C for 10-15 min. At this point the lysate should become brownish during incubation with buffer B3. When processing old or clotted blood the incubation time can be increased up to 30 min with once or twice vortexing during incubation.

  - Adjust DNA binding conditions
    Add 210 µl ethanol (96-100%) to each sample and vortex again.

- Tissue samples
  - Preparation
    Check if Proteinase K has been prepared. Set a heating block to 56°C ± 5°C.

  - Lysis of sample
    Cut 40 mg of animal tissue into small pieces using a scalpel and a petri dish and place the sample in a microtube. Add 180 µl of buffer T1 and 25 µl Proteinase K solution. Add a drop of silicon carbide beads and vortex to mix. Place the samples in the TissueLyser for 6 min at 25 Hertz. Incubate the samples at 56°C overnight.
    The following morning set a heating block to 70°C ± 5°C and preheat the Elution buffer BE to 70°C. Prepare Lysis buffer B3+EC (1/100).

Vortex the samples and add 200 µl of lysis buffer B3+EC. Vortex vigorously and incubate at 70°C ± 5°C for 10 min. If insoluble particles are visible, centrifuge for 5 min at high speed and transfer the supernatant to a new microcentrifuge tube.

- Adjust DNA binding conditions
  Add 210 µl ethanol (96-100%) to each sample and vortex again.

5.3.5. DNA extraction

- Liquid samples

  - DNA Binding

For each sample, place one NucleoSpin blood Column (red) into a collection tube. Apply the sample to the column. Centrifuge 1 min at 11 000g. Discard the collection tube with flow-through and place the column in a new collection tube. If a sample is not drawn through the column completely repeat the centrifugation at higher g-force (<15 000g).

  - Wash steps:

Add 500 µl buffer BW and centrifuge 1 min at 11 000g. Discard the flow-through and reuse the collection tube. Add 600 µl buffer B5 and centrifuge 1 min at 11 000g. Discard the flow-through and reuse the collection tube.

  - Drying of the silica membrane:

Centrifuge 1 min at 11 000g to remove residual ethanol.

  - DNA elution:

Place the NucleoSpin blood column in a 1.5 ml microtube and add 100 µl of preheated Buffer BE (@70°C). Dispense the buffer directly onto the silica membrane and incubate at 20°C ± 5°C for 1 min. Centrifuge 1 min at 11 000g.

- Tissue samples
  - DNA binding:
    For each sample, place one NucleoSpin Tissue Column (green) into a collection tube. Apply the sample to the column. Centrifuge for 1 min at 11 000g. Discard the collection tube with the flow-through and place the column in a new collection tube. If a sample is not drawn through the column completely repeat the centrifugation at 11 000g.

  - Wash steps:
    Add 500 µl buffer BW. Centrifuge for 1 min at 11 000g. Discard the flow-through and place the column back into the collection tube. Add 600 µl of buffer B5 to the column and centrifuge for 1 min at 11 000g. Discard the fow-through and place the column back into the collection tube.

  - Drying of the silica membrane:

Centrifuge 1 min at 11 000g to remove residual ethanol.

  - DNA elution:

Place the NucleoSpin tissue column in a 1.5 ml microtube and add 100 µl of preheated buffer BE (@70°C). Dispense the buffer directly onto the silica membrane and incubate at 20°C ± 5°C for 1 min. Centrifuge 1 min at 11 000g.

5.3.6. Preservation of DNA

Following extraction, the DNA can be stored at 5°C ± 3°C for up to 4 hours or at <-72°C for up to 1 year.

5.3.7. Decontamination and cleaning of the laboratorial material

Used tips, tubes, colums, etc are collected in trash bags, which are subsequently autoclaved. Used scalpels are deposited in “sharp collectors” and sterilized in a formol sterilizer. Work surfaces are decontaminated.