For use in quality control/manufacturing process only.

Residual DNA *E. coli* Kit

Kit for determination of residual DNA *E.coli*

Version: 04

Content Version: March 2025

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

Cat. No. 07 728 735 001 1 kit
96 PCRs each with a 20 µL final reaction volume

Store the kit at −15 to −25°C.

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1. General Information

1.1. Contents

Vial / bottle	Cap	Label	Function / description	Content
1	white	Workflow Negative Control	To exclude false-positive results.	1 vial, 700 µL
2	white	Dilution Buffer	For preparation of DNA standard dilutions.	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, 5x conc.		1 vial, 600 µL
4	purple	DNA Stock Solution	50 µg/mL solution.	1 vial, 40 µL
5	blue	$MgCl_2$, M-grade, 25 mM	For preparation of master mix.	1 vial, 200 µL
6	green	Detection Mix, 10x conc.	Primers and FAM-labeled detection probe with BHQ2 Quencher.	1 vial, 200 µL
7	white	PCR Negative Control	To exclude false-positive results.	1 vial, 100 µL
8	white	PCR Water M-grade	▪ For preparation of working solution. ▪ For preparation of master mix.	1 vial, 1 mL

1.2. Storage and Stability

Storage Conditions (Product)

The kit is shipped on dry ice.
When stored at −15 to −25°C, the kit is stable through the expiration date printed on the label.
The kit is stable at +2 to +8°C for 1 week.

Vial / bottle	Cap	Label	Storage
1	white	Workflow Negative Control	After opening, store at −15 to −25°C. ⚠ ***Avoid repeated freezing and thawing.***
2	white	Dilution Buffer	
3a	red	FastStart Enzyme	
3b	red	FastStart Reaction Mix, 5x conc.	
4	purple	DNA Stock Solution	
5	blue	$MgCl_2$, M-grade, 25 mM	Store at −15 to −25°C.
6	green	Detection Mix, 10x conc.	After opening, store at −15 to −25°C. ⚠ ***Keep protected from light.***
7	white	PCR Negative Control	After opening, store at −15 to −25°C. ⚠ ***Avoid repeated freezing and thawing.***
8	white	PCR Water M-grade	Store at −15 to −25°C.

1.3. Additional Equipment and Reagent required

Standard laboratory equipment

⚠ ***To minimize the risk of nuclease contamination, autoclave all vessels and use alcohol wipes.***

- Nuclease-free, DNA-free, aerosol-resistant pipette tips
- Nuclease-free, DNA-free vials
- Alcohol wipes
- Biosafety cabinet class II
- Vortex mixer
- Centrifuge

For the nucleic acid isolation (manual sample preparation)

- QC Sample Preparation Kit*
- 1 M Tris buffer
- Thermomixer

For the PCR workflow

- Laminar flow hood
- Bleach, ethanol, or other disinfectants
- Real-time PCR instrument for detection in FAM channel including accessories and disposables, such as the LightCycler® 480 Instrument II*
- Multiwell plates: LightCycler® Multiwell Plate 96, white*
- Standard swinging-bucket centrifuge containing a rotor for multiwell plates with suitable adaptors

1.4. 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.

1.5. Preparation Time

Assay Time

Hands-on time PCR setup: approximately 1 hour.
Total time-to-result (without sample preparation): approximately 1.5 hours.

2. How to Use this Product

2.1. Before you Begin

General Considerations

Number of tests

The kit contains sufficient reagents to run 96 PCRs each with a final reaction volume of 20 µL.

Precautions

To avoid contamination, perform the workflow setup under DNA-free conditions:
- Prepare and pipette all solutions with nuclease-free, DNA-free equipment and disposables.
- UV-treat the laminar flow hood prior to pipetting.
- Use sterile single-use gloves and freshly laundered laboratory coats.
- Close all reaction vials immediately after pipetting.
- To avoid cross-contamination, a spatial segregation of the sequential steps is essential.

Room Type	Workflow Step
Sample preparation	Extraction and purification of test samples, including preparation of recovery control sample.
Master mix preparation	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.

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.

Prevention of Carryover Contamination

The Residual DNA *E. coli* Kit is designed to prevent PCR carryover contamination using the uracil-DNA glycosylase (UNG) provided 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.

Working Solution

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 #:

1. 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.

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2. Prepare a 1 M Tris Buffer pH 7.5.

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3. Prepare 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 must be discarded or can be aliquoted and stored at −80°C for a maximum of 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.

2.2. Protocols

Manual sample preparation

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

⚠ ***Note that due to different types of matrices, that is, 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.***

Procedure for preparing DNA from 100 µL test sample

Figure 1 describes the protocol for preparing DNA from 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.

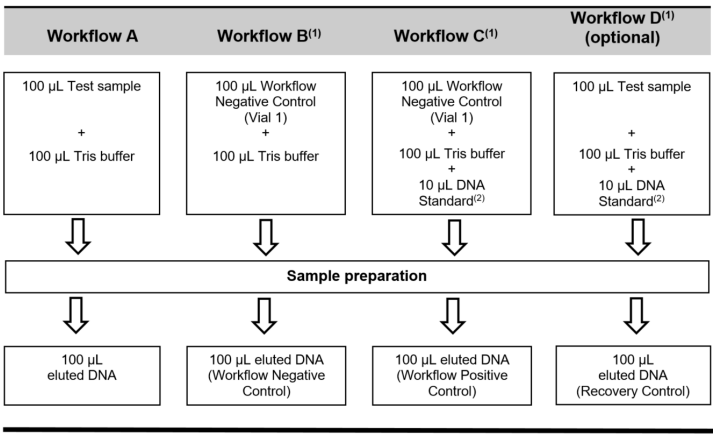

Fig. 1: Experimental overview of the sample preparation.

(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, **Preparation of the DNA standard dilutions for the standard curve**.

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 the *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 QC Sample Preparation Kit*. The Reaction Vials from the QC Sample Preparation Kit are marked with #.

See section, **Working Solution***, for information on preparing solutions.*

1. Equilibrate the thermomixer to +56°C.

2. Label 4 Reagent Vials# for each workflow according to Figure 1.
   – In each Reagent Vial#, add 100 µL 1 M Tris buffer for pH correction (pH 7.5) to:
   – 100 µL sample or to 100 µL Workflow Negative Control (Vial 1).
   – For Workflows C and D, add 10 µL DNA Standards A, B, or C (as Recovery Control[(1)]).

3. 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.

4. Add 220 µL of the Poly(A) working solution, see section, **Working Solution, Step 3**, to each Reagent Vial.

5. Close the Reaction Vials and vortex 3 times 5 seconds.

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

7. Remove the Reaction Vials.
   – Equilibrate thermomixer to +80°C.

8. Add 290 µL Precipitation Reagent (Vial 3#) to each Reaction Vial.
   – Close the Reaction Vials, invert 20 times, and vortex for 5 seconds.

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

10. Add 450 µL Washing Buffer (Vial 4#).

11. Close the Reaction Vials and invert 5 times.
    – Immediately centrifuge for 3 minutes at 16,000 × *g* and carefully remove all the supernatant.

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

13. Dry the DNA pellet for approximately 10 minutes.
    – Add 100 µL Dissolution Buffer (Vial 5#).

14. Close the Reaction Vials and incubate for 30 minutes at +80°C and 900 rpm in the thermomixer.
    – Remove the samples from the thermomixer.

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

16. Transfer the samples to the PCR room.

(1) 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, **Preparation of the DNA standard dilutions for the standard curve**.

PCR setup

Preparation of the DNA standard dilutions for the standard curve

Use the following guidelines 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 separated from the sample preparation area.
- Use nuclease-free, DNA-free vials.

Preparation of DNA standard solutions

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).

2. Prepare additional serial dilutions for Standards A to G starting with Dilution 1, according to the following table:

Volume [µL]	Volume Dilution Buffer [µL]	DNA Conc.	Dilution Name
–	–	50 µg/mL	DNA Stock Solution (Vial 4)
5 (Vial 4)	245	1 µg/mL	Dilution 1
10 (Dilution 1)	90	100,000 pg/mL	Standard A
10 (Dilution A)	90	10,000 pg/mL	Standard B
10 (Dilution B)	90	1,000 pg/mL	Standard C
10 (Dilution C)	90	100 pg/mL	Standard D
10 (Dilution D)	90	10 pg/mL	Standard E
5 (Dilution D)	95	5 pg/mL	Standard F[(1)]
10 (Dilution E)	90	1 pg/mL	Standard G[(2)]

3. The Standards A to G can be stored at +2 to +8°C for just the day of the preparation.

   Alternatively, store at −15 to −25°C and use within one week.

(1) Standard F = Quantification Limit.
(2) Standard G = Detection Limit.

Plate setup and number of PCR

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).

Reactions	Standards A to G	Sample PCR	Spiked Sample PCR	Workflow Positive Control	Workflow Negative Control	NTC (PCR Negative Control)	Total Reactions	Master Mix Preparation + 2 Additional Reactions[1]
Number of PCR	21	3	3	3	3	3	36	38

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

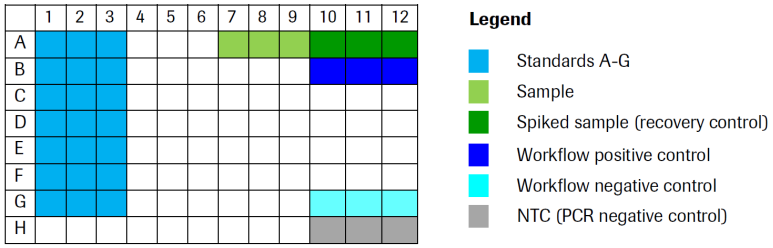

Fig. 2: Plate configuration proposal for one sample.

PCR with the LightCycler® 480 Instrument II

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					
Block type			**Reaction volume [µL]**		
96			20		
Detection format	**Excitation filter**		**Emission filter**		
Mono Color Hydrolysis Probe / UPL Probe					
FAM	465		510		
Programs					
Program name	**Cycles**		**Analysis mode**		
UNG	1		None		
Pre-Incubation	1		None		
Amplification	45		Quantification		
Cooling	1		None		
Temperature targets					
	Target [°C]	**Acquisition mode**	**Hold [hh:mm:ss]**	**Ramp rate [°C/s]**	**Acquisitions [per °C]**
UNG	40	None	00:10:00	4.4	–
Pre-Incubation	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 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 protected from light. Do not touch the surface of the LightCycler® 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) into a laminar flow hood, and thaw at +15 to +25°C.

4. Vortex and spin briefly before opening.

5. Change tip after each pipetting step.

6. For the plate setup (Figure 2), prepare the master mix for 38 PCRs according to the following table.
   – Use nuclease-free, DNA-free vials.

Vial	Reagent	1 Reaction [µL]	38 Reactions [µL] (1)
3a	FastStart Enzyme	0.28	10.64
3b	FastStart Reaction Mix, 5x conc.	4.0	152.0
6	Detection Mix, 10x conc.	2.0	76.0
5	$MgCl_2$ (25 mM)	0.2	7.6
8	PCR Water M-grade	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 PCR Negative Control (Vial 7) to the NTC wells.

9. Transfer the 96-well plate to the PCR room.

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

Preparation of the PCR plate and PCR run

1. Perform laminar flow hood cleaning using bleach, then ethanol or other disinfectant reagents in the PCR room.

2. Wipe pipetting tools with 10% bleach followed by 70% ethanol or other disinfectants.

3. Add 10 µL of the sample material (sample, standards, Positive Control) to the corresponding well prefilled with 10 µL master mix.

4. Load the prepared 96-well plate into the LightCycler® 480 Instrument II and start the run.

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

Experiment Properties	Description
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 µL
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 [hh:mm:ss]	Cycles
Hold Stage	Step 1: UNG Incubation	40	00:10:00	1
	Step 2: Initial Denaturation	95	00:10:00	1
PCR Stage	Step 1: Amplification	95	00:00:10	45
	Step 2: Data Collection (to be set on)	63	00:00:30	
Hold Stage	Cooling	40	00:00:30	1

2.3. Parameters

Sensitivity

Limit of Detection (LOD): 1 pg/mL
Limit of Quantification (LOQ): 5 pg/mL
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.

Specificity

Escherichia coli DNA is quantified using a real-time 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.

3. Results

LightCycler® 480 Instrument II

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.

Results interpretation

The following results (Figures 3 and 4) were obtained using the Residual DNA *E. coli* Kit on the LightCycler® 480 Instrument II.

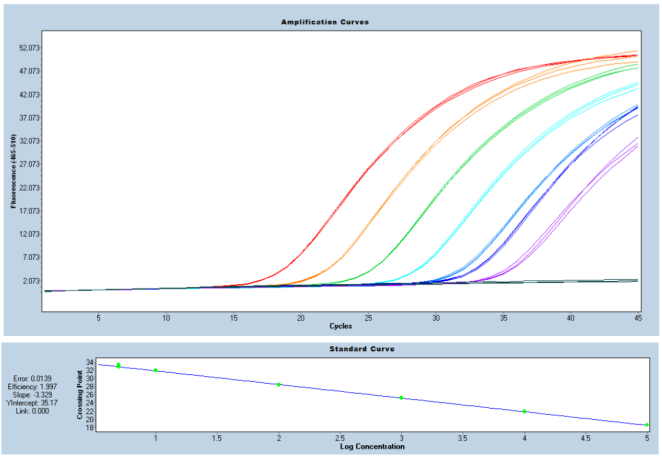

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Fig. 3: Typical analysis result with Standards.
Standard A: far left curve
Standard G: far right curve
NTC: flat line
As expected, the standard curve is linear (only Standards A to F are used for the calculation) and the PCR efficiency is 1.99. Generally, 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:

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

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

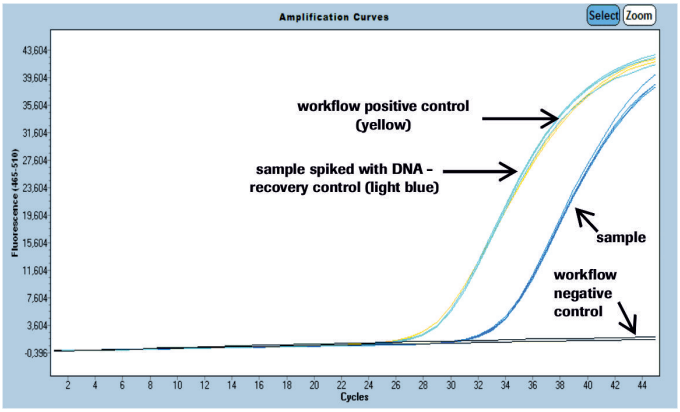

Fig. 4: Typical analysis result for one sample, one sample spiked with DNA, one Workflow Positive Control, and one Workflow Negative Control. DNA recovery = 90%.

Applied Biosystems® QuantStudio™ 6 Flex

Data analysis

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

Parameter	Setting
Plot Type	Rn vs Cycle
Graph Type	Log

Results interpretation

The criteria for the PCR experiment (linearity of the standard curve, PCR efficiency, and negative controls, see Figures 5 and 6) all remain the same as those with the LightCycler® 480 Instrument II, see section, **Results with the LightCycler® 480 Instrument II**.

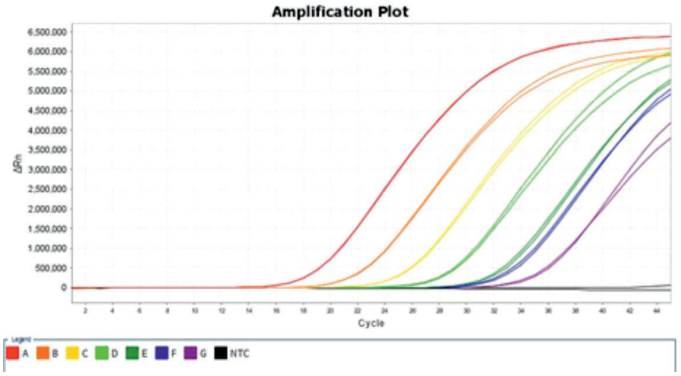

Fig. 5: Typical analysis results with Standards A to G.

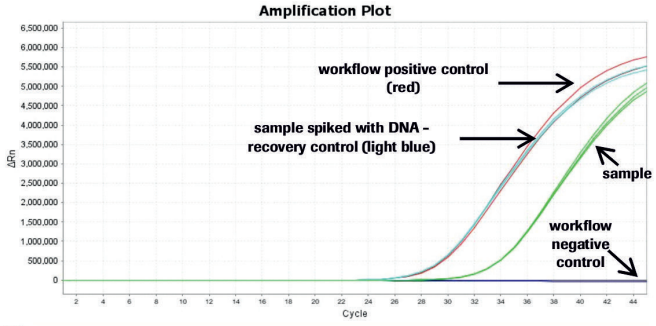

Fig. 6: Typical analysis result for one sample, one sample spiked with DNA, one Workflow Positive Control, and one Workflow Negative Control.

4. Troubleshooting

Observation	Possible 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 protected from light.***
		Store the reagents at −15 to −25°C. ⚠ ***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 present.	Remake all critical reaction mixes.
		Be sure to use special pre-PCR setup working areas.

5. Additional Information on this Product

5.1. Test Principle

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.

- 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.
- 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 also included.

5.2. 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

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

6.2. Changes to previous version

The product name LightCycler® 480 Multiwell Plate 96, white has been changed to LightCycler® Multiwell Plate 96, white.

6.3. Trademarks

LIGHTCYCLER and FASTSTART are trademarks of Roche.
All other product names and trademarks are the property of their respective owners.

6.4. License Disclaimer

Consult product detail pages at **custombiotech.roche.com** for patent license limitations, if available.

6.5. Regulatory Disclaimer

For use in quality control/manufacturing process only.

6.6. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

6.7. Contact and Support

For additional documentation such as certificates and safety data sheets, please visit **documentation.roche.com**.

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