HANDBOOK

Bio-T kit® BoNT all types

Cat. N° BIOTK061 - Pack (BIOTK062, BIOTK063, BIOTK064, BIOTK065)
Containing one kit of each following references:

Bio-T kit® BoNT A & F	Cat. N° BIOTK062 – 50 reactions
Bio-T kit® BoNT B & E	Cat. N° BIOTK063 – 50 reactions
Bio-T kit® BoNT C & D	Cat. N° BIOTK064 – 50 reactions
Bio-T kit® BoNT G	Cat. N° BIOTK065 – 50 reactions

Detection of Botulinum Neurotoxins (BoNT)
types A & F, B & E, C & D and G
by real-time PCR (qPCR)
with exogenous internal positive control (IPC)

Sample types

- Food for humans and animals
- Environmental samples (sludge, manure ...)
- All other matrices likely to contain botulinum neurotoxin genes
- Individual analysis

Not for diagnosic use

DOCUMENTS MANAGEMENT

The Bio-T kit® BoNT all types has this qPCR handbook, presenting the instruction information to perform the qPCR.
The last versions in use for this handbook is indicated on the certificate of analysis (CA) provided with the Bio-T kit® BoNT all types.

MODIFICATIONS MANAGEMENT

BioSellal indicates modifications done to this document by highlighting them using the rules presented in the Table below:

MODIFICATIONS MANAGEMENT			
Type of modification Highlighting color	**Minor modifications**	**Type 1 Major modifications**	**Type 2 Major modifications**
Impact on revision / version	**Change of revision date** **No change of version**	**Change of revision date** **+ change of version**	**Change of revision date** **+ change of version**
Examples of modifications	Corrections: typographical, grammatical or turns of phrase	EPC reference modification	Modification of Master Mix composition
	Addition of new sample type for extraction	Exogenous IPC reference modification	Modification of validated extraction protocol
	Addition of information giving more details or alternative protocol		
	Addition/Suppression of optional information		

PRESENTATION

Recommendations for sampling, shipping and storage of samples

Real-time PCR is a powerful technique allowing the detection of few amounts of pathogen genome. Genome can be rapidly degraded depending on the pathogen nature (bacteria / parasites, enveloped viruses...), the genome nature (DNA / RNA) and the sample type (presence of DNase / RNase). Thus, BioSellal recommends the following instructions to guarantee an optimal diagnosis.

Sampling

To prevent cross-contamination between samples leading to false positive results, it is mandatory to use disposable materials for single use and to avoid direct contact between specimens.

Shipping

According to the recommendations of French National Reference Center (NRC) for anaerobic bacteria and botulism (Institut Pasteur, Paris), food or environmental samples can be sent at room temperature, preferably in an airtight container without delay conditions. For other country, shipping must comply with the requirement of the relevant legislation and by default, with the specification of OIE manual.

Storage after reception

Storage must comply with the requirement of the relevant legislation of your country and by default, with the specification of OIE manual.

FOOD Line

The Bio-T kit® BoNT all types is a pack of 4 kits, belonging to the FOOD line. This FOOD line gathers a set of kits dedicated to the detection of pathogens or genes coding for proteins responsible for health disorders in humans or animals and which shared common PCR protocols It is compatible with BioSellal's other kits except with the ones belonging to the PIG and AVIAN lines. (information available on www.biosellal.com).

Description of the Bio-T kit® BoNT all types

The **Bio-T kit® BoNT all types** (Cat. N° BIOTK061) is composed of 4 kits:

- Bio-T kit® BoNT A & F Cat. N° BIOTK062 – 50 reactions
- Bio-T kit® BoNT B & E Cat. N° BIOTK063 – 50 reactions
- Bio-T kit® BoNT C & D Cat. N° BIOTK064 – 50 reactions
- Bio-T kit® BoNT G Cat. N° BIOTK065 – 50 reactions

Description of the Bio-T kit® BoNT A & F

The Bio-T kit® BoNT A & F (Cat. N° BIOTK062) contains a ready to use **PCR Master Mix** allowing the detection **in the same reaction well of**:

- **The coding gene for Botulinum Neurotoxins (BoNT) type A** with a 6-FAM labelling
- **The coding gene for Botulinum Neurotoxins (BoNT) type F** with a VIC labelling
- **An exogenous internal positive control IPC DNA,** with a Cy5 labelling, to add at the extraction step to assess nucleic acids extraction quality and absence of PCR inhibitors.

Description of the Bio-T kit® BoNT B & E

The Bio-T kit® BoNT B & E (Cat. N° BIOTK063) contains a ready to use **PCR Master Mix** allowing the detection **in the same reaction well of**:

- **The coding gene for Botulinum Neurotoxins (BoNT) type B** with a 6-FAM labelling
- **The coding gene for Botulinum Neurotoxins (BoNT) type E** with a VIC labelling
- **An exogenous internal positive control IPC DNA,** with a Cy5 labelling, to add at the extraction step to assess nucleic acids extraction quality and absence of PCR inhibitors.

Description of the Bio-T kit® BoNT C & D

The Bio-T kit® BoNT C & D (Cat. N° BIOTK064) contains a ready to use **PCR Master Mix** allowing the detection **in the same reaction well of**:

- **The coding gene for Botulinum Neurotoxins (BoNT) type C** with a 6-FAM labelling
- **The coding gene for Botulinum Neurotoxins (BoNT) type D** with a VIC labelling
- **An exogenous internal positive control IPC DNA,** with a Cy5 labelling, to add at the extraction step to assess nucleic acids extraction quality and absence of PCR inhibitors.

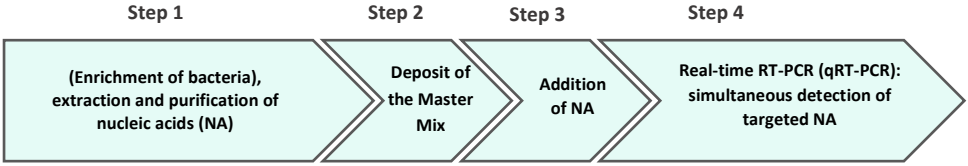
Description of the Bio-T kit® BoNT G

The Bio-T kit® BoNT G (Cat. N° BIOTK065) contains a ready to use **PCR Master Mix** allowing the detection **in the same reaction well of**:

- **The coding gene for Botulinum Neurotoxins (BoNT) type G** with a 6-FAM labelling
- **An exogenous internal positive control IPC DNA,** with a Cy5 labelling, to add at the extraction step to assess nucleic acids extraction quality and absence of PCR inhibitors.

These 4 kits are based on qualitative dectection of BoNT types A & F, B & E, C & D and G (detected or not detected) on food for humans and animals, environmental samples (sludge, manure ...) and all other matrices likely to contain botulinum neurotoxin genes. They have been developed and validated according to the **French regulatory standard NF U47-600-2 edited by AFNOR.**

Description of the whole process



Extraction method must comply with the requirement of the relevant legislation of your country and by default, with the specification of OIE manual 5µl of exogenous IPC (pink cap) must be added for each sample. This IPC is shared bewteen each BoNT kits. BioSellal recommends to add the IPC in the lysis solution.	qPCR handbook of the Bio-T kit® BoNT all types		
	Ready-to-use Master Mix MMBoNTAF-A MMBoNTBE-A MMBoNTCD-A MMBoNTG-A	Samples NC/NCS Process positive control EPC	Dyes: FAM/VIC/Cy5 for A&F, B&E, C&D FAM/Cy5 for G Passive reference: ROX Program : Classical program without RT

Kits contents and storage

Bio-T kit® BoNT A & F Cat. N° BIOTK062 - 50 reactions

Table 1. Description of the BoNT A & F kit contents

Description	Reference	Volume/tube	Presentation	Storage
Master Mix (MM) Ready to use	MMBoNTAF-A	750 µl	tube white cap Bag A	≤-16°C Protected from light, « MIX » Area
Exogenous Internal Positive Control (IPC)	IPC-A	250 µl	tube pink cap Bag B	≤-16°C « Extraction » Area
External Positive Control (EPC) Positive PCR control of BoNT A & F	EPCBoNTAF-A	110 µl	tube orange cap Bag C	≤-16°C « Addition of Nucleic acids » Area
Water RNase/DNase free	Aqua-A	1 ml	tube blue cap Bag C	≤-16°C « Addition of Nucleic acids » Area

Bio-T kit® BoNT B & E Cat. N° BIOTK063 - 50 reactions

Table 2. Description of the BoNT B & E kit contents

Description	Reference	Volume/tube	Presentation	Storage
Master Mix (MM) Ready to use	MMBoNTBE-A	750 µl	tube white cap Bag A	≤-16°C Protected from light, « MIX » Area
Exogenous Internal Positive Control (IPC)	IPC-A	250 µl	tube pink cap Bag B	≤-16°C « Extraction » Area
External Positive Control (EPC) Positive PCR control of BoNT B & E	EPCBoNTBE-A	110 µl	tube orange cap Bag C	≤-16°C « Addition of Nucleic acids » Area
Water RNase/DNase free	Aqua-A	1 ml	tube blue cap Bag C	≤-16°C « Addition of Nucleic acids » Area

Bio-T kit® BoNT C & D Cat. N° BIOTK064 - 50 reactions

Table 3. Description of the BoNT C & D kit contents

Description	Reference	Volume/tube	Presentation	Storage
Master Mix (MM) Ready to use	MMBoNTCD-A	750 µl	tube white cap Bag A	≤-16°C Protected from light, « MIX » Area
Exogenous Internal Positive Control (IPC)	IPC-A	250 µl	tube pink cap Bag B	≤-16°C « Extraction » Area
External Positive Control (EPC) Positive PCR control of BoNT C & D	EPCBoNTCD-A	110 µl	tube orange cap Bag C	≤-16°C « Addition of Nucleic acids » Area
Water RNase/DNase free	Aqua-A	1 ml	tube blue cap Bag C	≤-16°C « Addition of Nucleic acids » Area

Table 4. Description of the BoNT G kit contents

Description	Reference	Volume/tube	Presentation	Storage
Master Mix (MM) Ready to use	MMBoNTG-A	750 µl	tube white cap Bag A	≤-16°C Protected from light, « MIX » Area
Exogenous Internal Positive Control (IPC)	IPC-A	250 µl	tube pink cap Bag B	≤-16°C « Extraction » Area
External Positive Control (EPC) Positive PCR control of BoNT G	EPCBoNTG-A	110 µl	tube orange cap Bag C	≤-16°C « Addition of Nucleic acids » Area
Water RNase/DNase free	Aqua-A	1 ml	tube blue cap Bag C	≤-16°C « Addition of Nucleic acids » Area

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

Main critical points

- Wear appropriate personal protective equipment (lab coat, disposable gloves frequently changed).
- Work in dedicated and separate areas to avoid contamination: "Extraction" (unextracted samples storage, extraction equipment area), "Mix" (ready to use MM storage, qRT-PCR plates preparation), "Nucleic acids (NA) Addition" (Nucleic Acids storage and addition of extracted NA and controls in the qRT-PCR plate), "PCR" (final area containing the thermocycler(s)).
- Use dedicated equipment for each working area (gloves, lab coat, pipettes, vortex, ...).
- Use filter tips.
- Before use, thaw all components at room temperature.
- Vortex and spin briefly (mini-centrifuge) all reagents before use.
- Avoid the repetition of freezing-thawing cycles for samples, lysates, extracted NA.

DETECTION OF BoNT A & F, B & E, C & D AND G BY qPCR WITH BIOTK061 (BIOTK062, BIOTK063, BIOTK064, BIOTK065)

Global Procedure

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

- **Negative Control Sample (NCS)**: water (or PBS) replaces the sample from the first step of sample preparation.
  This control is mandatory for each extraction series.

- **Negative Amplification Control (NC)**: 5 µl of water RNase/DNase free (Aqua-A tube, **blue** cap) replaces sample Nucleic Acids extract on qPCR plate.
  This control is recommended when using the kit for the first time or to verify the absence of Master Mix contamination.

- **External Positive Control of BoNT (EPC)** : Synthetic DNA provided (tubes **EPCBoNTAF-A, EPCBoNTBE-A, EPCBoNTCD-A, EPCBoNTG-A : orange** cap), containing specific target of BoNT A and BoNT F; BoNT B and BoNT E ; BoNT C and BoNT D ; BoNT G.
  These controls are mandatory.

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

2) qPCR plate preparation

In the "MIX" dedicated area

1. After thawing, vortex and rapid centrifugation, **transfer 15 µl Master Mix** MMBoNTAF-A, MMBoNTBE-A, MMBoNTCD-A **or** MMBoNTG-A (**White** cap) in each well of interest (samples and controls).

In the "Nucleic Acids addition" dedicated area

2. **Add 5 µl of extracted nucleic acids (or NCS, water or EPC: orange** cap tube) 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: if the exogenous IPC was not added during sample extraction, it can be added directly in the qPCR plate:
- Add 1 µl of IPC (**pink** cap) with the extracted nucleic acids
- Or add directly the IPC (1 µl per reaction) in an aliquot of Master Mix before the deposits of 16 µl of this mix into each well of interest. Then add 5 µl of extracted nucleic acids.
The reaction volume will be increased to 21 µl, without impacting the effectiveness of the qPCR.

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 Table 5, Table 6, Table 7 and Table 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. Start the qPCR program. Approximate run time: 70 min.

3) Thermocycler settings

This kit was developed and validated on ABI PRISM® 7500 Fast (Applied Biosystems) in standard ramping and confirmed on AriaMx™ (Agilent Technologies, Fast ramping by default). For other thermocyclers, contact our technical support.

Table 5. Thermocycler configuration

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

For Bio-T kit® BoNT A & F, Bio-T kit® BoNT B & E, Bio-T kit® BoNT C & D

Table 6. Thermocycler Settings

Target	Detectors		Final Volume / well
	Reporter	Quencher	
BoNT A or BoNT B or BoNT C	FAM	NFQ-MGB ou None*	**20 µl** = 15 µl Master Mix + 5 µl extracted nucleics acids or controls†
BoNT F or BoNT E or BoNT D	VIC	NFQ-MGB ou None*	
Exogenous 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)
† Controls are NC (water), NCS (extracted water) and/or EPC.

For Bio-T kit® BoNT G

Table 7. Thermocycler Settings

Target	Detectors		Final Volume / well
	Reporter	Quencher	
BoNT G	FAM	NFQ-MGB ou None*	**20 µl** = 15 µl Master Mix + 5 µl extracted nucleics acids or controls†
Exogenous 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)
† Controls are NC (water), NCS (extracted water) and/or EPC.

Table 8. CLASSICAL Amplification program settings without RT†		
Ramping Standard ou Fast		
Cycles	Temps	Température
1 cycle	5 min	95°C
40 cycles	15 sec	95°C
	30 sec* + data acquisition	60°C

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

† optional step, in case of simultaneous detection of RNA genomes. Achieving a reverse-transcription (RT) step prior to PCR for the amplification of RNA genomes has no impact on the effectiveness of the Bio-T kit® BoNT all types.

NB: This amplification program is compatible with all Bio-T kits® except for ones belonging to the PIG and AVIAN LINES.

RESULTS INTERPRETATION

To analyze and interpret the signals obtained by qPCR, the Threshold must be set up.

The threshold must be assigned carefully in order to obtain the most reproducible result between different manipulations according to the requirements defined in Annex C of the French Standard **NF U47-600 (part 1)**. A consistent set of positives controls, usually an In-house Reference Material (MRI) or the EPC, is used to set the threshold value above the baseline and in the exponential amplification phase of the plot.

The Threshold Cycle, named « Ct » or « Cq » (depending on 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 analyzed in the same series.

The qPCR series is validated if the controls (EPC, NCS and NC) present valid results, then the result of each sample can be interpreted.

Main Scenarios

Controls Reading for Bio-T kit® BoNT A & F, Bio-T kit® BoNT B & E, Bio-T kit® BoNT C & D

Table 9. PCR Controls results interpretation

	Targets			
	BoNT A or BoNT B or BoNT C (FAM)	**BoNT F or BoNT E or BoNT D** (VIC)	**Exogenous IPC** (Cy5)	**Interpretation**
NCS Negative Control Sample **MANDATORY**	Neg	Neg	Pos	Valid
	At least one of the two targets **Pos**		Pos	Contamination with a positive sample during extraction step or during qPCR plate preparation.
	Neg	Neg	Neg	Omission of exogenous IPC DNA addition? Defective extraction?
NC Negative PCR Control **OPTIONAL**	Neg	Neg	Neg	Valid
	At least one of the three targets **Pos**			Contamination with a negative or a positive sample during PCR plate preparation? or Master Mix / Water contamination?
EPC PCR external positive control for BoNT A and F or BoNT B and E or BoNT C and D **MANDATORY**	Pos*	Pos*	Neg	Valid
	Neg	Neg	Neg	Problem during qPCR plate preparation: Master Mix error? EPC omission?
	Pos*	Pos*	Pos	Contamination with a sample during qPCR plate preparation?

* The Ct value obtained must be conform with the value indicated on the Certificate of Analysis (CA).

Controls Reading for Bio-T kit® BoNT G

Table 10. PCR Controls results interpretation			
	Targets		Interpretation
	BoNT G (FAM)	Exogenous IPC (Cy5)	
NCS Negative Control Sample **MANDATORY**	Neg	Pos	Valid
	Pos	Pos	Contamination with a positive/negative sample during extraction step or during qPCR plate preparation.
	Neg	Neg	Omission of exogenous IPC DNA addition? Defective extraction?
NC Negative PCR Control **OPTIONAL**	Neg	Neg	Valid
	At least one of the two target Pos		Contamination with a negative or a positive sample during PCR plate preparation? or Master Mix / Water contamination?
EPC BoNT G PCR external positive control **MANDATORY** *IN ABSENCE OF MRSI*	Pos*	Neg	Valid
	Neg	Neg	Problem during qPCR plate preparation: Master Mix error? EPC omission?
	Pos*	Pos	Contamination with a sample during qPCR plate preparation?

* The Ct value obtained must be conform with the value indicated on the Certificate of Analysis (CA).

Samples Reading for Bio-T kit® BoNT A & F, Bio-T kit® BoNT B & E, Bio-T kit® BoNT C & D

Table 11. Different types of results obtained for the samples			
Targets			
BoNT A or BoNT B or BoNT C (FAM)	BoNT F or BoNT E or BoNT D (VIC)	Exogenous IPC (Cy5)	Interpretation
Neg	Neg¥	Pos*	**Negative or Undetected**
Pos¥	Pos		**Positive or Detected**
Pos	Pos	Neg or Ct>35	**Positive or Detected** Problem during the IPC addition? Presence of inhibitors ǂ? Competition with the mains targets?
One of the targets is Neg		Neg or Ct>35	**Positive or Detected for the positive target** **Uninterpretable** = Repeat the analysis for the negative target Exogenous IPC omission during the extraction and/or qPCR? Presence of inhibitors ǂ? Nucleic acids degradation in the sample? Extraction problem? Competition with other target?
Neg	Neg	Neg or Ct>35	**Uninterpretable** = Repeat the analysis Nucleic acids extract omission or extract not in contact with Master Mix ? Presence of inhibitors ǂ? Nucleic acids degradation in the sample? Problem during the IPC addition? Extraction problem?

* The obtained Ct value depends on the thermocycler, the sample type and the used extraction protocol. This value must be, at least, included within the specified range in the certificate of analysis (CA). Ct values for IPC are available upon request. BioSellal recommends you to determine your own maximal IPC Ct value depending on your own extraction method and thermocycler.

ǂ In case of inhibition suspicion, 1) Repeat the qPCR with the dilution of extracted nucleic acids at 1/10 or 1/100 in the DNase/RNase free water. 2)Restart the analysis from the extraction step.

¥ In case of samples positive for both BoNT A and BoNT F, and if BoNT A Ct value is low, the curve shape for BoNT F can be impacted due to a competition phenomenon. Thus, BioSellal recommends to pay particular attention for the curve shape of BoNT F curves in case of BoNT A strong positive sample.

Samples Reading for Bio-T kit® BoNT G

Table 12. Different types of results obtained for the samples		
Targets		Interpretation
BoNT (FAM)	Exogenous IPC (Cy5)	
Neg	Pos*	**Negative ou Undetected**
Pos		**Positive ou Detected**
Pos	Neg or Ct>35	**Positive ou Detected** Problem during the IPC addition? Presence of inhibitors ǂ? Competition with the main target?
Neg	Neg or Ct>35	**Uninterpretable** = Repeat the analysis for the negative target Nucleic acids extract omission or extract not in contact with Master Mix? Presence of inhibitors ǂ? Nucleic acids degradation in the sample? Problem during the IPC addition? Extraction problem?

* The obtained Ct value depends on the thermocycler, the sample type and the used extraction protocol. This value must be, at least, included within the specified range in the certificate of analysis (CA). Ct values for IPC are available upon request. BioSellal recommends you to determine your own maximal IPC Ct value depending on your own extraction method and thermocycler.

ǂ In case of inhibition suspicion, 1) Repeat the qPCR with the dilution of extracted nucleic acids at 1/10 or 1/100 in the DNase/RNase free water. 2)Restart the analysis from the extraction step.

biosellal

www.biosellal.com

Technical Support

tech@biosellal.com +33 (0) 4 26 78 47 62

Information and orders

contact@biosellal.com +33 (0) 4 26 78 47 60