



BIOTOOLS

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BIOFOOD QL Kits

Kits for detection and identification of vertebrate species in food by Real-Time DNA Amplification

SHEEP ID KIT

Equipment: BioRad iCycler

Cat. No. 91.902B

Instructions for Use

PLEASE READ THE INSTRUCTIONS FOR USE THOROUGHLY BEFORE USING THE KIT, ESPECIALLY IF YOU ARE NOT FAMILIAR WITH THE PROTOCOL

1. GENERAL INFORMATION

BIOFOOD kits allow the detection and, depending on the specific kit, the precise identification of a high number of animal species in fresh or processed food and feed samples. The detection method is based on the stability of nucleic acids, that endure the processes used in food industry (temperature, vacuum, drying, etc.). The kit has been tested with fresh and highly processed samples (including cheese and animal feed).

BIOFOOD QL SHEEP ID Kit detects by Real-Time the presence or absence of *Ovis* material. The method is based on the detection of species-specific DNA sequences from cytochrome b. DNA from *Ovis* species present in positive samples is amplified by primers (specific for cytochrome b). SYBR® Green binds to double-stranded PCR product formed during each amplification cycling and emits a fluorescence signal. The positivity of the sample to cytochrome b gene is confirmed via dissociation curve analysis (melting curve, T_m).

BIOFOOD QL SHEEP ID Kit gives high sensitivity, the minimum material from *Ovis* species detected with the kit is 0.1 %.

RESEARCH USE ONLY

Some of the applications which may be performed with this product may be in certain countries under an applicable patent. The purchase of this product does not include or provide a license to perform patented applications. Users may be required to obtain a license depending on the country and/or application. Biotools does not encourage the unlicensed use of patented applications.

PLEASE CHECK INTEGRITY OF KIT AND REAGENTS BEFORE USE. DETERIORATED KITS MAY CAUSE EQUIVOCAL RESULTS.

2. PRINCIPLE

DNA is obtained either from fresh or processed samples¹ using an optimised method¹ (we recommend using Genomic DNA Extraction Kit - Cat. No. 21.002, 24 tests, or Cat. No. 21.003, 48 tests, available in our catalogue). Use of other methods is possible. However, user must confirm that the purified DNA can be used with the kit (concentration 50-100 ng/ μ l, $A_{260/280}=1.8 - 2.0$, absence of inhibitors that may affect the result of the amplification reaction, etc.). It is recommended to check the quality and suitability of the purified DNA for amplification reactions, e.g. performing control amplifications in parallel.

BIOFOOD QL SHEEP ID kit is based on the amplification of a cytochrome b region from *Ovis* species and its direct detection by SYBR® Green. The kit can be used with heterogeneous samples (more than one component).

3. REAGENTS

Kit contains enough reagents in liquid format to perform 48 rxns (Cat. No. 91.902B). To minimize the risk of contamination and facilitate the use of the kit several times the Kit is presented in two set of 24 reactions. Sample Kit (Cat. No. 91.901B) contains one set of 24 reactions. Store all reagents at -15 ± 8 °C (except diluted SYG solution which should be stored at 4 °C). Thaw and handle on ice. Do not freeze/thaw repeatedly. For frequent use, we recommend the aliquoting of the vial contents.

- **MASTER MIX** Two vials: 2 x 270 μ l
 A Tris-HCl solution, containing <10 % glycerol, KCl, <0.001 % dATP, dCTP, dGTP, dTTP and primers. Master Mix includes all amplification reagents for the detection of cytochrome b from *Ovis* species, except $MgCl_2$ and DNA polymerase, in the adequate ratios.
- **$MgCl_2$ SOLUTION (50mM)** Two vials: 2 x 1.8 ml
 Mix well before use.
- **DNA POLYMERASE** Two vials: 2 x 30 μ l
 Add to reaction mixtures shortly before introduction of vials in thermal cyclers.
- **SHEEP AMPLIFICATION CONTROL** Two vials: 2 x 40 μ l
 Sheep positive control which consists of DNA amplified product from *Ovis aries* at 10^6 copies/ μ l.
- **SYG** Two vials: 2 x 0.35 μ l
 A concentrated solution containing the intercalating dye SYBR Green I. **TO BE DILUTED WITH 500 μ l OF SYG BUFFER. Avoid exposure to light.** Add diluted SYG solution to reaction vials shortly before introduction in thermal cyclers. **Once diluted SYG vial should be stored at 4 °C.**
- **SYG BUFFER** Two vials: 2 x 600 μ l
 For diluting concentrated SYG vial.

¹ Food and feed samples, due to their composition (additives, colourings, preservatives) have a high amount of components that may inhibit amplification reactions. Therefore, it is a must that the DNA purification method eliminate these inhibitors, keeping DNA integrity.

4. MATERIALS REQUIRED BUT NOT PROVIDED

NOTE

For all equipments, regular maintenance and calibration is necessary. Follow manufacturer's instructions, and check working parameters regularly, specially for thermal cyclers and pipettes. Maintenance and calibration of instruments allows its correct functioning, and helps detecting problems that may render an incorrect analysis result.

Pre-amplification area

- Equipment, reagents and disposable material necessary for DNA purification (depending on the method, follow manufacturer's instructions)
- Timer
- Automatic pipettes² (10, 20 and 200 µl), filter or positive displacement tips, RNase-free³
- Disposable examination gloves, powder-free
- Sterile bidistilled water
- Screw cap polypropylene tubes, 1.5 ml capacity, non siliconised, conical, sterile, RNase-free. It is recommended to use screw cap tubes, in order to avoid the potential contamination of samples and controls
- Racks for 1.5 ml vials
- Containers for disposal of potentially-infectious material
- Disposable filter paper for working surface, cleaning paper for accidental spills
- Termini-DNA-Tor⁴ or equivalent, in order to remove DNA from working surfaces

Amplification area

- Real-Time thermal cycler: BioRad iCycler. Use of this kit in other equipments has not been tested. For further information, contact our Technical Dpt. (info@biotools.eu)
- Laminar flow cabinet
- Racks for reaction vials
- Real -Time amplification vials (as per manufacturer's instructions)
- Sterile bidistilled water (Cat. No. 20.033 or equivalent)
- Automatic pipettes (10, 20 and 200 µl), filter or positive displacement tips, RNase-free
- Disposable examination gloves, powder-free
- Containers for disposal of potentially-infectious material
- Disposable filter paper for working surface, cleaning paper for accidental spills
- Termini-DNA-Tor or equivalent, in order to remove DNA from working surfaces

5. PROTOCOL

A PREPARATION OF REACTION MIXTURE AND SAMPLES

In order to obtain the best results, it is essential to keep the reaction vials refrigerated until their introduction in the thermal cycler. For standard conical vials introduce them on ice or on refrigerating blocks, being careful not to wet the optical cap. Use of reaction mixtures and vials in non-refrigerated conditions may cause a drastic decrease in sensitivity and quality of the obtained fluorescence curves.

1.- Dilute SYG vial as indicated in "Reagents Section".

2.- Final reaction volume is 25 µl. Calculate the necessary volume of Master Mix + SYG (diluted) + MgCl₂ + DNA polymerase to perform analysis of the samples and the controls. It is recommended to perform one (1) Positive Control and one (1) Negative Control in each analysis round (this must be taken into account for volume calculations).

NOTE

Before starting the procedure check that all reagents contain enough volume to perform the necessary reactions. Thaw all vials on ice and Keep them on ice while on use.

SYG vial must be protected from light. Its exposure to light may inactivate it and affect the performance of the kit dramatically.

² Precision of automatic pipettes must be in the range of 3 % of the indicated volume. If necessary, calibrate and check regularly, following manufacturer's instructions. It is recommended to use RNase-free filter tips and positive displacement tips, in order to avoid cross contamination between samples and amplicons.

³ It is recommended to use different sets of pipettes for each reaction step (pre-amplification, amplification), in order to avoid contaminations that may render false positive results.

⁴ Available in Biotools' catalogue (Cat. No. 22.001).

3.- Mix the required volume of each reagent in a 1.5 ml vial, according to the number of reactions to be performed. **Perform this process in a laminar flow cabinet.** Keep the reaction mixture (reaction mixture = Master Mix + SYG diluted + MgCl₂ + DNA Polymerase + sterile bidistilled water) on ice. **Protect SYG vial from light at all times.**

Reagent	1 Reaction
Master Mix	10 µl
SYG (diluted)	0.4 µl
DNA Polymerase	0.5 µl
MgCl ₂	1 µl
Sterile bidistilled H ₂ O	10,6 µl

To ensure sufficient volume for all reactions prepare reaction mixture for n + 1

4.- Aliquot 22.5 µl of reaction mixture in each amplification vial⁵.

5.- Remove tubes from the laminar flow cabinet. **Addition of DNA to reaction mixtures should be performed in the pre-amplification area in order to avoid contaminations (never introduce DNA in the laminar flow cabinet).** Add 2,5 µl of DNA purified from samples (sample DNA concentration 20 ng/µl) and/or controls (Sheep Amplification Control) to each amplification vial. For samples where DNA cannot be quantified it is recommended to standardise a fixed volume to add (1-5 µl) and complete up to 25 µl final volume with sterile bidistilled water.

NOTE

Amplification must be started in the next 10 minutes after adding the purified DNA and controls to the amplification mix.

6.- Close amplification vials. When using optical caps, be careful not to leave traces of powder, dust, etc. Place vials in thermal cycler. Store remaining of all reagents at the indicated temperatures.

B PROGRAMMING THE BIORAD ICYCLER™

NOTE

Check thermal cycler regularly. Non-existent or poor calibration of the equipment may render equivocal results.

1.- Ensure that the iCycler apparatus is switched on and is properly connected to the computer. Allow 30 minutes for the camera to warm-up before performing the analysis. For further information on the installation of software and instrumentation setup, please consult the operator's manual.



2.- Click on the BioRad icon. The iCycler Frontscreen should appear.



3.- Select Workshop Module icon.

4.- Enter filename for protocol in Protocol Filename field.

Protocol Filename: **BioFood QL**

5.- Check the "Melt Curve" in the Show Options field.

Show options:

- Infinite Hold
- Gradient
- Melt Curve
- Increment Temperature
- Decrement Temperature

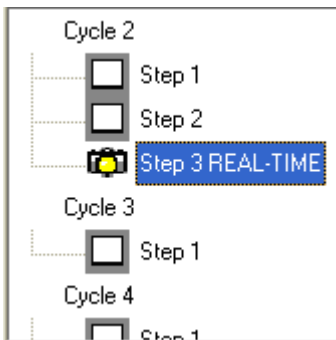
⁵ Amplification vials will be that specifically recommended by each Real-Time thermal cycler.

6.- Program the thermal cycle parameters according to the following instructions and table as shown in the software screen.

- chose "Insert Cycle" button for 3 cycles.
- in Cycle 2 choose "Insert Step" button for 3 steps.
- in Cycle 6 check the "Melt Curve" box.
- enter data as shown below.


Insert Cycle		Delete Cycle		Insert Step			Delete Step		
Cycle	Repeats	Step	Dwell Time	Setpoint	Melt Curve	+ Temp	- Temp	+ Time	
1	1	1	01:30	94.0	<input type="checkbox"/>				
2	30	1	00:10	94.0	<input type="checkbox"/>				
		2	00:30	62.0	<input type="checkbox"/>				
		3	00:40	72.0	<input type="checkbox"/>				
3	1	1	03:00	72.0	<input type="checkbox"/>				
4	1	1	01:00	94.0	<input type="checkbox"/>				
5	1	1	00:30	60.0	<input type="checkbox"/>				
6	70	1	00:10	60.0	<input checked="" type="checkbox"/>	0.5			


7.- Collect data in real time at the 72°C extension step, and during the melting curve. Under the "Select data collection step(s):" double-click over appropriate steps (Cycle 2 Step 3). A camera with a yellow lens should appear with a REAL TIME message next to it.



8.- Select the **Save this protocol** button.

9.- Select **Edit Plate Setup** window. The **Samples: Whole Plate loading** screen should appear. Select wells to be analysed.

a) Standards selected with the  Standard icon.

b) Unknown samples selected with the  Unknown icon.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		1		2		3		4		5		
C												
D			1		2		3		4			
E												

c) Label samples and define standard concentrations in right hand fields.

Standard Quantity:	<input type="text"/>
Sample Identifier:	<input type="text" value="Sample B2"/>

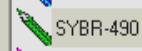
10.- Select the **View Quantities/Identifiers** window to designated which wells are to be analysed.

a) Select SYBR-490 as fluorophore and deselect all others.

1. Select or deselect a fluorophore:

<input type="checkbox"/> ROX-575	<input type="checkbox"/> TexasRed-575
<input checked="" type="checkbox"/> SYBR-490	<input type="checkbox"/> VIC-530

b) Assign a colour (traditionally green).



c) Select the wells that are to be analysed fluorogenically (should be the same as in step 9).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												

11.- Select the **Run Prep** window.

a) Ensure that 25 µl are read.

b) Check "PCR Quantification Melt Curve" as run purpose.

c) Check "Experimental Plate" for well factor source.

Edit Protocol	Edit Plate Setup	View Quantities/Identifiers	Run Prep
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Sample volume: ul

Select run purpose:

PCR Quantification Melt Curve

Pure Dye Calibration

Select well factor source:

Experimental Plate

Well Factor Plate

12.- Select Begin Run Button.

Perform the amplification following the indicated program:

Cycle 1:	(1x)	Denature		
	Step 1:		94 °C	01:30
Cycle 2:	(30x)	Amplification		
	Step 1		94°C	00:10
	Step 2		62 °C	00:30
	Step 3		72°C	00:40
	Data collection and real-time analysis enabled.			
Cycle 3:	(1x)			
	Step 1:		72°C	3:00
Cycle 4:	(1x)			
	Step 1:		94°C	1:00
Cycle 5:	(1x)			
	Step 1 :		60°C	00 :30
Cycle 6:	(70x)			
	Step 1:		60°C	00:10
	Increase setpoint temperature after cycle 2 by 0,5°C			
	Melt curve data collection and analysis enabled.			
Cycle 7:	(1x)	Cooling		
	Step 1:		4°C Hold	

6. INTERPRETATION OF RESULTS

Use of thermal cyclers with coupled fluorescence capture systems allows the monitoring in Real-Time and in each amplification cycle of the generated amplification products. Interpretation of results will be based on the specific software for the used equipment, following the manufacturer's instructions and recommendations.

Fluorescence curves

Real-Time amplification systems will render fluorescence increase curves depending on the amplification cycle. Thus, positive samples will render an increasing fluorescence curve. Cycle where fluorescence increase starts is proportional to the quantity of target material present in the sample. On the contrary, negative samples will not render a fluorescence increase during the process, rendering a basal fluorescence line.

The interpretation of results is performed based on the melting curve analysis:

1. There should not be melting curves for Negative Controls (Amplification and DNA purification blanks).
2. If a sample contains *Ovis* DNA material a melting peak should appear at the same temperature of the sheep positive control included in the Kit (81-86.5 °C).

NOTE

The melting temperature may vary depending on the employed instrument. The presence of inhibitors in the DNA extracted from the sample may cause variations in the expected melting values.

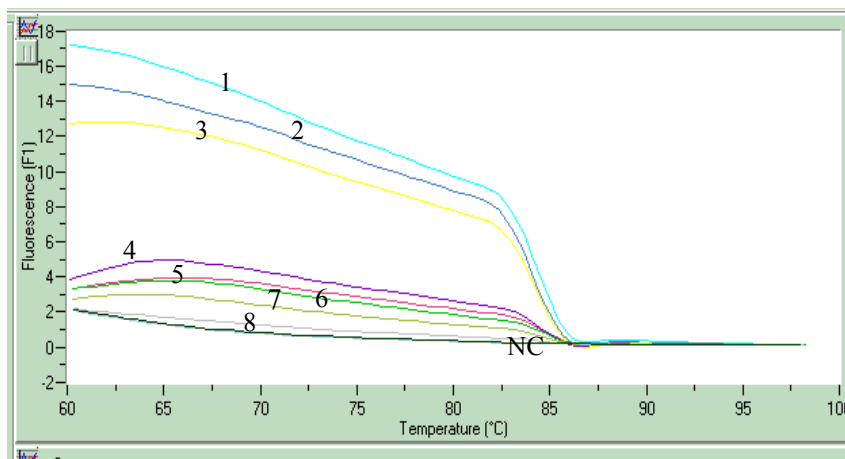


Figure 1. Melting curve obtained using a Positive Control containing 20 ng/ μ l of DNA from *Ovis aries*. Sample 1- 1:2 dilution; Sample 2- 1:10 dilution; Sample 3- 1:20 dilution; Sample 4- 1:50 dilution; Sample 5- 1:100 dilution; Sample 6- 1:200 dilution; Sample 7- 1:500 dilution; Sample 8- 1:1000 dilution; Sample NC-Negative Control (Double distilled water).

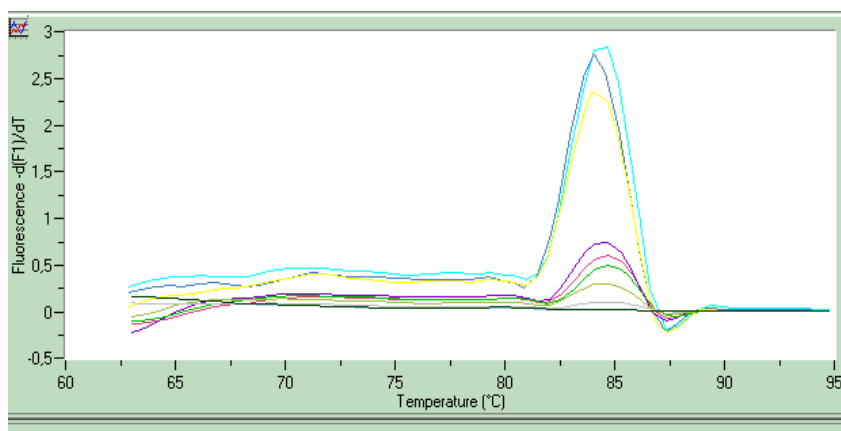


Figure 2. T_m analysis of the experiment shown in figure 1. A peak at 84 °C can be observed, there is a decrease in the fluorescence level for samples with higher dilutions.

NOTE

It must be considered that the test detects DNA, not protein or percentage of target material. The concentration of DNA is not always correlated with the percentage of target material, specially in processed samples, or materials containing trace amounts of DNA (e.g. bone, fat).

7. QUALITY CONTROL

It is recommended that at least one (1) Positive Control and one (1) Negative Control be run each time the test is performed. As with any new laboratory procedure, novel users should consider performing additional controls (both positive and negative) until a high degree of confidence is reached.

The Positive Controls must render the corresponding peaks in the Melting Analysis (see 'Interpretation of Results' chapter). Vials containing negative control (sterile bidistilled water) must render no peaks. Any analysis not fulfilling any of these results must be completely invalidated and discarded. It is necessary to repeat the process from its beginning, including DNA purification, processing other aliquot of the original sample. A failure of instruments during the test, indicated by error messages, also means that the test has not been valid. Repeat all the procedure for each sample from the amplification step.

8. PROCEDURAL PRECAUTIONS

1. Laboratory workflow must be unidirectional, from pre-amplification area to amplification area. Pre-amplification tasks must be initiated with the preparation of the reagents and sample purification. Equipments, materials and reagents must be dedicated and they must not be used for other activities or be transferred from one to another area. Gloves must be worn in each area, and must be discarded before proceeding to the next area. Equipments and materials used for setting-up of reactions must not be used for other activities, or for pipetting or processing amplified DNA or other DNA sources.
2. As with any analytical procedure, it is fundamental to use a good laboratory practice to obtain good results with this technique. Due to the high analytical sensitivity of the test, extreme care must be taken in order to keep the purity of all kit reagents and all reaction mixtures. All reagents must be carefully checked in order to ascertain their purity. Discard all suspect reagents.
3. Instructions must be followed in order to obtain correct results. Should the user have any questions, please contact our Technical Dpt. (info@biotools.eu).
4. This test has been validated for use with the reagents provided by the kit. The use of other amplification methods, or the use of equipment not fulfilling the specifications, may render equivocal results. User is responsible for validating the modifications for this test, in any of the indicated parameters.
5. Use powder-free examination gloves while handling reagents or samples, as well as lab coat. Wash hands thoroughly after performing the test.
6. Open and close reagent vials carefully. Observe temperature and light exposure instructions. After use, close vials and store at indicated temperatures.
7. Do not use product after expiry or best before date.
8. Kit components have been tested as a whole. **Do not interchange components** with other kits, or components from different lots.
9. Nucleic acids are very sensitive to degradation by nucleases. Nucleases are present in human skin and surfaces that have been in contact with humans. Wash with Termi-DNA-Tor and cover working surfaces with suitable paper. Use powder-free examination gloves throughout the whole process.
10. Extreme care must be taken when aliquoting the different volumes in each reaction step. Mix well after addition of each reagent, unless otherwise noted. Read instructions for use of automatic pipettes.
11. Do not pipette by mouth.
12. Packaging material included with the kit is resistant to the indicated storage conditions. Storage at different conditions can cause breakage of the material, and possible contamination of kit contents.
13. Plastic material included with the kit is resistant in the normal conditions of use. Use of plastic material in extreme conditions may cause its breakage, and therefore, impossibility to use the kit.
14. False negative results may be obtained due to polymerase inhibition. It is recommended to perform control reactions to distinguish between inhibition and true negatives.
15. Cross contamination between samples and exogenous DNA can only be avoided by following good laboratory practice. Instructions in this document must be strictly followed.

16. Use of this product is limited to qualified professional personnel, experienced in DNA purification and DNA amplification techniques.
17. It is important to pipet the indicated amounts, and mix well after each reagent addition. Check pipettes regularly.
18. Biotoools laboratories participate in a regular and satisfactory way in intercomparison studies (ring tests) recognised internationally (USDA-GIPSA, GeMMA Scheme, FAPAS & FEPAS etc.). On the other hand, Biotoools is an active member of different standardization and regulation committees (AENOR, CEN).

9. WARRANTY

Products are guaranteed to conform to the quality and content indicated on each vial and external labels during their shelf life. BIOTOOLS obligation and purchaser's rights under this warranty are limited to the replacement by BIOTOOLS of any product that is shown defective in fabrication, and that must be returned to BIOTOOLS, freight prepaid, or at BIOTOOLS' option, replacement of the purchasing price. Any complaint on damaged goods during transport must be directed to the handling or transport agent.

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Manufactured by:

BIOTOOLS, Biotechnological & Medical Laboratories, S.A. has been evaluated and certified to accomplish ISO 9001:2000 requirements for the following activities: Research and development of biotechnology products and manufacture of biotechnology and in vitro products.
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