



foodproof® *Escherichia coli* and *Shigella* Detection Kit

Revision A, January 2024

PCR kit for the qualitative detection of *Escherichia coli* and *Shigella* spp. DNA using the LightCycler® Carousel-Based or 480 II System.

Product No. KIT230034

Kit for 96 reactions

Store the kit at -15 to -25 °C

For food testing purposes.

FOR *IN VITRO* USE ONLY



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1. What this Product Does

1.1 Number of Tests

The detection kit is designed for 96 reactions with a final reaction volume of 20 µL each. Up to 30 samples (single sample preparation) plus positive and negative control reactions can be analyzed per LightCycler® Carousel-Based System run (i.e., the complete detection kit allows analysis of a maximum of 90 samples). Using the LightCycler 480 System, up to 94 samples can be analyzed.

1.2 Storage and Stability

- Store the kit at -15 to -25 °C through the expiration date printed on the label.
- Once the kit is opened, store the components as described in the following Contents table:

1.3 Kit Contents

Vial / Cap Color	Label	Contents / Function / Storage
1 - yellow cap	foodproof® <i>E. coli</i> and <i>Shigella</i> - Master Mix	• 3 x 420 µL • Ready-to-use primer and Hybridization Probe mix - specific for <i>E. coli</i> and <i>Shigella</i> • DNA and <i>E. coli</i> and <i>Shigella</i>-specific Internal Control (IC). • For amplification and detection of <i>E. coli</i>- and <i>Shigella</i>-specific sequences • Store at -15 to -25 °C. • Avoid repeated freezing and thawing! • Protect from light!
2 - red cap	foodproof <i>E. coli</i> and <i>Shigella</i> - Enzyme Solution	• 3 x 32 µL • Contains DNA-free Taq DNA Polymerase and Uracil-DNA Glycosylase (heat labile) for prevention of carry-over contamination • Store at -15 to -25 °C
3 - white cap	foodproof <i>E. coli</i> and <i>Shigella</i> - Internal Control	• 3 x 32 µL • Contains a stabilized solution of plasmid DNA. • For use as an internal amplification control. • Store at -15 to -25°C. • After first thawing, store at 2 to 8 °C for up to one month.
4 - purple cap	foodproof <i>E. coli</i> and <i>Shigella</i> - Control Template	• 1 x 50 µL • Contains a stabilized solution of plasmid DNA. • For use as a PCR run positive control. • Store at -15 to -25°C. • After first thawing, store at 2 to 8 °C for up to one month.
5 – clear, colorless cap	H ₂ O, PCR-grade	• 1 x 1 mL • Nuclease-free, PCR-grade H₂O. • For use as a PCR run negative control. • Store at -15 to -25°C.



1.4 Additional Equipment and Reagents Required

- LightCycler Carousel-Based System (LightCycler 1.x, 2.0 Instrument)
- LightCycler 20 µL Capillaries
- Standard benchtop microcentrifuge containing a rotor for 2.0 mL reaction tubes

Note: The LightCycler Carousel-Based System provides adapters that allow LightCycler Capillaries to be centrifuged in a standard microcentrifuge rotor.

or

- LC Carousel Centrifuge 2.0 for use with the LightCycler 2.0 Sample Carousel (optional).

If you use a LightCycler Instrument version below 2.0, you also need the LC Carousel Centrifuge 2.0 Bucket 2.1.

To adapt the LightCycler 2.0 Sample Carousel to the former LC Carousel Centrifuge, you need the LC Carousel Centrifuge 2.0 Rotor Set.

or

- LightCycler 480 II System
- LightCycler 480 II compatible PCR plate and sealing foil
- LightCycler Color Compensation Set
- foodproof StarPrep One Kit (Product No. KIT230175)

or

- foodproof ShortPrep II Kit (Product No. KIT230094)
- Nuclease-free, aerosol-barrier pipette tips
- Pipettes
- Sterile reaction tubes for preparing PCR mixes and dilutions

1.5 Applicability Statement

The foodproof *Escherichia coli* and *Shigella* Detection Kit is intended for the rapid detection of *E. coli* and *Shigella* DNA isolated from enrichment cultures prepared by various valid methods and inoculated with a variety of foods that are potentially contaminated with *E. coli* or *Shigella* spp.

The kit must not be used in diagnostic procedures.

The user should take care with enrichment cultures as they may contain high numbers of pathogenic organisms. It is strictly recommended to follow all necessary precautions for handling pathogenic organisms during the whole analysis.

The kit described in this Instruction Manual has been developed for the LightCycler Carousel-Based System and the LightCycler 480 System II (96-well block type). The LightCycler 480 I System should not be used for this kit.



2. How to Use this Product

2.1 Before You Begin

2.1.1 Precautions

Detection of *E. coli* and *Shigella* DNA using the foodproof *Escherichia coli* and *Shigella* Detection Kit requires DNA amplification by PCR. The kit provides all the 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:

- Prepare appropriate aliquots of the kit solutions and keep them 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.
- Do not touch the surface of the capillaries. Always wear gloves when handling the capillaries.
- Physically separate the workplaces for DNA preparation, PCR set up, and PCR to minimize risk of carry-over contamination.
- In order to avoid cross-contamination, close all capillaries that contain sample DNA and negative controls before pipetting positive controls.

Keep the foodproof *Escherichia coli* and *Shigella* Master Mix (vial 1, yellow cap) away from light.

2.1.2 Waste Disposal

Place any waste and biohazard material potentially contaminated with pathogenic bacteria in an appropriate plastic contaminated waste bag and label as follows: "CONTAMINATED waste, room number, date and initials." The bag should be autoclaved and then disposed of according to local regulations.

2.1.3 Sample Material

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

2.1.4 DNA Extraction

Hygiena Diagnostics provides sample preparation kits for all kinds of foods and raw materials (see "Additional Equipment and Reagents Required"). For more product information, please see www.hygiena.com.

2.1.5 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 *E. coli* and *Shigella* Control Template (vial 4, purple cap)] or with a positive sample preparation control DNA. Always close capillaries with template DNA and negative controls before adding positive control DNA.



2.1.6 Negative Control

Always run a negative control with the samples. To prepare a negative control, replace the template DNA with H₂O, PCR-grade water (vial 5, 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.

2.1.7 Color Compensation

The use of a previously generated color compensation object is a prerequisite for the unambiguous discrimination of *E. coli*/*Shigella* DNA and internal control (IC) DNA amplification in this dual-color experiment. A suitable color compensation object can be generated using dedicated reagents available as LightCycler Color Compensation Sets. As color compensation is instrument-specific, it is necessary to generate a CC object for every LightCycler Instrument. A new object has to be created after the optical system has been repaired. For additional information on color compensation, please refer to the manual for the respective LightCycler Instrument.

2.2 PCR Procedure

The following procedures are optimized for the LightCycler 480 II System and the LightCycler 2.0 Carousel-Based System. Program the LightCycler Systems before preparing the reaction mixes. The protocols contain the following programs:

- Pre-incubation to prevent carry-over contamination (UNG), to activate Taq polymerase and for DNA-denaturation
- Amplification of the target DNA
- Cooling of the LightCycler System

**2.2.1 LightCycler 480 II System Protocol**

The following procedure is optimized for use with the LightCycler 480 II System. Program the LightCycler before preparing the reaction mixes. Use the following LightCycler 480 System PCR program for the foodproof *Escherichia coli* and *Shigella* Detection Kit (for details on how to program the experimental protocol, see the LightCycler 480 II System Operator's Manual):

Setup		
Detection Format	Block Type	Reaction Volume
Multi Color HybProbe	96	20 µL
Filter Setting	Dynamic mode, LC480 II: Fluos (465-510, Red 640 (498-640) and Cy 5/ Cy 5.5 (498-660)	
Programs		
Program Name	Cycles	Analysis Mode
Pre-Incubation	1	None
Amplification	45	Quantification
Cooling	1	None

Temperature Targets							
	Target [°C]	Acquisition Mode	Hold [hh:mm:ss]	Ramp Rate [°C/s]	Secondary Target Temperature [°C]	Step Size [°C]	Step Delay [cycles]
Pre-Incubation							
Segment 1	37	None	00:02:00	4.4	0	0.0	0
Segment 2	95	None	00:10:00	4.4	0	0.0	0
Amplification							
Segment 1	95	None	00:00:05	4.4	0	0.0	0
Segment 2	59	Single	00:00:35	2.2	0	0.0	0
Segment 3	72	None	00:00:15	4.4	0	0.0	0
Cooling							
	40	None	00:00:30	2.2	0	0.0	0

**2.2.2 LightCycler Carousel-Based System Protocol**

The following procedure is optimized for use with the LightCycler Carousel-Based System. Program the LightCycler Carousel-Based System before preparing the reaction mixes. A LightCycler Carousel-Based System protocol that uses the foodproof *Escherichia coli* and *Shigella* Detection Kit contains the following programs (for details on how to program the experimental protocol, see the LightCycler Instrument Operator's Manual):

Pre-incubation		
Programs/Cycle Program Data	Value	
Cycles	1	
Analysis Mode	None	
Temperature Targets	Segment 1	Segment 2
Target/Target Temperature [°C]	40	95
Hold/Incubation Time [h:min:s]	00:02:00	00:10:00
Ramp Rate/Temperature Transition Rate [°C/s]	20	20
Sec Target/Secondary Target Temperature [°C]	0	0
Step Size [°C]	0.0	0.0
Step Delay [cycles]	0	0
Acquisition Mode	None	None

Amplification			
Programs/Cycle program data	Value		
Cycles	45		
Analysis Mode	Quantification		
Temperature Targets	Segment 1	Segment 2	Segment 3
Target/Target Temperature [°C]	95	59	72
Hold/Incubation Time [h:min:s]	00:00:00	00:00:30	00:00:05
Ramp Rate/Temperature Transition Rate [°C/s]	20	20	20
Sec Target/Secondary Target Temperature [°C]	0	0	0
Step Size [°C]	0.0	0.0	0.0
Step Delay [cycles]	0	0	0
Acquisition Mode	None	Single	None



Cooling	
Programs/Cycle program data	Value
Cycles	1
Analysis Mode	None
Temperature Targets	Segment 1
Target/Target Temperature [°C]	40
Hold/Incubation Time [h:min:s]	00:00:30
Ramp Rate/Temperature Transition Rate [°C/s]	20
Sec Target/Secondary Target Temperature [°C]	0
Step Size [°C]	0.0
Step Delay [cycles]	0
Acquisition Mode	None

2.2.3 Fluorescence and Run Setup Parameters

Parameter	Setting	
All LightCycler Software Versions		
Seek Temperature	30°C	
LightCycler Software (Prior to Version 3.5)		
Display Mode	Fluorescence channel F2 or F3	
Fluorescence Gains	Fluorimeter	Gain Value
	Channel 1 (F1)	1
	Channel 2 (F2)	15
	Channel 3 (F3)	30
LightCycler Software Version 3.5		
Display Mode - during run - for analysis	- Fluorescence channel F2 or F3 - F2/Back-F1 or F3/Back-F1	
Fluorescence Gains	Not required In data created with LightCycler Software Version 3.5, all fluorescence values are normalized to a fluorescence gain of “1”. This produces a different scale on the Y-axis than that obtained with previous LightCycler Software Versions. This difference does not affect the crossing points nor any calculated concentrations obtained.	
LightCycler Software Version 4.x		



Default channel • during run • for analysis	• Fluorescence channel 640 or 705 • 640/Back 530 or 705/Back 530
Fluorescence Gains	• Not required
“Max. Seek Pos”	Enter the number of samples, including controls
“Instrument Type”	“6 Ch.”: for LightCycler 2.0 Instrument (selected by default) “3 Ch.”: for LightCycler 1.5 Instrument and instrument versions below
“Capillary Size”	Select “20 µL” as the capillary size for the experiment. (For the “6 Ch.” instrument type only).



2.2.4 Preparation of the PCR Mix

Proceed as described below to prepare a 20 µL standard reaction.

Do not touch the surface of the capillaries. Always wear gloves when handling the capillaries. For LightCycler 480 users, do not touch the upper surface of the PCR multiwell plate.

1. Depending on the total number of reactions, place the required number of LightCycler Capillaries in centrifuge adapters or in a LightCycler Sample Carousel in a LC Carousel Centrifuge Bucket. For LightCycler 480 instruments, use a suitable multiwell plate.
2. Thaw the solutions and, for maximal recovery of contents, briefly spin vials in a microcentrifuge before opening. Mix carefully by pipetting up and down.
3. In a 1.5 mL reaction tube, prepare the PCR Mix for one 20 µL reaction by adding the following components in the order mentioned below, then mix gently by pipetting up and down.

The volumes indicated below are based on a single 20 µL standard reaction. Prepare the PCR mix by multiplying the amount in the "Volume" column by the number of reactions plus one positive and one negative control to be cycled plus one or two additional reactions to cover pipetting losses.

Component	Volume
foodproof <i>E. coli</i> and <i>Shigella</i> Master Mix, (vial 1, yellow cap)	13 µL
foodproof <i>E. coli</i> and <i>Shigella</i> Enzyme Solution (vial 2, red cap)	1 µL
foodproof <i>E. coli</i> and <i>Shigella</i> Internal Control (vial 3, white cap)	1 µL
Total volume	15 µL

4. Mix carefully by pipetting up and down. Do not vortex.
 - Pipet 15 µL PCR mix into each LightCycler capillary or plate well respectively.
 - For the samples of interest, add 5 µL sample DNA to a capillary or a well (LC 480), seal the capillary with a stopper.
 - For the negative control, add 5 µL H₂O, PCR-grade (vial 5, colorless cap); seal the capillary with a stopper.
 - For the positive control, add 5 µL foodproof *E. coli* and *Shigella* Control Template (vial 4, purple cap); seal the capillary with a stopper.
5. **For LightCycler Carousel Based System:** Place the adapters (containing the capillaries) in a standard benchtop microcentrifuge. (Place the centrifuge adapters in a balanced arrangement within the centrifuge.)
 - Centrifuge at 700 x g for 5 s (3,000 rpm in a standard benchtop microcentrifuge).
 - Alternatively, use the LC Carousel Centrifuge for spinning the capillaries.
 - Transfer the capillaries to the LightCycler.
6. **For LightCycler 480 II System:** Seal the plate accurately with an optical sealing foil. Place the plate in a swing bucket centrifuge and centrifuge at 1,500 x g for 30 s.
7. Cycle the samples as described above.



2.3 Analysis

Analyze real-time PCR results in channels F2/Back-F1 and F3/Back-F1 (LightCycler Software 3.5 and software versions below), in channels 640/Back 530 and 705/Back 530 (LightCycler Software 4.x) or channels Red 640 (498-640) and Cy 5/ Cy 5.5 (498-660) (LightCycler 480 Software 1.5) respectively, using the Quantification module (LightCycler Software 3.5 and software versions below), the Qualitative Detection module (LightCycler Software 4.x) or the Abs Quant/2nd Derivative Max analysis type (LightCycler 480 Software 1.5) of the LightCycler Analysis Software. Check for a positive result of the Internal Control (visible signal in channel F3, 705 or Cy5 / Cy 5.5 (498-660)) for each sample that is negative for *E. coli* and *Shigella* DNA (no signal in channel F2, 640 or Red 640 (498-640)). Compare the results from channel F2, 640 or Red 640 (498-640) (*E. coli* and *Shigella*) and channel F3, 705 or Cy 5 / Cy 5.5 (498-660) (Internal Control) for each sample, and interpret the results as described in the table below:

<i>E. coli</i> and <i>Shigella</i> Channel F2/Back-F1, Channel 640/Back 530 or Red 640 (498-640)	Internal Control Channel F3/Back-F1, Channel 705/Back 530 or Cy 5 / Cy 5.5 (498-660)	Result Interpretation
Positive	Positive	Positive
Negative	Positive	Negative
Positive	Negative	Positive
Negative	Negative	Invalid

Notes:

For LightCycler 480 II System: Use the “High Sensitivity” setting of the LightCycler Software to calculate results. For LightCycler 2.0 and LightCycler 480 II System: Always verify the software results (“positive”, “negative”, “uncertain”) for plausibility by inspection of the amplification curves.



3. Troubleshooting

Observation	Possible Cause	Recommendation
No signal increase is observed, even with positive controls.	Incorrect detection channel has been chosen.	- Set Channel Settings to F2/Back-F1 [640/Back 530, Red 640 (498-640)] or F3/Back-F1 [705/Back 530, Cy 5 / Cy 5.5 (498-660)]. - For the carousel-based systems, fluorescence data is acquired for all channels during the run, regardless of the channel settings. If the incorrect channel is selected, there is NO need to abort and redo the run.
	Pipetting errors or omitted reagents.	- Check for correct pipetting scheme and reaction setup. Repeat the PCR run. - Always run a positive control along with your samples.
	No data acquisition programmed.	- Check the cycle programs. - Select acquisition mode "single" at the end of each annealing segment of the PCR program.
No signal increase in Channel F3/Back-F1 [705/Back 530, Cy 5 / Cy 5.5 (498-660)] is observed.	Inhibitory effects of the sample material (e.g., caused by insufficient purification).	- Use the recommended DNA sample preparation kit to purify template DNA. - Dilute samples or pipet a lower amount of sample DNA (e.g., 2.5 µL instead of 5 µL). - Perform a sub-cultivation of the enrichment culture (e.g., 1:10 in BPW) to dilute the portion of food matrix in the sample.
Fluorescence intensity is too low.	Inappropriate storage of kit components.	- Store the foodproof <i>E. coli</i> and <i>Shigella</i> Master Mix (vial 1, yellow cap) at -15 °C to -25°C, protected from light. - Avoid repeated freezing and thawing.
	foodproof <i>E. coli</i> and <i>Shigella</i> Master Mix (vial 1, yellow cap) is not homogeneously mixed.	Mix the foodproof <i>E. coli</i> and <i>Shigella</i> Master Mix (vial 1, yellow cap) thoroughly before pipetting.
	Low initial amount of target DNA.	Increase the amount of sample DNA. Depending on the chosen DNA isolation method, inhibitory effects may occur.
Negative control samples are positive.	Carry-over contamination.	- 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. - Add positive controls after sample capillaries and negative control capillaries have been sealed with stoppers.



Fluorescence intensity varies.	Insufficient centrifugation of the capillaries or PCR plate.	Always centrifuge capillaries or PCR plate (loaded with the PCR mix) as described.
	Outer surface of the capillary tip or the sealing foil is dirty (e.g., by direct skin contact).	Always wear gloves when handling the capillaries or the sealing foil.

4. Additional Information on this Product

4.1 How this Product Works

The foodproof *Escherichia coli* and *Shigella* Detection Kit provides primers and Hybridization Probes (for sequence-specific detection), convenient premixed 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 supplied with the kit (vial 3, white cap). The IC has to be added to each reaction. Hybridization Probes were designed to bind specifically the IC, allowing detection in channel F3 (LightCycler Software 3.5 and versions below), 705 (LightCycler Software 4.x) or Cy 5 / Cy 5.5 (498-660) (LightCycler 480 Software 1.5), whereas the *E. coli* and *Shigella* DNA is detected in channel F2 (LightCycler Software 3.5 and versions below), 640 (LightCycler Software 4.x) or Red 640 (498-640) (LightCycler 480 Software 1.5). In the 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. Therefore, a negative result for the sample DNA of interest and amplification of the IC clearly indicates the absence of *E. coli* and *Shigella* DNA in the sample. The foodproof *Escherichia coli* and *Shigella* Detection Kit minimizes contamination risk and contains all reagents (except for template DNA) needed for the detection of *E. coli* and *Shigella* DNA. The kit is specifically adapted for PCR using the LightCycler System. Primers and Hybridization Probes provide specific detection of *E. coli* and *Shigella* DNA in food preparations. The kit described in this Instruction Manual has been developed for the LightCycler System.

4.2 Test Principle

1. Using the kit's supplied sequence-specific primers in a polymerase chain reaction (PCR), the LightCycler Carousel-Based System and its associated reagents amplify and simultaneously detect fragments of *E. coli* and *Shigella* genomic DNA.
2. The LightCycler Carousel-Based System detects these amplified fragments in real time through fluorescence generated by their corresponding pair of sequence-specific Hybridization Probes. For each amplicon, one probe is labeled at the 5'-end with an acceptor fluorophore and, to avoid extension, is modified at the 3'-end by phosphorylation. The other oligonucleotide probe is labeled at the 3'-end with a donor fluorophore.
3. During the annealing phase of each PCR cycle, these probes hybridize to an internal amplicon sequence. Only while hybridized in close proximity to each other do these probes result in fluorescence resonance energy transfer (FRET) between the two fluorophores. During FRET, the light source of the LightCycler Carousel-Based System excites the donor fluorophore and part of the excitation energy is transferred to the acceptor fluorophore.
4. The LightCycler Instrument measures the emitted fluorescence of the acceptor fluorophore.



4.3 Prevention of Carry-Over Contamination

The heat-labile Uracil-DNA 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 *E. coli* and *Shigella* 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 *Escherichia coli* and *Shigella* Detection Kit, decontamination can be achieved with the provided reagents.

4.4 Quality Control

The foodproof *Escherichia coli* and *Shigella* Detection Kit is function tested using the LightCycler Carousel-Based System.

5. Supplementary Information

5.1 Ordering Information

Hygiena Diagnostics offers a broad range of reagents and services. For a complete product overview and for more information, please visit our website at www.hygiena.com.

5.2 License Notice

The purchase price of this product includes limited, nontransferable rights under U.S. Patent No. 7,687,247 owned by Life Technologies Corporation to use only this amount of the product to practice the claims in said patent solely for activities of the purchaser for bioburden testing, environmental testing, food testing, or testing for genetically modified organisms (GMO) in accordance with the instructions for use accompanying this product. No other rights are conveyed, including no right to use this product for *in vitro* diagnostic, therapeutic, or prophylactic purposes. Further information on purchasing licenses under the above patent may be obtained by contacting the Licensing Department, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, CA 92008. Email: outlicensing@lifetech.com.

5.3 Trademarks

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5.4 Contact and Support

If you have questions or experience problems with this or any other product of Hygiena Diagnostics GmbH, please contact our Technical Support staff (www.hygiena.com/support). 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.



5.5 Reference Number

The reference number and original Hygiena Diagnostics GmbH article number: R 300 09

6. Change Index

Version 1, August 2009

First version of the package insert.

Version 2; March 2017

License Notice changed.

Version 3; September 2017

License Notice changed.

Revision A, January 2024

Rebranding and new layout.

R 300 09 20 -> INS-KIT230034-RevA



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