Cat. # RR102A

For Research Use

TaKaRa

O-157 (Verocytotoxin Genes) One Shot PCR Screening Kit Ver.2

Product Manual

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Also available from TaKaRa

PCR Kits & Primers Set for detection of pathogenic bacterial genes

Multiplex PCR O-157/Verocytotoxin Genes Detection Kit ..... Cat. #RR107A**
O-157 (Verocytotoxin Genes) One Shot PCR Typing Kit ..... Cat. #RR106A**
O-157 (Verocytotoxin Genes) PCR Screening Set ..... Cat. #RR100**
O-157 (Verocytotoxin Genes) PCR Typing Set ..... Cat. #RR105A**
PCR Pathogenic Bacterial Primers Set* ..... Cat. #S001-S027**
Positive Control Template for PCR Pathogen Detection ..... Cat. #S031-S046**
TaKaRa PCR Mycoplasma Detection Set ..... Cat. #6601**
TaKaRa PCR Human Papillomavirus Detection Set ..... Cat. #6602
TaKaRa PCR Human Papillomavirus Typing Set ..... Cat. #6603

* : Manufactured by SHIMADU CORPORATION.
** : Not available in all geographic locations. Check for availability in your region.

PCR Related Products

TaKaRa Taq™ DNA Polymerase ..... Cat. #R001A/B/C/D
TaKaRa Taq™ DNA Polymerase (with Mg^{2+} free buffer) ..... Cat. #R001AM/BM/CM
TaKaRa Ex Taq® DNA Polymerase ..... Cat. #RR001A/B/C/D
TaKaRa LA Taq® DNA Polymerase ..... Cat. #RR002

I. Description

Enterohemorrhagic *E. coli* (EHEC) strains, including O157:H7 serotype, cause hemorrhagic colitis accompanied by bloody excrement and heavy abdominal pain, and further lead to hemolytic uremic syndrome. These severe symptoms are caused by Verocytotoxin, which is produced by EHEC. When detecting EHEC, it is important to discriminate a pathogenic strain which is capable of producing Verocytotoxin from non-pathogenic ones, accurately in a quick method.
PCR is a simple and powerful method for amplification of target DNA, using a small amount of template DNA. By cycling 3 steps at different temperature, target DNA can be significantly amplified in a couple of hours. (See VI. Principle for the details of PCR.)
This kit enables simple detection of EHEC by specifically detecting genes of Verocytotoxin utilizing PCR method. One Shot PCR solution contains all the reagents required to perform PCR in a 0.2 ml PCR tube. This kit applies *TaKaRa Ex Taq* HS, which achieves higher yields of PCR products more efficiently, than conventional *Taq*, so that highly sensitive detection is possible in a less time. Furthermore, template EC3 is supplied as a positive control in each tube of this kit to prevent false negative detection. As the size of amplified fragments obtained from this template is completely different from those from Verocytotoxin gene, the existence of Verocytotoxin gene can be easily verified by gel electrophoresis.

II. Components (For 48 reactions)

2X One Shot PCR solution tube* (supplied in 0.2 ml PCR tube)

25 μl x 48 tubes

* : 2X One Shot PCR solution tube contains the followings:

1. Primer EVC-1 & 2
   - Target gene: enterohemorrhagic *E. coli* (EHEC) Verocytotoxin gene VT1, VT2, VT2vha, VT2vhb, VT2vpl
   - Amplified size: 171 bp

2. Control template EC3
   This kit includes positive control template to assure the PCR performance. By performing PCR using primer EVC-1 & 2 and this template, 685 bp of amplified fragment will be obtained.

3. *TaKaRa Ex Taq* HS
   As *TaKaRa Ex Taq* HS is a thermostable polymerase for PCR with a proof reading activity, resulting in more efficient amplification than a conventional *Taq*, it works effectively for detection of Verocytotoxin gene.

4. *Ex Taq* Buffer

5. dNTP Mixture

III. Storage

Stored and shipped at -20℃.
Avoid vigorous mixing and repeating freeze-thaw cycles because each tube includes *TaKaRa Ex Taq* HS.

IV. Materials Required but not Provided

[Reagents]
- Sterile purified water
- NuSieve™ 3:1 Agarose (Lonza, Cat. #50091)*
- Electrophoresis buffer
  Tris-Acetate-EDTA Buffer (TAE) 50x Powder, pH8.3 (Cat. #T9131)
  TBE powder (Cat. #T905)
- DNA marker
  pHY Marker (Cat. #3404A/B)
  φ X174 *Hin*c II digest (Cat. #3406A/B)
  100 bp DNA Ladder (Cat. #3407A/B)
- Loading buffer (6X : 36% glycerol, 0.05% bromophenol blue, 0.05% xylene cyanol). This loading buffer is supplied with Takara's DNA marker.
- DNA staining
  Ethidium bromide

[Equipment]
- Heating block (applicable at 95℃)
- Refrigerated centrifuge, compatible for 1.5 ml tube
- Thermal cycler
  TaKaRa PCR Thermal Cycler Dice Gradient/Standard (Cat. #TP600/TP650)*
  TaKaRa PCR Thermal Cycler Dice mini (Cat. #TP100)*
- Electrophoresis apparatus
  Mupid®-2plus (Cat. #AD110)
  Mupid®-exU (Cat. #AD140)
- Ultraviolet transilluminator (Wavelength 300 nm)
- Polaroid camera to photograph stained gel
  Mupid®-Scope WD (Cat. #MS-WD)*

[Other]
- 1.5 ml tube or 0.2 ml PCR tube [0.2 ml Hi-Tube Dome Cap (Cat. #NJ200)*]
- Micropipettes for 20 μl and for 200 μl and tips (sterile)
- Tray for staining agarose gel

* : Not available in all geographic locations. Check for availability in your region.

V. Precautions for Operation

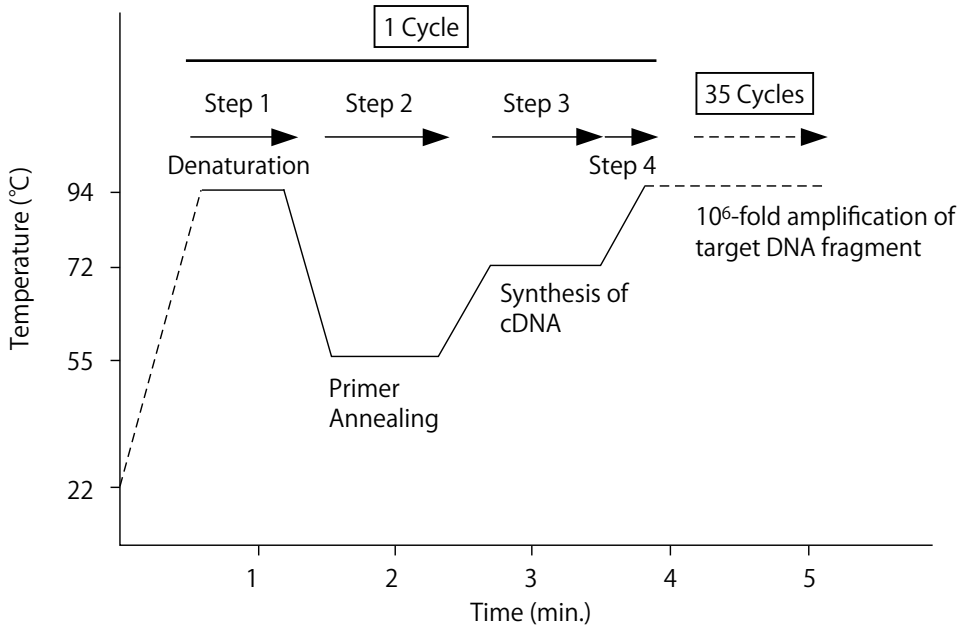
When handling thermal cycler, be sure to follow the instruction for the instrument.

1) If a primer is degraded by contamination with nuclease, exact analysis cannot be performed. Sweat or saliva of an operator can cause contamination with nuclease. Extreme caution should be exercised during operation.
2) When sample is judged positive, confirm with other microbiological method.
3) PCR reaction is of extremely high sensitivity. In order to prevent contamination, it is recommended to set the separate 3 areas described below in a flow from preparation of solution to detection, which are physically separated from one another.
   - ○ Area 1 : Preparation of reaction solution and addition to tubes and preparation of samples
   - ○ Area 2 : Addition of samples to reaction solution
   - ○ Area 3 : Reaction and detection by electrophoresis

   Do not open the cap of tubes which contains amplified substance in areas other than Area 3.

VI. Principle

PCR (Polymerase Chain Reaction) process is a simple and powerful method which allows *in vitro* amplification of DNA fragments through a succession of three incubation steps at different temperatures. The double-stranded DNA is heat denatured (denaturation step), the two primers complementary to the 3' region of the target segment are annealed at low temperature (annealing step), and then extended at an intermediate temperature (extension step). One set of the three consecutive steps is referred to as one cycle. PCR process is based on the repetition of the cycle and can amplify DNA fragments significantly, around 10^6-fold amplification in a couple of hours.



Step1: Denature the target double-stranded DNA fragment in the reaction mixture containing primer, dNTP, and polymerase : 94℃, 1 min.
Step2: Anneal primer to obtained single-stranded DNA : 55℃, 1 min.
Step3: Synthesize cDNA with DNA polymerase : 72℃, 1 min.
Step4: Return to Step 1 - to denature the amplified double-stranded DNA again to yield single-stranded DNA : 94℃, 1 min.

One set of the consecutive 1 - 4 steps is referred as one cycle and perform 35 cycles.

VII. Protocol

Experimental example: Detection using a heat-extracted bacterial sample

A. Preparation of heat-extracted sample

Method-1

1) Add 4 μl of enriched culture into a 1.5 ml tube or 2 μl of that into a 0.2 ml PCR tube.
2) Dispense 196 μl of sterile purified water into a 1.5 ml tube or 98 μl of that into 0.2 ml PCR tube and mix.
3) Heat at 95℃ for 5 min. (When using 0.2 ml PCR tube, it is convenient to use a PCR Thermal Cycler.)
4) Centrifuge at 12,000 rpm at 4℃ for 10 min. and collect supernatant. The 25 μl of that is used as the heat-extracted sample. To get high sensitivity, try with Method-2.

Method-2

1) Add 1 ml of enriched culture (Novobiocin-supplemented medium etc) into 1.5 ml tube.
2) Centrifuge at 5,000 rpm at 4℃ for 5 min. and waste supernatant.
3) Add 100 μl of sterile purified water into the precipitate (bacterial cells etc) and mix.
4) Heat at 95℃ for 5 min.
5) Centrifuge at 12,000 rpm at 4℃ for 5 min. and collect supernatant. The 25 μl of that is used as the heat-extracted sample.

Note: When the sample prepared by Method-2 inhibit PCR reaction, try the protocol of (1) or/and (2) below for additional purification.
(1) The precipitate at step 2 are washed with PBS buffer for 2 or 3 times (briefly, suspend with 500 μl PBS, centrifuge, and discard the supernatant. Repeat these 2 or 3 times).
(2) The heat-extracted sample prepared can be diluted for 10-fold or 100-fold with sterile purified water. Then the 25 μl of the dilution can be used for PCR reaction.

※ Enrichment culturing is performed according to a standard protocol appropriate for each sample. The heat-extracted sample can be stored at -20℃.

B. PCR reaction

1) Add 25 μl of the heat-extracted sample into a 2X One Shot PCR solution tube. Prepare a negative control by adding 25 μl of sterile purified water instead of the sample.
2) Secure tightly a cap of each tube and set in a thermal cycler. Perform PCR under the following condition.

94℃	1 min.	┐
55℃	1 min.	├ 35 cycles
72℃	1 min.	┘
72℃	10 min.	1 cycle

The reaction completes in about 2.5 hours. The PCR reactants can be stored at 4℃ or -20℃.

C. Preparation of agarose gel

1) Dispense electrophoresis buffer into a triangle flask and slowly add NuSieve™ 3:1 Agarose to the concentration of 3% (w/v) with mixing.
2) Heat for 2 - 3 min. in a microwave. After heating, mix well and confirm that the agarose is uniformly solved. Heat the slurry again for the minimum time required to allow all of the grains of agarose to dissolve.
3) Set up the gel board.
4) After the agarose gel solution cools to 50 - 60℃, pour the solution into the gel board and insert a comb to generate slots. Leave for 30 min to 1 hour at room temperature and harden the gel.

*When staining the gel with ethidium bromide before applying samples.
Cool the solution to 50 - 60℃ and add ethidium bromide solution in a final concentration of 0.5 μg/ml and mix gently to be dissolved uniformly.

5) After the gel hardens enough, mount the gel in a electrophoresis tank.
6) Pour the electrophoresis buffer into the electrophoresis tank so that the gel is completely immersed.
7) Remove the comb carefully not to break the gel.

D. Gel electrophoresis

1) Connect the electrical leads carefully not to mistake the electrodes, between anode and cathode. As DNA is charged with negative, it migrates from cathode to anode.
2) Add 2 μl of Loading buffer to each tube containing 10 μl of PCR reactant and mix. Slowly load the mixture into the slots of the submerged gel using a micropipette. DNA Marker of known size should be loaded into slots on both the right and left sides of the gel.
3) Apply a constant voltage of 50 - 150 V and run the gel until the bromophenol blue* have migrated 3 - 4 cm in front of the comb.
   * : Bromophenol blue migrates faster.

E. Verification of stained band

When the gel has been prestained with ethidium bromide, perform only 3).

1) Prepare 1 μg/ml ethidium bromide solution in the amount enough to submerge the gel, and keep it in a tray for staining agarose gel.
2) Put the gel in the tray and leave it for 20 - 30 min.
3) Set the gel on an ultraviolet transilluminator and photograph the gel. Verify the size of the DNA bands of reactants comparing with DNA Marker.

CAUTION : Gloves should be worn in handling Ethidium bromide and the stained gel.

VIII. Interpretation

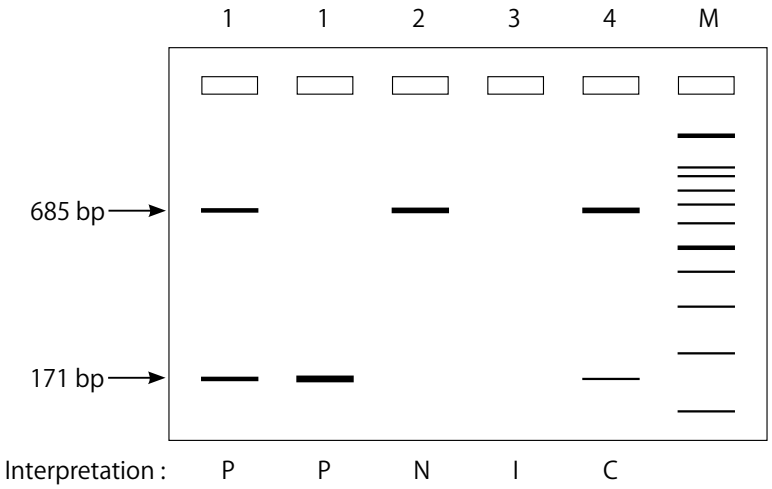
When a sample is considered positive for Verocytotoxin gene, the amplified fragments of 171 bp can be detected. A band of 685 bp indicates that there has been no PCR inhibition.

1. A sample is considered **POSITIVE** for Verocytotoxin gene if a 171 bp band appears in the sample lane, whether or not 685 bp of band appears. (The bands of positive control disappears when Verocytotoxin gene exists a lot in a sample.)

2. A sample is considered **NEGATIVE** for Verocytotoxin gene if no 171 bp band appears, but 685 bp band appears.

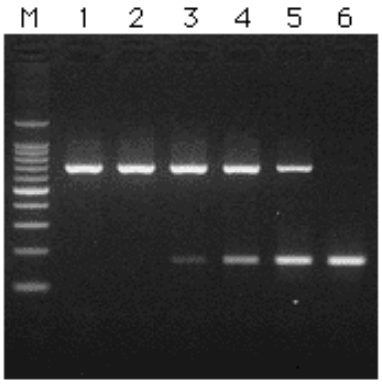
3. An **INDETERMINATE** test result occurs when neither 171 bp band nor 685 bp band appears. As it is assumed that the PCR reaction was not carried out normally, you need to perform PCR again.

4. If the 171 bp band appears in the negative control lane, it indicates that the process was **CONTAMINATED**. Disinfect the reagents, the instruments, and the place where the reaction mixture was prepared. And then, perform the detection again.



* Interpretation
P: POSITIVE
N: NEGATIVE
I: INDETERMINATE
C: CONTAMINATED

Electrophoresis Result



Lane M: 100 bp DNA Ladder
1: Negative control
2: 10^0 cell/tube
3: 10^1 cells/tube
4: 10^2 cells/tube
5: 10^3 cells/tube
6: 10^4 cells/tube
3% NuSieve™ 3:1 Agarose gel
Prestained with Ethidium bromide

XI. Experimental Example of Sample Preparation from Food

A: Sample preparation

Food
↓
Weighing 25 g
↓
Enrichment culture, in 225 ml mEC media containing novobiocin
Incubate at 42 - 43℃ for 18 - 24 hrs

mEC media containing novobiocin	
Tryptone	20 g/L
bile salts #3	1.12 g/L
lactose	5.0 g/L
K_2HPO_4	4.0 g/L
KH_2PO_4	1.5 g/L
NaCl	5.0 g/L
sodium novobiocin	20 mg/L
Adjust pH7.0	

Secondary enrichment culturing in
Tryptic soy broth media. Incubate at
37℃ for 18 - 24 hrs.
↓
Detection by PCR

Detection by PCR

B. Pretreatment for PCR

Dispense 4 μl of enrichment culture in a 1.5 ml tube or 2 μl of that in a 0.2 ml tube.
↓
Add 196 μl of TE buffer [10 mM Tris-HCl, 0.1 mM EDTA (pH8.0)] or 98 μl of that and then mix.
↓
Heat at 95℃ for 5 min.
↓
Centrifuge at 12,000 rpm at 4℃ for 10 min. and collect the supernatant
↓
Apply 25 μl of supernatant to the subsequent PCR.
↓
Perform One Shot PCR

X. Related Products

PCR Pathogenic Bacterial Primers Set EVT-1, EVT-2 (VT1 gene of EHEC) (Cat. #S006)*
PCR Pathogenic Bacterial Primers Set EVS-1, EVS-2 (VT2 gene of EHEC) (Cat. #S007)*
PCR Pathogenic Bacterial Primers Set EVC-1, EVC-2 (VT genes of EHEC) (Cat. #S008)*
Positive Control Templates for PCR Pathogen Detection (Cat. #S031 - S046)*
TaKaRa Ex Taq® (Cat. #RR001A/B/C)

* Not available in all geographic locations. Check for availability in your region.

XI. References

1) Takao, T., T. Tanabe, Y.-M. Hong, Y. Shimonishi, H. Kurazono, T. Yutsudo, C. Sasakawa, M. Yoshikawa and Y. Takeda (1988) *Microb. Pathog*. **5**: 357-369.
2) Jackson, M.P., R.J. Neil, A.D. O'Brien, R.K. Holmes and J.W. Newland (1987) FEMS *Microbio. Lett*. **44**: 109-114.
3) Ito, H., A. Terai, H. Kurokawa, Y. Takeda and M. Nishibuchi (1990) *Microb. Pathog*. **8**: 47-60.
4) Weinstein, D.L., M.P. Jackson, J.E. Samuel, R.K. Holmes and A.D. O'Brien (1955) *J. Bacteriol*. **170**: 4223-4230.