Primerdesign™ Ltd

Escherichia coli O104:H4 genomes

genesig® Advanced kit
150 tests

GENESIG
Kits by Primerdesign

For general laboratory and research use only

Introduction to Escherichia coli O104:H4

Escherichia coli O104:H4 is a rare enterohemorrhagic strain of the bacterium *Escherichia coli*, and the cause of the 2011 *Escherichia coli* O104:H4 outbreak. The "O" in the serological classification identifies the cell wall lipopolysaccharide antigen, and the "H" identifies the flagella antigen.

Analysis of genomic sequences obtained by BGI Shenzhen show that the O104:H4 outbreak strain is an enteroaggregative *E. coli* (EAEC or EAggEC) type that has acquired Shiga toxin genes.

Specificity

The primerdesign genesig kit for *Escherichia coli* O104:H4 genomes is designed for the in vitro quantification of E.coli O104:H4 genomes.

It has been shown that detection of the 4 target genes with this kit provides a unique genetic signature that can correctly identify strains of E.coli O104:H4.

If you require further information, or have a specific question about the detection profile of this kit then please send an email to enquiry@primerdesign.co.uk and our bioinformatics team will answer your question.

Kit contents

- E.coli_terC specific primer/probe mix (150 reactions **BROWN**)
  FAM labelled

- E.coli_fliC specific primer/probe mix (150 reactions **BROWN**)
  FAM labelled

- E.coli_nnaA primer/probe mix (150 reactions **BROWN**)
  FAM labelled

- E.coli_stx2A primer/probe mix (150 reactions **BROWN**)
  FAM labelled

- E.coli_O104:H4 positive control template (**RED**)

- 4x Internal extraction control primer/probe mix (150 reactions **BROWN**)
  VIC labelled

- Internal extraction control DNA (150 reactions **BLUE**)

- Endogenous control primer/probe mix (150 reactions **BROWN**)
  FAM labelled

- RNase/DNase free water (**WHITE**)
  For resuspension of primer/probe mixes

- Template preparation buffer (**YELLOW**)
  For resuspension of internal extraction control template and positive control template

Reagents and equipment to be supplied by the user

- **Real-time PCR Instrument**

- **DNA extraction kit**
  This kit is recommended for use with the genesig EASY DNA/RNA extraction kit. However, it is designed to work well with all processes that yield high quality DNA with minimal PCR inhibitors**.**

- **oasig™ Lyophilised or Precision®PLUS 2X qPCR Master Mix**
  This kit is intended for use with oasig or PrecisionPLUS 2X qPCR Master Mix.

- **Pipettors and Tips**

- **Vortex and centrifuge**

- **Thin walled 1.5 ml PCR reaction tubes**

Kit storage

This kit is stable at room temperature but should be stored at -20ºC on arrival. Once the lyophilised components have been resuspended they should not be exposed to temperatures above -20ºC for longer than 30 minutes at a time and unnecessary repeated freeze/thawing should be avoided. The kit is stable for six months from date of resuspension under these circumstances.
Primerdesign does not recommend using this kit after the expiry date stated on the pack.

Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and RNA/DNA integrity (An internal PCR control is supplied to test for non specific PCR inhibitors). Always run at least one negative control with the samples. To prepare a negative control, replace the template RNA sample with RNase/DNase free water.

Dynamic range of test

Under optimal PCR conditions genesig E.coli_O104:H4 detection kits have very high priming efficiencies of >95% and can detect less than 100 copies of target template.

Notices and disclaimers

This product is developed, designed and sold for research purposes only. It is not intended for human diagnostic or drug purposes or to be administered to humans unless clearly expressed for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. During the warranty period Primerdesign genesig detection kits allow precise and reproducible data recovery combined with excellent sensitivity. For data obtained by violation to the general GLP guidelines and the manufacturer's recommendations the right to claim under guarantee is expired. PCR is a proprietary technology covered by several US and foreign patents. These patents are owned by Roche Molecular Systems Inc. and have been sub-licensed by PE Corporation in certain fields. Depending on your specific application you may need a license from Roche or PE to practice PCR. Additional information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at Roche Molecular Systems, 1145 Atlantic Avenue, Alameda, CA 94501 or Applied Biosystems business group of the Applera Corporation, 850 Lincoln Centre Drive, Foster City, CA 94404. In addition, the 5' nuclease assay and other homogeneous amplification methods used in connection with the PCR process may be covered by U.S. Patents 5,210,015 and 5,487,972, owned by Roche Molecular Systems, Inc, and by U.S. Patent 5,538,848, owned by The Perkin-Elmer Corporation.

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The PCR process is covered by US Patents 4,683,195, and 4,683,202 and foreign equivalents owned by Hoffmann-La Roche AG.
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GeneAmp®, TaqMan® and AmpliTaqGold® are registered trademarks of Roche Molecular Systems, Inc.,
The purchase of the PrimerDesign reagents cannot be construed as an authorization or implicit license to practice PCR under any patents held by Hoffmann-LaRoche Inc.

Principles of the test

Real-time PCR

4x E.coli specific primer and probe mixes are provided and the target sequences can be detected through the **FAM** channel.

The primer and probe mixes provided exploit the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the pathogen DNA/cDNA. A fluorogenic probe, is included in the same reaction mixture which consists of an oligonucleotide labeled with a 5`-reporter dye and a downstream, 3`-quencher, During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of qPCR platforms.

Positive control

The positive control can be used at a single dilution for a qualitative analysis of the samples. Each time the kit is used, at least one positive control reaction must be included on the run. A positive result indicates that the primers and probes for quantification of the target pathogen gene are working properly in that particular experimental scenario. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false-positive results. This can be achieved by handling this component in a Post PCR environment. Care should also be taken to avoid cross-contamination of other samples when adding the positive control to the run. This can be avoided by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

Negative control

To validate any positive findings a negative control reaction should be included every time the kit is used. For this reaction the RNase/DNase free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run.

Internal DNA extraction control

When performing DNA extraction, it is often advantageous to have an exogenous source of DNA template that is spiked into the lysis buffer. This control DNA is then co purified with the sample DNA and can be detected as a positive control for the extraction process. Successful co-purification and PCR of the control DNA also indicates that PCR inhibitors are not present at a high concentration.

A separate primer and probe mix are supplied with this kit to detect the exogenous DNA using PCR. The primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control DNA does not interfere with detection of the pathogen target DNA even when present at low copy number. The Internal control is detected through the **VIC** channel and gives a CT value of 28+/-3.

Endogenous ACTB control

To confirm extraction of a valid biological template a primer and probe mix is included to detect an endogenous gene. Detection of the endogenous control is through the FAM channel and it is **NOT** therefore possible to perform a multiplex with the pathogen primers. A poor endogenous control signal may indicate that the sample did not contain sufficient biological material.

Resuspension protocol

To minimize the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR lab or PCR cabinet. Barrier tips are recommended for all pipetting steps.

1. **Pulse-spin each tube in a centrifuge before opening.**
   This will ensure lyophilised primer and probe mix is in the base of the tube and is not spilt upon opening the tube.

2. **Resuspend the primer/probe mixes in the RNase/DNase free water supplied, according to the table below:**
   To ensure complete resuspension, vortex each tube thoroughly.

Component – resuspend in water	Volume
Pre-PCR pack	
All primer/probe mixes (**BROWN**)	165 µl
Internal extraction control primer/probe mix (**BROWN**)	165 µl
Endogenous control primer/probe mix (**BROWN**)	165 µl

3. **Resuspend the internal control template and positive control template in the template preparation buffer supplied, according to the table below:**
   To ensure complete resuspension, vortex the tube thoroughly.

Component – resuspend in template preparation buffer	Volume
Post-PCR heat-sealed foil	
Internal extraction control DNA (**BLUE**)	600 µl
Post-PCR heat-sealed foil	
E.coli_O104:H4 Positive control template (**RED**) *	500 µl

* This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

DNA extraction

The internal extraction control DNA can be added either to the DNA lysis/extraction buffer or to the DNA sample once it has been resuspended in lysis buffer.

DO NOT add the internal extraction control DNA directly to the unprocessed biological sample as this can lead to degradation and a loss in signal strength.

1. **Add 4µl of Internal extraction control DNA (BLUE) to each sample in DNA lysis/extraction buffer**

2. **Complete DNA extraction according to the manufacturer's protocols**

qPCR detection protocol

1. **For each DNA sample prepare a reaction mix for each target gene according to the table below:**
   Include sufficient reactions for positive and negative controls.

Component	1 reaction
oasig or PrecisionPLUS 2X qPCR Master Mix	10 µl
Pathogen primer/probe mix (**BROWN**)	1 µl
Internal extraction control primer/probe mix (**BROWN**)	1 µl
RNase/DNase Free water (WHITE)	3 µl
Final volume	**15 µl**

2. **For each DNA sample prepare an endogenous control reaction according to the table below (Optional):**

Component	1 reaction
oasig or PrecisionPLUS 2X qPCR Master Mix	10 µl
Endogenous control primer/probe mix (**BROWN**)	1 µl
RNase/DNase Free water (**WHITE**)	4 µl
Final volume	**15 µl**

3. **Pipette 15µl of this mix into each well according to your qPCR experimental plate set up.**

4. **Prepare sample DNA templates for each of your samples.**

5. **Pipette 5µl of DNA template into each well, according to your experimental plate set up.**
   For negative control wells use 5µl of RNase/DNase free water. The final volume in each well is 20µl

6. **Pipette 5µl of positive control template into each well, according to your experimental plate set up.**
   The final volume in each well is 20µl.

qPCR amplification protocol

Amplification conditions using oasig or PrecisionPLUS 2X qPCR Master Mix.

	Step	Time	Temp
Cycling x50	Enzyme Activation	2 min	95°C
	Denaturation	10 s	95°C
	DATA COLLECTION*	60 s	60°C

* Fluorogenic data should be collected during this step through the FAM and VIC channels

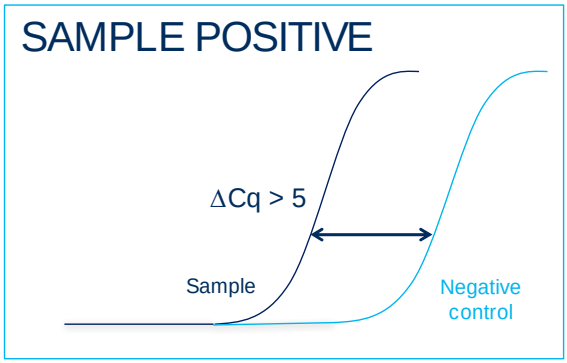
Interpretation of results

For each target the following table should be used to interpret whether a result can be called as positive or negative.

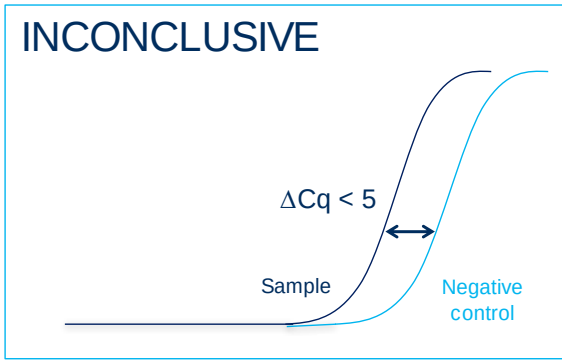
Target (FAM)	Internal control (VIC)	Positive control	Negative control	Interpretation
≤ 30	+ / -	+	-	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	+	+	-	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	-	+	-	**POSITIVE QUALITATIVE RESULT** do not report copy number as this may be due to poor sample extraction
-	+	+	-	**NEGATIVE RESULT**
+ / -	+ / -	+	≤ 35	**EXPERIMENT FAILED** due to test contamination
+ / -	+ / -	+	> 35	*
-	-	+	-	**SAMPLE PREPARATION FAILED**
+ / -	+ / -	-	+ / -	**EXPERIMENT FAILED**

Positive control template (**RED**) is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

*Where the test sample is positive and the negative control is positive with a Cq > 35, the sample must be reinterpreted based on the relative signal strength of the two results:



If the sample amplifies > 5 Cq earlier than the negative control then the sample should be reinterpreted (via the table above) with the negative control verified as negative.



If the sample amplifies < 5 Cq earlier than the negative control then the positive sample result is invalidated and the result should be determined inconclusive due to test contamination. The test for this sample should be repeated.

Once the results from each target have been interpreted individually, the following table can be used to determine presence of E.coli O104:H4 strains:

fliC	nnaA (O-antigen)	stx2A	terC	Interpretation
-	**-**	**-**	**-**	E.coli of unknown classification may be present
+	**+**	**+**	**+**	E.coli O104:H4
n/a	**n/a**	**n/a**	**+**	Tellurite resistant E.coli
n/a	**n/a**	**+**	**n/a**	Shiga toxin producing E.coli

Internal PCR control

The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency, the quantity of DNA added to the PCR reaction and the individual machine settings. Cq values of 28±3 are within the normal range. When amplifying a E.coli_O104:H4 sample with a high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.

Endogenous ACTB control

The signal obtained from the endogenous control primer and probe set will vary according to the amount of biological material present in a given sample. An early signal indicates the presence of a good yield of biological material. A late signal suggests that little biological material is present in the sample.