

Cat. # RR240A

For Research Use

TAKARA

**Bacteria (*tuf* gene)
Quantitative PCR Kit**

Product Manual

v202203Da

Table of Contents

I.	Description.....	3
II.	Components	4
III.	Storage	4
IV.	Materials Required but not Provided	5
V.	Precautions for Use.....	5
VI.	Precautions	6
VII.	Protocol.....	7
VIII.	Analysis.....	11
IX.	Appendix	13
X.	References.....	15
XI.	Related Products	15

I. Description

The Bacteria (*tuf* gene) Quantitative PCR Kit is intended for the quantification of bacterial number using real-time PCR. This kit can generate results more quickly than conventional methods that require 2 - 4 days of culture, and can be used for bacterial detection in the food safety and environmental research fields. Furthermore, with conventional methods, differences in optimal culture conditions for different bacteria make it necessary to use specialized culture conditions and/or media to detect various types of bacteria present in the specimen ¹⁻³. This kit allows the total number of a wide variety of bacterial strains to be measured without any specialized culture conditions.

Target Genes

PCR detection of general bacterial strains using the 16S rRNA gene is possible. But this analysis is not suitable for precise quantification of bacterial number because the copy number of 16S rRNA varies among bacterial strains. The target gene of this kit is the protein elongation factor Tu (*tuf*) gene. The *tuf* gene has a high degree of conservation among various bacterial strains, and has a chromosomal copy number of one or two ⁴.

Real-Time PCR

Real-time PCR is a method of gene detection that monitors incorporation of fluorescent moieties in real time during the PCR amplification process. This intercalator method offers excellent speed and quantitative performance. The TB Green® *Premix Ex Taq*™ GC reagent that is included with this kit uses TB Green as an intercalator for detection and is capable of efficiently amplifying sequences with high GC content, making it suitable for detection of the *tuf* gene in a wide range of strains.

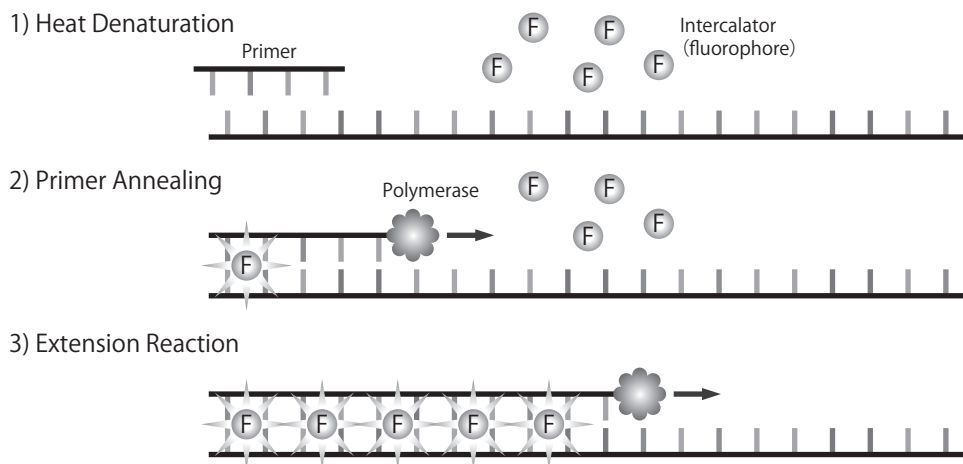


Figure 1. Fluorescent intercalator detection method.

The intercalator fluoresces when incorporated into dsDNA during PCR amplification.

Note: Drs. Bon Kimura and Hajime Takahashi of Tokyo University of Marine Science and Technology and Dr. Yuichiro Tanaka of TOYO SUISAN KAISHA, LTD. participated in the development of this kit.

II. Components (25 μ l volume, 100 Reactions)

TB Green <i>Premix Ex Taq</i> GC*1	2X conc.	625 μ l x 2
TUF Primer Mix	5X conc.	250 μ l x 2
dH ₂ O		1 ml
ROX Reference Dye* 2	50X conc.	50 μ l
ROX Reference Dye II* 2	50X conc.	50 μ l
TUF Positive Control	1 x 10 ⁵ copies/ μ l	100 μ l
EASY Dilution (for Real Time PCR)		1 ml x 2

*1 Includes *TAKARA Ex Taq*[®] HS, dNTP Mixture, Mg²⁺, and TB Green.

*2 This component is to be used for analyses using a device that corrects fluorescent signals between wells, such as the real-time PCR devices by Applied Biosystems.

- ◆ Use the ROX Reference Dye
 - Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)
- ◆ Use the ROX Reference Dye II
 - Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific)
- ◆ Do not use this component
 - Thermal Cycler Dice™ Real Time System II (Cat. #TP900/TP960: discontinued)
 - Thermal Cycler Dice Real Time System *Lite* (Cat. #TP700/TP760: discontinued)

III. StorageTB Green *Premix Ex Taq* GC (Package 1)

4°C (stable for 6 months)

-80°C (for long-term storage)

Note: Avoid storage at -20°C. Once the product is thawed, store at 4°C and use within 6 months.

Please be sure to shield from light during storage. Furthermore, be aware of the risk of contamination when storing at 4°C.

Other Components (Package 2)

Store at -20°C

IV. Materials Required but not Provided

<Preparation of Sample Suspensions>

Dilution solution (0.1% peptone in physiological saline, etc. based on the specimen type)
Stomacher and Stomacher bag

<DNA Extraction>

NucleoSpin Tissue (Cat. #740952.10/.50/.250)*
Ethanol (> 99%)
Heat block (Set to 56°C and 70°C)
Micropipette
Micropipette tips (with hydrophobic filters)
High-speed microcentrifuge
20 mg/ml lysozyme in 20 mM Tris-HCl, 2 mM EDTA, 1% Triton X-100 (pH 8.0)

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

<Real-Time PCR>

Amplification equipment for real-time PCR and tubes
Thermal Cycler Dice Real Time System // (Cat. #TP900/TP960: discontinued)
Thermal Cycler Dice Real Time System *Lite* (Cat. #TP700/TP760: discontinued)
Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific)
StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)
Benchtop Centrifuge

V. Precautions for Use

These are precautions for using this kit. Be sure to read before use.

1. Intended Use : This kit is a product for use in food and environmental testing. This is intended for research use only.
2. Results of Assay : This product is designed for detection of the target gene DNA, and detects not only living bacteria but also inactivated bacteria. The target gene may not be detected when there is mutation, deletion, or insertion in the genomic sequences correspond with the primers. (Takara Bio is not responsible for any actions taken as a result of analytical determinations made with this product.)
3. Disposal : Samples should be handled according to regulations governing use of potentially infectious materials. Dispose of materials according to the safety regulations for your facility and in accordance with any applicable local, state, or federal regulations. Keep the work area sanitized at all times and sterilize all samples and equipment used during the experiment. Sterilization may be conducted by autoclaving at 121°C for at least 20 min or by treatment with 2.5% sodium hypochlorite, followed by processing materials according to guidelines for potentially infectious waste. Process and dispose of plastic and filter paper reagent containers and instruments according to regulations concerning the handling of hazardous materials.

VI. Precautions

1. TB Green *Premix Ex Taq* GC contains enzymes. Before use, make sure the reagent is evenly mixed by gently inverting several times without creating bubbles; if inadequately mixed, it may not provide sufficient reactivity. Do not mix by vortexing.

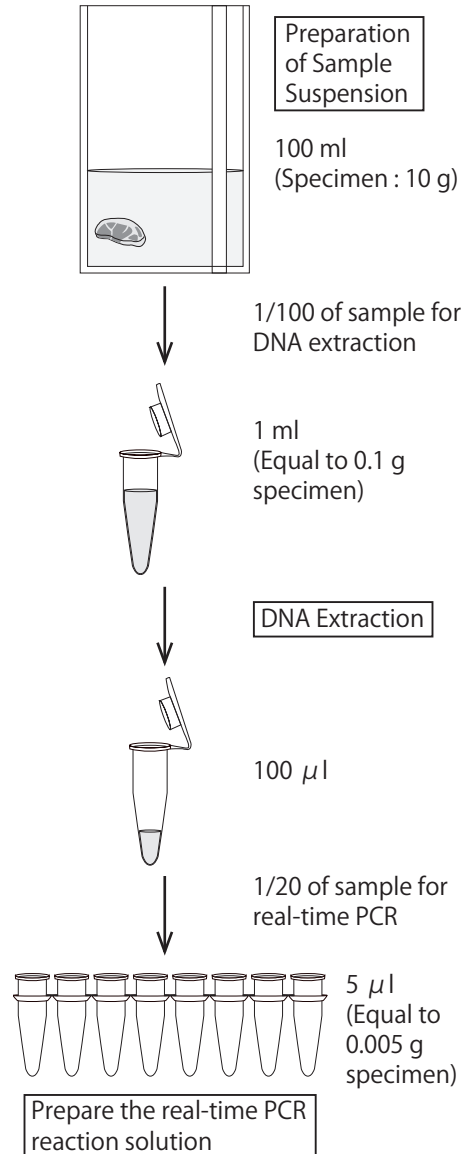
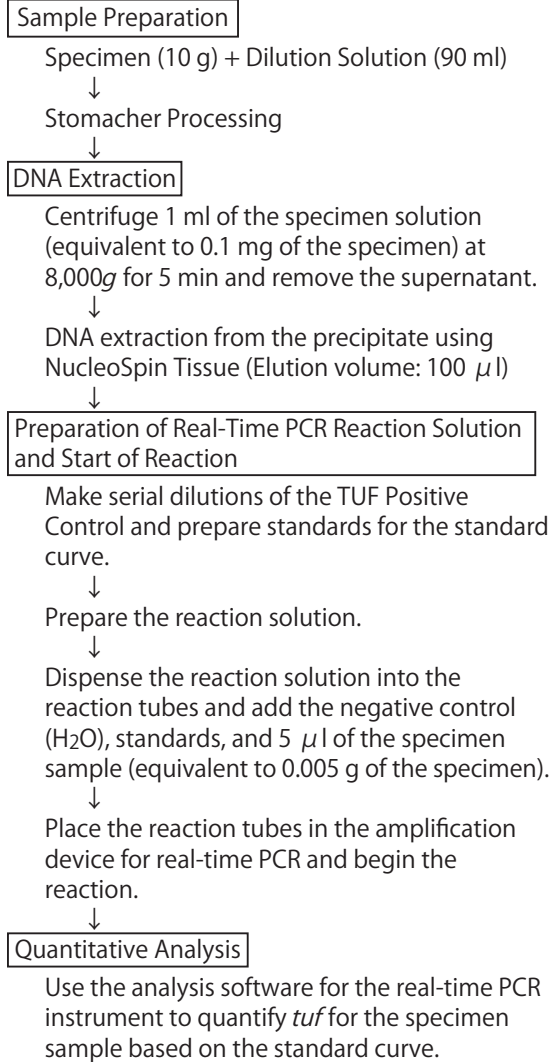
Please note that a white to yellowish-white precipitate may form when TB Green *Premix Ex Taq* GC is stored at -80°C. This will dissolve completely when the solution is hand warmed gently or when it is left at room temperature for a short period (protect from light) and then mixed by inversion. Failure to redissolve the precipitate may result in unevenness of the reagent components; be sure to mix until homogenous before use.

2. Place reagents on ice when preparing the reaction solution.
3. TB Green *Premix Ex Taq* GC includes TB Green. Do not to expose to strong light during preparation of the reaction solution.
4. We recommend that the three following areas be established from preparation of reaction mixture to addition of samples in the laboratory working space and be physically separated from each other. 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

This kit allows the amplification reaction and detection to take place in real time. Therefore, the amplification products require no electrophoresis or other treatment after the reaction is complete. Never remove amplification products from tubes, as doing so may cause nucleic acid contamination in the laboratory workspace.

VII. Protocol

Overview



VII-1. Preparation of the Sample Suspension (Work in area 2)

1. Weigh 10 g of the specimen and transfer to a Stomacher bag, etc.
2. Add 90 ml of dilution solution (such as 0.1% peptone in physiological saline) (equivalent to 9 times the specimen).
3. Homogenize as necessary using a Stomacher, etc. and use as the specimen solution for DNA extraction.

VII-2. DNA Extraction (Work in area 2)

Perform DNA extraction according to the NucleoSpin Tissue protocol for bacteria (Gram-positive bacteria) and elute in 100 μ l.

1. Dispense 1 ml of the specimen solution into a 1.5 ml microtube.
2. Centrifuge for 5 min at 8,000g and remove the supernatant.
3. Suspend the pellet in 180 μ l of 20 mg/ml lysozyme in 20 mM Tris-HCl, 2 mM EDTA, 1% Triton X-100 (pH 8.0)*¹ and incubate for 30 - 60 min at 37°C.
4. Add 25 μ l of Proteinase K*² and incubate at 56°C for 1 - 3 hours (or overnight) until completely dissolved.
5. Agitate the sample. Add 200 μ l of Buffer B3, agitate strongly, and then incubate for 10 min at 70°C. If undissolved material remains, centrifuge for 5 min at 11,000g and transfer the supernatant to a new tube.
6. Add 210 μ l of ethanol (> 99%) to the supernatant and mix well.
7. Place the NucleoSpin Tissue Column in the Collection Tube. Transfer the above solution to the Column and centrifuge for 1 min at 11,000g. After discarding the filtrate, place the Column in the same Collection Tube.
8. 1st Wash: Add 500 μ l of Buffer BW to the Column and centrifuge for 1 min at 11,000g. After discarding the filtrate, place the Column in the same Collection Tube.
2nd Wash: Add 600 μ l of Buffer B5*³ to the Column and centrifuge for 1 min at 11,000g. After discarding the filtrate, place the Column in the same Collection Tube.
9. Centrifuge the Column for 1 min at 11,000g.
10. Place the Column in a 1.5 ml microtube (provided by the user). Add 100 μ l of Buffer BE that has been heated to 70°C, incubate for 1 min at room temperature, and centrifuge for 1 min at 11,000g.
Store the eluted DNA solution at 4°C and use as the template sample for real-time PCR. For long-term storage, store at -20°C, and avoid repeated freezing and thawing.

*1 Not included in NucleoSpin Tissue.

*2 Preparation of Proteinase K solution

In the case of NucleoSpin Tissue Cat. #740952.10:

Add 260 μ l of Proteinase Buffer PB to 6 mg of Proteinase K (lyophilized) and dissolve completely. Store the prepared Proteinase K solution at -20°C (stable for 6 months).

*3 Preparation of Buffer B5 solution

In the case of NucleoSpin Tissue Cat. #740952.10:

Add 16 ml of ethanol (96 - 100%) to 4 ml of Wash Buffer B5 (concentrate).

VII-3. Real-Time PCR

1. Preparation of the samples for the standard curve (Work in area 3)

Make serial dilutions of the TUF Positive Control using EASY Dilution to obtain the samples for the standard curve.

- (1) 1×10^5 copies/ μ l (TUF Positive Control stock solution)
- (2) 1×10^4 copies/ μ l (5 μ l TUF Positive Control stock solution + 45 μ l EASY Dilution)
- (3) 1×10^3 copies/ μ l (5 μ l of 1×10^4 copies/ μ l solution + 45 μ l EASY Dilution)
- (4) 1×10^2 copies/ μ l (5 μ l of 1×10^3 copies/ μ l solution + 45 μ l EASY Dilution)

Using these samples, it is possible to obtain quantitative values in terms of copy number of *tuf* gene per gram of specimen from the real-time PCR data. (Refer to the section "VIII. Analysis" for the calculation methods.)

Table 1. Comparison of Copies/Reaction and Copies/g specimen

	copies/reaction (Copy Number per Reaction)	copies/g specimen (Copy Number per Gram of Specimen)
1	5×10^5	1×10^8
2	5×10^4	1×10^7
3	5×10^3	1×10^6
4	5×10^2	1×10^5

2. Preparation of the Reaction Solution (Work in area 1)

Prepare the reaction mixture on ice.

Prepare enough for the required number of tubes plus some extra, dispense 20 μ l into each PCR reaction tube, and close the caps lightly. Add 5 μ l of sterile purified water in place of the DNA sample to one tube as a negative control and close the cap tightly.

[For the Thermal Cycler Dice Real Time System]

Reagent	(per reaction)
TB Green <i>Premix Ex Taq</i> GC (2X conc.)	12.5 μ l
TUF Primer Mix (5X conc.)	5.0 μ l
dH ₂ O	2.5 μ l
DNA sample or standard curve sample	(5.0 μ l)*2
Total	25.0 μ l

[For Applied Biosystems Real-Time PCR Instruments]

Reagent	(per reaction)
TB Green <i>Premix Ex Taq</i> GC (2X conc.)	12.5 μ l
TUF Primer Mix (5X conc.)	5.0 μ l
ROX Reference Dye or ROX Reference Dye II*1	0.5 μ l
dH ₂ O	2.0 μ l
DNA sample or standard curve sample	(5.0 μ l)*2
Total	25.0 μ l

*1 ROX Reference Dye is used for the StepOnePlus, and ROX Reference Dye II is used for the 7500 Fast Real-Time PCR System.

*2 The DNA sample and standard curve samples should be added in step 3, NOT in this step.

[Caution] Do not touch tubes and caps with bare hands; doing so may introduce noise during detection of fluorescent signal.

3. Addition of the Sample (DNA Solution) (Work in area 3)
Add 5 μ l of sample (specimen DNA sample, standard curve samples, etc.) to the PCR reaction solution and close the caps tightly. Centrifuge briefly in a benchtop centrifuge and place in the real-time PCR amplification instrument.
4. Real-Time PCR Reaction
Perform the following PCR conditions.
 - * Please refer to the instruction manual for the real-time PCR amplification instrument for specific operating procedures.

Initial Denaturation

95°C 30 sec

2 Step PCR

35 cycles

95°C 5 sec

60°C 30 sec (Detection of Fluorescence: FAM)

Melt curve analysis

Note: The sequence of PCR products amplified using samples and the TUF Primer Mix may differ from that of the TUF Positive Control. In dissociation analysis of real-time PCR, the T_m values of the PCR products differ between the sample and the TUF Positive Control. The copy number can also be correctly calculated under these conditions.

VIII. Analysis

VIII-1. Principles of Quantification

TUF Positive Control

The TUF Positive Control included in this kit is plasmid DNA that contains the *tuf* gene region and is adjusted to 1×10^5 copies/ μ l (based on the OD₂₆₀). When serially diluted, it can be used to obtain a standard curve and quantify experimental samples.

Note : This kit detects both viable and non-viable bacteria. If you need to detect only viable bacteria, perform a culture test as well.

Calculation of the Copy Number per Gram of Specimen

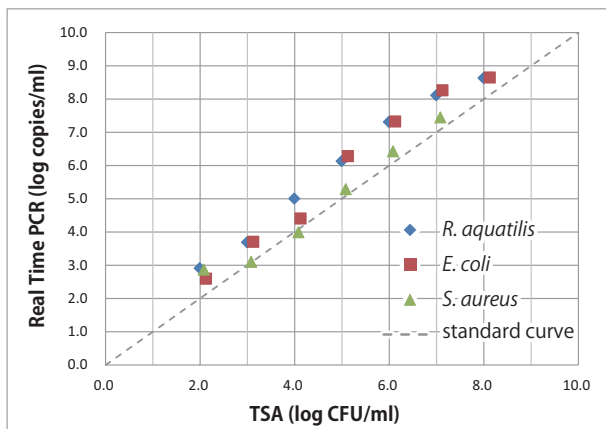
When this product is used according to the instructions, an amount equivalent to 0.005 g of the specimen is used for a real-time PCR reaction, and the value of *tuf* gene copies/reaction can be multiplied by 200 to obtain the copy number of *tuf* gene per gram specimen.

- 10 g of the specimen + 90 ml of dilution solution
- ↓ Stomacher treatment
- 1 ml of the specimen solution (equivalent to 0.1 g of the specimen)
- ↓ DNA extraction
- 100 μ l of the DNA solution (equivalent to 0.1 g of the specimen)
- ↓ 1/20 to real-time PCR
- 5 μ l per reaction used in real-time PCR (equivalent to 0.005 g of the specimen)

VIII-2. Relationship between the Gene Copy Number and Bacterial Cell Number

Cultured Bacteria

Rahnella aquatilis, *Escherichia coli*, and *Staphylococcus aureus* bacterial strains were cultured overnight in TSB media and each was serially diluted using physiological saline. Viable cell number was determined by culturing on TSA agar plates. Additionally, 1 ml of the bacterial suspensions were analyzed by real-time PCR analysis according to the instructions for this kit.



The cell number on TSA plates (colony forming units: CFU) are plotted on the X-axis, and the gene copy number obtained from real-time PCR are plotted on the Y-axis. There is good correlation between bacterial cell number and gene copy number. While there was a tendency for the copy number calculated to be somewhat high, the difference was limited to no more than one order of magnitude.

Figure 2. The relationship between *tuf* gene copy number and bacterial cell number.

Food Specimens

10% suspensions of 9 vegetables, 6 salads (including cut vegetables), and 8 meats were prepared. Measurement of cell number using TSA plate cultures (30°C, 48 hours) and real-time PCR analysis using this kit were carried out.

Table 2. Gene Copy number and cell number (food specimens)

	qPCR	TSA
	[log copies/g]	[log CFU/g]
Cabbage	1.51	0.90
Mizuna	3.38	3.20
Sprouts	4.61	3.30
Bok Choy	6.63	6.57
Cabbage	7.08	5.60
Lettuce	7.27	6.33
Mizuna	8.02	7.24
Sprouts	8.29	7.07
Mitsuba	8.65	7.10
Shredded Cabbage	2.66	2.48
Cut Lettuce	3.27	2.96
Cut Lettuce	5.09	4.41
Mixed Vegetable Salad	6.55	5.39
Green Salad	7.13	6.21
Mixed Salad	8.20	6.53
Beef	1.55	1.99
Pork	3.50	2.78
Sliced Pork	4.86	5.15
Ground Meat	5.47	4.89
Ground Chicken	5.53	4.17
Pork Loin	6.08	5.41
Chicken Thigh	6.20	4.97
Sliced Beef	6.28	6.25

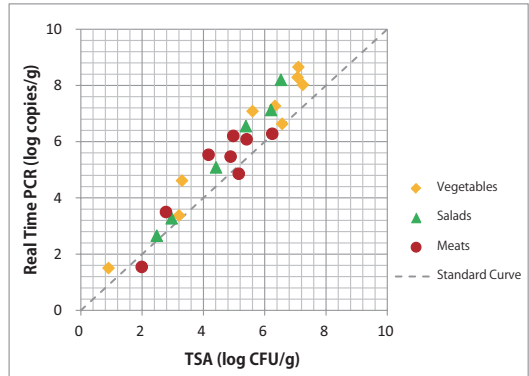


Figure 3. Relationship between *tuf* copies and cell number in food specimens.

The results from TSA cultures (CFU/g) are plotted on the X-axis, while the results of real-time PCR (copies/g) are plotted on the Y-axis. There is good correlation between the results from the cultures and real-time PCR. There was a tendency for the copy number to be 1 - 2 orders of magnitude higher than the bacterial cell number. This may be due to the presence of dead cells and/or bacterial strains that are difficult to detect by culturing.

IX. Appendix

Detectable strains

Bacterial strains that have been detected with this kit are listed in Table 3. These strains showed a Ct value of 20 or less when real-time PCR was performed using 10 ng of purified genomic DNA as the template ⁴⁾.

Table 3. Detectable bacterial strains

Bacterial strain ^a	Strain	Source	Accession number	<i>tuf</i> -qPCR amplification	Ct ^b
Gram negative					
Bacteroidetes					
<i>Chryseobacterium formosense</i>	FI55	Chub mackerel	AB472770	+	16.0
<i>Flavobacterium hercynium</i>	FI48	Spotted mackerel	AB472772	+	17.5
<i>Flavobacterium johnsoniae</i>	JCM 8514		AB472806	+	18.0
<i>Sejongia antarctica</i>	FI18	Spotted mackerel	AB472786	+	17.3
<i>Sphingobacterium kitahiroshimense</i>	FI23	Stone flounder	AB472789	+	16.9
γ-Proteobacteria					
<i>Acinetobacter baumannii</i>	JCM 6841		AB472793	+	15.8
<i>Acinetobacter baumannii</i>	FI63	Flatfish (meita)	AB472767	+	14.3
<i>Aeromonas hydrophila</i>	JCM 1027		AB472795	+	16.5
<i>Aeromonas molluscorum</i>	FI56	Horse mackerel	AB472768	+	15.0
<i>Alteromonas macleodii</i>	JCM 20772		AB472797	+	16.7
<i>Citrobacter freundii</i>	IAM 12471		AB472801	+	15.4
<i>Colwellia aestuarii</i>	FI04	Chub mackerel	AB472771	+	14.1
<i>Enterobacter aerogenes</i>	IAM 1183		AB472802	+	15.8
<i>Erwinia carotovora</i>	IAM 12633		AB472804	+	16.1
<i>Escherichia coli</i>	IAM 1137		AB472805	+	14.2
<i>Klebsiella pneumoniae</i>	IAM 1063		AB472808	+	15.3
<i>Morganella morganii</i>	ATCC 35200		AB472816	+	15.9
<i>Photobacterium phosphoreum</i>	FI59	Horse mackerel	AB472777	+	14.7
<i>Proteus mirabilis</i>	JCM 1669		AB472817	+	17.5
<i>Pseudoalteromonas haloplanktis</i>	FI01	Spotted mackerel	AB472780	+	14.9
<i>Pseudomonas fluorescens</i>	FI28	Stone flounder	AB472781	+	15.8
<i>Psychrobacter immobilis</i>	ATCC 43116		AB472818	+	16.8
<i>Psychromonas arctica</i>	FI26	Chub mackerel	AB472783	+	16.0
<i>Rahnella aquatilis</i>	JCM 1683		AB472819	+	15.9
<i>Raoultella planticola</i>	JCM 7251		AB472820	+	17.5
<i>Salmonella serover Typhimurium</i>	ATCC 13311		AB472822	+	15.1
<i>Schineria larvae</i>	FI13	Spotted mackerel	AB472788	+	15.0
<i>Serratia marcescens</i>	IAM 1104		AB472823	+	16.2
<i>Shewanella frigidimarina</i>	FI20	Spotted mackerel	AB472787	+	15.5
<i>Shewanella japonica</i>	JCM 21433		AB472824	+	15.8
<i>Shewanella putrefaciens</i>	NBRC 3908		AB492873	+	14.4
<i>Vibrio diazotrophicus</i>	FI52	Horse mackerel	AB472791	+	16.2
<i>Vibrio parahaemolyticus</i>	ATCC 17802		AB472827	+	16.3
<i>Xanthomonas euvesicatoria</i>	FI22	Chub mackerel	AB472792	+	15.5

<i>Xanthomonas oryzae</i>	JCM 20241		AB472828	+	19.6
<i>Yersinia enterocolitica</i>	ATCC 9610		AB472829	+	15.9
<i>α-Proteobacteria</i>					
<i>Paracoccus denitrificans</i>	FI34	Spotted mackerel	AB472779	+	17.0
<i>β-Proteobacteria</i>					
<i>Alcaligenes faecalis</i>	JCM 20522		AB472796	+	17.9
<i>Burkholderia caledonica</i>	JCM 21561		AB472799	+	16.3
Gram positives					
Bacilli					
<i>Aerococcus sanguinicola</i>	JCM 11549		AB472794	+	18.4
<i>Bacillus subtilis</i>	IAM 1076		AB472798	+	16.8
<i>Brochothrix thermosphacta</i>	NBRC 12167		AB492875	+	15.3
<i>Carnobacterium divergens</i>	JCM 5816		AB472800	+	15.7
<i>Carnobacterium maltaromaticum</i>	NBRC 15684		AB492874	+	16.2
<i>Enterococcus faecalis</i>	JCM 5803		AB472803	+	14.8
<i>Lactococcus lactis</i>	NRIC 1174		AB472811	+	18.1
<i>Leuconostoc carnosum</i>	JCM 9695		AB472812	+	17.9
<i>Listeria monocytogenes</i>	ATCC 15113		AB472813	+	18.4
<i>Planomicrobium chinense</i>	FI41	Chub mackerel	AB472778	+	16.0
<i>Staphylococcus aureus</i>	ATCC 12600		AB472826	+	16.1
<i>Staphylococcus pasteurii</i>	FI64	Flatfish (meita)	AB472790	+	16.0
Actinobacteria					
<i>Rothia dentocariosa</i>	JCM 3067		AB472821	+	16.1
<i>Rothia nasimurium</i>	FI43	Horse mackerel	AB472784	+	18.2
<i>Salinibacterium amurskyense</i>	FI36	Flatfish (meita)	AB472785	+	17.2

- a : Classification was based on the National Center for Biotechnology Information taxonomy.
b : The cycle threshold value (Ct) for the reaction containing 10 ng of genomic DNA per reaction.

Note: It should be possible to detect additional bacterial strains based on *tuf* gene sequence information. 10,855 gene sequences named 'tuf' or 'elongation factor' were extracted from 1561 bacterial genome sequences registered in GenBank (as of November 21, 2011), and those with 100% matches for this product's primer sequence are listed. The list is available on the product webpage.
https://www.takara-bio.co.jp/research/cycleave/elongation_factor.xlsx

X. References

- 1) Takeo Fujii. Viable Cell Measurement Methods for Aquatic Foods I: Culture Medium Components, Culture Temperature and Plating Methods. Tokai Suiken Jo. (1989) **118**: 71-79. (Japanese)
- 2) Tsubasa Fukuda, Manabu Furushita, and Tusneo Shiba. A Comparison of Viable Cell Counts in Fresh Fish with the Official 35°C Culture Method and the 20°C Cell Culture Method. *Journal of National Fisheries University*. (2012) **60**:183-188. (Japanese)
- 3) Masataka Satomi, Kan Oikawa, and Yutaka Yano: Fisheries Science Series 141: The Quality and Freshness of Seafood and Advanced Preservation Techniques (Edited by Jun'ichi Nakazoe and Hideaki Yamanaka) 6. Microbiological Quality Evaluation. (Japanese)
- 4) Tanaka Y, Takahashi H, Simidu U, and Kimura B. Design of a new universal real-time PCR system targeting the *tuf* gene for the enumeration of bacterial counts in food. *J Food Prot.* (2010) **73**: 670-679.

XI. Related Products

TB Green® *Premix Ex Taq*™ GC (Perfect Real Time) (Cat. #RR071A/B)*
NucleoSpin Tissue (Cat. #740952.10/.50/.250)*
Enterobacteriaceae (*rplP* gene) Quantitative PCR Kit (Cat. #RR241A)*

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

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Premix Ex Taq and Thermal Cycler Dice are trademarks of Takara Bio Inc.

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