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EURL-AP Standard Operating Procedure

DNA extraction using the "Wizard® Magnetic DNA purification system for Food" kit

Experts for edition and revision	
Version 1.0	**Last major revision**
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Content

1. SUMMARY

This SOP is a binding complement to point 2.2.4. of Annex VI to Commission Regulation (EC) No 152/2009 as lastly amended by Commission Regulation (EU) No 51/2013 and describes the DNA extraction method to be used for the detection of animal DNA in feed samples as matrix.

The extraction consists of a lysis step that aims to bring the DNA into solution. The liberated DNA is recovered using silica coated on magnetic beads. This allows purification of DNA from several other compounds like proteins, glycosidic compounds or carbohydrates.

The SOP is based on two test portions per sample yielding two independent DNA extracts which can be used for PCR analysis. Controls to be included are also described.

2. SCOPE AND PURPOSE

The purpose of the DNA extraction method is to provide DNA for subsequent PCR based detection methods. The method has to yield DNA of sufficient quality and quantity but is also required to be suitable for routine use in terms of ease of operations, sample throughput and cost.

This protocol describes the DNA extraction procedure for the detection of animal DNA in a feed sample as the matrix (feed materials or compound feed). Two procedures are presented: a semi-automated one and a manual one.

This SOP is a binding complement to point 2.2.4. of Annex VI to Commission Regulation (EC) No 152/2009 as lastly amended by Commission Regulation (EU) No 51/2013.

Important remark: Because of the fact that the PCR results can be dependent on the DNA extraction method used, it is mandatory that DNA extracts used with the EURL-AP validated PCR methods are produced according to this SOP.

3. DEFINITIONS

3.1. DNA extraction and DNA purification

According to EN ISO 24276:2006, DNA extraction involves the steps to isolate the DNA from a matrix while DNA purification covers the steps increasing the purity of a DNA extract.

3.2. Controls

Definitions of the several controls to be used (“positive DNA extraction control”, “extraction blank control”) are the same as the ones considered in ISO 24276:2006.

3.3. PCR grade water

The protocol requires PCR grade water. When linking this to technical standards on water established by professional organizations, this might correspond either to Type I water (ASTM classification in ASTM D1193-91-06:2011) which is also called ultrapure water or Grade I water according to ISO 3696:1987.

3.4. Abbreviations used

- CEN : European Committee for Standardisation
- DNA : deoxyribonucleic acid
- ISO : International Organization for Standardisation
- NRL : national reference laboratory
- NA : not applicable
- p.a. : pro analysi (reagent grade)
- PAP : processed animal proteins
- RNAse : ribonuclease
- rpm : rotations per minute
- SOP : standard operating procedure

4. VALIDATION STATUS AND PERFORMANCE CHARACTERISTICS

4.1. Collaborative trials

The method was used in an implementation study organised by the EURL-AP to assess its implementation by the NRL network. In total 25 NRLs participated and 21 submitted results. The sample set received by the participants consisted of 10 blind samples but only 4 feed samples (blanks or feed matrices fortified with terrestrial processed animal proteins) were submitted to the DNA extraction protocol while the 6 others were ready to use DNA extracts.

The report of this implementation study can be found under :
http://eurl.craw.eu/img/page/interlaboratory/EURL_AP_PCR_ILS_2012_final_version.pdf

4.2. Limit of detection

The method detects 0.1% of PAP in feed as determined in an in-house validation (Fumière et al., 2006).

4.3. Specificity

NA

5. HEALTH AND SAFETY WARNINGS

Take into consideration all safety measures advised by the manufacturer of equipment for its use. Keep track of safety data sheets of all reagents involved in the process and take into consideration the safety warnings they contain.NA

6. EQUIPMENT AND MATERIALS

6.1. General instructions and precautions

- Laboratory organisation should follow the guidelines given by relevant authorities like EN ISO 24276 (General requirements and definitions).
- The DNA extraction step shall be executed in a laboratory or a space dedicated to this procedure and physically separated from rooms or workspaces where the other steps of the analysis are realized.
- Exposure of the work surface to UV-radiation for a period of time may be a helpful complementary way to decontaminate the bench area.
- Air conditioning shall be stopped before starting the extraction. Windows shall be closed to avoid air movements in the room.
- All handling of reagents and controls shall conform to ISO 9001:2000 or ISO17025 standards or equivalent.
- Handling of reagents and controls require dedicated equipment – especially pipettes.

- Reagents of the Promega kit have to be used within a defined temperature range (22°C-25°C). This is very important because temperature has a direct impact on the affinity of DNA towards silica. Not respecting the prescribed temperature range may result in poor yield of the DNA extract. On the other hand, Promega also advices to store the kit in a given temperature range (20°C-25°C). The temperature ranges provided by Promega are rather conservative. From the experience of EURL-AP for the purpose of PAP detection, this range can be extended. Use of the kit can be done in the range 18°C-25°C and storage in the range 15°C-25°C. **See at section 8 what to do if you are out of these temperature ranges.**
- The work surface is decontaminated by using e.g. HCl 0.1N or 10 % sodium hypochlorite solution (bleach with ~3% of active chlorine) before any extraction. This step can be followed by a cleaning of the surface with denatured ethanol (allows a more rapid drying)
- All the equipment used must be cleaned prior to use, for instance with DNA Erase or an equivalent treatment to remove any residual DNA. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for molecular biology applications. They must be DNase-free, DNA-free, sterile and shall not absorb proteins or DNA.
- In order to avoid contamination, filter pipette tips protected against aerosol shall be used.
- Use only powder-free gloves and change them frequently.
- Pipettes shall be checked regularly for precision and recalibrated if necessary.
- In order to avoid batch contaminations, aliquots of reagents shall be prepared.
- Vigorously mix the magnetic bead solution to get the beads in suspension but don’t vortex it.
- Paper towel holders must be enclosed to avoid contamination.

6.2. Equipment

- DNA extraction instrument (optional).
- Plastic reaction vessels suitable for the DNA extraction instrument (optional).
- Microcentrifuge.
- Micropipettes.
- Vortex.
- Rack for reaction tubes.
- 1.5/2.0 ml tubes (also called microcentrifuge tubes or vials).
- Special racks or microfuge tube holders with a magnet (magnetic separation stand).

6.3. Reagents

- DNA extraction kit “Wizard ® Magnetic DNA purification system for Food” (Promega, Madison, WI, USA – www.promega.com)
- Absolute ethanol p.a. grade or equivalent.
- HCl p.a. grade or any equivalent DNA degrading reagent.
- Isopropanol : 2-propanol p.a. grade.
- PCR grade water.

7. STEP BY STEP PROCEDURE

7.1. Sample preparation

The sample must be ground and homogenised before weighing of the test portions. The laboratory will avoid risk of cross-contamination of the samples by a careful cleaning of grinding equipment. Two representative test portions of 100 mg (100-105 mg) will each be weighed in a microcentrifuge tube of 2 ml.

In addition, each series of extraction will include:

- one positive DNA extraction control made of a sample adulterated at a level of ≤ 0.1 % in mass fraction of PAP* (e.g. 0.05 %).
- one extraction blank control made of PCR grade water

that will be submitted to the same extraction process as the samples.

7.2. Principle of the extraction method

The extraction method is based on the principle of the use of silica-coated magnetic beads. During a first lysis step, remaining cellular structures release their DNA content in the solution. This DNA will bind to the silica coated on the surface of the magnetic beads. In defined conditions of temperature, pH and ionic strength, DNA can show a high affinity towards silica. The buffers used are playing on this affinity so that the beads will catch the DNA out of the solution or in some circumstances release it. These beads on which DNA is bound are washed in order to remove undesired compounds and in a final step DNA is removed from the beads and recovered in PCR grade water.

7.3. Manual DNA extraction protocol

1. Weigh 100 mg (100-105 mg) of the sample in a microcentrifuge tube of 2 ml.
2. Add 500 µl of Lysis Buffer A + 5 µl of RNAse A (4 mg/ml).
3. Mix by vortexing for 10 to 15 seconds.
4. Add 250 µl of Lysis Buffer B.
5. Mix by vortexing for 10 to 15 seconds.
6. Incubate for 10 minutes at room temperature (22-25°C).
7. Add 750 µl of Precipitation Solution.
8. Mix by vortexing for 10 to 15 seconds.
9. Centrifuge for 10 minutes at 15870 g.
10. Transfer 1000 µl of the supernatant to a new microcentrifuge tube. With some absorbent samples, the volume of available supernatant is below 1000 µl; in that case, a maximum of supernatant will be transferred to a microcentrifuge tube. A special care will be taken to avoid the simultaneous transfer of the pellet.
11. Vigorously mix (but don’t vortex) the bottle of Magnesil® (magnetic beads) and add 50 µl of magnetic beads to the supernatant.
12. Resuspend the magnetic beads in the supernatant by pipetting or by inverting.
13. Add a volume of isopropanol equivalent to 0.8 volume of the transferred supernatant (e.g. 800 µl of isopropanol/1000 µl of supernatant).

* The PAP to consider should consist of material in relation to the animal target that is looked for. If a ruminant PCR has to be performed, the PAP should consist of ruminant material.

14. Invert the closed tube 5 to 6 times to mix and incubate 5 minutes at room temperature.
15. Mix by pipetting or by inverting for 90 seconds.
16. Insert the tubes into the magnetic separation stand for 1 minute.
17. Discard the liquid phase.
18. Add 250 µl of Lysis Buffer B.
19. Mix by pipetting or by inverting for 30 seconds.
20. Insert the tubes into the magnetic separation stand and discard the liquid phase after 60 seconds.
21. Add 1000 µl of ethanol 70%.
22. Mix by pipetting or by inverting for 30 seconds.
23. Insert the tubes into the magnetic separation stand and discard the liquid phase after 60 seconds.
24. Add 1000 µl of ethanol 70%.
25. Mix by pipetting or by inverting for 30 seconds.
26. Insert the tubes into the magnetic separation stand and discard the liquid phase after 60 seconds.
27. Open the tubes during 5 min at room temperature (evaporation of ethanol).
28. Add 300 µl of PCR grade water.
29. Mix by pipetting or by inverting for 60 seconds.
30. Insert the tubes into the magnetic separation stand and incubate during 60 seconds at room temperature.
31. Recover the liquid phase and transfer it to a new tube.
32. Check that there are no more magnetic beads in the liquid by placing the tubes into the magnetic separation stand for 60 seconds, recover the liquid and transfer it to a new tube.

Important remark: Although the original protocol provided by Promega mentions two incubation steps, the present procedure prescribes only one incubation step (step 6) as it was validated in this way during the PCR implementation study conducted in 2012. This procedure must be followed strictly.

7.4. Semi-automated DNA extraction protocol using a KingFisher mL

1. Weigh 100 mg (100-105 mg) of the sample in a microcentrifuge tube of 2 ml.
2. Add 500 µl of Lysis Buffer A + 5 µl of RNAse A (4 mg/ml).
3. Mix by vortexing for 10 to 15 seconds.
4. Add 250 µl of Lysis Buffer B.
5. Mix by vortexing for 10 to 15 seconds.
6. Incubate for 10 minutes at room temperature (22-25°C).
7. Add 750 µl of Precipitation Solution.
8. Mix by vortexing for 10 to 15 seconds.
9. Centrifuge for 10 minutes at 15870 g.
10. Transfer 1000 µl of the supernatant to a new microcentrifuge tube. With some absorbent samples, the volume of available supernatant is below 1000 µl; in that case, a maximum of supernatant will be transferred to a microcentrifuge tube. A special care will be taken to avoid the simultaneous transfer of the pellet.
11. Vigorously mix (but don’t vortex) the bottle of Magnesil® (magnetic beads) and add 50 µl of magnetic beads to the supernatant.
12. Resuspend the magnetic beads in the supernatant by pipetting or by inverting.

13. Add a volume of isopropanol equivalent to 0.8 volume of the transferred supernatant (e.g. 800 µl of isopropanol/1000 µl of supernatant)[†].
14. Invert the tube 5 to 6 times to mix and incubate 5 minutes at room temperature.
15. Prepare the racks to place in the KingFisher

    Well1: 1.8 ml of sample with the magnetic beads

    Well 2: 250 µl of Lysis Buffer B

    Well 3: 1000 µl of ethanol 70%.

    Well 4: 1000 µl of ethanol 70%.

    Well 5: 300 µl of PCR grade water.
16. Start the following protocol of the KingFisher (Software version: BindIt Software 3.1 for KingFisher Instruments)[‡]:

	Sub-step	Parameters	Position (well number)	Layout (reagents)
1	DNA **binding**	***Beginning of step:*** Precollect No Release beads No ***Mixing/pause parameters*** Pause for manual handling No 1. Mixing time [hh:mm:ss] 00:01:00 1. Mixing speed Medium 2. Mixing time [hh:mm:ss] 00:01:00 2. Mixing speed Medium Loop count 1 ***End of step:*** Postmix No Collect count 3 Collect time [s] 2	A or 1	Name Microbeads Volume [µl] 50 Type Reagent Name Lysate Volume [µl] 500 * Type Reagent Name Isopropanol Volume [µl] 400 * Type Reagent * BindIt Software 3.1 does not accept a total volume of the reagents in the well > 1000 µl even if it can be up to 1800 µl. The real volume in the well 1 is indicated at point 15.
2	**Wash** in Buffer Lysis B	***Beginning of step:*** Precollect No Release beads Yes ***Mixing/pause parameters*** Pause for manual handling No 1. Mixing time [hh:mm:ss] 00:00:15 1. Mixing speed Medium 2. Mixing time [hh:mm:ss] 00:00:20 2. Mixing speed Slow Loop count 1 ***End of step:*** Postmix No Collect count 3 Collect time [s] 2	B or 2	Name Buffer Lysis B Volume [µl] 250 Type Reagent
3	**Wash** in ethanol 70%	***Beginning of step:*** Precollect No Release beads Yes ***Mixing/pause parameters*** Pause for manual handling No 2. Mixing time [hh:mm:ss] 00:00:15 3. Mixing speed Medium 4. Mixing time [hh:mm:ss] 00:00:20 3. Mixing speed Slow Loop count 1 ***End of step:***	C or 3	Name Ethanol 70 % Volume [µl] 1000 Type Reagent

[†] Technical specifications of the KingFisher advice not to exceed 1000 µl per well in order to decrease contamination risk. However, experience of EURL-AP showed that the protocol as it is never led to any contamination.

[‡] Protocol to adapt to other DNA extraction instruments

		Postmix No Collect count 3 Collect time [s] 2		
4	**Wash 2** in ethanol 70%	***Beginning of step:*** Precollect No Release beads Yes ***Mixing/pause parameters*** Pause for manual handling No 3. Mixing time [hh:mm:ss] 00:00:15 5. Mixing speed Medium 6. Mixing time [hh:mm:ss] 00:00:20 4. Mixing speed Slow Loop count 1 ***End of step:*** Postmix No Collect count 3 Collect time [s] 2	D or 4	Name Ethanol 70 % Volume [µl] 1000 Type Reagent
5	**Drying**	Dry time [hh:mm:ss] 00:04:56 Tip position Outside well / tube	Above well 5	
6	**Elution** in PCR grade H_2O	Release time [hh:mm:ss] 00:00:47 Release speed Medium	E or 5	Name PCR grade H_2O Volume [µl] 300 Type Reagent
7	**Collecting** microbeads	Collect count 3 Collect time [s] 5	E or 5	
8	**Discarding of** microbeads	Release time [hh:mm:ss] 00:00:10 Release speed Medium		

17. Recover the liquid and transfer it to a new tube.
18. Check that there are no more magnetic beads in the liquid by inserting the tubes into the magnetic separation stand for 60 seconds, recover the liquid and transfer it to a new tube.

Important remark: Although the original protocol provided by Promega mentions for two incubation steps, the present procedure prescribes only one incubation step (step 6) as it was validated in this way during the PCR implementation study conducted in 2012. This procedure must be followed strictly.

8. INTERPRETATION OF RESULTS

Interpretation of results can only be done once the PCR step is completed.

It is important that the expected results for both extraction controls are correct. The positive DNA extraction control should give a positive PCR result (Ct < cut-off value) for an animal target that it is supposed to contain. The extraction blank control also called negative extraction control should be negative (Ct ≥ cut-off value or no amplification) for the animal targets under analysis.

A positive DNA extraction control containing a 0.1% mass fraction of PAP[§] or lower must be included in each series of extraction as well as an extraction blank control.

[§] The PAP to consider should consist of material in relation to the animal target that is looked for. If a ruminant PCR has to be performed, the PAP should consist of ruminant material.

Note: If for some reason the kit is stored or used at an incorrect temperature, the positive extraction control is very important. If it is still positive for a content somewhat above the LOD, it validates that conditions of use and/or storage of the kit, even if they were out of the recommended temperature range, have no impact on the results.

9. REFERENCES

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http://www.promega.com/~/media/files/resources/protocols/technical%20bulletins/0/wizard%20magnetic%20dna%20purification%20system%20for%20food%20protocol.pdf?la=en but as version 04/09 (consulted the 20-02-2013).

Version	Publication date	Application date	Repeal date
1.0	29.03.2013	29.04.2013	30.06.2014
1.1	24.06.2014	01.07.2014	