IDENTIFICATION OF *Echinococcus granulosus* COMPLEX AT GENOTYPE/SPECIES LEVEL FROM HYDATID CYSTS BY PCR AND SEQUENCING

INDEX

1. Aim and field of application .......................................................... 2
2. Principle of the method ................................................................. 2
3. References ..................................................................................... 3
4. Definitions .................................................................................... 4
5. Devices/instruments ..................................................................... 5
6. Reagents and chemicals ............................................................... 6
7. Procedure ....................................................................................... 7
   7.1 Sample preparation .................................................................. 7
   7.2 DNA extraction ....................................................................... 8
   7.3 PCR amplification ................................................................... 9
   7.4 Display of PCR products ....................................................... 10
   7.5 Result interpretation ............................................................. 11
   7.6 PCR products purification ..................................................... 11
   7.7 Display of purified PCR products ......................................... 12
   7.8 Sequencing ............................................................................. 12
8. Interpretation of sequencing results ............................................. 13
9. Characteristics of the method ....................................................... 16
10. Safety measures .......................................................................... 16
1. **AIM AND FIELD OF APPLICATION**

This document describes a method that allows to identify the following genotypes/species belonging to the *E. granulosus* complex:

**Table A** – Genotypes and species belonging to the *Echinococcus granulosus* complex identifiable by this method.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1, G1BC, G2, G3</td>
<td><em>E. granulosus</em> sensu stricto</td>
</tr>
<tr>
<td>G4</td>
<td><em>E. equinus</em></td>
</tr>
<tr>
<td>G5</td>
<td><em>E. ortleppi</em></td>
</tr>
<tr>
<td>G6, G7, G8, G10</td>
<td><em>E. canadensis</em></td>
</tr>
</tbody>
</table>

This method can be applied to samples collected from hydatid cysts (protoscoleces or germinal layer) of human or animal origin preserved in ethanol.

2. **PRINCIPLE OF THE METHOD**

The PCR is a molecular biology technique that allows the amplification of specific nucleic acid fragments, which initial and terminal nucleotide sequences are known (oligonucleotide pair). If a species (or genotype) has its own characteristic DNA portion, due to its composition and/or dimension, it is possible to choose an oligonucleotide pair allowing its amplification. The PCR amplification is characterized by a high sensitivity and specificity. DNA sequencing is the process of determining the exact primary structure of a given DNA fragment (i.e. Adenine, Cytosine, Guanine and Thymine).

The classification and taxonomy of *Echinococcus* spp. has been controversial for several decades. However, based on phylogenetic studies, the status of species has been re-evaluated and confirmed for *E. granulosus*, *E. multilocularis*, *E. oligarthrus* and *E. vogeli*. Moreover, *E. felidis* and *E. shiquicus* have been confirmed as sister species of *E. granulosus* sensu stricto and *E. multilocularis*, respectively.

*Echinococcus granulosus* is a complex of species/genotypes which differ in terms of life-cycle patterns and hosts. The biological variants of *E. granulosus* have been designated as strains; molecular genetic studies support this designation and resulted in the identification of ten genotypes included in this taxon (from G1 to G10). Based on mitochondrial DNA (mtDNA) analysis, the *E. granulosus* complex has been split into *E. granulosus* sensu stricto (genotypes G1, G2 and G3), *E. equinus* (G4), *E. ortleppi* (G5) and the still controversial taxon *E. canadensis* (G6-G10). Variants within *E. granulosus* s. s. include the sheep (G1), the Tasmanian sheep (G2) and the buffalo (G3) strains. Variants within *E. canadensis* include the camel strain (G6), the pig strain (G7), the north American cervid strain (G8), the human polish strain (G9), and the fennoscandian cervid strain (G10). Nevertheless, since the molecular data available on G9 genotype and the *E. felidis* species are still very limited, these two taxa will not be taken into account in this method. Moreover, as reported in table A, the method includes a variant of the G1 genotype, the G1BC.

The genotypes of the *E. granulosus* complex can be differentiated by the composition of the nucleotide sequences of the mitochondrial marker used (cytochrome c oxidase subunit 1). By comparing the sequencing products with the reference sequences registered in the GenBank, it is possible to unambiguously identify all the species/genotypes belonging to the *E. granulosus* complex.
The method consists of the following phases:

1. DNA extraction from the hydatid cyst
2. Amplification reaction (PCR)
3. Electrophoresis 1
4. Amplicon purification
5. Electrophoresis 2
6. Marker reaction (PCR)
7. Sequence purification
8. Sequencing
9. Analysis of the results

3. REFERENCES


ISO/FDI 20837:2006(E). Microbiology of food and animal feeding stuffs – Polymerase chain reaction (PCR) for the detection of food-borne pathogens - Requirements for sample preparation for qualitative detection.
ISO/FDI 20838:2006(E). Microbiology of food and animal feeding stuffs – Polymerase chain reaction (PCR) for the detection of food-borne pathogens - Requirements for amplification and detection for qualitative methods.


4. DEFINITIONS

**Amino acids** - group of organic acids containing one or more aminic groups forming the proteins.

**Amplicon** - DNA sequence (or DNA fragment) amplified by PCR.

**Codon** - sequence of 3 nucleotides (triplet) codifying for a specific amino acid.

**CO1** - Cytochrome c oxidase subunit 1.

**Consensus sequence** - sequence derived from the alignment of the *forward* and *reverse* sequences.

**DNA/Metacestode** - DNA extracted from a single test sample.

**Forward sequence** - Nucleotide sequence resulting from using the primer *forward*.

**G1_AB033407_Reference** - mitochondrial sequence codifying for CO1 extracted from the genotype G1 sequence of the *E. granulosus* sensu stricto species and registered in GenBank (n° AF297617; from 436 to 786 base pairs).

**Negative control for the amplification** - Reagent grade water. This control is used in the metacestode DNA amplification session to verify the efficacy of the PCR system.

**Oligonucleotide** - short sequence (15/30 nucleotide bases) used to amplify a DNA specific fragment.

**Positive control for the DNA extraction** - a reference cyst analysed in the same working session of test samples, to verify the efficacy of the DNA extraction session.

**Positive control for the amplification** - mitochondrial DNA extracted from reference hydatid cysts. It is used in the metacestode DNA amplification sessions to verify the proper functioning of the PCR system.

**PCR** - Polymerase Chain Reaction.
Reference DNA - DNA extracted from reference hydatid cysts and identified at genotype level by amplification and sequencing of the CO1 marker.

Reference hydatid cysts - Hydatid cysts belonging to the G1, G2, G3, G4 and G5 genotypes preserved in ethanol and sent by the Parasitological Institute of the University of Bern, Switzerland.

Reference sequence - Mitochondrial sequence codifying for CO1 "[G1]_AB033407_Reference".

Reverse sequence - Nucleotide sequence resulting from using the primer reverse.

Test sample - Hydatid cyst preserved in ethanol, to be identified.

The definitions and terminology used in the UNI EN ISO 22174 standard are applied in this document.

5 DEVICES/INSTRUMENTS

5.1 Adjustable volume pipettes, volume range: 1-10µL, 2-20µL, 20-100µL, 50-200µL, 200-1000µL.
5.2 Analytical balance, readability 0.1g.
5.3 Analytical grade water system production, resistivity ≥ 18 Mohm/cm.
5.4 Bench top refrigerated centrifuge for 1.5 mL tubes, minimum 10,000xg.
5.5 Bench top refrigerated centrifuge for 12 mL tubes.
5.6 Biohazard hood.
5.7 Chemical hood.
5.8 Digital imaging system.
5.9 DNA sequencer equipped with a capillary apparatus and POP fixed phase with management and sequence analysis software.
5.10 Freezer ≤/15°C.
5.11 Horizontal electrophoretic apparatus.
5.12 Magnetic separation stand.
5.13 PCR cabinet.
5.14 PCR thermocycler.
5.15 Refrigerator, temperature range +1 ÷ +8°C.
5.16 Stereo microscope, magnification 60÷100x.
5.17 Thermoblock with vibration, temperature range 25+100°C.
5.18 UV transilluminator.
5.19 Vortex.
6 REAGENTS AND CHEMICALS

6.1 **Proteinase K.** Commercial solution. Store according to the manufacturer’s recommendations.

6.2 **DTT (Ditiotreitol).** Commercial solution. Store at -20°C. Store according to the manufacturer’s recommendations.

6.3 **Lysis buffer A.** Commercial solution: Wizard Magnetic DNA Purification System for Food, Promega. Store according to the manufacturer’s recommendations.

6.4 **RNase A.** Commercial solution: Wizard Magnetic DNA Purification System for Food, Promega. Store according to the manufacturer’s recommendations.

6.5 **Lysis Buffer B.** Commercial solution: Wizard Magnetic DNA Purification System for Food, Promega. Store according to the manufacturer’s recommendations.

6.6 **Precipitation Solution.** Commercial solution: Wizard Magnetic DNA Purification System for Food, Promega. Store according to the manufacturer’s recommendations.

6.7 **Magnesil.** Commercial solution: Wizard Magnetic DNA Purification System for Food, Promega. Store according to the manufacturer’s recommendations.

6.8 **Isopropanol.** Commercial solution. Store according to the manufacturer’s recommendations.

6.9 **Ethanol 70%.** Preparation of 50 mL: take 35 mL of the Ethanol 100% solution and add 15 mL of distilled water. Store up to 12 months.

6.10 **Ethanol 100%.** Commercial solution. Store according to the manufacturer’s recommendations.

6.11 **Milli-Q grade water.** Resistivity ≥ 18 Mohm/cm.

6.12 **Reference DNA.** Genomic purified DNA from reference hydatid cyst. Store at -20°C (5.3) up to 5 years.

6.13 **DNA/Metacestode.** DNA preparation extracted from a single hydatid cyst. Store frozen for up to 5 years.

6.14 **PCR master mix.** Commercial solution for PCR amplification assays. Store according to the manufacturer’s recommendations.

6.15 **CO1.F and CO1.R Oligonucleotide.** Commercial preparation (table B). The lyophilised products is reconstituted with TE 0.1x solution (6.19), according to the manufacturer’s recommendations, at a concentration of 100 pmol/µL; the lyophilised product can be stored frozen for up to 5 years; the reconstituted product can be stored frozen up to 24 months.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>code</th>
<th>marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’-TTT.TTT.GGC.CAT.CCT.GAG.GTT.TAT-3’</td>
<td>CO1.F</td>
<td>CO1</td>
</tr>
<tr>
<td>5’-TAA.CGA.CAT.AAC.ATA.ATG.AAA.ATG-3’</td>
<td>CO1.R</td>
<td></td>
</tr>
</tbody>
</table>

6.16 **Reference hydatid cyst.** Hydatid cysts belonging to G1, G2, G3, G4 and G5 genotypes provided by the Institute of Parasitology, Bern, Switzerland.

6.17 **2x PCR master mix.** 2x commercial solution (composition: dATP 400 µM, dCTP 400 µM, dGTP 400 µM, dTTP 400 µM, MgCl2 3mM, Taq DNA polymerase 50 U/mL). Store according to the manufacturer’s recommendations.

6.18 **TE 1x solution.** Commercial product 10mM Tris-HCl (pH 8.0), 1mM EDTA-Na₂, pH 7.9–8.1 at 25°C. Store refrigerated for up to 12 months.

6.19 **TE 0.1x solution.** TE 1x diluted solution. For 100 mL preparation: take 10 mL from 1x solution and
add 90 mL water. Filter with 0.22 µm filters and prepare aliquots of 10 mL. Store frozen for up to 24 months.

6.20 **Loading buffer 6x.** Commercial product allowing DNA molecule electrophoresis to be performed. Store according to the manufacturer’s recommendations.

6.21 **Agarose.** Commercial product suitable for performing DNA molecule electrophoresis. Store at room temperature for up to 24 months.

6.22 **TAE solution 50x.** Commercial product (2M Tris-acetate, 50mM EDTA, pH 8.2–8.4 at 25°C). Store at room temperature for up to 24 months.

6.23 **TAE solution 1x.** 1000 mL preparation: take 20 mL of the 50x solution and bring to 1000 mL with water. Store at room temperature for up to 1 month.

6.24 **Ethidium bromide solution.** Commercial product 10 mg/L. To prepare the working solution, dilute 1:100,000; for 100 mL solution, add 1.0 µL. Store in the dark at room temperature for up to 24 months. **NOTE:** Ethidium bromide is potentially mutagenous, carcinogenic and teratogenic; wear disposable gloves and handle the solution containing this substance very carefully.

6.25 **L50.** Commercial product containing markers for DNA molecular weight multiple of 50 bp. All commercial products containing molecules of multiples of 50 bp within the 50-500 bp range can be used. Store refrigerated according to manufacturer’s recommendations.

6.26 **Membrane Binding Solution.** Commercial product: Wizard SV Gel and PCR Clean-Up System. Store according to the manufacturer’s recommendations.

6.27 **Wizard SV Minicolumns.** Commercial product: Wizard SV Gel and PCR Clean-Up System. Store according to the manufacturer’s recommendations.

6.28 **Collection Tubes.** Commercial product: Wizard SV Gel and PCR Clean-Up System.

6.29 **Membrane Wash Solution.** Commercial product: Wizard SV Gel and PCR Clean-Up System. Store according to the manufacturer’s recommendations.

6.30 **Centri-Sep.** Applied Biosystem commercial product for the purification of sequencing. Store according to the manufacturer’s recommendations.


6.32 **BUFFER 10X.** Buffer for electrophoretic runs. Store according to the manufacturer’s recommendations.

6.33 **POP Polymer.** Applied Biosystem commercial product: polymer for electrophoresis. Store according to the manufacturer’s recommendations.

7 **PROCEDURE**

7.1 **SAMPLE PREPARATION**

Test samples are checked to verify their conservation status. Test samples can be complete hydatid cyst (7.1.1), germinal layer (7.1.2) or protoscolex (7.1.3).

7.1.1 Complete hydatid cyst

a. Transfer the hydatid cyst in a Petri dish and observe under the stereo microscope (5.16) in order to identify the presence of protoscoleces.

b. Aspire with a 10 mL syringe the content of the cyst and subsequently cut a piece of germinal layer.
Transfer both samples in a 2 mL tube.

c. Centrifuge tube (5.4) for 3 min at 3,000 x g, and discard the liquid phase.

d. Add deionised water (Milli-Q) (6.11) in the 2 mL tube.

e. Re-suspend the solution by vortexing.

f. Repeat step c) and d) for a total of 3 times.

g. Collect 200 mg of pellet and transfer in a 2 mL tube.

7.1.2 Germinal layer

a. Cut a piece of germinal layer. Transfer the germinal layer to a 2 mL tube.

b. Follow the steps 7.1.1 c)-f).

c. Collect 200 mg of tissue and transfer in a 2 mL tube.

7.1.3 Protoscolecetes

a. Aspire the protoscolecetes with a 10 ml syringe. Transfer the protoscolex to a 2 mL tube.

b. Follow the points 7.1.1 from c) to f).

c. Collect 200 mg of protoscolecetes and transfer in a 2 mL tube.

Store the tubes frozen. Under these conditions, larvae can be stored for the DNA extraction for up to 10 years.

7.2 DNA EXTRACTION

If not otherwise specified, the procedure is carried out at room temperature.

a. Unfreeze the test samples.

b. Add to the sample 400 µL of Lysis Buffer A (6.3), 50 µL of Proteinase K (6.1) and 50 µL of Dtt (6.2) and resuspend the solution by vortexing (5.19).

c. Incubate the solution for 15-20 min in the thermoblock (5.7) at 60°C (850 rpm).

d. Incubate the solution for 10 min in the thermoblock (5.17) at 90°C (850 rpm).

e. Add to the sample 250 µL of Lysis Buffer B (6.5), 5 µL of Rnase A (6.4) and resuspend the solution by vortexing (5.19).

f. Incubate the solution for 10 min at room temperature.

g. Add 750 µL of Precipitation solution (6.6) and resuspend the solution by vortexing (5.19).

h. Centrifuge the sample at 13,000 x g (5.19) for 7 min.

i. Transfer 1,0 mL of supnatant into a 2 mL tube.

j. Add 50 µL of magnetic resin (MagneSil) (6.7) to the supnatant and resuspend the solution by vortexing (5.19).

k. Add 0.8 vol of isopropanol (6.8), mix ten times the tube and incubate for 7 min.

l. Place the tube in the magnetic separation stand MagneSphere (5.12) and wait for 30-60 sec, so that the paramagnetic resin particles are blocked by the magnetic field on the tube wall.
m. Discard the liquid phase by aspirating, avoiding the dislodging of the resin particles.

n. Remove the tube from the MagneSphere, add 250 µL of Lysis Buffer B (6.5) and invert the tube 2-3 times.

o. Place the tube in the magnetic separation stand MagneSphere and wait for 30-60 sec.

p. Discard the liquid phase by aspirating, avoiding the dislodging of the resin particles.

q. Remove the tube from the MagneSphere, and add 1.0 mL of 70% ethanol (6.9).

r. Place the tubes in the magnetic separation stand MagneSphere and wait for 30-60 sec.

s. Repeat steps p)-r) for 3 times in total.

t. Incubate the tube for 15 min at 65°C in the thermoblock (5.17) or 30 min at room temperature.

u. Add 100 µL Nuclease-Free water (6.11), gently resuspend the resin particles, do not vortex and incubate for 5 min at 65°C in the thermoblock (5.7).

v. Place the tubes in the magnetic separation stand MagneSphere (5.6) and wait for 30-60 sec. Collect the liquid phase (80-90 µL) and transfer to a 1.5 mL tube.

w. If the final volume is less than 100 µL, add Nuclease-Free water.

The resulting extract is defined as “DNA/Metacestodes” (6.13). Under these conditions, it can be stored for up to 5 years.

### 7.3 PCR AMPLIFICATION

Unless otherwise clearly stated, store tubes on ice; use tips with barrier and wear disposable gloves. At each working session use a positive control for the DNA extraction (DNA extracted from a Reference hydatid cyst, 6.18), a positive control for the amplification (reference DNA, 6.12) and a negative control for the amplification (water, 6.11) in order to verify the efficacy of PCR system.

The following procedure involves the use of a 2x MasterMix PCR concentration.

a. Thaw: DNA/Metacestodes (6.13), 2x PCR MasterMix (6.14), CO1.F and CO1.R (6.15), positive control for the DNA extraction (6.16), positive control for the amplification (6.12), negative control for the DNA extraction (6.11).

b. Prepare an adequate cumulative volume of the amplification mix. Evaluate the volume on the basis of a single sample amplification mix (table C) and of the total number of samples plus 4 (1 for the DNA extraction, 1 for the positive amplification control, 1 for the negative one and 1 extra sample every 12 test samples).

<table>
<thead>
<tr>
<th>Table C – mix of amplification for a single sample: compounds and volumes.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2x PCR MasterMix</strong></td>
</tr>
<tr>
<td><strong>H₂O Milli-Q</strong></td>
</tr>
<tr>
<td><strong>CO1.F marker</strong></td>
</tr>
<tr>
<td><strong>CO1.R marker</strong></td>
</tr>
<tr>
<td><strong>Total</strong></td>
</tr>
</tbody>
</table>
c. Mark with a progressive number an adequate number of 0.2 µL PCR tubes.
d. Mix the amplification mix by vortexing and centrifuge at maximum speed for a few sec.
e. Transfer 48 µL of the cumulative amplification mix to each PCR tube.
f. Add 2 µL of the DNA/Metacestodes (6.13) to be tested to each tube.
g. Close the tubes, mix by vortexing and centrifuge at maximum speed for a few sec.
h. Start the amplifying cycle (Table D) on the thermocycler device; wait for the temperature to reach 94°C and insert the tubes in the thermoblock by pausing the instrument.

Table D – PCR cycle for the identification of the test sample.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>7 min/94°C</td>
</tr>
<tr>
<td>Amplification</td>
<td>30 s/94°C; 30 s/55°C; 30 s/72°C</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>38</td>
</tr>
<tr>
<td>Final extension</td>
<td>5 min/72°C</td>
</tr>
</tbody>
</table>

i. Keep tubes on ice or refrigerated until starting electrophoresis.

7.4 DISPLAY OF PCR PRODUCTS

Electrophoresis
a. Assemble the electrophoresis apparatus according to the manufacturer’s recommendations. For the gel preparation, use a comb suited for the number of samples to be tested.
b. Add 2 gr agarose (6.21) in 100 mL TAE 1x (6.23) in a glass beaker.
c. Gently resuspend the powder by rotation.
d. Boil the agarose suspension for 30 sec. If the solution is not homogeneous, continue to boil for another 30 sec.
e. Restore with water the volume lost by boiling.
f. Allow the agarose solution to cool.
g. Before it solidifies (at about 47°C), add 1.0 µL of ethidium bromide solution (6.24).
h. Shake gently to dissolve uniformly the ethidium bromide and pour the agarose in the gel tray previously prepared (point “a”).
i. Wait for the gel to solidify, which requires at least 30 min.
j. Place the tray with the gel in the electrophoresis apparatus.
k. Cover the gel with TAE 1x buffer (6.23) and gently pull out the comb.
l. The first and last wells are loaded with 15 µL of the L50 solution (6.25).
m. Load in each well 20 µL of the amplification product, respecting the progressive numbering of the tubes (point 7.3 “c”).
n. Connect the electrophoresis apparatus with the power supply and set 10 v/cm of gel.
o. Run the gel for about 30 min or until the fastest dye, contained in the loading buffer (6.20), reaches a distance of 1 cm from the gel border.
p. After 30 min, switch off the power supply, place the gel under UV illumination and check the band separation. The electrophoresis run is adequate if it is possible to easily distinguish all bands of molecular weight marker ranging from 50 and 500 bp. If the separation is incomplete, continue the run.

q. At the end of the run, transfer the gel to the imaging system and print the result.

7.5 RESULT INTERPRETATION

The size of the amplification bands revealed by the electrophoresis is evaluated by their comparison with the reference molecular weight L50. Since all the genotype/species belonging to *E. granulosus* complex display a PCR product of 460 bp, thus the amplification test will be considered as valid if:

i) The positive control of amplification displays a band of 460 bp. If a 460 bp band is not present or different bands in size are present, repeat the PCR amplification using new reagents;

ii) The negative control of amplification do not show any amplification product or, eventually, only bands related to unused oligonucleotides and/or primer dimer. If other bands are present, repeat the PCR amplification using new reagents;

iii) The positive DNA extraction control display a band of 460 bp. If a 460 bp band is not present or different bands in size are present, repeat the PCR amplification using new reagents.

Evaluation of tested sample: if the band of 460 bp is not present, thus repeat the PCR amplification. If the second PCR results negative, thus repeat the DNA extraction of the sample. If the third PCR results negative, the sample will be considered as “not determinable”.

7.6 PCR PRODUCTS PURIFICATION

a. Add to the PCR product an equal volume of *Membrane Binding Solution* (6.26).
b. Transfer the solution in the *SV Minicolumn* (6.27), insert the column in the *collection tube* and incubate for 1 min.
c. Centrifuge the *SV Minicolumn* (5.5) at 16.000 x g for 1 min.
d. Remove the *SV Minicolumn* and discard the liquid phase from the *collection tube*.
e. Reinsert the *SV Minicolumn* in the *collection tube*.
f. Wash the *SV Minicolumn* adding 700 µL of *Membrane Wash Solution* (6.29) (with 95% ethanol added).
g. Centrifuge the *SV Minicolumn* (5.5) at 16.000 x g for 1 min.
h. Remove the *SV Minicolumn* and discard the liquid phase from the *collection tube*.
i. Wash the *SV Minicolumn* adding 700 µL of *Membrane Wash Solution* (6.29)
j. Centrifuge the *SV Minicolumn* (5.5) at 16.000 x g for 5 min.
k. Remove the *SV Minicolumn* and discard the liquid phase from the *collection tube*.
l. Centrifuge the *SV Minicolumn* (5.5) at 16.000 x g for 1 min to dry the binding matrix.
m. Transfer the *SV Minicolumn* in a new 1,5 mL tube. Add 50-100 µL of room temperature Nuclease-Free Water (6.11). Incubate for 1 min at room temperature.
n. Centrifuge the *SV Minicolumn* (5.5) at 16.000 x g for 1 min.
o. Discard the *SV Minicolumn*, transfer the 1,5 mL tube in a refrigerator (5.10).
7.7 DISPLAY OF PURIFIED PCR PRODUCTS

Test samples are displayed as described in paragraph 7.4. DNA concentration will be determined by comparing test sample with the reference marker.

7.8 SEQUENCING

Purified test samples (step 7.6) can be:
- send to a qualified laboratory for sequencing;
- analysed as described in paragraphs 7.8.1-7.8.3.

7.8.1 Preparation of the sequencing reaction

Unless otherwise clearly stated, store tubes on ice; use tips with barrier and wear disposable gloves. Sequencing reaction will be performed in 0.2 mL tubes, preparing two different reaction mixtures. Positive control for the amplification (6.12) will be used in the analysis.

PCR cycles are as follow:

<table>
<thead>
<tr>
<th>96°C for 10”</th>
<th>50°C for 5”</th>
<th>60°C for 2’</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 cycles</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. For each test sample, prepare two reaction mixtures (one for each primer used).
b. For each sample of the CO1.F primer reaction mixture, add the following reagents in a 1.5 mL:
   a. 2.5 µL of ABI 5x Sequencing Buffer;
   b. 1.5 µL of BigDye Terminator Mix;
   c. 0.5 µL of CO1.F Primer (3.2 pmol/µL);
   d. Add 4.5 µL of the mixture in a 0.2 mL tube. Add 5.5 µL of DNA (3-10ng) in each tube.
d. For each sample of the CO1.R primer reaction mixture, add the following reagents in a 1.5 mL:
   a. 2.5 µL of ABI 5x Sequencing Buffer;
   b. 1.5 µL of BigDye Terminator Mix;
   c. 0.5 µL of CO1.R Primer (3.2 pmol/µL);
e. Add 4.5 µL of the mixture in a 0.2 mL tube. Add 5.5 µL of DNA (3-10ng) in each tube.
f. Insert the samples in a thermocycler at 96°C and start the PCR program.

7.8.2 Purification (Removal of Dye Terminators prior to Sequencing)

*Column Hydration*

a. Gently tap the column (6.30) to insure that the dry gel has settled in the bottom of the spin column.
b. Remove the top column cap and reconstitute the column by adding 0.8 mL of reagent grade water or buffer. Leave the column end stopper in place so the column can stand up by itself. Replace the column cap and hydrate the gel by shaking and inverting the column or vortexing briefly. It is important to hydrate all of the dry gel.

c. Wait at least 30 minutes at room temperature before using the columns. Reconstituted columns may be stored refrigerated at 4°C (5.15) for several days. Longer storage can be accomplished in 10 mM sodium azide (NaN₃). Allow refrigerated columns to warm at room temperature before continuing this procedure.

Removal Of Interstitial Fluid

a. Remove air bubbles from the column gel by inverting and sharply tapping the column, allowing the gel to slurry to the opposite end of the column. Stand the column up and allow the gel to settle while in a microtube rack.

b. After the gel has settled and is free of bubbles, first, remove the top column cap and then remove the column end stopper from the bottom.

c. Allow excess column fluid to drain (gravity) into a wash tube (2 mL). If the fluid does not begin to flow immediately through the end of the column, use a 2 mL latex pipet bulb to apply gentle air pressure to the top of the column to force the fluid to start passing through the column filter. The column will stop draining on its own. Approximately 200–250 µL will drain from the column. Discard this fluid.

d. Spin the column and wash tube in a variable speed centrifuge at 750 × g for 2 min to remove interstitial fluid. If you use a micro-centrifuge, it is important to keep track of the position of the column using the orientation mark molded into the column.

e. Approximately 300 µL of fluid will be removed. If there is a drop at the end of the column, blot it dry. Discard the wash tube and the interstitial fluid. Do not allow the gel material to dry excessively. Process the sample within the next few min.

Sample Processing

a. Hold the column up to the light. Transfer 20 µL of completed DyeDeoxy terminator reaction mixture to the top of the gel. Carefully dispense the sample directly onto the centre of the gel bed at the top of the column, without disturbing the gel surface. Do not contact the sides of the column with the reaction mixture or the sample pipet tip, since this can reduce the efficiency of purification and possibly ruin the analysis due to excess dyes.

b. Place the column into the sample collection tube (1.5 mL) and place both into the rotor. Maintain proper column orientation. The highest point of the gel media in the column should always point toward the outside of the rotor (see Figure 1). Spin the column and collection tube at 700-800 × g for 2 min (5.4). The purified sample will collect in the bottom of the Sample Collection Tube. Discard the spin column and proceed with the ABI sample preparation procedure.

7.8.3 Electrophoretic run in the sequencer

Add 15 µL of formamide HiDi to each test sample (5 µL) to a final volume of 20 µL. Run the samples in the sequencer. Plates can be stored at -20°C (5.10).

8 Interpretation of sequencing results

The forward and the reverse sequences are considered acceptable if peaks are well defined and not superimposed with a low background noise that permit to unequivocally identify the single nucleotides (Figure 1).
**Figure 1** – electropherogram of the reference sequence codifying for CO1.

a. Create the **consensus** sequence between the **forward** and **reverse** sequences using the program Accelrys gene 2.5 (Accelrys, San Diego, CA, USA). Align the **consensus** with the reference sequence “[G1A]_AB033407_Reference” (Figure 2). **Consensus** is considered acceptable if the overlapping of the **forward** and **reverse** sequence do not create any ambiguity in the identification of the reference codons. If any ambiguous codon is present, thus repeat the “**Preparation of the sequencing reaction**” (7.8.1).

b. Analyse the **consensus** file of the test sample defining the 5 amino acid that are codified by the codons: 16 (positions 46-47-48), 18 (positions 52-53-54), 20 (positions 58-59-60), 84 (positions 250-251-252) and 87 (positions 259-260-261). The positions of the codons refer to the reference sequence “[G1A]_AB033407_Reference” (Figure 2).

**Figure 2** – Reference nucleotide sequence “[G1]_AB033407_Reference”. The Amino acid composition of the 5 codons (evidenced in brackets) determine the assignation of genotype/species belonging to the E. granulosus complex.

The interpretation of the amino acid codified by the triplets refers to table E.
### Table E – genetic code for the interpretation of amino acid codifying for the triplets.

<table>
<thead>
<tr>
<th>Second letter</th>
<th>T</th>
<th>C</th>
<th>A</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATT Ile [I]</td>
<td>ACT Thr [T]</td>
<td>AAT Asn [N]</td>
<td>AGT Ser [S]</td>
<td>T</td>
</tr>
<tr>
<td>ATC Ile [I]</td>
<td>ACC Thr [T]</td>
<td>AAC Asn [N]</td>
<td>AGC Ser [S]</td>
<td>C</td>
</tr>
<tr>
<td>ATA Ile [I]</td>
<td>ACA Thr [T]</td>
<td>AAA Asn [N]</td>
<td>AGA Ser [S]</td>
<td>A</td>
</tr>
</tbody>
</table>

The amino acids that are present in table F identify the genotype/species belonging to *E. granulosus* complex.

### Table F – Amino acid utilised for the identification of the test sample at genotype/species level belonging to *E. granulosus* complex.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Species</th>
<th>Amino acid (Codon 16)</th>
<th>Amino acid (Codon 18)</th>
<th>Amino acid (Codon 20)</th>
<th>Amino acid (Codon 84)</th>
<th>Amino acid (Codon 87)</th>
</tr>
</thead>
</table>
In the case the amino acid composition of the test sample results as that of *E. canadensis* (G6 or G7 genotypes), the genotype identification will be evaluated using the codon 2 (*positions 4-5-6*) respect to the reference sequence "[G1]_AB033407_Reference". In that case the identification follows table G.

**Table G** – nucleotide composition of codon 2 for the identification of the genotypes G6 e G7 belonging to the species *E. canadensis*.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Species</th>
<th>Codon 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6</td>
<td><em>E. canadensis</em></td>
<td>[CCT]</td>
</tr>
<tr>
<td>G7</td>
<td><em>E. canadensis</em></td>
<td>[CCC]</td>
</tr>
</tbody>
</table>

### 9 CHARACTERISTICS OF THE METHOD

This method has been characterised in terms of repeatability and specificity. The results of the validation protocol were used to confirm that the method is suitable for the expected aim and are reported in the validation report, which can be received upon request.

### 10 SAFETY MEASURES

This method has to be carried out only by authorized personnel. The operator should wear individual protection devices during the test performance. For general safety measures, refer to the CDC guidelines.