HostDetect™ *E. coli* PCR DNA Quant Kit User Guide

v 2.0

For Research Use Only.
Not for use in diagnostic procedures.

Follow the protocol included with the kit.

Table of symbols

	Store at -25 ℃ to -15 ℃
	Consult instructions for use
	This way up
	Recyclable
	Contains sufficient for (n) reactions
	Catalogue number
	Lot number
	Manufacturer
	Use by date
	Fragile
	Date of manufacture

Table of Contents

Product Name

HostDetect™ *E. coli* PCR DNA Quant Kit (DXMDX-RGT-1005)

Intended Use

The HostDetect™ *E. coli* PCR DNA Quant Kit is a real-time PCR test intended for the quantitative detection of residual host genomic DNA of *E. coli* in biopharmaceutical products. Use the kit after extracting DNA from the testing samples. Adjust the concentration of all test samples within the analytical range of the assay.

The HostDetect™ *E. coli* PCR DNA Quantification kit is sensitive and specific for DNA from the *E. coli* genome and is not subject to detection of human or environment DNA that might be introduced during sample handling. The kit is designed to meet the sensitivity requirements defined by WHO (10 ng *E. coli* DNA per therapeutic dose)[1].

To generate the standard curve used for DNA quantification in test samples, the *E. coli* assay requires 5 dilutions from reference standard DNA. USP *E. coli* genomic DNA standard (Cat# 1231557) and the Cygnus *E. coli* genomic DNA (Cat# D412-1) are recommended for reference standard to prepare the standard curve.

Principles of the Assay

The HostDetect™ *E. coli* PCR DNA Quant kit is a real-time polymerase chain reaction test. The reagents utilize sequence-specific primers and TaqMan® probe to amplify the 16s rRNA gene of *E. coli* genomic DNA for residual host genomic identification. A primer/probe set to detect Internal Control (IC) is also included for monitoring the entire process from extraction to real-time PCR, or for real-time PCR only.

The probes for the *E. coli* genomic DNA and Internal Control detection are labeled with FAM and HEX/VIC fluorescent dyes, respectively, to generate target-specific signal. ROX is used for Passive Reference for instruments wherever applicable (see Appendix for details).

The assay also uses a dUTP/UNG carryover prevention system to avoid contamination of PCR products and subsequent false positive results.

Samples need to go through nucleic acid extraction before starting the PCR procedure. Standards do not need nucleic acid extraction.

Kit Contents and Storage

The kit contains sufficient reagents to run 192 PCR reactions with a final reaction volume of 15 µL.

Component Name	Specifications & Loading		Main Ingredients	Storage Conditions*
Reagent A	950 µL	× 1 tube	Buffers, dNTPs, Mg^{2+}	-25 to -15 °C
E. coli Reagent B	230 µL	× 1 tube	TE buffer, primers, probes	-25 to -15 °C
Reagent C	150 µL	× 1 tube	Taq DNA polymerase, UNG	-25 to -15 °C
Negative Control	1.4 mL	× 2 tubes	TE buffer	-25 to -15 °C
Internal Control	1.4 mL	× 2 tubes	Synthetic plasmid of internal control.	-25 to -15 °C

*Completely thaw reagents before use. Reagents are stable within six cycles of freeze-thaw. Reagent A may precipitate upon thawing. Mix well to ensure complete resuspension before use. Once thawed, store at 2 - 8 °C for up to a week.

Note that Reagent C (an enzyme mix) must always be stored at -25 to -15 °C.

Maximum Number of Tests per Kit

192 Tests for 15 µL PCR

Materials Required but Not Provided

Item	Source
Instrument	
Real-time PCR Instruments	Revvity™ Eonis™ Q System , Applied Biosystems™ 7500 Fast or Fast Dx Real-Time PCR System, Applied Biosystems™ QuantStudio™ 3, 5, 6, 7 Flex, or Dx Real-Time Instrument, BioRad® CFX96™ Touch Real-Time PCR Detection System
Reagents	
E. coli genomic DNA	Cygnus Technologies, Cat# D412-1 USP, Cat# 1231557
Consumables	
96-Well Plates	Compatible 96-well plates for the corresponding Real-Time PCR Instruments
Optical Adhesive Film	Compatible optical film for the corresponding Real-Time PCR Instruments

Miscellaneous items	
Micropipettes	Any major lab supplier
Non-aerosol pipette tips (Low retention tips)	Any major lab supplier
Microcentrifuge tube (Low DNA binding tubes)	Any major lab supplier
Nuclease-free water	Any major lab supplier
1X TE buffer, pH 8.0	Any major lab supplier

Warnings and Precautions

1. Keep the kit upright during storage and transportation.
2. Before using the kit, check tubes for leakage or damage. Thaw each component in the kit at room temperature, thoroughly mix, and then centrifuge before use.
3. Cross-contamination may occur due to inappropriate handling of reference materials and specimens, which will cause inaccurate results. Use sterile disposable filter-tips to aspirate reagents and specimens.
4. After the operation, disinfect the work area and the instrument surface with a freshly prepared 10% sodium hypochlorite solution, and then clean with 70% ethanol. Finally, turn on UV light to disinfect working surfaces for 30 minutes.
5. Calibrate the qPCR instruments regularly according to instrument's instructions to eliminate crosstalk between channels.
6. This kit uses PCR-based technology and experiments should be conducted in three separate areas: reagent preparation area, specimen preparation area, amplification area. Protective equipment accessories (goggles, work clothes, hats, shoes, gloves, etc.) should be worn during operation and protective equipment accessories should be changed when entering and leaving different work areas. Protective equipment accessories in each work area are not interchangeable.
7. Do not use reagents after the expiration date.
8. Do not use the kit if the outer box sealing label is broken upon arrival.
9. Do not use reagents if the tube caps are open or broken upon arrival.
10. Dispose of waste according to local, state, and federal regulations.

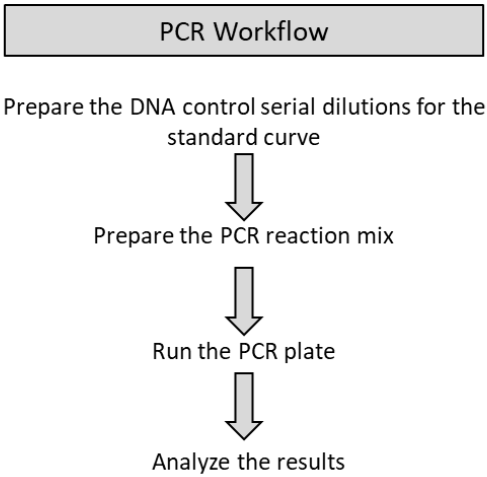
Safety Precautions

1. Wear appropriate Personal Protective Equipment (PPE), including (but not limited to) disposable powder-free gloves, hats, protective lab coats, and goggles. Change gloves often when handling reagents or samples.
2. Wash hands thoroughly after handling specimens and reagents.
3. Follow national biological safety recommendations for handling biological samples.

Laboratory Precautions for Contamination Prevention

1. Prior to processing samples, thoroughly clean the work area with freshly prepared 10% bleach or 70% ethanol. Then wipe the work area with water.
2. Avoid excessive handling of high concentration *E. coli* genomic DNA to avoid contamination.
3. Change gloves after handling the Positive Control or high concentration *E. coli* genomic DNA.
4. After amplification is complete, immediately place the PCR plates in a sealable bag; ensure the bag is sealed, then discard the plates in a biohazard container.
5. Change gloves after handling a post PCR plate.
6. All materials used in one area should remain in that area and should not be moved or used in other areas. Never bring post PCR plates to other areas, such as PCR set up area or sample preparation area.

Workflow



Standard stock preparation

1. Guidelines for standard dilutions
    a. Prepare the standard curve and the test samples in different areas of the lab.
    b. If possible use different sets of pipettors for test sample preparation and for standard curve preparation and aliquoting to avoid cross-contamination of test samples.
    c. Briefly vortex each tube to mix the contents thoroughly before each dilution step.
    d. Briefly centrifuge to collect all the liquid at the bottom before making the next dilution.
2. Prepare and aliquot the stock of *E. coli* genomic DNA standard.
    a. Label three 0.5 mL microcentrifuge tubes: PRE 1, PRE 2, and PRE 3.
    b. Prepare the dilutions of the DNA concentrate according to the table below. It is recommended to use 1X TE buffer pH 8.0 as the diluent.

Tube #	Sample Dilution	Concentration in Sample
PRE 1	NA	10 ng/µL Stock
PRE 2	5 µL PRE 1 + 3.3 µL diluent	6 ng/µL
PRE 3	5 µL PRE 2 + 45 µL diluent	0.6 ng/µL

    c. If the standard stock is higher than 10 ng/µL, first dilute it to 10 ng/µL and aliquot at least 7 µL to PRE 1 tube. Store aliquoted standard stocks at -80 °C for long term storage.
    d. If the standard stock is lower than 10 ng/µL, please adjust the dilution table accordingly.
    e. Aliquot at least 7 µL PRE 3 tube to prepare the standard serial dilution for

standard curve generation.

Prepare the E. coli genomic DNA dilutions for the standard curve

1. Prepare the standard serial dilutions
   a. Label five 0.5mL microcentrifuge tubes: STD 1, STD 2, STD 3, STD 4, and STD 5.
   b. Prepare the standard curve by making 10-fold dilutions of the DNA Concentrate according to the table below. Dilutions STD 1-STD 5 will be used for standard curve preparation in the PCR.
   c. Store the DNA dilution tubes at 4 °C for same day usage.

Tube #	Sample Dilution	Concentration in Sample	Final input in PCR reaction
PRE 3	NA	0.6 ng/µL	NA
STD 1	**5 µL PRE 3 + 45 µL diluent**	**60 pg/µL**	**300 pg**
STD 2	**5 µL STD 1 + 45 µL diluent**	**6 pg/µL**	**30 pg**
STD 3	**5 µL STD 2 + 45 µL diluent**	**0.6 pg/µL**	**3 pg**
STD 4	**5 µL STD 3 + 45 µL diluent**	**0.06 pg/µL**	**0.3 pg**
STD 5	**5 µL STD 4 + 45 µL diluent**	**0.006 pg/µL**	**0.03 pg**

Setup real-time PCR reactions

Setup PCR manually for 15 µL PCR Reactions with FAM™ and HEX™/VIC™ channels with ROX used as passive reference where appliable.

1. Calculate the total number of reactions needed in triplicate for the standards, controls, and test samples.
2. Prepare PCR master mix in Reagent Preparation Area according to the following table. It is recommended to prepare 110% of the calculated amount of PCR mix to account for pipetting carryovers.

   Note: Reagent A may precipitate. Thaw completely at room temperature and mix well to ensure complete resuspension before use**.**

Component	Volume/ test	Volume for n* Samples	110% of volume
Reagent A	3.75 µL	3.75 x 3 x n µL	4.125 x 3 x n µL
ECO Reagent B	0.75 µL	0.75 x 3 x n µL	0.825 x 3 x n µL
Reagent C	0.50 µL	0.50 x 3 x n µL	0.55 x 3 x n µL
Internal Control	1 µL	1 x 3 x n µL	1.1 x 3 x n µL
Water or TE	4 µL	4 x 3 x n µL	4.4 x 3 x n µL

*n equals the total number of test samples, extraction controls, standard dilutions, negative template control.

3. Briefly vortex the prepared PCR mix to ensure it is fully mixed, then centrifuge briefly to collect the liquid in the bottom of the tube.
4. Pipette 10 µL of the PCR mix into assigned well of a 96- or 384-well PCR plate.
5. Add 5 µL of the **extracted** nucleic acid of samples into assigned wells containing PCR mix.
6. Add 5 µL of the *E. coli* genomic DNA standard dilutions (STD 1 -STD 5) to the standards well-containing PCR mix.
7. Add 5 µL of the kit negative control into the no template control (NTC) well-containing PCR mix.

Note: Set up a 96-well PCR plate using the example plate layout below.

8. Seal the PCR plate with an appropriate film.
9. Vortex the sealed plate for 10 seconds, and centrifuge for 3 minutes at 350x g.

Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	NTC	NTC							STD 1	STD 1	STD 1
B	NTC	NTC	NTC							STD 2	STD 2	STD 2
C	NEC	NEC	NEC							STD 3	STD 3	STD 3
D	S1	S1	S1							STD 4	STD 4	STD 4
E	S2	S2	S2							STD 5	STD 5	STD 5
F	S3	S3	S3									
G	S4	S4	S4									
H	PEC	PEC	PEC									

S= Sample;
PEC: Positive Extraction Control, recommended.
NEC: Negative Extraction Control, recommended.
NTC: No Template Control;
STD: Standard Dilutions.
The plate layout is a suggested plate layout. Adjust the layout according to the number of test samples to be run.

Amplification and detection

Set up and run the PCR instrument according to the instrument reference guide. Note: Make sure to select “ROX” on the passive reference setting wherever applicable (refer to Appendix “Instrument configuration” for details).

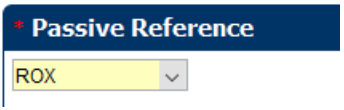
The following instructions are using QuantStudio™ Dx 96-well instrument as an example. For other instruments, please refer to the respective instrument user guide.

1. Create a new experiment, enter the experiment name in the Experiment Name field.
2. Select Fast 96-Well for the block setting.

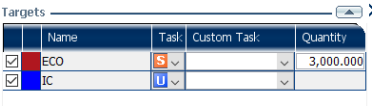
Note: Select "Fast" run mode if Fast mode is available on instrument with 384-well platform or select "Fast 96-well" with 96-well platform for QuantStudio™ 3, 5, 6, 7 Flex. Select "7500 Fast (96 wells)" for ABI 7500 Fast systems.

3. Select the Standard Curve mode, TaqMan® Reagents, and Fast mode.
4. In the Define panel, enter the Target Name. Select FAM and HEX/VIC in the Reporter Dye drop drop-down list. Select None in the Quencher Dye drop-down list. Select ROX in the Passive Reference panel.

Target Name or Detector	Channel
E. coli	FAM
Internal Control	HEX/VIC



5. Set up the standard curve as shown in the Plate Layout. Assign the tasks ( ) and enter the Quantity for each set of triplicates (STD 1 -STD 5, 300 pg, 30 pg, 3 pg, 0.3 pg, 0.03 pg).



6. Set up the qPCR Run Method according to the table below. Select "Lid Heating".

Step	Temperature	Time	Number of Cycles
1	37 °C	2 minutes	1
2	94 °C	10 minutes	1
3	94 °C	10 seconds	40
	61 °C*	1 minute	

** Collect fluorescence signal during the final 61 °C step.*

7. Make sure the reaction volume setting is set to 15 µL.
8. Double check all settings and start the run.

Interpretation of the results

After the qPCR run is finished, follow the general procedure to analyze the results.

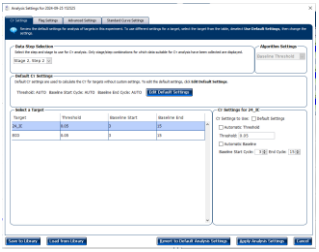
The setting of parameters should be adjusted according to the specific instrument user guide and software version.

The Ct values of the standards are used to construct a standard curve with values reported by the instrument in pg host cell DNA/reaction. When the test sample Ct value is outside the bound of the standard curve, it should not be used to calculate the test sample concentration. The concentration of host cell DNA can be mathematically transformed for reporting residual DNA in ng/mL, ng/mg of tested products or in ng/dose.

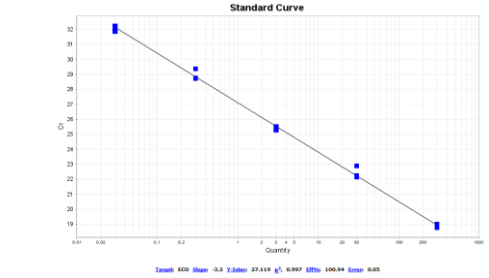
Data analysis using Applied Biosystems™ QuantStudio™ Dx Real-Time Instrument is shown as an example below:

1. In the QuantStudio™ Test Development Software, open your experiment, then navigate to the **Analysis** tab.

2. In the **Analysis Settings** tab, adjust the baseline and threshold settings. Refer to the Appendix for further explanation of baseline and threshold settings.



3. In the Analysis tab, review the Standard Curve plot. Verify the values for the slope, Y-intercept, R^2, and Efficiency.



4. Navigate to the Export tab to export the results.

The acceptance criteria of results are shown in the following list:

1. The standard curve: $R^2 \geq 0.98$, Eff%= 100±20%. If the specified criteria are not satisfied, it is permissible to exclude up to two data points, provided they are not from the same triplicate. Then rerun the analysis.
2. The detection result of NTC should be undetermined or at least 3 Ct value more than the lowest standard curve concentration.

Assay performance

Linearity and LoD

Linearity is demonstrated by analysis of standard DNA from *E. coli* ranging from 300 pg/reaction to 0.03 pg/reaction. Limit of Detection (LoD) is 0.01 pg/reaction.

Accuracy and Limit of Quantification

Limit of Quantification (LoQ) is 0.03 pg/reaction for the PCR assay.

Specificity

The assay is specific to *E. coli* DNA and is unaffected by the presence of unrelated DNA.

Precision

Assay repeatability is assessed on two instruments by analyzing 10 repeats of STD 3 (3 pg/rxn) and STD 5 (0.03 pg/rxn). To demonstrate intermediate precision, three replicates of STD 3 and STD 5 were analyzed by 2 operators (operator-to -operator precision) and by the same operator over 3 days (day-to-day precision).

Precision of HostDetect™ *E. coli* PCR DNA Quant Assay

Validation parameter	Validation target /CV	Samples	Validation result /CV	
			Instrument 1	Instrument 2
Repeatability (Intra run)	< 15%	3 pg/rxn	0.5%	0.3%
		0.03 pg/rxn	0.6%	0.4%
Intermediate Precision (operator-to -operator)	< 15%	3 pg/rxn	0.5%	0.3%
		0.03 pg/rxn	1.1%	1.3%
Intermediate Precision (day to day)	< 15%	3 pg/rxn	0.4%	0.3%
		0.03 pg/rxn	0.9%	0.7%

References

1. Guidelines on the quality, safety, and efficacy of biotherapeutic protein products prepared by recombinant DNA technology, World Health Organization, 2013.

Appendix

Instrument configuration

1. ROX Reference Selection

   - **Instruments that require ROX reference**: ABI 7500 Fast or Fast Dx, QuantStudio™ 3, QuantStudio™ 5, QuantStudio™ 6, QuantStudio™ 7 Flex, QuantStudio™ Dx, and Eonis™ Q.

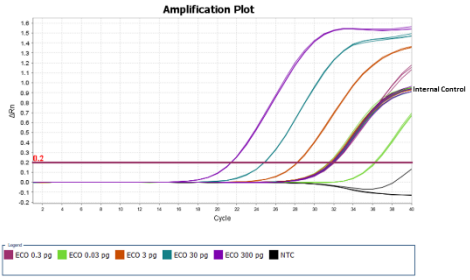
   - **Instruments that do not require ROX reference**: CFX™ 96 and CFX™ 384.

2. Baseline and threshold setting for ABI 7500 Fast or Fast Dx, QuantStudio™ 3, QuantStudio™ 5, QuantStudio™ 6, QuantStudio™ 7 Flex, QuantStudio™ Dx, CFX™ 96, CFX™ 384, and Eonis™ Q.

   - Set baseline for each target: The horizontal part of the baseline is used for the baseline range, which normally starts from 3-5 cycles and ends at 15-20 cycles. Baseline setting is normally automatically done by instrument. Manual baseline 3-15 is recommended in general.

   - Set threshold for each target: Thresholds should be adjusted to fall within exponential phase of the fluorescence curves and above any background signal (refer to the background signal of true negative samples). The threshold value for different instruments varies due to different signal intensities.

   One result from QuantStudio™ Dx instrument is shown below as an example.



Revision history:

Revision	Date	Description
1.0	Jan 25 2024	New document for E. coli DNA Quantification Kit
2.0	Dec 16 2024	Product name change

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