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Print Protocol | Print Changes to Previous Version | Print Safety Precautions

Residual DNA *E. coli* Kit

Version 02

Content version: September 2017

Quantitative detection of residual DNA from *E. coli* in products derived from fermentation processes

Cat. No. 07 728 735 001 1 kit

96 reactions of 20 µl final volume each

Store the kit at –15 to –25°C.

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Table of Contents

1. What this Product Does

Number of Tests

The kit contains sufficient reagents to run 96 PCR reactions each with a final reaction volume of 20 µl.

Kit Contents

Vial / Bottle	Cap	Label	Function / Description	Content
1	white	Workflow Negative Control		1 vial, 700 µl
2	white	Dilution Buffer		5 vials, 1 ml each
3a	red	FastStart Enzyme	▪ Ready-to-use hot start PCR reaction mix. ▪ Contains FastStart Taq DNA Polymerase, reaction buffer, uracil-N-glycosylase, and dNTP mix (with dUTP instead of dTTP).	1 vial, 30 µl
3b	red	FastStart Reaction Mix, 5× conc.		1 vial, 600 µl
4	purple	DNA Stock Solution (50 µg/ml)		1 vial, 40 µl
5	blue	$MgCl_2$, M-grade, 25 mM		1 vial, 200 µl
6	green	Detection Mix, 10× conc.	Primers and FAM-labeled detection probe with BHQ2 Quencher.	1 vial, 200 µl
7	white	PCR Negative Control		1 vial, 100 µl
8	white	PCR Water, M-grade		1 vial, 1 ml

Storage and Stability

- This product is shipped on dry ice.
- This kit is stable until the expiration date printed on the label when stored at –15 to –25°C.
- This kit is stable at +2 to +8°C for 1 week.

Once the kit is opened, store the kit components as described in the following table:

Vial / Bottle	Cap	Label	Storage
1	white	Workflow Negative Control	▪ Store at –15 to –25°C. ⚠ Avoid repeated freezing and thawing.
2	white	Dilution Buffer	
3a	red	FastStart Enzyme	
3b	red	FastStart Reaction Mix	
4	purple	DNA Stock Solution (50 µg/ml)	
7	white	PCR Negative Control	
5	blue	$MgCl_2$ M-grade, 25 mM	▪ Store at –15 to –25°C.
8	white	PCR Water, M-grade	
6	green	Detection Mix, 10× conc.	▪ Store at –15 to –25°C. ⚠ After opening the kit, store vial protected from light.

Assay Time

Hands-on time PCR setup: approximately 1 hour.

Total time-to-result (without sample preparation): approximately **1.5 hours**

Additional Equipment and Reagents Required

- Standard laboratory equipment
- Nuclease-free, DNA-free aerosol-resistant pipette tips
- Nuclease-free, DNA-free vials to prepare working solution, dilutions, and master mixes
- To minimize risk of nuclease contamination, autoclave all vessels and use alcohol wipes

For nucleic acid isolation (manual sample preparation)

- QC Sample Preparation Kit*

For the PCR workflow

- Laminar flow hood
- Real-time PCR instrument for detection in FAM channel including accessories and consumables. We recommend the LightCycler® 480 Instrument II*.
- Multiwell plates: LightCycler® 480 Multiwell Plate 96 white*.
- Standard swinging-bucket centrifuge containing a rotor for multiwell plates with suitable adaptors.

Application

The Residual DNA *E. coli* Kit is designed for the quantification of residual DNA in products derived from fermentation processes of *Escherichia coli* cells (*E. coli*).

Purified DNA control from *E. coli* cells (DNA Stock Solution, Vial 4) is included in the kit to generate a standard curve for quantification of the DNA in test samples.

Specificity and Sensitivity

Escherichia coli DNA is quantified using a real-time polymerase chain reaction (PCR) assay. The PCR-based assay is sensitive and specific for DNA from *E. coli* cells and not subject to detection of human or environmental DNA that might be introduced during sample handling.

The kit was developed to meet the sensitivity requirements defined by WHO (10 ng *E. coli* DNA per therapeutic dose).

Linearity is demonstrated by analysis of standard DNA from *E. coli* ranging from 5 pg/ml to 10 µg/ml.

Limit of Detection (LOD): 1 pg/ml

Limit of Quantification (LOQ): 5 pg/ml

2. How to Use this Product

2.1 Before You Begin

Precautions

To avoid contamination, perform the workflow setup under DNA-free conditions. This includes:

- Prepare and pipette all solutions with nuclease-free, DNA-free equipment and consumables.
- UV-treat the laminar flow hood prior to pipetting.
- Use sterile single-use gloves and freshly laundered laboratory coats.
- Close vials immediately after pipetting.
- Spatial segregation of the sequential workflow steps.

Rooms	Workflow Step
Sample preparation room	Extraction and purification of test samples, including preparation of recovery control sample.
Master mix preparation room	Master mix preparation and pipetting of PCR Negative Control to the NTC wells.
PCR room for setup and amplification run	Dilution and pipetting of samples and PCR Positive Control to the PCR plate. Running the LightCycler® 480 Instrument II.

2.2 Manual Sample Preparation

In combination with this kit, a manual sample preparation with the QC Sample Preparation Kit* is recommended.

⚠ Note that due to different types of matrices (i.e. high protein amounts or very high DNA amount), the test samples should be appropriately diluted before running the sample preparation. For this purpose, use the Dilution Buffer provided in this kit (Vial 2).

⚠ The correct pH of the sample solutions (pH 7.5) is very important for good DNA recovery.

Preparation of the Working Solutions

In addition to the ready-to-use solutions supplied with the QC Sample Preparation Kit*, the following working solutions must be prepared first. The vials from the QC Sample Preparation Kit are marked with #.

❶	Turn on the biosafety cabinet and perform cleaning before use. Wipe pipetting tools with 10% bleach followed by 70% ethanol or other appropriate disinfectants. Clean other items before placing them into the hood.
❷	Prepare a 1 M Tris Buffer pH 7.5.
❸	Preparation of the Poly(A) working solution: ▪ Dissolve 2 mg Poly A (1 bottle, Vial 7#) in 200 µl PCR Water M-grade (Vial 8). ⚠ This Poly(A) solution has to be discarded or can be aliquoted and stored at -80°C for a maximum for 4 weeks. ▪ Add 9 µl of this solution to 211 µl Lysis Buffer (Vial 2#) to prepare 220 µl Poly(A) working solution. A 220 µl aliquot is needed to prepare one test sample.

#Vial from the QC Sample Preparation Kit.

Procedure for Preparing DNA from 100 µl Test Sample

Figure 1 describes the protocol for preparing DNA from the 100 µl test sample.

Use Workflow A for each test sample to be analyzed.

Run once per sample preparation:

- Workflow B: to prepare the Workflow Negative Control.
- Workflow C: to prepare the Workflow Positive Control.
- Optional: Workflow D to prepare the Recovery Control.

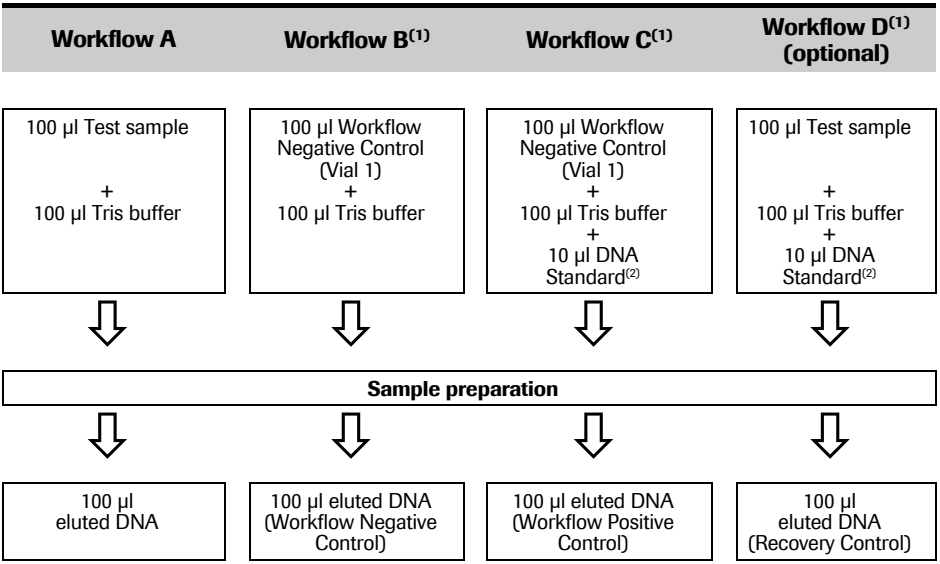


Fig. 1: Experimental overview of the Sample preparation.

(1) 1× in every experiment
(2) The spike of a DNA Standard depends on the concentration of residual DNA in the test samples: For low or very low DNA concentration, use the Standard C; for high DNA concentration, use Standards A or B (for Standards A, B, and C, see Section 2.3.1).

For the recovery control, use a DNA concentration which is 5 to 10 times higher than in the test sample.

The following protocol describes the sample preparation for different matrices derived from *E. coli* cells, in order to obtain a DNA sample as PCR template in good yield.

⚠ Instead of the Workflow Negative Control (Vial 1), you can also use the Dilution Buffer (Vial 2).

Use the Reagent Vials provided in the QC Sample Preparation Kit[#].

❶ Equilibrate thermomixer to +56°C.

❷ Label 4 Reagent Vials[#] for each workflow according to the Figure 1. In each Reagent Vial[#], add 100 µl 1 M Tris buffer for pH correction (pH 7.5):
- to100 µl sample,
- for to 100 µl Workflow Negative Control (Vial 1).

For Workflows C and D: Add 10 µl DNA Standard A, B, or C (as Recovery Control, see [(2)] above).

❸ In each Reagent Vial, add 30 µl Proteinase K (Vial 1[#]) to 200 or 210 µl of the corresponding solution (as prepared in Step 2 above).

❹ Add 220 µl of the Poly(A) working solution (see Step 3 in "Preparation of the Working Solutions") to each Reagent Vial.

❺ Close the Reagent Vials and vortex 3 times 5 seconds.

❻ Incubate for 30 minutes at +56°C and 900 rpm in the thermomixer.

❼
- Remove the Reaction Vials.
- Equilibrate the thermomixer to +80°C.

❽
- Add 290 µl Precipitation Reagent (Vial 3[#]) to each Reaction Vial.
- Close the Reagent Vials, invert 20 times, and vortex for 5 seconds.

❾
- Centrifuge for 3 minutes at 16,000 × *g*.
- Decant supernatant without removing pellet.

❿ Add 450 µl Washing Buffer (Vial 4[#]).

⓫
- Close the Reagent Vials and invert 5 times.
- Centrifuge immediately for 3 minutes at 16,000 × *g* and carefully remove all off the supernatant.

⓬ Centrifuge for 3 seconds at 16,000 × *g* and carefully remove the residual supernatant.

⓭
- Dry the DNA-pellet for approximately 10 minutes.
- Add 100 µl Dissolution Buffer (Vial 5[#]).

⓮
- Close the Reagent Vials and incubate for 30 minutes at +80°C and 900 rpm in the thermomixer.
- Remove the samples from the thermomixer.

⓯
- Cool down the samples and centrifuge for 3 seconds at 16,000 × *g*.
- Vortex the samples until the pellet is completely dissolved.

⓰ Transfer the samples to the PCR room.

[#]Vial from the QC Sample Preparation Kit

2.3 Setting Up the PCR Experiment

2.3.1 Preparation of the DNA Standard Dilutions for the Standard Curve

The following guidelines are recommended to avoid carryover contamination and to ensure proper sample preparation and quantitative PCR (qPCR) of samples:

- Use pipettes for the serial dilutions that are different from those used for sample preparation or PCR setup.
- Prepare the serial dilutions in an area physically separate from the sample preparation area.
- Use nuclease-free, DNA-free vials.

Preparation of Dilution 1: Dilute DNA Standard Stock Solution (Vial 4) 1:50 with Dilution Buffer (Vial 2) by adding 5 µl DNA Stock Solution to 245 µl Dilution Buffer (final concentration of DNA 1 µg/ml).

Further serial dilutions for Standards A to G can be prepared starting with Dilution 1, according to Table 1.

The Standards A to G can be stored at +2 to +8°C for only the day of the preparation; otherwise store at -15 to -25°C and use within one week.

Volume (µl)	Vol. Dilution Buffer (µl)	DNA Conc.	
		50 µg/ml	DNA Stock Solution (Vial 4)
5 µl Vial 4	245	1 µg/ml	Dilution 1
10 µl Dilution 1	90	100,000 pg/ml	**Standard A**
10 µl Dilution A	90	10,000 pg/ml	**Standard B**
10 µl Dilution B	90	1,000 pg/ml	**Standard C**
10 µl Dilution C	90	100 pg/ml	**Standard D**
10 µl Dilution D	90	10 pg/ml	**Standard E**
5 µl Dilution D	95	5 pg/ml	**Standard F**
10 µl Dilution E	90	1 pg/ml	**Standard G**

Table 1: DNA Standard Dilutions A to G;
Standard F = Quantification Limit; Standard G = Detection Limit

2.3.2 Plate Setup and Number of PCR Reactions

Figure 2 illustrates an example of a plate setup for only one sample, including all standards and controls. For accurate quantitative results, run triplicates for each PCR. Always run negative controls (NTC). To prepare a negative control (NTC), replace the template DNA with the PCR Negative Control (Vial 7).

	Standards A to G	Sample PCR	Spiked Sample PCR	Workflow Positive Control	Workflow Negative Control	NTC (PCR Negative Control)	Total Reactions	Master Mix Preparation [1]
Number of PCR reactions	**21**	**3**	**3**	**3**	**3**	**3**	**36**	**38**

[1] Calculated by adding two additional reactions to compensate for a slight loss of liquid during the pipetting steps (e.g., 36 reactions + 2 = 38 reactions)

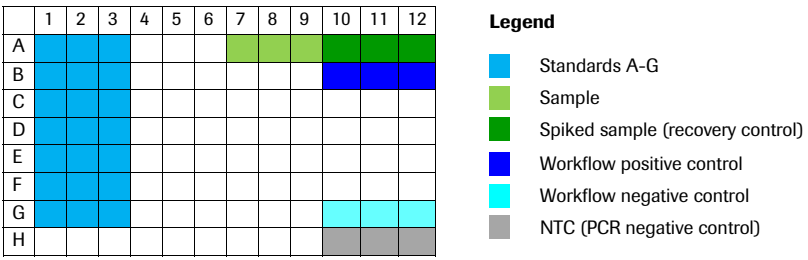


Fig. 2: Plate configuration proposal for one sample.

2.3.3 PCR with the LightCycler® 480 Instrument II

LightCycler® 480 Instrument II PCR Profile

For details on how to program the experimental protocol, see the current LightCycler® 480 Instrument II Operator's Manual.

Program the LightCycler® 480 Instrument II before preparing the reaction mixes.

Program the PCR profile as indicated below and save it as a template file for reuse.

Setup			
Detection Format		**Reaction Volume**	**Block Type**
MonoColor Hydrolysis Probe or UPL Probe		20 µl	96
Programs			
Program Name		**Cycles**	**Analysis Mode**
Initial Denaturation		1	None
Amplification		45	Quantification
Cooling		1	None
Temperature Targets			
Target [°C]	**Acquisition Mode**	**Hold [hh:mm:ss]**	**Ramp Rate [°C/s]**
Initial Denaturation			
40	None	00:10:00	4.4
95	None	00:10:00	4.4
Amplification			
95	None	00:00:10	4.4
63	Single	00:00:30	2.2
Cooling			
40	None	00:00:30	2.2

Preparation of the Master Mix

Since real-time PCR is an extremely sensitive method to detect traces of DNA, follow the appropriate guidelines for preparing PCR master mixes.

⚠ Keep Vial 6 away from light. Do not touch the surface of the LightCycler® 480 Multiwell Plate during handling.

1. Perform laminar flow hood cleaning (using bleach, then ethanol or other disinfectant reagents) in the master mix room.
2. Wipe pipetting tools with 10% bleach followed by 70% ethanol or other disinfectants. Wipe all other items with 70% ethanol before bringing into the hood.
3. Place the reagents (see Step 6) in a laminar flow hood, and let them thaw at +15 to +25°C.
4. Vortex and spin down briefly before opening.
5. Change tip after each pipetting step.
6. For the plate setup (Figure 2), prepare the master mix for 38 PCR reactions according to the table below. Use nuclease-free, DNA-free vials.

Vial	Component	1 reaction [µl]	38 reactions [µl][(1)]
3a	FastStart Enzyme	0.28	10.64
3b	FastStart Reaction Mix (5×)	4.0	152.0
6	Detection Mix (10×)	2.0	76.0
5	$MgCl_2$ (25 mM)	0.2	7.6
8	PCR Water	3.52	133.76
	Total volume	10	380

7. Distribute 10 µl of the respective master mix into the respective well of a 96-well plate.
8. Add 10 µl of the PCR Negative Control (Vial 7) to the NTC wells.
9. Transfer the 96-well plate to the PCR room.

(1) Calculated by adding two additional reactions to compensate for a slight loss of liquid during the pipetting steps (e.g., 36 reactions + 2 = 38 reactions)

Preparation of the PCR Plate and PCR Run

❶	Perform laminar flow hood cleaning (using bleach, then ethanol or other disinfectant reagents) in the PCR room.
❷	Wipe the pipetting tools with 10% bleach followed by 70% ethanol or other disinfectants.
❸	Add 10 µl of the sample material (sample, standards, positive control) to the corresponding well prefilled with 10 µl master mix.
❹	Load the prepared 96-well plate into the LightCycler® 480 Instrument II and start the run.

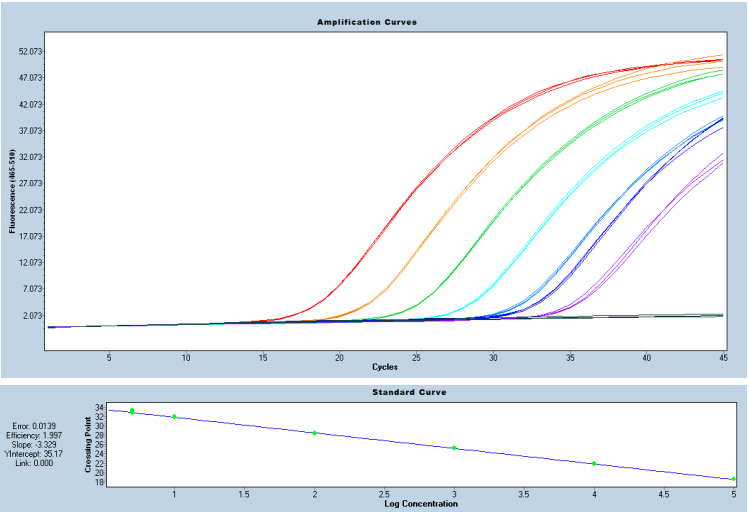
Data Analysis

For the data analysis with the LightCycler® 480 Instrument II, Abs Quant/2nd Derivative Max for All Samples is recommended. For more information, refer to the LightCycler® 480 Instrument II Operator's Manual.

3. Results

3.1 Results with the LightCycler® 480 Instrument II

The following results were obtained using the Residual DNA *E. coli* Kit on the LightCycler® 480 Instrument II.



Error: 0.0139| Efficiency: 1.997 | Slope: -3.329 | YIntercept: 35.17 | Link: 0.000

Fig. 3: Typical analysis result with Standards;
Standard A: far left curve; Standard G: far right curve; NTC: flat line.

As expected, the standard curve is linear (only Standards A to F are used for the calculation) and the PCR efficiency is 1.99.
Generally, the PCR efficiency should be at least 1.85 or higher.

The PCR Negative Control is negative, the Workflow Negative Control is also negative, respectively. The Cp value for the Standard G corresponds to the detection limit of Cp approximately >35.

DNA recovery can be determined by including samples spiked with known DNA amounts which are prepared from the corresponding DNA standards. With the help of the standard curve, the recovery of DNA (percentage) can be calculated from the PCR results according to the following equation:

$$recovery\,(\%) = \frac{DNA\_conc\_spiked\ sample\,(pg/ml) - DNA\_conc\_sample\,(pg/ml)}{DNA\_conc\_spike\_solution\ \ (pg/ml)/10} * 100$$

Typically, the range for this value varies from 80% to 100%.

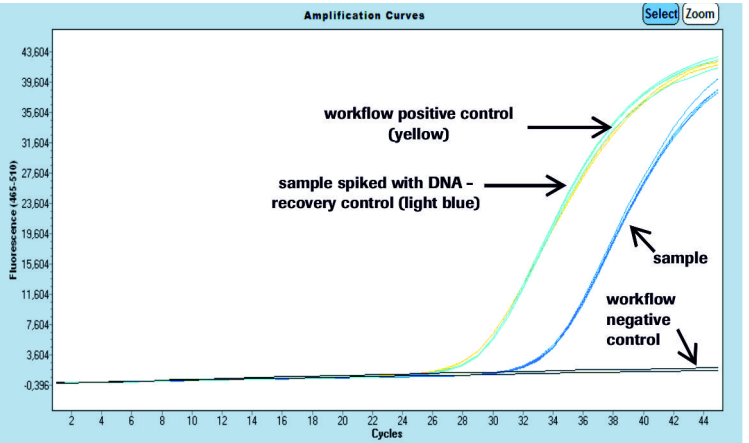


Fig. 4: Typical analysis result for one sample, one sample spiked with DNA, one workflow positive control, and one workflow negative control;
DNA recovery = 90%.

3.2 PCR with the Applied Biosystems® QuantStudio™ 6 Flex

The Residual DNA *E. coli* Kit can also be used with the QuantStudio™ 6 Flex PCR System from Applied Biosystems®.

PCR Profile

Prepare the instrument according to the Operator's Manual of the QuantStudio™ 6 Flex PCR System.
Use the protocol as defined below:

Experiment Properties	
Instrument Type	QuantStudio™ 6 Flex System
Block	96-well (0.2 ml)
Type of Experiment	Standard Curve
Reagents	Other
Properties for Instrument Run	Standard
Define	
Set up Sample Volume	20 ml
Reporter/ Quencher	FAM/NFQ-MGB
Assign	

Define and set up the standards according to the Operator's Manual.

Run Method

Program the protocol as indicated below and save it as a template file for reuse.

⚠ Runs performed with the instrument default protocol will produce invalid results.

Stage	Step	Temp (°C)	Duration	Cycles
1. Hold Stage	Step 1: UNG Incubation	40	10:00 min	1
	Step 2: Initial Denaturation	95	10:00 min	1
2. PCR Stage	Step 1: Amplification Step 2: Data Collection (to be set on)	95 63	00:10 min 00:30 min	45
3. Hold Stage	Cooling	40	00:30 min	1

Data Analysis

Perform data analysis according to the Operator's Manual of the QuantStudio™ 6 Flex PCR System. Choose automated baseline and threshold.

Plot Type: Rn vs Cycle

Graph Type: Log

Results

The criteria for the PCR experiment (linearity of the standard curve, PCR efficiency, and negative controls) all remain the same as those with the LightCycler® 480 Instrument II (see Section 3.1).

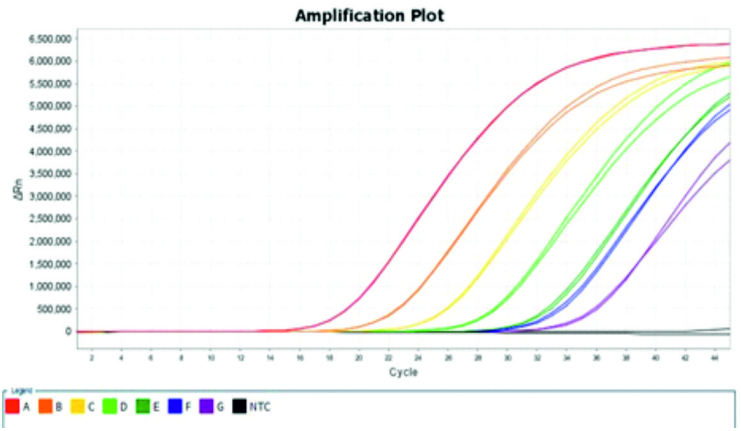


Fig. 5: Typical analysis results with Standards A to G.

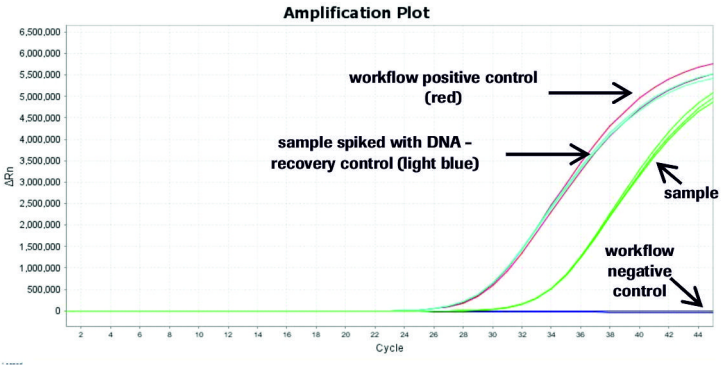


Fig. 6: Typical analysis results for one sample, one sample spiked with DNA, one workflow positive control and one workflow negative control.

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Limitations

The Residual DNA *E. coli* Kit was evaluated using the LightCycler® 480 Instrument II. Results obtained may also be valid for other real-time PCR instruments, but must be verified empirically.

4. Troubleshooting

Problem	Cause	Recommendation
Fluorescence intensity varies.	Some of the reagent is still in the upper part of the microwell or an air bubble is trapped in the microwell.	Repeat centrifugation, but allow sufficient centrifugation time so all reagent is at the bottom of the microwell and air bubbles are expelled.
	Skin oils or dirt on the surface of the microwell plate.	Always wear gloves when handling the multiwell plate.
Fluorescence intensity is very low.	Low concentration or deterioration of dyes in the reaction mixtures because dye was not stored properly.	▪ Keep dye-labeled reagents away from light. ▪ Store the reagents at -15 to -25°C and avoid repeated freezing and thawing.
	Poor PCR efficiency (reaction conditions not optimized).	Always run a positive control along with your samples.
	DNA is degraded during isolation or improper storage.	▪ If possible, check DNA quality. ▪ Store DNA samples at -15 to -25°C.
	Pipetting errors and/or omitted reagents.	▪ Check for missing reagents. ▪ Check the pipetting procedure.
	Impure sample material inhibits reaction.	Dilute sample 1:10 and repeat the analysis.
Negative control sample gives a positive signal.	Contamination	Remake all critical reaction mixes. Be sure to use special pre-PCR setup working areas.

5. Additional Information on this Product

5.1 How this Product Works

The Residual DNA *E. coli* Kit is designed for fast, highly sensitive, and specific real-time PCR analysis of host-cell residual DNA from the *Escherichia coli* cells in biomanufacturing processes. Host cell impurities such as residual DNA can be easily quantified with this kit during the purification process.

The broad range allows testing of samples such as in-process controls with higher amounts of DNA and bulk samples with very low amounts of DNA. The assay is accurate and reliable across a broad range of sample types, from in-process samples to final product.

5.2 Principle

The kit uses specific PCR of highly conserved regions within DNA from the *E. coli* cells. Highly specific primers and one hydrolysis probe are included in the detection mix. The probe is labeled with a fluorescent dye (FAM) detected by real-time PCR instruments.

In the kit, a ready-to-use hot start reaction mix for detecting DNA targets with hydrolysis probes is included. The chemically modified polymerase enzyme is inactive during initial PCR setup, thereby avoiding nonspecific elongation of primer-template hybrids forming at lower temperature. The polymerase is irreversibly activated by an initial activation step at higher temperature.

To exclude false-positive results, a Workflow Negative Control and a PCR Negative Control are included.

The kit is designed to prevent PCR carryover contamination, using the uracil-DNA glycosylase (UNG) already included in the FastStart Reaction Mix (Vial 3b). The incorporation of deoxyuridine triphosphate (dUTP) occurs during PCR, creating dUTP-containing amplicons. These can be digested by pretreatment of successive PCR mixtures with UNG. UNG removes uracil from DNA molecules by cleaving the N-glycosylic bond. Resulting abasic sites are hydrolyzed due to the high temperatures during the initial PCR denaturation step. Hydrolyzed DNA can no longer serve as a PCR template. UNG is inactivated during the initial denaturation step. Native DNA does not contain uracil, and is therefore not degraded by UNG-mediated denaturation.

5.3 Quality Control

Each lot of the Residual DNA *E. coli* Kit is function tested using the LightCycler® 480 Instrument II. PCR is performed on a dilution series of DNA amplified using the PCR protocol of the Residual DNA *E. coli* Kit.

6. Supplementary Information

6.1 Conventions

Text Conventions To make information consistent and easy to understand, the following text conventions are used throughout this document:

Text Convention	Usage
Numbered instructions labeled ❶, ❷ *etc.*	Steps in a procedure that must be performed in the order listed.
Asterisk*	Denotes a product available from Roche Diagnostics.

Symbols In this document, the following symbols are used to highlight important information:

Symbol	Description
	Information Note: Additional information about the current topic or procedure.
⚠	Important Note: Information critical to the success of the procedure or use of the product.

6.2 Changes to Previous Version

New protocol for the preparation of samples (Section 2.2).

6.3 Ordering Information

Roche offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our homepage www.lifescience.roche.com.

- LightCycler® 480 System: www.lightcycler480.com

Product	Pack Size	Cat. No.
LightCycler® 480 Instrument II, 96 well	1 instrument (96 well)	05 015 278 001
LightCycler® 480 Block Kit 96 Silver	1 block kit for 96-well PCR multiwell plates	05 015 219 001
LightCycler® 480 Multiwell Plate 96, white	5 x 10 ml plates with sealing foils	04 729 692 001
LightCycler® 480 Sealing Foil	50 foils	04 729 757 001
LightCycler® 480 Software, Version 1.5	1 software package	04 994 884 001
QC Sample Preparation Kit	1 kit	08 146 829 001

6.4 Trademarks

FASTSTART and LIGHTCYCLER are trademarks of Roche.

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6.5 Regulatory Disclaimer

For use in quality control/manufacturing process only.

Published by
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For more information about this product, as well as documentation such as Instructions for Use and Safety Data Sheets, please visit custombiotech.roche.com

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