

FOR *IN VITRO* USE ONLY

foodproof[®] *Clostridium botulinum* Detection LyoKit **– 5´Nuclease –**

Version 3, March 2017

PCR kit for the qualitative detection of botulinum type **A, B, E** and **F** neurotoxin-producing Clostridia (*Clostridium botulinum*, *C. baratii* and *C. butyricum*) using real-time PCR instruments.

Order No. R 602 40-1 / R 602 40-2

**Kit for 96 reactions (lyophilized) for a maximum of
94 samples**

Store the kit at 2 to 8 °C

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1. What this Product Does

Number of Tests

The kit is designed for 96 reactions with a final reaction volume of 25 µl each. Up to 94 samples (single sample preparation) plus positive and negative control reactions can be analyzed per run.

Storage and Stability

- Store the kit at 2 °C to 8 °C through the expiration date printed on the label.
- Once the kit is opened, store the kit components as described in the following Kit Contents table:

Kit Contents

Component	Label	Contents / Function / Storage
foodproof[®] Clostridium botulinum Detection LyoKit Microplate, prefilled with 96 reactions (lyophilized)	Aluminum bag containing a 8-tube strip mat <ul style="list-style-type: none">• R 602 40-1 with white low profile tubes*• R 602 40-2 with clear regular profile tubes*	<ul style="list-style-type: none">• 96 prefilled reactions (lyophilized).• Ready-to-use PCR mix containing primer and probes specific for DNA of the botulinum neurotoxin types A, B, E and F and the Internal Control (IC) as well as Taq DNA Polymerase and Uracil-N-Glycosylase (UNG, heat labile) for prevention of carry-over contamination.• For amplification and detection of botulinum neurotoxin type A, B, E and F specific sequences.• Store at 2 °C to 8 °C in the aluminum bag (sealed).• Protect from light and moisture!
Control Template	Vial 2 (purple cap)	<ul style="list-style-type: none">• 1 x 250 µl• Contains a stabilized solution of DNA.• For use as a PCR run positive control.• Store at 2 to 8 °C.
H ₂ O PCR-grade	Vial 3 (colorless cap)	<ul style="list-style-type: none">• 2 x 1 ml• Nuclease-free, PCR-grade H₂O.• For use as a PCR run negative control.• Store at 2 to 8 °C.
Cap strips	Plastic bag containing 8-cap strips	<ul style="list-style-type: none">• 12 x 8-cap strip• For use in real-time PCR after addition of samples.

*Tube profile and instrument compatibility chart is available online: www.bc-diagnostics.com/compatibility-chart

Additional Equipment and Reagents Required

- Real-time PCR cycler suitable for detection of FAM-, HEX-, and ROX- labeled probes and capable of performing a melting curve analysis. Without a melting curve analysis, the four botulinum neurotoxin types can still be detected but not differentiated. In cases the strip tubes don't fit for the instrument the samples have to be transferred to appropriate PCR vessels after resuspension of the lyophilized PCR mix.
- Sample Preparation Kit
 - **foodproof[®] StarPrep Two Kit** (Order No. S 400 08)¹ **or**
 - **foodproof[®] ShortPrep II Kit** (Order No. S 400 02)¹ **or**
 - **foodproof[®] Sample Preparation Kit II** (Order No. S 400 05)¹
- Resuspension Reagent (Order No. A 500 14)¹
- Sterile double-distilled water
- Nuclease-free, aerosol-resistant pipette tips
- Pipettes
- Vortex centrifuge Multispin MSC-6000 for PCR-strips (Order No. D 110 66)¹ **with**
- SR-32, Rotor for MSC-3000/6000 (Order No. D 110 65)¹ **or**
- Vortex centrifuge CVP-2 for PCR-plates (Order No. D 110 67)¹

¹ Available from BIOTECON Diagnostics; see Ordering Information for details



Applicability Statement

The **foodproof**[®] *Clostridium botulinum* Detection LyoKit – 5’Nuclease – is intended for the qualitative detection of botulinum type **A, B, E** and **F** neurotoxin-producing Clostridia (*Clostridium botulinum*, *C. baratii* and *C. butyricum*) isolated from enrichment cultures prepared by valid methods and inoculated with all relevant kinds of samples that are potentially contaminated with these microorganisms.
 The kit must not be used in diagnostic procedures.

The kit described in this Instruction Manual has been developed for real-time PCR instruments with a FAM, a HEX, and a ROX detection channel which are capable of performing a melting curve analysis. The performance of the kit was tested with the following real-time PCR instruments: LightCycler[®] 480, LightCycler[®] 96, LightCycler[®] 2.0 (Roche Diagnostics), Mx3005P[®] (Agilent Technologies), ABI 7500 Fast (Applied Biosystems), iQ5 (Bio-Rad), and PikoReal[®] 24 (Thermo Scientific).

Note: A Color Compensation (Color Compensation Set 3; Order No. A 500 10) is necessary and will be supplied by BIOTECON Diagnostics for users of the LC 480 Systems I and II. Please contact BIOTECON Diagnostics for further information.

2. How to Use this Product

2.1 Before You Begin

Precautions

Detection of DNA using the **foodproof**[®] *Clostridium botulinum* Detection LyoKit requires DNA amplification by PCR. The kit provides all reagents required for the PCR. However, in order to achieve reliable results, the entire assay procedure must be performed under nuclease-free conditions. Follow the instructions below to avoid nuclease-, carry-over-, or cross-contamination:

- Keep the kit components separate from other reagents in the laboratory.
- Use nuclease-free labware (e.g., pipettes, pipette tips, reaction vials).
- Wear gloves when performing the assay.
- To avoid cross-contamination of samples and reagents, use fresh aerosol-preventive pipette tips.
- To avoid carry-over contamination, transfer the required solutions for one experiment into a fresh tube, rather than directly pipetting from stock solutions.
- Physically separate the workplaces for DNA preparation, PCR setup, and PCR to minimize the risk of carry-over contamination. Use a PCR hood for all pipetting steps.

Keep the foodproof[®] *Clostridium botulinum* Detection lyophilized PCR Mix away from light and moisture.

Sample Material

Use any sample material suitable for PCR in terms of purity, concentration, and absence of inhibitors. For preparation of genomic DNA from various sample enrichments, refer to the corresponding product package inserts of a suitable sample preparation kit (see “Additional Equipment and Reagents Required”).

DNA Extraction

BIOTECON Diagnostics provides sample preparation kits suitable for all kind of food samples and PPS (see “Additional Equipment and Reagents Required”).
 For more product information please refer to www.bc-diagnostics.com.

Positive Control

Always run a positive control with the samples. To prepare a positive control, replace the template DNA with the provided control DNA [**foodproof**[®] *Clostridium botulinum* Detection Control Template (vial 2, purple cap)] or with a positive sample preparation control.

Negative Control

Always run a negative control with the samples. To prepare a negative control, replace the template DNA with H₂O PCR-grade (vial 3, colorless cap). Include a negative control during sample preparation to monitor reaction purity and cross-contamination. This extraction control can be used as an additional negative control reaction.

2.2 Procedure

Program Setup

The following procedure is optimized for a real-time PCR instrument with a FAM (amplification of the botulinum neurotoxin types BoNT A and E, melting curve identification of BoNT E), HEX (amplification of BoNT B and F, melting curve identification of BoNT F), and ROX (amplification of the Internal Control) detection channel. Program the PCR instrument before preparing the PCR samples. Use the following real-time PCR-protocol for the **foodproof[®] Clostridium botulinum** Detection LyoKit. For details on how to program the experimental protocol, see the Instrument Operator’s Manual of your real-time PCR-cycler:

<u>Pre-incubation</u>	1 cycle
Step 1:	37 °C for 4 minutes
Step 2:	95 °C for 5 minutes
 <u>Amplification</u>	 50 cycles
Step 1:	95 °C for 5 seconds
Step 2*:	60 °C for 60 seconds

* Fluorescence detection in step 2

<u>Melting Curve</u>	1 cycle
Step 1:	95 °C for 50 seconds
Step 2:	37 °C for 50 seconds
Step 3*:	ramp up to 80 °C

* Fluorescence detection during 37 – 80 °C ramp with 1 – 2 measurements/ °C

Notes:

- For some real-time PCR instruments the type of the probe quencher as well as the usage of a passive reference dye has to be specified. The **foodproof[®] Clostridium botulinum** Detection LyoKit contains probes with a non-fluorescent (“dark”) quencher and no passive reference dye.
- For users of the Agilent Mx3005P instrument: Choose Experiment Type “SYBR[®] Green (with Dissociation Curve)” and add FAM, HEX and ROX channels for data collection in the setup section.
- Please contact BIOTECON Diagnostics, if you have questions how to program your cycler.

Preparation of the PCR Mix

Proceed as described below to prepare a 25 µl standard reaction. Always wear gloves when handling strips or caps. Use any sample material suitable for PCR in terms of purity, concentration, and absence of inhibitors.

Note: The PCR strips must be stored in the provided aluminum bag with the silica gel pads to avoid liquid absorption.

1. Take the needed number of PCR tube strips out of the aluminum bag. Use scissors or scalpel to cut the strips apart. Tightly seal the bag afterwards and store away at the recommended conditions.
2. Place the PCR tube strips containing the lyophilized reagents in a suitable PCR tube rack. Check that the reagent pellets are at the bottom of the tubes. If not, briefly centrifuge or flick the pellets to the bottom before proceeding.
3. Decap the tube strips cautiously and discard the cap strips.

Note: Do not leave strips open for extended periods of time. To avoid unwanted liquid absorption, open strips only shortly before filling.

4. Pipet 25 µl sample into each PCR-vessel:
 - For the samples of interest, add 25 µl sample DNA (if using less volume, add PCR-grade H₂O to achieve 25 µl).
 - For the negative control, add 25 µl PCR-grade H₂O (vial 3, colorless cap).
 - For the positive control, add 25 µl **foodproof**[®] *Clostridium botulinum* Detection Control Template (vial 2, purple cap).

Note: To reduce the risk of cross-contamination, it is recommended to prepare only one PCR tube strip at a time.

5. Seal the vessels accurately and tightly with the colorless cap strips.
6. Mix thoroughly using a vortex centrifuge.

Note: BIOTECON Diagnostics recommends vortex centrifuges Multispin MSC-3000 (D 110 64) for PCR-strips or vortex centrifuge CVP-2 for PCR-plates (D 110 67). Dedicated protocols are available for this centrifuge.

Note: Alternatively resuspend the pellet by manual mixing. This may be achieved by cautiously pipetting the sample up and down multiple times during step 4 or flipping the tube strips after sealing while pressing down the cap strip.

7. Spin the PCR tube strips for 30 seconds at 150 – 200 g in a suitable centrifuge.

Note: If your centrifuge exceeds 200 g, do not centrifuge for more than 5 seconds. Avoid centrifugation at forces exceeding 1000 g!

8. Place the samples in your PCR cycler and run the program as described above.

Note: For using any LightCycler 480 instrument, a special adapter (Order No. Z 100 24) is necessary. For some PCR instruments, the PCR strips should be placed in a balanced order into the cycler block. For example two strips can be placed in column 1 and 12.

2.3 Data Interpretation

The kit is intended for the qualitative detection of botulinum type **A, B, E** and **F** neurotoxin-producing Clostridia (*Clostridium botulinum*, *C. baratii* and *C. butyricum*).

Amplification

The amplification of **botulinum neurotoxin types BoNT A and E** is analyzed in the fluorescence channel suitable for FAM labeled probes detection. The amplification of **BoNT B and F** is analyzed in the fluorescence channel suitable for HEX. The amplification of the **Internal Control** is analyzed in the fluorescence channel suitable for ROX.

Compare the results from channels FAM, HEX and ROX for each sample, and interpret the results as described in the table below.

Channel FAM	Channel HEX	Channel ROX	Result Interpretation
Positive	Negative	Positive or Negative	Positive for BoNT A and/or E Negative for BoNT B and F
Negative	Positive	Positive or Negative	Negative for BoNT A and E Positive for BoNT B and/or F
Positive	Positive	Positive or Negative	Positive for BoNT A and/or E Positive for BoNT B and/or F
Negative	Negative	Positive	Negative for BoNT A, B, E, F
Negative	Negative	Negative	Invalid

Note: The Control Template contains a mixture of all four target sequences and therefore usually generates significantly higher fluorescent values than samples that are positive for only one or two of the targets. This can affect positive/negative calls in automatic analysis of amplification curves by the respective instrument software. Always check results visually for plausibility.

Melting curves

Samples that show a positive amplification signal in the FAM or HEX detection channel can be further differentiated using a melting curve analysis in these channels.

A prerequisite for the unambiguous discrimination of the botulinum neurotoxin types in this multi-color experiment is a suitable calibration of the PCR instrument for channels FAM, HEX and ROX. Please refer to the Operator's Manual of your real-time PCR cyler for further information.

The following table lists the detectable botulinum neurotoxin types in the respective channels and their expected melting peak temperatures (± 2 °C dependent on the real-time PCR instrument):

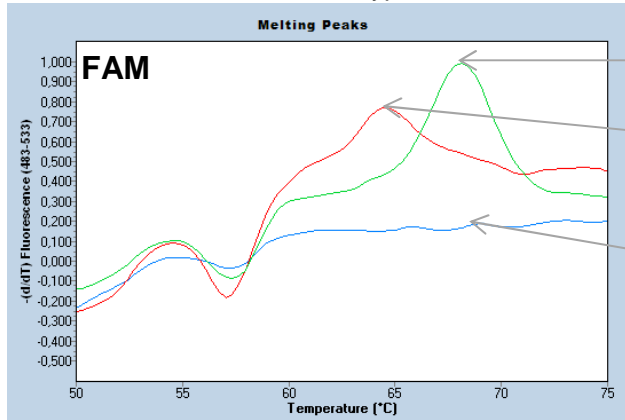
Botulinum neurotoxin type (BoNT)	FAM channel – melting peak temperature	HEX channel – melting peak temperature
A	none	none
B	none	none
E	68 °C \pm 2 °C	none
F	none	55 °C \pm 2 °C and 64,5 °C \pm 2 °C
Control Template	(64 °C \pm 2 °C)	55 °C \pm 2 °C and 62,5 °C \pm 2 °C

Note: The melting peak temperature ranges given in the above table mainly reflect the variability between instruments and their respective analysis software. The Control Template contains a mixture of all target sequences but its melting peaks are distinguishable from the expected melting peaks of BoNT E and F samples. However, single sequence variants of BoNT E or F may differ in their melting peak temperature. Furthermore, food and sample preparation matrices can slightly shift melting peak temperatures.

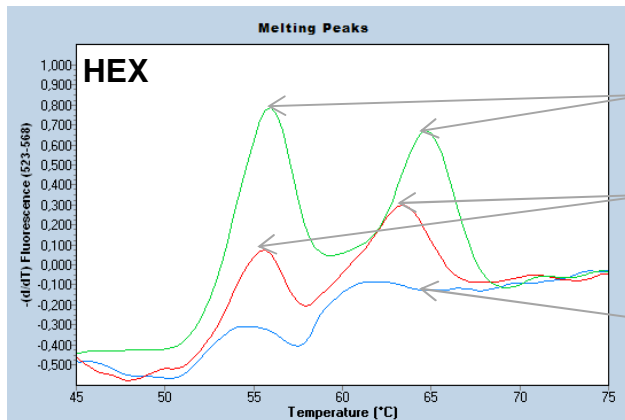


The screenshots show typical melting curves on a LightCycler 480 instrument.

BoNT = Botulinum neurotoxin type



- BoNT E showing melting peak at 68 °C
- Control Template showing no melting peak or minor melting peak at 64 °C
- BoNT A showing no melting peak



- BoNT F showing melting peak at 55 °C and 65 °C
- Control Template showing melting peak at 55 °C and 63 °C
- BoNT B showing no melting peak

Melting peak temperature greater than 70 °C can occur, but are not attributed to one of the target botulinum neurotoxin types and can safely be ignored.

The peak height of positive samples may vary according to the initial cell concentration. Note that the presence or absence of specific melting peaks should be checked manually for all positive samples as the peak finding algorithms of the respective PCR instrument software may not detect all relevant maxima of the melting curve. A guarantee for the identification via melting curves cannot be given.



3. Troubleshooting

Observation	Possible Reason	Recommendation
No signal increase is observed, even with positive controls.	Incorrect detection channel has been chosen.	<ul style="list-style-type: none">• Set channel settings to the appropriate detection channel.
	Pipetting errors.	<ul style="list-style-type: none">• Check for correct reaction setup. Repeat the PCR run.• Always run a positive control along with your samples.
	No data acquisition programmed.	<ul style="list-style-type: none">• Check the cycle programs.
No signal increase in channel ROX is observed.	Inhibitory effects of the sample material (e.g., caused by insufficient purification).	<ul style="list-style-type: none">• Use the recommended DNA sample preparation kit to purify template DNA.• Dilute samples or pipet a lower amount of sample DNA (e.g., 5 µl instead of 25 µl).
Fluorescence intensity is too low.	Inappropriate storage of kit components.	<ul style="list-style-type: none">• Store the foodproof[®] <i>Clostridium botulinum</i> Detection lyophilized PCR Mix at 2 °C to 8 °C, protected from light and moisture.
	Low initial amount of target DNA.	<ul style="list-style-type: none">• Increase the amount of sample DNA. Depending on the chosen DNA isolation method, inhibitory effects may occur.
Strong decrease of fluorescence baseline.	Resuspension of lyophilized PCR mix not complete.	<ul style="list-style-type: none">• Always resuspend lyophilized PCR mix thoroughly.
Negative control samples are positive.	Carry-over contamination.	<ul style="list-style-type: none">• Exchange all critical solutions.• Repeat the complete experiment with fresh aliquots of all reagents.• Always handle samples, kit components and consumables in accordance with commonly accepted practices to prevent carry-over contamination.
Fluorescence intensity varies.	Insufficient centrifugation of the PCR strips. Resuspended PCR mix is still in the upper part of the vessel.	<ul style="list-style-type: none">• Always centrifuge PCR strips.
	Outer surface of the vessel or the seal is dirty (e.g., by direct skin contact).	<ul style="list-style-type: none">• Always wear gloves when handling the vessels and seal.
Pellets are difficult to dissolve.	The lyophilized PCR mix started to rehydrate.	<ul style="list-style-type: none">• Store the lyophilized PCR mix always in the aluminum bag with the silica gel pad.• Open strip shortly before filling.
Amplification positive and melting curve negative, or vice versa.	Low initial amount of target DNA.	<ul style="list-style-type: none">• Prolong the enrichment time and perform a new sample preparation.• Increase the amount of sample DNA. Depending on the chosen DNA isolation method, inhibitory effects may occur.

4. Additional Information on this Product

How this Product Works

The **foodproof**[®] *Clostridium botulinum* Detection LyoKit[®] provides all necessary reagents and a Control Template for reliable interpretations of results. To ensure maximum reliability of the kit and to prevent misinterpretation of negative results due to inhibition of the amplification, an Internal Control (IC) is included. A hydrolysis probe was designed to bind specifically the IC, allowing detection in the ROX channel, whereas the botulinum neurotoxin DNA is detected in channel FAM and HEX. In case of a negative result due to inhibition of the amplification by the sample DNA of interest, the amplification of the IC is suppressed as well, whereas a negative result for the sample DNA of interest and amplification of the IC clearly indicates the absence of DNA of the target organisms in the sample. The **foodproof**[®] *Clostridium botulinum* Detection LyoKit minimizes contamination risk and contains all reagents (except for template DNA) needed for the detection of DNA of the target botulinum neurotoxin types. Primers and probes provide specific detection in food samples. The described performance of the kit is guaranteed for use on the real-time PCR instruments listed above only.

Test Principle

1. Using the kit's sequence-specific primers in a polymerase chain reaction (PCR), the PCR instrument and the supplied reagents amplify fragments of botulinum neurotoxin specific sequences.
2. The PCR instrument detects these amplified fragments in real time through fluorescence generated by cleavage of the hybridized probes due to the 5'-nuclease activity of the Taq DNA polymerase.
3. During the ramp phase of the PCR (melting curve), additional probes hybridize to an internal sequence of the amplicons which separates the reporter dye from the quencher dye, increasing the reporter dye signal.
4. The PCR instrument measures the emitted fluorescence of the reporter dye.

Prevention of Carry-Over Contamination

The heat-labile Uracil-N-Glycosylase (UNG) is suitable for preventing carry-over contamination between PCRs. This technique relies on the incorporation of deoxyuridine triphosphate (dUTP) during all amplification reactions, and the pretreatment of all successive PCR mixtures with the heat-labile UNG. The UNG cleaves DNA at any site where a deoxyuridine residue has been incorporated. The resulting abasic sites are hydrolyzed due to the high temperatures during the initial denaturation step, and can no longer serve as PCR templates. The heat-labile UNG is inactivated during the initial denaturation step. Native DNA (e.g., the isolated *Clostridium* genomic DNA) does not contain uracil and is therefore not degraded by this procedure. Since dTTP is replaced with dUTP and UNG is included in the **foodproof**[®] *Clostridium botulinum* Detection LyoKit, decontamination can be achieved with the provided reagents.

Background Information

Botulism is a potentially lethal paralytic disease caused by botulinum neurotoxin, which is the most poisonous naturally occurring substance known [1]. Different anaerobic, Gram-positive spore-forming *Clostridium* species including *Clostridium botulinum* and some strains of *Clostridium baratii* and *Clostridium butyricum* are able to produce botulinum neurotoxins [2].

Botulism can occur in three forms: food-borne botulism, infant botulism and wound botulism. Food-borne botulism is the classical form of botulism caused by the consumption of food containing preformed neurotoxin. Foods most frequently involved are home-canned foods such as cured meats, canned vegetables, and fermented fish products. Outbreaks caused by commercial foods included sausages, sauces, vacuum-packed seafood and other kind of preserved or canned food products.

Infant botulism can happen to infants ingesting spores of toxin-producing clostridia, which are able to germinate and develop in the intestine since babies younger than one year possess a poorly developed gut microflora. The bacteria release the toxin into the intestine followed by absorbance of the toxin into the bloodstream causing paralysis by blocking neuron cells. Honey and infant milk powder have been associated with infant botulism [4].

Wound botulism is a rare form developing when botulinum toxin producing spores germinate and grow in profound wounds or abscesses that provide anaerobic conditions.

The botulinum neurotoxins are classified into eight serotypes designated A–H [2], [3], of which A, B, E, and F are shown toxic to humans [4]. Identification of botulinum neurotoxin types A, B, E and F with the **foodproof**[®] *Clostridium botulinum* Detection LyoKit is in accordance with ISO/TS 17919 [5]. Not the neurotoxins itself but the neurotoxin genes are detected by the molecular technique used.

References

- [1] D. M. Gill, "Bacterial toxins: a table of lethal amounts," *Microbiological reviews*, vol. 46, no. 1, pp. 86-94, 1982.
- [2] M. D. Collins and A. K. East, "Phylogeny and taxonomy of the food-borne pathogen *Clostridium botulinum* and its neurotoxins.," *Journal of applied microbiology*, vol. 84, no. 1, pp. 5-17, Jan. 1998.
- [3] J. Barash and S. Arnon, "A novel strain of *Clostridium botulinum* that produces type B and type H botulinum toxins," *The Journal of infectious diseases*, vol. 209, no. 2, pp. 183-91, 2014.

- [4] M. Lindström and H. Korkeala, "Laboratory diagnostics of botulism," *Clinical Microbiology Reviews*, vol. 19, no. 2, pp. 298-314, 2006.
- [5] ISO/TS17919, "Microbiology of food, animal feed, and environmental samples – Polymerase chain reaction (PCR) for the detection of food-borne pathogens – Detection of botulinum type A, B, E, and F neurotoxin-producing clostridia." 2012.

Quality Control

The **foodproof**[®] *Clostridium botulinum* Detection LyoKit is function tested using the LightCycler[®] 480 System (R 602 40-1) and the Mx3005P[®] (R 602 40-2).

5. Supplementary Information

5.1 Ordering Information

BIOTECON Diagnostics is offering a broad range of reagents and services. For a complete overview and for more information, please visit our website at www.bc-diagnostics.com.

5.2 License

License Notice

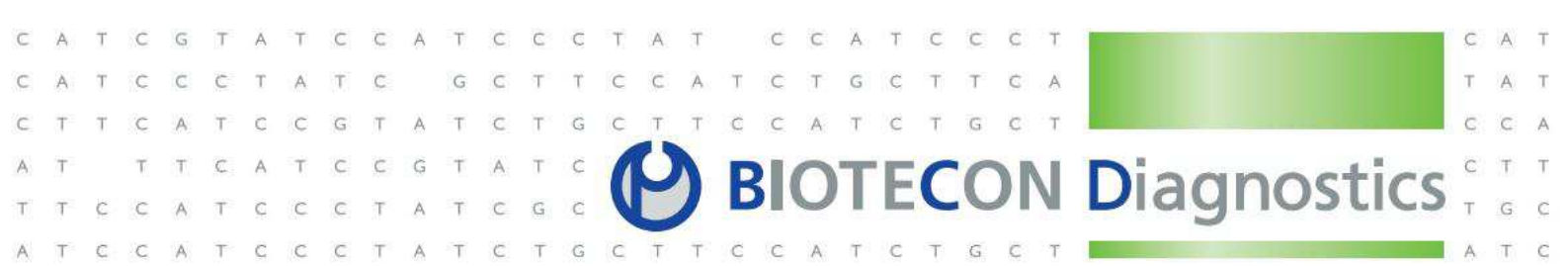
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5.3 Trademarks

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5.4 Contact and Support

If you have questions about this or any other product of BIOTECON Diagnostics, please contact our Technical Support staff (for details see www.bc-diagnostics.com). Our scientists commit themselves to providing rapid and effective help. We also want you to contact us if you have suggestions for enhancing our product performance or using our products in new or specialized ways. Such customer information has repeatedly proven invaluable to us and the worldwide research community.



6. Change Index

Version 1, April 2015

First version of the manual.

Version 2, June 2016

Addition of Resuspension Reagent and sterile double-distilled water in Chapter 1 "Additional Equipment and Reagents Required"

Version 3, March 2017

License Notice changed.

Introduction of vortex centrifuges into the PCR Setup Procedure.

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