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

INDEX

1. AIMS AND FIELD OF APPLICATION

This document describes a method that allows the identification of the following genotypes/species of *E. granulosus* complex:

Table A *– Genotypes and species of* Echinococcus granulosus *complex identifiable by this method.*

Genotypes	Species
G1, G1BC, G2, G3	*E. granulosus* sensu stricto
G4	*E. equinus*
G5	*E. ortleppi*
G6, G7, G8, G10	*E. canadensis*

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

2. PRINCIPLE OF THE METHOD

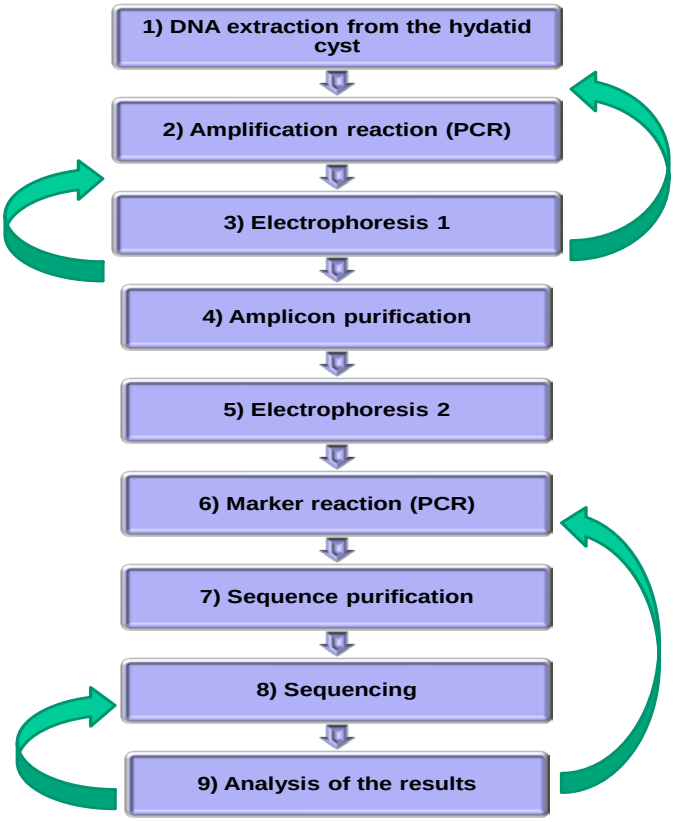
PCR is a molecular biology technique that allows the amplification of specific nucleic acid fragments, for which initial and terminal nucleotide sequences are known (oligonucleotide pair). If a species (or genotype) has its own characteristic DNA portion, due to its composition and/or dimension, it is possible to choose an oligonucleotide pair allowing its amplification. The PCR amplification is characterized by 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) whereas the taxon including *E. canadensis* (G6-G10) is still controversial. 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 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 *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 *E. granulosus* species complex.

The method consists of the following phases:



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UNI EN ISO 22174:2005. Microbiologia di alimenti e mangimi per animali – reazione a catena di polimerizzazione (PCR) per la ricerca di microrganismi patogeni negli alimenti – requisiti generali e definizioni.

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 *forward* primer.

G1_AB033407_Reference - mitochondrial sequence codifying CO1 DNA 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 – Molecular grade PCR water. This control is used in the PCR 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 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 received from 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 *reverse* primer.

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 manufacturer's recommendations.

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

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

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

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

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

6.7 **Magnesil**. Commercial solution: Wizard Magnetic DNA Purification System for Food, Promega0. Store according to manufacturer's recommendations.

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

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

6.10 **Ethanol 100%**. Commercial solution. Store according to 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) for 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 for up to 24 months.

Table B *Oligonucleotide sequences used in the analysis.*

Sequence	code	marker
5'-TTT.TTT.GGC.CAT.CCT.GAG.GTT.TAT-3'	CO1.F	CO1
5'-TAA.CGA.CAT.AAC.ATA.ATG.AAA.ATG-3'	CO1.R	

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 manufacturer's recommendations.

6.18 **TE 1x solution**. Commercial product 10mM Tris-HCl (pH 8.0), 1mM EDTA-Na_2, 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: 10 mL from 1x solution are added to 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: 20 mL of the 50x solution are brought up 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 manufacturer's recommendations.

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

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

6.31 **Kit BigDye Terminator**. Applied Biosystem commercial product for sequencing: Ready Reaction Mix, BigDye Terminator v1.1/3.1, Sequencing Buffer (5X). Store according to manufacturer's recommendations.

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

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

7 PROCEDURE

7.1 SAMPLE PREPARATION

Test samples are checked to verify their 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 onto 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 contents of the cyst and subsequently cut a piece of germinal layer. Transfer both samples into 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) to 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 into 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 into a 2mL tube.

7.1.3 Protoscoleces

a. Aspire the protoscoleces 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 protoscoleces and transfer in a 2 mL tube.

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

7.2 DNA EXTRACTION

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

a. Defrost 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 surnatant into a 2 mL tube.
j. add 50 µL of magnetic resin (*MagneSil*) (6.7) to the surnatant and resuspend the solution by vortexing

(5.19).

k. Add 0,8 vol of isopropanol (6.8), mix ten times 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 dislodging 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 stated, store tubes on ice; use barrier tips and disposable gloves. At each working session use a positive control for the DNA extraction (DNA extracted from a Reference hydatid cyst, 6.16), 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 reagent.

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 for 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 C *– Mix of amplification for a single sample: compounds and volumes.*

2x PCR MasterMix	25 μL
H_2O Milli-Q	18 μL

2x PCR MasterMix	25 µL
CO1.F marker	2,5 µL (12.5 pmol)
CO1.R marker	2,5 µL (12.5 pmol)
Total	48 µL

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.*

Initial denaturation	7 min/94°C
Amplification	30 s/94°C; 30 s/55°C; 30 s/72°C
Number of cycles	38
Final extension	5 min/72°C

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

7.4 VISULISATION 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) to 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 using 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 INTERPRETATION OF RESULTS

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 band sizes are present, repeat the PCR amplification using new reagents;

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

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

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

7.6 PURIFICATION OF PCR PRODUCTS

a. Add to the PCR product an equal volume of *Membrane Binding Solution* (6.26).

b. Transfer the solution into 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* into 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,* Store the 1,5 mL tube in the refrigerator (5.10).

7.7 VISULISATION 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 stated, store tubes on ice; use barrier tips and 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:

96°C for 10''	50°C for 5''	60°C for 2'
25 cycles		

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);

c. Add 4.5 µL of the mixture into 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 into 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 micro-tube 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.

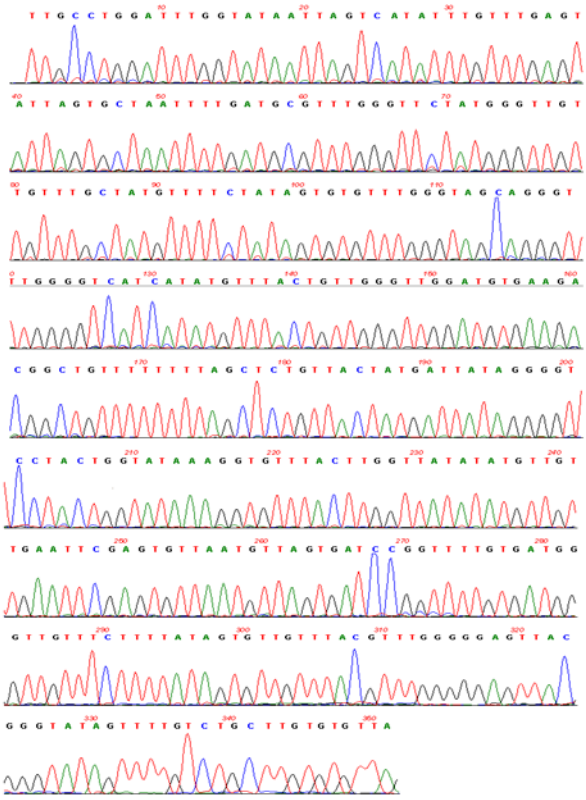
7.8.3 Electrophoretic run in the sequencer

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 permits unequivocal identification of 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, 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 assigning 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.*

		Second letter					
		T	**C**	**A**	**G**		
First letter	**T**	TTT Phe [F]	TCT Ser [S]	TAT Tyr [T]	TGT Cys [C]	**T**	**Third letter**
		TTC Phe [F]	TCC Ser [S]	TAC Tyr [T]	TGC Cys [C]	**C**	
		TTA Leu [L]	TCA Ser [S]	TAA Ter [end]	TGA Trp [W]	**A**	
		TTG Leu [L]	TCG Ser [S]	TAG Ter [end]	TGG Trp [W]	**G**	
	C	CTT Leu [L]	CCT Pro [P]	CAT His [H]	CGT Arg [R]	**T**	
		CTC Leu [L]	CCC Pro [P]	CAC His [H]	CGC Arg [R]	**C**	
		CTA Leu [L]	CCA Pro [P]	CAA Gln [Q]	CGA Arg [R]	**A**	
		CTG Leu [L]	CCG Pro [P]	CAG Gln [Q]	CGG Arg [R]	**G**	
	A	ATT Ile [I]	ACT Thr [T]	AAT Asn [N]	AGT Ser [S]	**T**	
		ATC Ile [I]	ACC Thr [T]	AAC Asn [N]	AGC Ser [S]	**C**	
		ATA Ile [I]	ACA Thr [T]	AAA Asn [N]	AGA Ser [S]	**A**	
		ATG Met [M]	ACG Thr [T]	AAG Lys [K]	AGG Ser [S]	**G**	
	G	GTT Val [V]	GCT Ala [A]	GAT Asp [D]	GGT Gly [G]	**T**	
		GTC Val [V]	GCC Ala [A]	GAC Asp [D]	GGC Gly [G]	**C**	
		GTA Val [V]	GCA Ala [A]	GAA Glu [E]	GGA Gly [G]	**A**	
		GTG Val [V]	GCG Ala [A]	GAG Glu [E]	GGG Gly [G]	**G**	

Amino acids shown 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.*

Genotype	**Species**	**Amino acid** (Codon **16**)	**Amino acid** (Codon **18**)	**Amino acid** (Codon **20**)	**Amino acid** (Codon **84**)	**Amino acid** (Codon **87**)
G1	*E. granulosus* s. s.	**Ala [A]**	**Phe [F]**	**Ala [A]**	**Ser [S]**	**Val [V]**

G1BC	*E. granulosus* s. s.	**Ala [A]**	**Phe [F]**	**Val [V]**	**Ser [S]**	**Val [V]**
G2	*E. granulosus* s. s.	**Ala [A]**	**Phe [F]**	**Val [V]**	**Ser [S]**	**Ala [A]**
G3	*E. granulosus* s. s.	**Ala [A]**	**Phe [F]**	**Ala [A]**	**Ser [S]**	**Ala [A]**
G4	*E. equinus*	**Ala [A]**	**Leu [L]**	**Val [V]**	**Asn [N]**	**Lys [K]**
G5	*E. ortleppi*	**Ala [A]**	**Leu [L]**	**Val [V]**	**Asn [N]**	**Arg [R]**
G6	*E. canadensis*	**Ser [S]**	**Leu [L]**	**Val [V]**	**Asn [N]**	**Ala [A]**
G7	*E. canadensis*	**Ser [S]**	**Leu [L]**	**Val [V]**	**Asn [N]**	**Ala [A]**
G8	*E. canadensis*	**Ala [A]**	**Leu [L]**	**Val [V]**	**Asn [N]**	**Gly [G]**
G10	*E. canadensis*	**Ser [S]**	**Leu [L]**	**Val [V]**	**Asn [N]**	**Ser [S]**

In case amino acid composition of the test sample is that of *E. canadensis* (G6 or G7 genotypes), the genotype identification will be evaluated using codon 2 (*positions 4-5-6*) in 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.

Genotype	Species	Codon 2
G6	*E. canadensis*	**[CCT]**
G7	*E. canadensis*	**[CCC]**

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 is to be conducted by authorized personnel only. The operator should wear individual protection devices during the test performance. For general safety measures, please refer to the CDC guidelines .