HANDBOOK

Bio-T kit® *Streptococcus agalactiae*

Cat. N° BioTK026
25 reactions

Detection and quantification of *Streptococcus agalactiae* in milk by real-time PCR (qPCR) with exogenous internal positive control (IPC)

ALL SPECIES

Sample types

- Milk with or without preservatives (eg : bronopol or glycerol 10%)
- Storage at 4°C (with bronopol) or frozen (with or without glycerol)

Recommended DNA Extraction

- Magnetic beads extraction (eg: BioSellal – BioExtract® SuperBall® Cat. N° BES384)
- Silica membrane columns (eg: BioSellal – BioExtract® Column Cat. N° BEC050 or BEC250)

Other extraction kits are available : contact our Technical Support for more information.

For veterinary use only



PRESENTATION

MILK™ line

BioSellal's qPCR MILK™ line consists of 11 qPCR detection kits targeting major mastitis responsible bacteria as *Streptococcus agalactiae* Bio-T kit® (BioTK026). The exogenous internal positive control (IPC) to be added during the extraction process is the same for all the qPCR MILK™ line kits.

Description of the Bio-T kit® *Streptococcus agalactiae*

Real-time PCR (qPCR) highlights the presence of targeted nucleic acid (NA) in an accurate and quick way. The Bio-T kit® *Streptococcus agalactiae* (BioTK026) is a duplex qPCR system enabling to simultaneously detect, in the same assay, the presence of:

- *Streptococcus agalactiae* (6-FAM labeled)
- the exogenous IPC (Cy5 labeled) added during the NA extraction process, enabling to assess NA extraction quality and absence of PCR inhibitors.

This kit can be used for the analysis of quarter milk, individual or composite milk samples including bulk tank milk samples.

Futhermore, an External Positive Control (EPC) is included within the kit: it is a quantified synthetic DNA plasmid containing one copy of the *S. agalactiae* targeted nucleotide sequence, titrated in number of genome equivalent (GE) per ml of milk*. This control is used as a PCR positive control for qualitative analysis (presence/absence) and can also be used either as a reference point to estimate the relative bacterial load within a positive sample or to establish a calibration line enabling to quantify the exact concentration of *S. agalactiae* detected in the sample, expressed in GE per ml of milk*.

** The S. agalactiae concentration expressed in GE per ml of milk is calculated from the sequence copy number detected per PCR reaction well. It takes into account the number of targeted sequence per bacterial genome as well as the extraction process yield (sample treatment and DNA extraction) established by BioSellal.*

Kit content and storage conditions

Table 1. kit contents description				
Description	**Reference**	**Volume /tube**	**Presentation**	**Storage**
Ready to use Master Mix (MM)	MMSAG-A	410 µl	1 tube **white** cap **Bag A**	-20°C in a dark place, « MIX » Zone
Exogenous Internal Positive Control (IPC)* Exogenous amplification control	IPC-A	140 µl	1 tube **pink** cap **Bag B**	-20°C « Extraction » Zone
External Positive Control (EPC)* *S. agalactiae* amplification positive control	EPCSAG-A	110 µl	1 tube **orange** cap **Bag C**	-20°C « Nucleic acids (NA) addition» Zone
Water RNase/DNase free	Aqua-A	1 ml	1 tube **blue** cap **Bag C**	4°C ou -20°C « Nucleic acids (NA) addition» Zone

* See quality control certificate of analysis (CA) for the reference values.

Kit reagents are stable until the expiration date stated on the label, subject to compliance with good storage conditions.

List of reagents and consumables not provided with the kit

Table 2. Reagents and consumables not provided with the kit			
Consumables / Reagent	**Description**	**Supplier***	**Cat. N°**
ATL lysis buffer	Cell lysis buffer	BioSellal	ATL19076
BioExtract® Column	Extraction column kit (DNA/RNA) (50)	BioSellal	BEC050
BioExtract® Column	Extraction column kit (DNA/RNA) (250)	BioSellal	BEC250
BioExtract® SuperBall®	Magnetic beads extraction kit (DNA/RNA) (4x96)	BioSellal	BES384

* Suppliers are given on an indicative purpose only

For consumables related to the thermocycler, refer to the instrument manual.

General precautions

- From sample pre-treatment to bacterial lysis steps, it is recommended to use a Biological Safety Cabinet.
- Wear appropriate personal protective equipment (lab coat, disposable gloves frequently changed,,…).
- Work in dedicated and separate areas to avoid contaminations: « Extraction » (unextracted samples storage, extraction equipment area), « MIX » (ready to use Master Mix storage, qPCR plates preparation), « Nucleic acids (NA) addition » (NA storage and addition of extracted NA and controls in the qPCR plate), « PCR » (final area containing the thermocycler(s)).
- Use dedicated equipment for each working area (gloves, lab coat, pipettes, vortex, racks…).
- Use filter tips.
- Before use, thaw all components at room temperature.
- Vortex and spin briefly (mini-centrifuge) all reagents before use.
- It is recommended not to exceed 3 freeze-thaw cycles of the reagents, samples, lysates and extracted nucleic acids. Depending on your use, we recommend you to aliquot at reception the ready-to-use Master Mix in adequate volume.

Outline of the steps to perform from milk sample treatment to qPCR result

Step 1
Sample preparation : cell/bacteria wall lysis. It is the sample processing.

Step 2
Nucleic acids (NA) Extraction/Purification.

Step 3
Addition of the Master Mix in the plate wells or in strips of qPCR, dilutions of the EPC then addition of nucleic acids and controls into qPCR wells or strips.

Step 4
Real-time PCR (qPCR): simultaneous amplification and detection of target DNA (*Streptococcus agalactiae* and IPC).

MILK SAMPLE PROCESSING

This step consists in the milk sample preparation to enable an efficient bacterial wall cell lysis, including for Gram-positive bacteria as *Streptococcus agalactiae*.

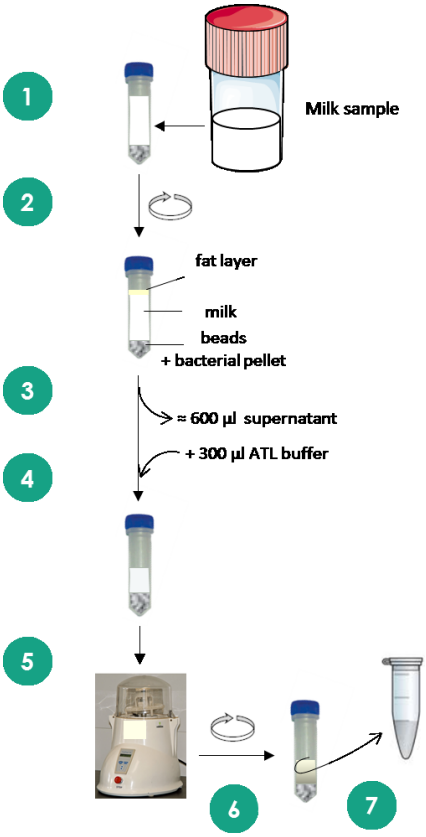
The recommended milk sample volume is of **800 µl**.

For an optimal Gram-positive bacterial wall cell lysis, sample grinding step is recommended (eg: use of FastPrep 24™ homogenizer and 0.1 mm zirconium beads).

The milk pre-treatment protocol requires the use of “ATL” lysis buffer, sold separately (BioSellal Cat. N° ATL19076).

It is mandatory to include a negative control sample (NCS) in order to validate the absence of samples cross-contamination during the whole process, from sample pre-treatment to qPCR result. For this control, milk sample is replaced by sterile water or PBS (RNase/DNase free) and will be processed in parallel of the other samples of interest.

Figure 1. Milk sample processing scheme prior nucleic acid extraction



MILK SAMPLE PROCESSING

1. Transfer of **800µl** milk sample in a 2 ml sterile tube containing grinding beads (eg: zirconium 0,1mm)
2. Centrifuge 5 min at 10 000 xg
3. Remove ≈ 600 µl of supernatant *1
4. Add 300 µl of ATL lysis buffer
5. Homogenizer :
   2x 45s at 6.5 m/s (eg FastPrep™)
   2x 45s at 6800 rpm (eg Precellys™)
   3-5 min at 30 Hz (eg Retsch™)
   5 min at 3000 rpm (eg Genie disruptor®)
6. Centrifuge 1 min at 10 000 xg
7. Take off **200 µl** of supernatant*2 and transfer into a 1.5 ml tube
   = lysate volume to process for nucleic acids extraction.

*1 : Discard the fat layer as much as possible, then throw out the supernatant without drying the pellet : leave a fine liquid film covering the pellet.

*2 : Take 200 µl of supernatant, avoid pipetting bubbles or residual fat from the upper part of the tube.

NUCLEIC ACIDS EXTRACTION

BioSellal recommends two extraction kits:

- **BioExtract® Column (Cat. N° BEC050 ou BEC250)** based on in silico membrane column, recommended for 1 to 12-20 samples extraction in parallel.
- **BioExtract® SuperBall® (Cat. N° BES384)** based on the use of magnetic beads and automated extraction system such as KingFisher™ Duo, mL or Flex, recommended for 12 or more samples extraction in parallel.

The protocol of each method is proposed below. For further information, contact our Technical Support or refer to the handbook on www.biosellal.com.

Note : **The exogenous IPC** (tube IPC-A, **pink** cap) provided with the Bio-T kit® *Streptococcus agalactiae*, must be used at this step. Note that the exogenous IPC is the same for all the qPCR kits belonging to the MILK™ line.

Silica Column

BioExtract® Column Kit

Cat. N° BEC050 or BEC250

Please refer to the extraction kit protocol for solutions preparation

1. Lysis and Adjustment of adsorption conditions

In a 1.5 ml micro-centrifuge tube, add:

20 µl Proteinase K

200 µl of processed sample. Replace by 200 µl of DNase/RNase free water or PBS for NCS.

100 µl of lysis solution LA-carrier + IPC prepared as indicated in Table 3:

Table 3. Lysis solution LA-carrier + IPC

Reagents	Samples number				
	1	6*	12*	24*	30*
LA Buffer	100 µl	660 µl	1.32 ml	2.64 ml	3.3 ml
Carrier RNA (1 µg/µl)	1 µl	6.6 µl	13.2 µl	26.4 µl	33 µl
Exogenous IPC (pink cap tube)	5 µl	33 µl	66 µl	132 µl	165 µl

* In order to ensure the pipetting volume, the prepared volume contains an additional volume of 10%.

Vortex and incubate 15 min at 20-25°C (room temperature). Centrifuge briefly (benchtop mini-centrifuge).

Add 350 µl of LB Buffer.
Vortex and centrifuge briefly.

2. Adsorption on the silica membrane

Carefully transfer the entire volume (670 µl) on the BioExtract® Mini Spin Column (placed into a clean 2 ml collection tube).

Centrifuge at **6 000 x g for 1 min**. Change the collection tube (Place the BioExtract® Mini Spin Column into a clean collection tube and discard the collection tube containing the filtrate).

3. Washes and Drying of the silica membrane

Add **600 µl of W1 Buffer**.

Centrifuge at **6 000 x g for 1 min**. Change the collection tube.

Add **600 µl of W2 Buffer**.

Centrifuge at **6 000 x g for 1 min**. Change the collection tube.

Centrifuge at **20 000 x g for 2 min** to dry the membrane.

4. Elution of Nucleic Acids

Place **the BioExtract® Mini Spin Column into a clean 1.5 ml micro-centrifuge tube**, and discard the collection tube containing the filtrate.

Add gently 90 µl of EL Buffer (at room temperature or pre-heated at 70°C) onto the center of the membrane.

Incubate at **room temperature (15–25°C) for 1 min**.

Centrifuge at **20 000 x g for 1 min**.

Conserve the eluate (90 µl) into the 1.5 ml labeled tube and discard the column.

The extracted DNA can be stored **at 4°C if the qPCR is performed within 4-8 hours following the extraction otherwise it is recommended to store it at <-20°C for 6 months or at <-70°C for a better conservation**.

Figure 2. Nucleic acids Purification using the BioExtract® Column Kit (Cat. N° BEC050 or BEC250)

1 **Lysis and Adjustment of adsorption conditions**		20 µl Proteinase K 200 µl processed sample 100 µl of LA-carrier +IPC lysis solution (pour 1 sample.: 100 µl LA Buffer + 1 µl carrier RNA + 5µl exogenous IPC) Room temperature (RT) 15 min 350 µl of LB Buffer
2 **Adsorption onto the silica membrane**		Load the BioExtract® Mini Spin Column carefully 6 000 x g 1 min
3 **Washes** **Drying the silica membrane**		1st Wash — 600 µl W1 — 6 000 x g, 1 min 2st Wash — 600 µl W2 — 6 000 x g, 1 min - — - — 20 000 x g, 2 min
4 **Elution of nucleic acids**		90 µl of EL Buffer (RT) RT 1 min 20 000 x g 1 min

Magnetic Beads

BioExtract® SuperBall® Kit

Cat. N° BES384

Using the KingFisher™ Flex, Duo or mL or equivalent workstation

Please refer to the extraction kit protocol for solutions preparation

1. Preparation of plates or strips

Prepare the consumables (see Table 4)

Flex : 4 plates Deep-well and 2 microplates. Annotate it depending on the element to add.
Duo : 1 plate Deep-well and 1 elution strip.
mL: 1 strip per sample. Get out the sliding worktable from the workstation and place the strips on it.

Add in the « Deep-well lysate » plate for KingFisher™ (Flex), in the Row A for KingFisher™ (Duo) or in Position A for KingFisher™ (mL) :

20 µl Proteinase K
200 µl of processed sample, previously vortexed
500 µl of LAB-SMB-carrier + IPC solution, previously thoroughly vortexed (30 sec).

Table 4 below presents the LAB-SMB-carrier + IPC lysis solution composition:

Table 4. LAB-SMB-carrier + IPC lysis solution

Reagents	Number of samples*						
	1	5	10	12	15	48	96
LA Buffer	100 µl	550 µl	1.1 ml	1.32 ml	1.65 ml	5.28 ml	10.56 ml
LB Buffer	400 µl	2.2 ml	4.4 ml	5.28 ml	6.6 ml	21.12 ml	42.24 ml
SMB (SuperBall Magnetic Beads)‡	25 µl	137.5 µl	275 µl	330 µl	412.5 µl	1.32 ml	2.64 ml
Carrier RNA (1 µg/µl)	1 µl	5.5 µl	11 µl	13.2 µl	16.5 µl	52.8 µl	105.6 µl
Exogenous IPC (pink cap tube)	5 µl	27.5 µl	55 µl	66 µl	82.5 µl	264 µl	528 µl

* In order to ensure the pipetting volume, the prepared volume contains an additional volume of 10%. The exceeding volume of lysis solution can be stored for maximum 8 days, beyond this duration, the solution has to be discarded.
‡ Thoroughly vortex for 3 minutes before first use and for 1 minute for the next uses.

Prefill deep-well plates and microplates according to Table 5 below :

Table 5. KingFisher™ Flex, Duo and mL configuration and Reagents volumes

Position on the strip or plate			Element to add	Volume per well (µl)
Flex	Duo*	mL		
Deep-well Lysate	Row A	Position A	Lysate†	720†
Deep-well Wash 1	Row E	Position B	W1 Buffer	700
Deep-well Wash 2	Row F	Position C	W2 Buffer	700
Deep-well Wash 3	Row G	Position D	Ethanol (96–100%)	750
Elution Microplate	Elution strip	Position E	EL Buffer	90
Rod Cover Microplate (Large 96-Rod Cover)	Row B	*Placed manually*	Rod cover	—

* Rows C, D and H are empty
† Includes 20 µl Proteinase K, 200 µl of processed sample and 500 µl of LAB-SMB-carrier+IPC Lysis solution.

2. Run the KingFisher™ program

Select the program « BioExtract_KF_Flex», « BioExtract_KF_Duo » or « BioExtract_KF_mL » and load the plates/strips into the workstation, then run the program.

At the end of the extraction program, keep the elution plate containing the extracted nucleic acids and discard the other plates/strips.

The extracted DNA can be stored at 4°C if the qPCR is performed within 4-8 hours following the extraction otherwise it is recommended to store it at <-20°C for 6 months or at <-70°C for a better conservation.

Figure 2. Nucleic acids Purification using the BioExtract® SuperBall® Kit (Cat. N° BES384)

	KingFisher™ Flex	KingFisher™ Duo	KingFisher™ mL	Element to add
1 Plates or Strips Preparation	Deep-well Lysate 	Row A 	Position A 	**Lysate :** 20 µl Proteinase K 200 µl Sample 500 µl LAB-SMB-carrier+IPC Lysis solution
	Deep-well Wash 1 	Row E 	Position B 	700 µl W1 Buffer
	Deep-well Wash 2 	Row F 	Position C 	700 µl W2 Buffer
	Deep-well Wash 3 	Row G 	Position D 	750 µl Ethanol (96-100%)
	Elution microplate	Elution strip	Position E 	90 µl EL Buffer
	Rod cover microplate	Row B  (Rows C, D and H are empty)	*Rod cover placed manually*	Rod Cover
2 KingFisher™	• Switch on the KingFisher™ Flex, Duo or mL. • Slide open the front door of the protective cover. • Select the "BioExtract_KF_Flex", "BioExtract_KF_Duo" or "BioExtract_KF_mL" program. • Press START and follow the messages to load the different slots of the worktable.			

To get the KingFisher™ program corresponding to the KingFisher™ system you are using (Flex, Duo or mL), please contact our technical support (tech@biosellal.com).

DETECTION OF *STREPTOCOCCUS AGALACTIAE* USING THE KIT BIOTK026

Global procedure

1) Establish a plate setup defining each sample position and **including the following controls** :

- **Negative Control Sample (NCS)**: DNase/RNase free water or PBS replaces the sample from the initial sample processing step.
  This control is mandatory for each run of extraction.

- **Negative PCR amplification Control (NC)**: water (eg Aqua-A tube, **blue** cap) replaces the extracted nucleic acids in the qPCR plate.
  This control is recommended when using the kit for the first time or to verify the absence of Master Mix contamination in case of non-compliant NCS result.

- ***S. agalactiae* External Positive PCR amplification Control** (**EPCSAG-A** tube, **orange** cap): quantified synthetic DNA harboring the *S. agalactiae* targeted nucleotide sequence. It must be diluted. Concentration and reference Ct values are reported in the certificate of analysis (CA).
  This control is mandatory for each PCR run.

△ CAUTION : *Handling this EPC tube represents a DNA contamination hazard, it is thus recommended to open and handle it in a restricted area, away from other PCR components and to take precautions to avoid cross-contamination with nucleic acids extracts during deposit on qPCR plate.*

2) Choose the bacterial load (GE/milk ml) quantification method (relative, absolute) and **preparation of the EPC**.

Choice 1 : **Relative Quantification**

For relative quantification, a **single "reference" point** (1 PCR well) corresponding to a given *S. agalactiae* load (in GE copies) and identified as a threshold facilitating the interpretation of the results is used. We recommend **10^4 GE / ml of milk**. The reference point (REF) is constituted using the EPC of the kit (**EPCSAG-A**, **orange** cap), diluted as indicated in Table 6 and Figure 3 below.

An interpretation grid is presented in Table 11.

Choice 2 : **Absolute Quantification**

For absolute quantification, a **5 points standard calibration line**, linking *S. agalactiae* bacterial load (GE/ml of milk) to Ct values, must be set up. This calibration line will be done using the provided quantified EPC following 4 serial 10-fold dilutions as described in Table 6 and Figure 3, to get 10^8 (pure EPC) to 10^4 GE / ml of milk decreasing concentrations.

The 5 PCR wells corresponding to the 5 EPC calibration decreasing concentrations (from 10^8 to 10^4 GE / ml of milk) must be assigned as "standard" during the thermocycler software configuration.

Table 6. Link between EPC dilutions and deduced bacterial load (GE/ml of milk)

EPC dilution factor	Non diluted	10^{-1}	10^{-2}	10^{-3}	**10^{-4}**
Corresponding *S. agalactiae* load (GE / ml of milk)	10^8	10^7	10^6	10^5	**10^4**
Relative Quantification	-	-	-	-	**x**
Absolute Quantification	x	x	x	x	x

Preparation of EPC for relative or absolute quantification

△ CAUTION : *DNA contamination hazard*

- **4** (relative or absolute quantification) **1:10 serial dilutions** (eg 5 µl in 45 µl of water (**blue** cap tube) or TE 1X) **of the provided pure EPC** (**orange** cap tube) must be performed.

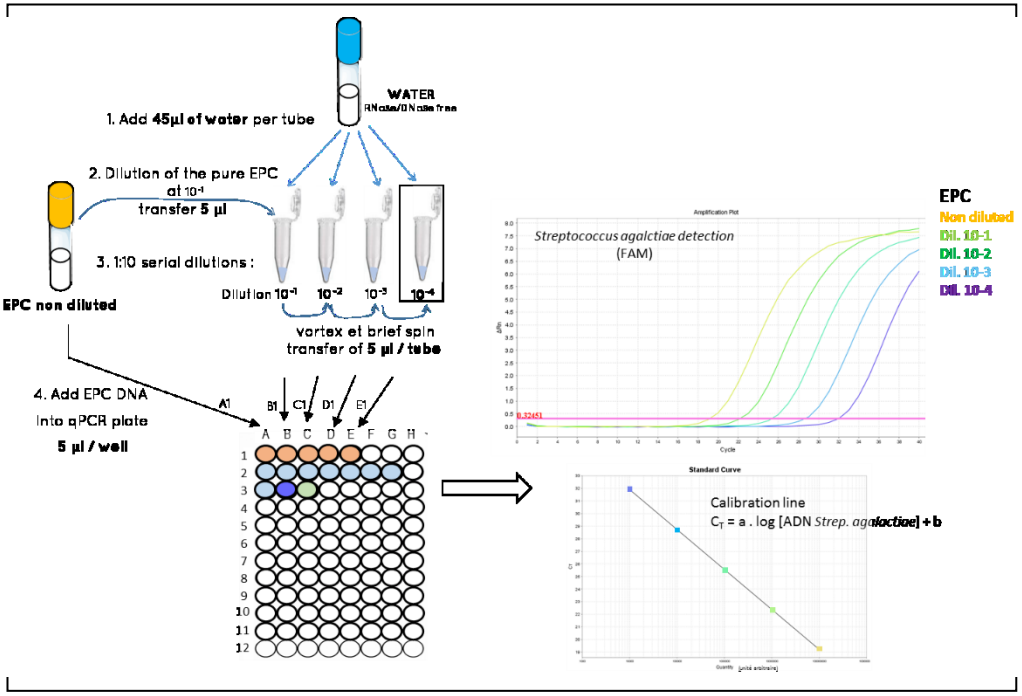
  Dilutions must be performed straight prior PCR plate preparation.

- Take care to **vortex and briefly spin each tube,** in between each dilution step.
- **5 µl of each EPC solution of interest**, depending on the chosen quantification method, will be transfered into PCR wells according to the defined plate plane.

Figure 3 presents the EPC dilution process to get relative or absolute quantification.

Figure 3. EPC preparation for relative or absolute bacterial load quantification.

For a relative quantification, only the 10^{-4} dilution of the pure EPC (boxed in the figure) corresponding to the bacterial load of 10^4 GE *S. agalactiae* / ml of milk will be used (1 single point). For an absolute quantification, the 5 EPC concentration levels will be analysed by PCR (5 points) to get a calibration line linking *S. agalactiae* bacterial load (GE/ml of milk) to Ct values as illustrated in this figure.



3) Extemporaneous qPCR plate preparation

In the «MIX » dedicated area

1. After thawing, vortex and brief centrifugation, transfer **15 µl Master Mix MMSAG-A** (**white** cap) in each well of interest (samples and controls).

In the «Nucleic Acid addition» dedicated area

2. **Add 5 µl extracted DNA (or NCS, or water or EPC)** in each well of interest. Make sure to pipet out in the bottom of the well, in the Master Mix, and to avoid the formation of bubbles.

Note : **in the case the addition of exogenous IPC was forgotten during the nucleic acids extraction process**, it is possible to add it during the qPCR plate preparation : **add 1 µl of IPC-A (pink** cap**) on top of the 5 µl of sample nucleic acid**.

3. **Seal the plate with an optically clear sealer** or **close the strip caps.**

In the PCR amplification dedicated area

4. **Define the thermocycler parameters** (see Tables 7-8).

5. It is recommended to **spin the plate down prior to place it in the thermocycler**, to prevent drops in the well pit walls.

6. **Run the program** (see Table 8). Approximate run time 65 min.

4) Thermocycler settings

This kit was developed and validated on ABI PRISM® 7500 Fast (Applied BioSystems) and AriaMx™ (Agilent Technologies). For other thermocyclers, please contact our technical support.

	ABI PRISM® 7500 Fast	AriaMx™
Mode	Quantitation – Standard curve	Quantitative PCR, Fluorescence Probe
Ramping	Ramping Standard or Fast	Ramping Fast by default
Passive Reference	ROX	ROX

Table 7. Thermocyclers settings

Target	Detectors		Final volume / well
	Reporter	Quencher	
S. agalactiae	FAM	NFQ-MGB ou None*	**20 µl** = 15 µl MM + 5 µl extracted NA or controls†
IPC	CY5	NFQ-MGB ou None*	
To assign to samples and controls†			

* Depends on the thermocycler model. Do not hesitate to contact the BioSellal Technical Support (tech@biosellal.com)

† The controls are NC (=water), NCS (=extracted water) and EPC.

Table 8. Amplification PROGRAM settings

Standard or Fast Ramping		
Cycles	Time	Temperature
1 cycle	5 min	95°C
40 cycles	15 sec	95°C
	30 sec* + data acquisition	60°C

* Set 31 seconds for some thermocyclers such as ABI PRISM® 7500.

NB : Achieving a step-reverse transcription (RT) prior to PCR for the amplification of RNA genomes has no impact on the effectiveness of qPCR Bio-T kit® *S. agalactiae*.

RESULTS INTERPRETATION

To analyse and interpret the signals obtained by qPCR, the « threshold line» as to be assessed. The threshold line has to be assigned carefully in order to obtain the most reproducible result between different manipulations according to the requirement defined in Annex C of the French standard NF U47-600 part 1. For this purpose, a coherent set of positive signals, at least the positive control (EPC), is used, and the threshold line is placed above the background noise, and in middle of the exponential amplification zone.

The threshold cycle, named "Ct" or "Cq" according thermocyclers, corresponds to the intersection between the amplification curves and the threshold line. It allows the relative measurement of the concentration of the target in the PCR reaction when a calibrated extract is analysed in the same series.

The qPCR serie is validated if the controls (EPC, NCS or NC) provide valid results, then the result of each sample can be interpreted qualitatively or quantitatively (relative or absolute).

Main Scenarios

Controls reading

Table 9. qPCR Controls interpretation

	Targets		Interpretation
	S. agalactiae (FAM)	Exogenous **IPC** (CY5)	
NCS Negative Control Sample **MANDATORY**	Neg	Pos*	Validated
	Neg	Neg	▪ No addition of the exogenous IPC DNA during NA extraction ? ▪ Problem during qPCR preparation: Master Mix error ? see EPC and NC controls results.
	Pos	Pos*	▪ Contamination with positive sample or EPC during qPCR plate preparation or Master Mix contamination.
NC Negative PCR Control OPTIONAL	Neg	Neg	Validated
	Pos	Neg/Pos	▪ Contamination with positive sample or EPC during qPCR plate preparation or Master Mix contamination.
EPC *S. agalactiae* External Positive Control **MANDATORY**	Pos*	Neg	Validated
	Neg	Neg	▪ Problem during qPCR preparation: Master Mix error ? Omission to add EPC?
	Pos	Pos	▪ Contamination during plate preparation.

* *Obtained Ct value must be compliant with values indicated on the certificate of analysis (CA).*

Samples reading

- **Qualitative Analysis: signals reading**

Table 10. Differents types of résults		
Targets		**Interpretation**
S. agalactiae (FAM)	**Exogenous IPC** (CY5)	
Neg	**Pos** Refenced Ct (see Certificate of Analysis)	**Negative or Non detected**
Pos		**Positive or Detected** Quantification possible
Pos	**Neg** or **Ct >35**	**Positive or Detected** ▪ Presence of PCR inhibitors ?* ▪ Problem during exogenous IPC addition ? ▪ Competition with the target ? ***Quantification is not possible***
Neg	**Neg** ou **Ct >35**	**Uninterpretable** ▪ Omission of extracted NA during plate setup or addition non in contact with the MM ? ▪ Presence of PCR inhibitors ?* ▪ Problem during extraction ? ▪ Degradation of NA in the sample ?

** In case of suspicion of inhibition, 1) repeat qPCR by pre-diluting the extracted NA to 1:10 or even 1:100 in DNase / RNase free water or 2) renew analysis from extraction step.*

- **Quantitative Analysis**

Relative Quantification

Comparative analysis of the *S. agalactiae* Ct values obtained for the samples versus the Ct value of the Reference Material **(REF)** at 10^4 GE/ml.

The analysed samples will not be quantified precisely but *S. agalactiae* load will be determined relatively to the position of the Ct value obtained with respect to the Ct value **of the Reference Point (REF)**. Table 11 below presents the reading and interpretation grid.

Table 11. Interpretation of qPCR results in Relative Quantification of *S. agalactiae*		
qPCR Results		**Interpretation**
Negative		**Not detected** Quantity of GE < LDpcr
Positive	Ct ≥ Ct REF 10^4 GE/ml	**Positive : +**
	Ct < Ct REF 10^4 GE/ml	**Highly Positive : ++**

An appended document proposes cow/herd management actions depending on the results obtained. This document is available on demand to BioSellal's Technical Support.

Absolute Quantification

To estimate *S. agalactiae* bacterial load in sample, expressed in GE/ml of milk, a calibration line must be drawn using the thermocycler software. It requires :

- To assign quantification values (GE/ml) for the 5 calibration EPC points defined as « standards ».
- The thermocycler software will automatically establish the calibration line and indicate the linear equation enabling to determine the bacterial load from Ct values.

The PCR efficiency E is computed too: it must be included between 85 and 115%.

- For each Ct value corresponding to samples NA, the software will give a bacterial load in GE/ml. This concentration can be calculated independently using the calibration linear equation.

For an easy interpretation, it is possible to refer to Table 11 interpretation grid.

An appended document proposes cow/herd management actions depending on the results obtained. This document is available on demand to BioSellal's Technical Support.

EPC preparation grid for all the Bio-T kit® from the MILK line

		Relative Quantification		Absolute Quantification	
Bio-T kit® Milk pathogen of interest	Concentration of the pure EPC provided with the kit (GE/ml)*	**REF**ERENCE EPC Concentration (GE/ml)*	Number of serial 1 :10 dilutions to be done to get the **REF** EPC value	EPC concentration range (GE/ml) for calibration line	Number of serial 1 :10 dilutions required for the calibration line
Staphylococcus aureus **(SAU) - BioTK007**	10^7	10^4	3	10^7 to 10^3	4
Staphylococcus spp **(SSP) - BioTK013**	10^7	10^4	3	10^7 to 10^3	4
Streptococcus uberis **(SUB) - BioTK011**	10^7	10^4	3	10^7 to 10^3	4
Streptococcus dysgalactiae **(SD) - BioTK012**	10^7	10^4	3	10^7 to 10^3	4
Enterobacteriaceae **(ETB) - BioTK009**	10^7	10^4	3	10^7 to 10^3	4
Escherichia coli **(EC) - BioTK008**	10^9	10^5	4	10^9 to 10^5	4
Klebsiella spp **(KSP) - BioTK010**	10^8	10^4	4	10^8 to 10^4	4
Pseudomonas spp **(PSP) - BioTK025**	10^7	10^4	3	10^7 to 10^3	4
Trueperella pyogenes **(TPY) - BioTK014**	10^8	10^4	4	10^8 to 10^4	4
Mycoplasma bovis **(MYB) - BioTK015**	10^8	10^4	4	10^8 to 10^4	4
Streptococcus agalactiae **(SAG) - BioTK026**	10^8	10^4	4	10^8 to 10^4	4

* Refer to Certificate of Analysis (CA) of each kit to know the expected Ct values.

Notes :

Notes :

www.biosellal.com

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