Cat. # CY233

For Research Use

TaKaRa

# CycleavePCR™ *Streptococcus agalactiae* (GBS) Detection Kit

Product Manual

v201903Da

# Table of Contents

## I. Description

*Streptococcus agalactiae* (also known as Group B hemolytic *Streptococcus* or GBS) is a gram-positive bacteria that is part of the normal flora in the human genitourinary and gastrointestinal tracts. GBS can cause opportunistic infections including meningitis in the elderly and sepsis immediately after birth in newborns. Given the severity of these infections, reliable and fast screening methods are essential. GBS detection using culture methods is time-consuming and the detection sensitivity is low.

The CycleavePCR *Streptococcus agalactiae* (GBS) Detection Kit is designed for rapid detection of the GBS histidine kinase-encoding gene, *dltS*, by real-time PCR.

This kit enables amplification products to be detected by cycling probe technology, which provides highly sensitive detection through the combined use of a RNA/DNA chimeric probe and RNase H. This enables efficient detection of specific sequences of the gene fragment during and after amplification. One end of the probe is labeled with a fluorescent moiety and the other end with a quencher. When intact, this probe does not emit fluorescence, due to the action of the quencher. However, when it forms a hybrid with the complementary sequence of an amplification product, RNase H cleaves RNA in the chimeric probe, resulting in strong fluorescent signal emission (see Figure 1). The amount of amplified product can be monitored by measuring the intensity of emitted fluorescence.

This kit contains a FAM-labeled probe for detecting the GBS *dltS* gene, and a ROX-labeled probe for detecting the internal control. By simultaneously monitoring two wavelengths, *dltS* gene detection and false negative monitoring through detection of the internal control DNA can be achieved in a single tube. The kit relies on real-time PCR detection, which requires no electrophoresis and yields results quickly.

This kit includes *TaKaRa Ex Taq*® HS, a hot-start PCR enzyme, which prevents non-specific amplifications caused by mispriming or primer dimer formation during reaction mixture preparation or other pre-cycling steps. The use of *TaKaRa Ex Taq* HS therefore allows high-sensitivity detection.

This kit was developed under the supervision of Dr. Kimiko Ubukata, Department of Infectious Diseases, Keio University School of Medicine.

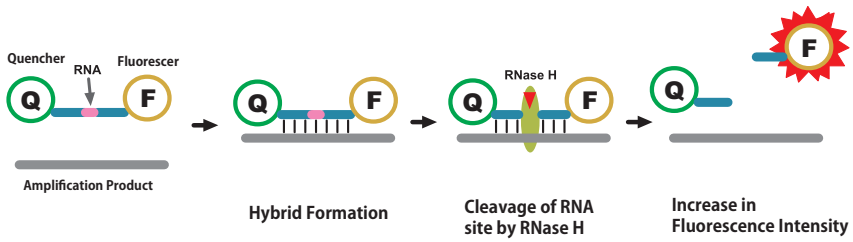



Figure 1. Principle of Cycling Probe Technology.

## II. Components (50 reactions, 25 μl volume)

| | | | |
|---|---|---|---|
| 1. | 2X Cycleave Reaction Mixture | 625 μl | |
| 2. | dltS Primer/Probe Mix (FAM, ROX)＊1 | 100 μl | |
| 3. | $dH_2O$ | 500 μl | |
| 4. | dltS Positive Control＊2 | 60 μl | (30 rxns) |

＊1 Contains a fluorescent-labeled probe; store protected from light.
＊2 To avoid contamination, store separately from components 1 - 3.

[Component Information]

**2X Cycleave Reaction Mixture**:
A PCR reagent containing enzymes, buffer, and a dNTP mixture.

**dltS Primer/Probe Mix (FAM, ROX):**
A mixture containing primers and probes for detecting the *dltS* gene and an internal control DNA.
The *dltS* gene (the target gene) or the internal control DNA is amplified with the appropriate primer. The FAM-labeled probe detects the *dltS* gene and the ROX-labeled probe detects the internal control DNA.

**Internal control DNA:**
The internal control DNA has no sequences related to the target gene. It functions to detect false negatives and is present in every reaction mixture supplied in this kit. When no target is detected, but the internal control DNA is detected, this indicates that there is no PCR inhibition and the concentration of target in the sample is below the detection limit. When neither the target nor the internal control DNA is detected, this indicates that the PCR reaction failed. When there is a large amount of the target DNA, the amplification of the target is prioritized, resulting in the delay, weakening, or absence of the internal control DNA signal. In such situations, the assay can be interpreted as positive.

**dltS Positive Control:** Positive control DNA for the *dltS* gene.

## III. Storage -20℃

## IV. Materials Required but Not Provided

[Real-time PCR]

- Real-time PCR instrument and tubes
  Thermal Cycler Dice™ Real Time System *III* (Cat. #TP900/TP960)*
  Thermal Cycler Dice Real Time System *Lite* (Cat. #TP700/TP760)*
  Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific), etc.
- Benchtop centrifuge
- 1,000-μl, 200-μl, 20-μl, and 10-μl micropipettes
- Micropipette tips (with hydrophobic filter)
- 0.2 ml 8-strip tube, individual flat caps (Cat. #NJ600)*

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

[Sample preparation]

- Nucleic acid extraction reagent (EXTRAGEN II (Tosoh, Co., Ltd.), etc.)
- Mutanolysin (Sigma-Aldrich, Cat. #M4782)

## V. Precautions

1. Operate real-time PCR amplification instruments in accordance with the manufacturer's instructions.
2. The chimeric probes and primers are susceptible to degradation by nuclease and, if degraded, cannot provide accurate detection. Take care to avoid nuclease contamination from such sources as perspiration or saliva introduced during sample handling.
3. Samples that test positive should be subjected to an additional microbiological test to verify the result.
4. We recommend designating and physically segregating three separate areas as described below for reaction mixture preparation, sample preparation, and performing detection reactions.

   Avoid opening/closing tubes containing amplification products in any of these areas.

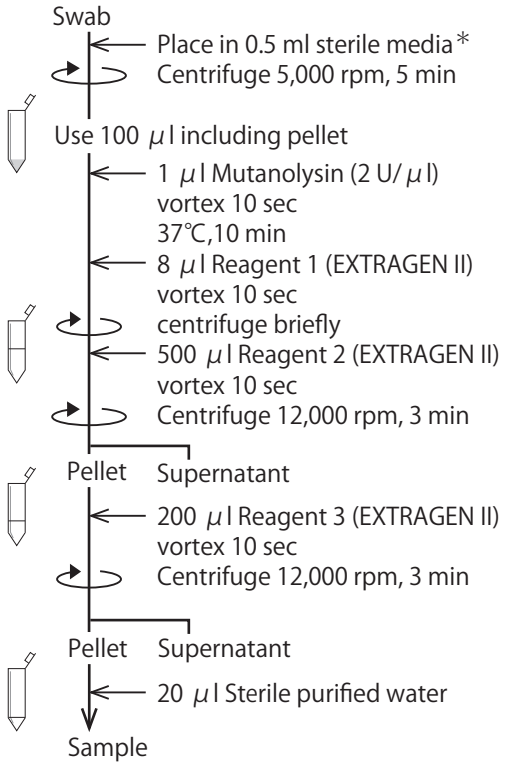
   Area 1: reaction mixture preparation and dispensing
   Area 2: sample preparation
   Area 3: addition of samples to reaction mixtures, reaction, and detection

   This kit allows amplification and detection to take place simultaneously in real time. Thus, no electrophoresis or other analytical methods are required after the reaction is complete. Never remove amplification products from tubes as doing so may introduce contamination.
5. Results obtained with this kit are analyzed using a real-time PCR amplification instrument. Failure of any of the auto functions on the real-time PCR amplification instrument may lead to erroneous analysis of results. Make sure that the settings on the real-time PCR amplification instrument are adjusted correctly, as described in the instrument manual.

## VI. Protocol

<Overview>

1. Sample Preparation (Work in Area 2)




2. Real-time PCR instrument setup
3. Reaction mixture preparation and reaction start

   Prepare the reaction mixture and dispense in tubes (Work in Area 1)

| Reagent | Volume |
|---|---|
| 2X CycleavePCR Reaction Mixture 🟡 | 12.5 µl |
| dltS Primer/Probe Mix (FAM, ROX) 🟤 | 2.0 µl |
| Sample, Positive Control, or $dH_2O$ | 2.0 µl |
| $dH_2O$ ○ | 8.5 µl |
| Total | 25 µl |

   Set the tubes in the real-time PCR instrument; start the reaction.

4. Results
5. Analysis of Results

＊ MUELLER HINTON BROTH, etc.

### VI-1. Sample Preparation (Work in Area 2)

Prepare template DNA from the sample using a reagent such as EXTRAGEN II (Tosoh, Co., Ltd.).

**Preparation example using EXTRAGEN II**

(1) Sterily place the sample swab in 0.5 ml of sterile media and suspend. Centrifuge at 5,000 rpm for 5 min. Remove 400 μl of the supernatant.

(2) Add 1 μl Mutanolysin (2 U/μl)* to the remaining 100 μl sample and vortex briefly to mix. Incubate for 10 min at 37℃.

* Preparing Mutanolysin: Dissolve Mutanolysin (Lyophilized, Sigma-Aldrich, Cat. #M4782) in sterile water to obtain a final concentration of 2 U/μl. Aliquot the solution and store at -20℃.

(3) Add 8 μl Reagent 1 (co-precipitation reagent), vortex for 10 sec to mix, and centrifuge briefly.

(4) Add 500 μl Reagent 2 (propanol, protein denaturant) and vortex for 10 sec to mix.

(5) Centrifuge at 12,000 rpm for 3 min. Discard the supernatant. Centrifuge briefly again and remove any residual supernatant.

(6) Add 200 μl Reagent 3 (propanol, potassium chloride) and vortex for 10 sec to mix.

(7) Centrifuge at 12,000 rpm for 3 min. Discard the supernatant. Centrifuge briefly again and remove any residual supernatant.

(8) Dissolve the pellet in 20 μl of sterile purified water. Keep sample on ice. If the sample will not be used immediately, store at -20℃.

**Note**: Use filtered tips for all steps.

### VI-2. Preparation of Reaction Mixture

This kit allows the simultaneous detection of amplification products from both the *dltS* gene and the internal control in a single reaction tube. **To obtain accurate detection results, perform the positive control reaction and the negative control reaction for the *dltS* gene simultaneously.**

* To maximize the reliability of your results, we recommend running at least 2 reactions per sample.

(1) Prepare the following reaction mixture on ice. (Work in Area 1)

Prepare a premix containing components other than the sample template in volumes sufficient for the required number of tubes plus a few extra. Dispense aliquots of 23 μl and cap loosely. Set up one of the tubes as a negative control by adding 2 μl of $dH_2O$ and capping the tube tightly.

The required number of tubes is defined as the number of samples + 2 (one for the negative control reaction and one for the positive control reaction).

| Reagent | Volume (1 reaction) | Final Conc. |
|---|---|---|
| 2X CycleavePCR Reaction Mixture 🟡 | 12.5 μl | 1X |
| dltS Primer/Probe Mix (FAM, ROX) 🟤 | 2.0 μl | 1X |
| Sample, Positive Control*1, or $dH_2O$ | (2.0 μl)*2 | |
| $dH_2O$ ○ | 8.5 μl | |
| Total | 25 μl | |

*1 For the positive control reaction, use dltS Positive Control 🔴.
*2 Add the sample or the positive control DNA in step (2), not in this step.

(Move to Area 3)

(2) Add sample (template). (Work in Area 3)

Add either the sample or the positive control to all tubes except the negative control tube and then cap tightly. Be sure to wear gloves.

Briefly centrifuge the tubes in a table top centrifuge and then place them in a real-time PCR instrument.

[Precaution]
Start reactions within 1 hour of preparing the reaction mixtures.

### VI-3. Amplification and Detection (Work in Area 3)

Operating procedures differ depending on the real-time PCR instrument used. For specific operating procedures, refer to the instrument manual.

**[ For the Thermal Cycler Dice Real Time System *II*]**

PCR Conditions
- Initial denaturation (Hold)
  - Cycle: 1
  - 95℃ 2 min
- 3-step PCR
  - Cycles: 40
  - 95℃ 10 sec
  - 50℃ 30 sec
  - 72℃ 20 sec (detection)

Detection Filter
- FAM
- ROX

Sample layout
- Internal Control Detection Filter
  - ROX
- Sample Types
  - Negative control
    - Sample Type: NC
  - Positive Control
    - Sample Type: PC
  - Test Sample
    - Sample Type: UNKN (unknown)

**[ For the Applied Biosystems 7500 Fast Real-Time PCR System, StepOnePlus Real-Time PCR System ]**

Use Quantification-Standard Curve mode. To change the default settings, use Advanced Setup.

PCR Conditions
- Initial Denaturation (Hold)
  - Cycle: 1
  - 95℃ 2 min
- 3-step PCR
  - Cycle: 40
  - 95℃ 10 sec
  - 50℃ 30 sec
  - 72℃ 25 sec＊(detection)

＊ Time varies depending on the real-time PCR device used. Refer to the instrument manual for reaction conditions.

Passive Reference
- none

Define Targets
- Target Name: dltS, Reporter: FAM, Quencher: (none)
- Target Name: IC, Reporter: ROX, Quencher: (none)

Define Samples
- Negative Control
  - Sample Type: NTC (No Template Control)
- Positive Control
  - Sample Type: Standard or Unknown
- Test Sample
  - Sample Type: Unknown

**Note:** The same procedure can be used for the StepOnePlus Real-time PCR System. However, since the detection sensitivity for ROX is low, the ROX (IC) amplification curve will appear small when all targets are displayed simultaneously. In order to analyze the ROX and FAM curves, display them separately.

## VII. Analysis of Results

Samples tested:

**Note:** Perform a final analysis of the assay based on all results including those of the control reactions.

| | | ROX (internal control) | |
|---|---|---|---|
| | Amplification Signal | (+) | (−) |
| FAM (dltS) | (+) | *dltS* positive＊1 | *dltS* positive＊1 |
| | (−) | Below the limit of detection＊2 | Inconclusive＊3 |

**Note:** If the Ct is 33 cycles or later, confirm specific target gene amplification by agarose gel electrophoresis. When performing electrophoresis, be careful to avoid contamination of the PCR products.

【 Amplification product size 】

*dltS* from samples: 199 bp
dltS Positive Control: 147 bp
Internal control: 134 bp

Positive Control reaction (using the dltS Positive Control)

| | | ROX (internal control) | |
|---|---|---|---|
| | Amplification Signal | (+) | (−) |
| FAM (dltS) | (+) | No problem with the reaction | No problem with the reaction |
| | (−) | Problem with the reaction＊4 | Inconclusive＊3 |

Negative Control reaction (using $dH_2O$)

| | | ROX (internal control) | |
|---|---|---|---|
| | Amplification Signal | (+) | (−) |
| FAM (dltS) | (+) | Possible contamination in the reaction＊5 | Possible contamination in the reaction＊5 |
| | (−) | No contamination in the reaction | Inconclusive＊3 |

＊1 Regardless of the results of the internal control DNA detection (+)/(−), *dltS* is positive. Verify the absence of contamination in the reaction system based on the result of the negative control reaction.

＊2 Verify that the result is (+) for the Positive Control detection (indicating no problem with the reaction).

＊3 The PCR reaction or the cycling probe detection failed. Perform the reaction again. Because the sample may contain a reaction inhibitor, it may be necessary to prepare a fresh sample.

＊4 Either there is a problem with the dltS Primer/Probe Mix, or the dltS Positive Control is degraded.

＊5 Decontaminate the bench area and apparatuses used for preparing reaction mixtures.

## VIII. Troubleshooting

- When the FAM filter (target gene detection) shows an amplification curve in the negative control reaction (NC).
  → Contamination may have occurred. Decontaminate the bench area used to prepare reaction mixtures as well as the apparatuses and instruments used. Then perform the reaction again.
- When both the FAM filter and the ROX filter (internal control detection) show no amplification curve in the positive control reaction (PC).
  → The PCR reaction or the cycling probe detection failed. Repeat the reaction.
- When the ROX filter shows an amplification curve but the FAM filter does not show an amplification curve in the positive control reaction (PC).
  → There may be a problem with the primer/probe mix or the positive control may have been degraded.
- When both the FAM filter and the ROX filter do not show an amplification curve in the sample reaction (UNKN).
  → The PCR or the cycling probe detection failed. Repeat the reaction.
  The sample may contain a reaction inhibitor. Perform the reaction again with diluted samples. Alternatively, prepare a fresh sample and then perform the reaction again.
- When, in the sample reaction (UNKN), the FAM filter yielded an amplification curve but the ROX filter did not.
  → When there is a large amount of target DNA, the amplification of the target may be prioritized, resulting in the competitive inhibition of the amplification reaction for the internal control DNA.
  The detection system performed properly.

## IX. Related Products

CycleavePCR™ GBS Capsular Typing Kit (Cat. #CY234)
Thermal Cycler Dice™ Real Time System *II* (Cat. #TP900/TP960)*
Thermal Cycler Dice™ Real Time System *Lite* (Cat. #TP700/TP760)*
0.2 ml 8-strip tube, individual Flat Caps (Cat. #NJ600)
96 well Hi-Plate for Real Time (Cat. #NJ400)
Sealing Film for Real Time (Cat. #NJ500)
48 well snap plate (Cat. #NJ700)
Flat cap for snap plate (Cat. #NJ720)
Plate Sealing Pads (Cat. #9090)

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