

Cat. #RR062A

For Research Use

TAKARA

Multiplex PCR Assay Kit Ver.2

Product Manual

v201510Da

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I. Description

Multiplex PCR allows simultaneous amplification of more than one PCR product by inclusion of multiple primer pairs in one PCR reaction. In addition to consuming fewer reagents, multiplex PCR facilitates time savings by simultaneous product amplification and makes it possible to use valuable samples efficiently.

The Multiplex PCR Assay Kit Ver.2 includes enzymes for high-speed priming and buffer composition optimized to increase the specificity of primer annealing. When compared to conventional multiplex PCR, this kit facilitates rapid, highly specific PCR amplification with minimal sequence-specific amplification bias. Depending on enzyme quantity and thermal cycling conditions, multiplex PCR with approximately 200 primer pairs may be performed. If desired, this kit can also be used for non-multiplex PCR amplification using otherwise problematic primer pairs that frequently generate extra bands, smears, or fail to amplify the target fragment, because the Multiplex PCR Assay Kit Ver.2 promotes higher specificity while requiring less experimental time for optimization of PCR conditions.

II. Components (100 reactions, 50 μ l volume)

2X Multiplex PCR Buffer (Mg ²⁺ , dNTP plus)*	1.25 ml x 2
Multiplex PCR Enzyme Mix	25 μ l

*: Contains the buffer and dNTP mixture required for the reaction. (Final Mg²⁺ concentration is 2 mM.)

III. Storage -20°C

IV. Precautions

These are precautions for the use of this kit. **Read prior to using the kit.**

- (1) When preparing PCR reactions, prepare sufficient master mix (including 2X Multiplex PCR Buffer, Multiplex PCR Enzyme Mix, and sterile water) for several reactions (as many as 10). Use of a master mix minimizes experimental variability by decreasing volume loss during pipetting, decreasing repetitive pipetting steps, preventing agitation of enzyme solutions, and allowing accurate dispensing of reagents.
- (2) Mix the 2X Multiplex PCR Buffer (Mg²⁺, dNTP plus) and Multiplex PCR Enzyme Mix gently to avoid foaming. Do not vortex.
Prior to pipetting, centrifuge briefly to collect reagents at the bottom of the tubes. Multiplex PCR Enzyme Mix is highly viscous (50% glycerol solution). Pipette slowly and carefully.
- (3) Store the 2X Multiplex PCR Buffer (Mg²⁺, dNTP plus) and Multiplex PCR Enzyme Mix at -20°C until needed. Return to -20°C immediately.
- (4) When dispensing reagents, use a fresh disposable tip and take care to prevent cross-sample contamination.
- (5) The PCR conditions given in this manual are for the TaKaRa PCR Thermal Cycler Dice™*. Optimal PCR conditions may differ for other instruments.

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

V. Designing Primers for Multiplex PCR

- Minimize the difference in the Tm values between primers.
- Maintain target length at or below 2 kb.
- Primers should be 22 - 30 nt in length with 50 - 60% GC composition. Avoid inclusion of GC- or AT-rich regions.
- Prior to use with this kit, test primer sets to confirm performance, including specificity and ability to amplify the intended target.

VI. Protocol

VI-1. Multiplex PCR

- (1) Prepare the following reaction solution in a 0.2 ml tube.

	Volume
2X Multiplex PCR Buffer (Mg ²⁺ , dNTP plus)	25 μl
Primers*1	As needed
Multiplex PCR Enzyme Mix	0.25 μl
Template DNA	As needed
dH ₂ O	to 50 μl

*1 : Typically, each primer is used at a final concentration of 0.2 μM. Optimizing primer concentration may improve results.
Primer Tm values should be 55°C or higher. Calculate Tm values using the following formula:

$$Tm = 4 \times (nG + nC) + 2 \times (nA + nT) *2 + 35 - 2 \times (\text{Total Nucleotide Number})$$

*2: n = number of each nucleotide

Note: If calculating Tm value based on the Nearest Neighbor method instead of the formula above, primer Tm values should be 60°C or higher.

- (2) Set tubes in the thermal cycler and perform PCR using the following conditions:

Thermal cycling condition for ≥ 10 primer pairs and targets < 2 kb long:

94°C,	60 sec.	
↓		
94°C,	30 sec.	}
57 - 60°C,	30 - 60 sec.	
72°C,	30 - 60 sec.	
↓		
72°C,	10 min.	

Thermal cycling conditions for > 10 primer pairs:

94°C,	60 sec.	
↓		
94°C,	30 sec.	}
60°C,	1 - 10 min.*3	
↓		
72°C,	10 min.	

*3 : Elongation time will vary based on primer number and performance. Set it at 4 min. initially when using 200 primer pairs.

- (3) After PCR is complete, analyze a portion of the sample by electrophoresis. For multiplex PCR products up to 1 kb long, good electrophoretic separation is possible with a 3 - 4% agarose gel.

VI-2. Increasing PCR Specificity Using a Single Primer Pair

- (1) Prepare the following reaction solution in a 0.2 ml tube.

	Volume
2X Multiplex PCR Buffer (Mg ²⁺ , dNTP plus)	25 μl
Primer 1 (20 pmol/μl)	0.5 μl
Primer 2 (20 pmol/μl)	0.5 μl
Multiplex PCR Enzyme Mix	0.25 μl
Template DNA	As needed
dH ₂ O	to 50 μl

- (2) Set tubes in the thermal cycler and perform PCR using the following conditions:

```

94°C,      30 sec.
  ↓
94°C,      30 sec.
57 - 60°C, 10 - 15 sec. *
72°C,      30 sec./kb  ] 30 - 40 cycles
OR,
  ↓
94°C,      30 sec.
65°C,      30 sec/kb  ] 30 - 40 cycles
    
```

*: Use as short an annealing time as possible.

- (3) After PCR is complete, analyze a portion of the reaction by electrophoresis.

VII. Experimental Examples (Multiplex PCR)

- (1) Example 1

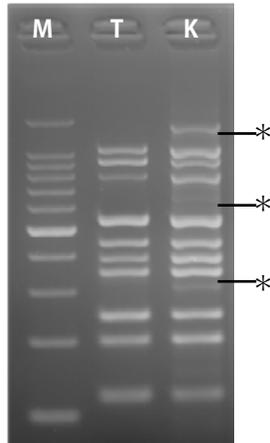
Multiplex PCR was carried out with 10 primer pairs (target product lengths: 122 bp, 201 bp, 247 bp, 353 bp, 395 bp, 449 bp, 548 bp, 801 bp, 955 bp, and 1,068 bp), using human genomic DNA as the template. 50 ng of human genomic DNA was added to a 50 μl reaction as template and the primers were used at final concentrations of 0.2 μM.

[PCR Reaction Conditions]

```

94°C,      60 sec.
  ↓
94°C,      30 sec.
57°C,      30 sec.
72°C,      30 sec.  ] 30 cycles
  ↓
72°C,      10 min.
    
```

[Results]



M: 100 bp DNA ladder (Cat. #3407A/B)
T : Multiplex PCR Assay Kit Ver.2
K : Company K reagent for multiplex PCR
(The reactions were carried out using the manufacturers' recommended conditions.)

While Multiplex PCR Assay Kit Ver.2 amplified the 10 desired targets specifically, Company K's reagent resulted in non-specific amplification.

*: Non-specific amplification

(2) Example 2

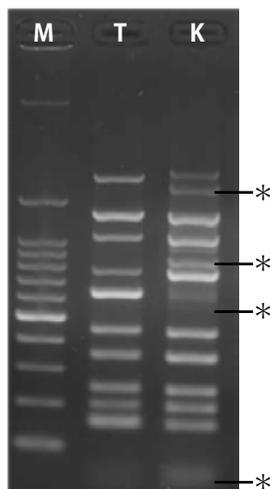
Multiplex PCR was carried out with 10 primer pairs (target lengths: 155 bp*, 201 bp, 247 bp, 353 bp, 449 bp, 604 bp*, 780 bp, 1,068 bp, 1,321 bp and 1,967 bp*), to amplify GC-rich regions of human genomic DNA. 50 ng of human genomic DNA was added to a 50 μ l reaction as template. Primers were used at final concentrations of 0.2 μ M.

*: Target regions with GC composition of 65% or higher

[PCR Reaction Conditions]

94°C, 60 sec.
↓
94°C, 30 sec.
57°C, 30 sec. } 30 cycles
72°C, 60 sec.
↓
72°C, 10 min.

[Results]



M: 100 bp DNA ladder
T : Multiplex PCR Assay Kit Ver.2
K : Company K reagent for multiplex PCR
(The reactions were carried out using the manufacturers' recommended conditions.)

While Multiplex PCR Assay Kit Ver.2 amplified the 10 targets specifically, Company K's reagent resulted in non-specific amplification and primer dimers.

*: Non-specific amplification

(3) Example 3

Multiplex PCR was carried out using 20 ng of human genomic DNA as template with the Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies, Cat. #4475346) (207 primer pairs).

1.5-fold of the standard amount of Multiplex PCR Enzyme Mix was used (0.15 μ l per 20 μ l reaction).

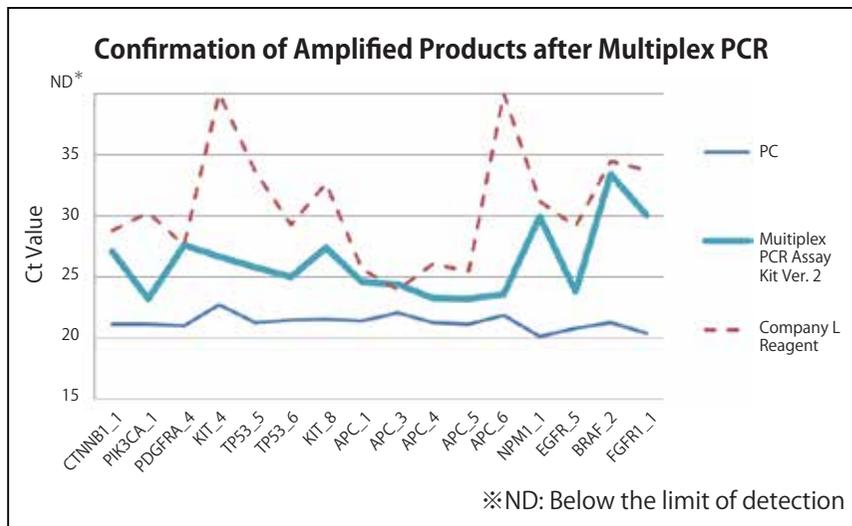
Each reaction was carried out using the manufacturer's recommended conditions.

[PCR Reaction Conditions]

94°C, 60 sec.
 ↓
 94°C, 30 sec.] 17 cycles
 60°C, 4 min.]
 ↓
 72°C, 10 min.

To validate the results, real-time PCR was performed to assess 16 arbitrarily chosen genes, allowing comparison of multiplex PCR products from this kit, Company L multiplex reagent PCR products, and human genomic DNA (PC) as templates.

[Results]



For real-time PCR reactions using the products generated with this kit, all 16 targets were amplified. The amplification showed less sequence bias than that obtained for products amplified by Company L's multiplex reagent.

VIII. Optimal Parameters

[For multiplex PCR]

- Optimize the annealing temperature by varying in increments of 1°C.
- While the standard annealing time is 30 seconds, the optimal time may differ for various PCR instruments. When using a high-speed PCR instrument with rapid temperature ramp speed, setting the annealing step to 60 seconds may improve PCR efficiency.
- For multiplex PCR using ≥ 10 primer pairs, the standard elongation time is 30 sec./kb. However, when using a high-speed PCR instrument with rapid temperature ramp speed, setting the elongation step to 60 sec./kb may improve PCR efficiency.
- For multiplex PCR using > 10 primer pairs, increasing the amount of enzyme (Multiplex PCR Enzyme Mix) may improve PCR efficiency.

[To increase specificity of PCR using a single primer pair]

- Optimize the annealing temperature by varying in increments of 1°C.
- Use a shorter annealing step during 3-step PCR. Longer annealing times may result in non-specific amplification.

IX. Troubleshooting

(1) If there are few or no amplification products:

- Decrease the annealing temperature by decrements of 1°C.
- Increase the quantity of template.
- Increase the number of PCR cycles.
- Increase the amount of primer used.
- Confirm the purity of the template DNA. Prepare new template.

(2) If nonspecific bands are observed:

- Increase the annealing temperature by increments of 1°C.
- Decrease the quantity of template.
- Decrease the number of PCR cycles.
- Decrease the amount of primer used.
- When cDNA is used as template, confirm that pseudogenes have not been amplified. Redesign primers if necessary.
- After PCR cycles are complete, add a final step to incubate at 72°C for 10 minutes.

X. Related Products

Takara PCR Thermal Cycler Dice™ *Touch* (Cat. #TP350)*
Takara PCR Thermal Cycler Dice™ *Gradient* (Cat. #TP600)*
0.2 ml Hi-Tube Dome Cap (Cat. #NJ200)*
100 bp DNA Ladder (Cat. #3407A/B)

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

Thermal Cycler Dice is a trademark of TAKARA BIO INC.

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