Table of Contents

I. Description .................................................................2
II. Kit Components ..........................................................2
III. Storage .................................................................2
IV. Reagents and instruments not supplied in the kit ..........3
V. Precautions for Operation ........................................3
VI. Preparation example of the environmental sample, such as food, feces, soil and water ..................4
VII. Protocol ...............................................................5
VIII. Judgement ...........................................................7
IX. References ............................................................8
X. Related Products .......................................................8
I. Description

Incidence of food poisonings caused by Campylobacter is becoming more frequent than ever in recent years. Campylobacter species are gram-negative, spiral-shaped bacteria that multiply under 3-10% oxygen microaerophilic condition. The main symptom caused by Campylobacter is enteritis. And about 90% of the pathogenic bacteria are Campylobacter jejuni (C. jejuni) and a few percent of those are Campylobacter coli (C. coli). Campylobacter fetus (C. fetus) is isolated from specimen of septicemia or meningitidis, and considered as one of pathogenic bacteria for such symptoms. As the symptoms are different according to the bacteria species, it is very important not only to detect presence of the bacteria but also to identify species of those. Currently, detection and identification of Campylobacter is done by culture method. Because the growth of those bacteria is slow, it would take at least 2 days for pre-culture of the bacteria and several days for identification test of bacteria species. Thus the period of about a week is required for certainly identification of Campylobacter species. It have been expected to develop rapid detection and identification method of Campylobacter applying gene detection technology such as PCR.

This kit is designed to specifically and rapidly detect and identify 3 kinds of bacterium species, C. jejuni, C. coli, and C. fetus, by PCR using B subunit gene (cdtB gene) or C subunit gene (cdtC gene) of the Cytolethal distending toxin (cdt genes) as targets. In a Campylobacter species, there are infrequently strains which have deletion or mutation on cdtB gene or cdtC gene. To increase detection precision, detection of both cdtB and cdtC genes will be performed. This kit allows to detect the target genes in highly sensitive because this kit contains enzyme for hot start PCR, Takara Ex Taq™ Hot Start Version, by precluding miss-priming during preparation of reaction solution and non-specific amplification derived from primer dimmer.

This kit is designed by TaKaRa through kindly cooperation with Osaka Prefecture University and Fuso Pharmaceutical Industries, Ltd.

II. Kit Components (for 100 reactions*1, 50 μl PCR)

1. Premix Ex Taq™ HS *2 (2X conc.)  500 μl x 5
2. cdtB Primer Mix  250 μl
3. cdtC Primer Mix  250 μl
4. dH2O  1 ml x 3
5. C. jejuni Positive Control  150 μl
6. C. coli Positive Control  150 μl
7. C. fetus Positive Control  150 μl

*1 : 50 reactions for cdtB gene detection and 50 reactions for cdtC gene detection.
*2 : 2X concentration of PCR reaction reagent which contains Takara Ex Taq™ HS, Reaction Buffer, and dNTP mixture. The activity might reduce by repeating the freeze and thaw process, thus divide 25 μl of it in PCR tube and store at -20°C after thawed out.

III. Storage

-20°C

Cat. #RR134A
v1102Da
IV. Reagents and instruments not supplied in the kit

【Reagents】
1. Sterilized distilled water
2. NuSieve® 3 : 1 Agarose (Lonza Inc.)
3. Electrophoresis buffer (TBE powder (Cat. #T905) or 20X TAE Buffer (Lonza Inc.))
4. DNA marker [100 bp DNA Ladder (Cat. #3407A/B)]
5. Loading buffer (6X : 36% glycerol, 0.05% bromophenol blue, 0.05% xylene cyanol, 30mM EDTA) (this loading buffer is supplied with DNA marker listed on 4)
6. DNA stain reagents [SYBR® Green I (Lonza Inc.) or Ethidium Bromide]

【Instruments】
1. Heating block (applicable at 95°C)
2. Refrigerated centrifuge, compatible with 1.5 ml tubes
3. Thermal Cycler for PCR
   TaKaRa PCR Thermal Cycler Dice™ (Cat. #TP600/TP650)
   TaKaRa PCR Thermal Cycler Dice™ mini (Cat. #TP100)
4. Electrophoresis apparatus
   Mupid®-2plus (Cat. #AD110)
   Mupid®-exU (Cat. #AD140)
5. Equipment to photograph electrophoresis gel

【Others】
1. 0.2 ml PCR tube [0.2 ml Hi-Tube Dome Cap (Cat. #NJ200), TaKaRa Micro PCR Tube (Cat. #9047)]
2. Micropipette for 200 μl, 20 μl, and 10 μl
3. Micropipette tips (with hydrophobic filter)

V. Precautions for Operation:
1) When handling thermal cycler, be sure to follow the instruction for the instrument.
2) 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.
3) When sample is judged positive, confirm with other microbiological method.
4) PCR reaction is of extremely high sensitivity. In order to prevent contamination, it is recommended to set the separate four 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
   ○ Area 2: Preparation of samples
   ○ Area 3: Addition of samples to reaction solution
   ○ Area 4: Reaction and detection by electrophoresis

Do not open the cap of tubes which contains amplified substance in areas other than Area 4.
VI. Preparation example of environmental samples, such as food, feces, soil and water

**PCR using bacteria isolated from a cultured bacteria mixture.**

(1) Directly mix samples to culture medium, or produce a mixture with equal amounts of solution by stomacher etc.

(2) Add the obtained mixture to Bolton medium or Preston medium of 10 fold volume. Culture the bacteria under microaerophilic condition (5% O₂, 10% CO₂, 85% N₂) for 24 - 48 hours at 37°C.

(3) Inoculate by spreading directly 100 µl of the bacteria cultured suspension on selective medium, such as Skirrows agar medium, or by filter method [place sterilized membrane filter (pore 0.45 µm) on blood agar plate, then spread the bacteria cultured suspension. Stand for 30 min at room temperature, then remove the filter]. Then incubate the plate for isolation of bacteria under microaerophilic condition (5% O₂, 10% CO₂, 85% N₂) for 48 hours at 37°C.

(4) Suspend a small quantity of bacteria from a colony on the plate (Skirrows agar medium etc) to TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH8.0) using sterilized platinum loop. The turbidity at 600 nm of bacterial suspension should be about 0.1.

(5) Treat for 10 min at 95°C.

(6) Centrifuge at 15,000 rpm for 10 min, and use the supernatant as heat-extracted sample.

**PCR using suspension culturing bacteria or specimen.**

(1) Centrifuge suspension of bacteria or sample suspension at 1,200 rpm for 5 min. Transfer the supernatant to a new tube and centrifuge at 15,000 rpm for 10 min.

(2) Add appropriate volume of TE buffer to the precipitates. After treat for 10 min at 95°C, centrifuge at 15,000 rpm for 10 min. Use the supernatant as heat-extracted sample. As other method, use DNA extracted by NucleoSpin® Tissue as PCR template.

VII. Protocol

1) **PCR reaction mixture preparation and reaction condition**

To obtain correct result, perform negative control and positive control reaction together.

1) Prepare the following master mixture on ice for necessary quantities (numbers of sample, negative control, positive control, and a few).

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premix Ex Taq™ HS</td>
<td>25 µl</td>
</tr>
<tr>
<td>cdtB Primer Mix or cdtC Primer Mix</td>
<td>5 µl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>15 µl</td>
</tr>
<tr>
<td>45 µl</td>
<td></td>
</tr>
</tbody>
</table>

2) Add 45 µl of the master mixture to PCR tubes on ice. And add 5 µl of heat extracted sample. Also, prepare negative control by adding 5 µl of sterilized distilled water instead of the sample and positive control by adding 5 µl of each Positive Control.

**[PCR condition]**

94°C, 30 sec.  
55°C, 30 sec.  
72°C, 30 sec.  
30 - 40 cycles  

* The reaction completes in about 2 hours. The PCR reaction solution can be stored at 4°C or -20°C.
(2) Preparation of agarose gel
1) Dispense electrophoresis buffer into a triangle flask and slowly add NuSieve® 3 : 1 Agarose to concentration of 3% (w/v) with mixing.
2) Heat for 2 - 3 minutes in a microwave. After heating, mix well and confirm that the agarose is uniformly solved. Heat the slurry again to allow all of agarose to dissolve.
3) Set up the gel tray.
4) After the agarose gel solution cools to 50 - 60°C, pour the solution into the gel tray and inset a comb to generate slots. Leave for 30 minutes to 1 hour at room temperature and harden the gel.

When making the gel with ethidium bromide.
Cool the gel solution to 50 - 60°C and add ethidium bromide solution in a final concentration of 0.5 μg/ml and mix gently to be dissolved uniformly. Pour the solution into the gel tray and inset a comb to generate slots. Leave for 30 minutes to 1 hour at room temperature and harden the gel.
5) After the gel harden enough, remove the comb carefully not to break the gel.
6) Set the gel with tray in the electrophoresis tank. Pour electrophoresis buffer into the electrophoresis tank so the gel is completely immersed.

(3) 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 1.0 μl of 6X loading buffer to each tube containing 5 μl of PCR reactant and mix. Slowly load the mixture into the slots of the submerged gel using a micropipette. DNA standard 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 (faster migrating dye) have migrated 2 - 3 cm in front of the comb.

(4) Verification of stained bands (when the gel is prestained with ethidium bromide, perform only 3.)
1) Prepare 1 μg/ml ethidium bromide solution, or SYBR® Green I *(Lonza Inc.) or Gel-Star® (Lonza Inc.) solution (pre-diluted by 104-fold with electrophoresis buffer) 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 without moving for 20 - 30 minutes.
3) Set the gel on an ultraviolet transilluminator and photograph the gel*. Verify the size of the bands of reactants comparing with the DNA marker.

*: When using SYBR® Green I or GelStar® for staining, please use the filter designated for use with each staining dye.

CAUTION: Gloves should be worn in handling Ethidium bromide, SYBR® Green I, GelStar® solution, or the stained gel.

(5) Notes
This kit does not contain internal control. Therefore, correct judgment cannot be done when PCR reaction is inhibited (false negative), according to a method of sample preparation. To avoid false negative, it is recommended to confirm that PCR reaction is not prevented by a heat-extracted sample absent from known Campylobacter bacteria. (For example: an amplified band could be detected by PCR reaction using 5 μl of negative heat-extracted sample and 5 μl of Positive Control.) When sample is judged as negative, it is recommended to determine final judgment together with the result obtained by microbiological method.
VIII. Judgement

The following shows the size of amplified products by PCR reaction.

The size of product by PCR reaction using the *cdtB* Primer Mix (*cdtB* gene)

<table>
<thead>
<tr>
<th>Species</th>
<th>PCR product from sample (genomic DNA)</th>
<th>Product from each Positive Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. jejuni</em></td>
<td>714 bp</td>
<td>274 bp</td>
</tr>
<tr>
<td><em>C. fetus</em></td>
<td>553 bp</td>
<td>203 bp</td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>413 bp</td>
<td>153 bp</td>
</tr>
</tbody>
</table>

The size of product by PCR reaction using the *cdtC* Primer Mix (*cdtC* gene)

<table>
<thead>
<tr>
<th>Species</th>
<th>PCR product from sample (genomic DNA)</th>
<th>Product from each Positive Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. jejuni</em></td>
<td>524 bp</td>
<td>274 bp</td>
</tr>
<tr>
<td><em>C. fetus</em></td>
<td>397 bp</td>
<td>202 bp</td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>313 bp</td>
<td>154 bp</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lane</th>
<th>Primer</th>
<th>Template</th>
<th>Amount</th>
<th>Amplified size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>cdtB</em> Primer Mix</td>
<td><em>C. jejuni</em> genomic DNA</td>
<td>5 pg</td>
<td>714 bp</td>
</tr>
<tr>
<td>2</td>
<td><em>cdtB</em> Primer Mix</td>
<td><em>C. fetus</em> genomic DNA</td>
<td>5 pg</td>
<td>553 bp</td>
</tr>
<tr>
<td>3</td>
<td><em>cdtB</em> Primer Mix</td>
<td><em>C. coli</em> genomic DNA</td>
<td>5 pg</td>
<td>413 bp</td>
</tr>
<tr>
<td>4</td>
<td><em>cdtB</em> Primer Mix</td>
<td><em>C. jejuni</em> genomic DNA</td>
<td>5 pg</td>
<td>524 bp</td>
</tr>
<tr>
<td>5</td>
<td><em>cdtB</em> Primer Mix</td>
<td><em>C. fetus</em> genomic DNA</td>
<td>5 pg</td>
<td>397 bp</td>
</tr>
<tr>
<td>6</td>
<td><em>cdtB</em> Primer Mix</td>
<td><em>C. coli</em> genomic DNA</td>
<td>5 pg</td>
<td>313 bp</td>
</tr>
<tr>
<td>7</td>
<td><em>cdtC</em> Primer Mix</td>
<td><em>C. jejuni</em> Positive Control</td>
<td>5 μl</td>
<td>274 bp</td>
</tr>
<tr>
<td>8</td>
<td><em>cdtC</em> Primer Mix</td>
<td><em>C. fetus</em> Positive Control</td>
<td>5 μl</td>
<td>203 bp</td>
</tr>
<tr>
<td>9</td>
<td><em>cdtC</em> Primer Mix</td>
<td><em>C. coli</em> Positive Control</td>
<td>5 μl</td>
<td>153 bp</td>
</tr>
<tr>
<td>10</td>
<td><em>cdtC</em> Primer Mix</td>
<td><em>C. jejuni</em> Positive Control</td>
<td>5 μl</td>
<td>274 bp</td>
</tr>
<tr>
<td>11</td>
<td><em>cdtC</em> Primer Mix</td>
<td><em>C. fetus</em> Positive Control</td>
<td>5 μl</td>
<td>202 bp</td>
</tr>
<tr>
<td>12</td>
<td><em>cdtC</em> Primer Mix</td>
<td><em>C. coli</em> Positive Control</td>
<td>5 μl</td>
<td>154 bp</td>
</tr>
<tr>
<td>M</td>
<td>100 bp DNA Ladder</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Campylobacter (cdt gene) PCR Detection and Typing Kit

- Confirm that no amplification product is obtained from Negative control reaction. If products having size listed above are detected, it is considered as contamination in reaction. After clean up the area preparing reaction solution and all the equipments used, then perform all the reactions again.

- Confirm that PCR product having size amplified from Positive Control primer is detected in Positive Control reactions. If no product with correct size is obtained, PCR reaction is not performed with some reasons. Trouble of instruments, miss of reagent preparation, or deterioration of reagents is thought as cause. Perform the reaction again.

- If correct results are obtained from Negative Control reaction and Positive Control reaction and a PCR band is detected in reaction using the cdtB and/or cdtC Primer Mix and sample (genomic DNA), bacteria species corresponding to the band size is present.

- If correct results are obtained from Negative Control reaction and Positive Control reaction and a PCR band is NOT detected in reaction using heat-extracted sample, it would be judged as negative (Undetectable).

Caution: It should be noted that no PCR product is detected if PCR inhibitory substances are included in the heat-extracted sample even though Campylobacter species are present in the sample examined, resulting in ‘Negative judgment’ (namely false negative). In order to avoid false negative, it is recommended to confirm that PCR product band derived from the Positive Control could be detected by PCR reaction using a mixture of one of Positive Controls with a heat-extracted sample as a template. When PCR product with properly size is not detected, it should be considered that the sample contains PCR inhibitory substances. In such case, it is recommended to perform sample preparation and then PCR reaction again.

IX. References

X. Related Products
- TBE (Tris-borate-EDTA) Powder (Cat. #T905)
- 100 bp DNA Ladder (Cat. #3407A/B)
- TaKaRa PCR Thermal Cycler Dice™ (Cat. #TP600/TP650)
- TaKaRa PCR Thermal Cycler Dice™ mini (Cat. #TP100)
- Mupid®-2plus (Cat. #AD110)
- Mupid®-exU (Cat. #AD140)
- 0.2 ml Hi-Tube Dome Cap (Cat. #NJ200)
- NucleoSpin® Tissue (Cat. #740952.10/.50/.250)
NOTICE TO PURCHASER: LIMITED LICENSE

[A1] PCR Notice
This product is covered by one or more of the following US patents and corresponding patent claims outside the US: 5,079,352, 5,789,224, 5,618,711, 6,127,155 and claims outside the US corresponding to US Patent No. 4,889,818. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product solely in Environmental Testing, Food Testing, Industrial Microbiology, including reporting results of purchaser’s activities for a fee or other commercial consideration, and also for the purchaser’s own internal research. No right under any other patent claim (such as the patented 5’ Nuclease Process claims in US Patents Nos. 5,210,015 and 5,487,972, and the dsDNA-binding dye process claims in US Patents Nos 5,994,056 and 6,171,785) is conveyed expressly, by implication, or by estoppel. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

[L15] Hot Start PCR
Licensed under U.S. Patent No. 5,338,671 and 5,587,287, and corresponding patents in other countries.

[L42] Campylobacter PCR Detection
This product is sold by Takara Bio Inc. under license from Fuso Pharmaceutical Industries, Ltd. and Osaka Prefecture University. This product is the subject of the pending US patent application and its foreign counterparts.

[M57] LA Technology
This product is covered by the claims 6-16 of U.S. Patent No. 5,436,149 and its foreign counterpart patent claims.

NOTE: This product is for research use only. It is not intended for use in therapeutic or diagnostic procedures for humans or animals. Also, do not use this product as food, cosmetic, or household item, etc. Takara products may not be resold or transferred, modified for resale or transfer, or used to manufacture commercial products without written approval from TAKARA BIO INC. If you require licenses for other use, please contact us by phone at +81 77 543 7247 or from our website at www.takara-bio.com.