KOD SYBR® qPCR Mix

QKD-201T 1 ml × 1
QKD-201 1.67 ml × 3

Store at -20°C, protected from light

Contents

CAUTION

All reagents in this kit are intended for research purposes. Do not use for diagnosis or clinical purposes. Please observe general laboratory precautions and safety procedures while using this kit.

JAPAN
TOYOBO CO., LTD.
Tel (+81)-6-6348-3888
www.toyobo.co.jp/e/bio
tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel (+86)-21-58794900.4140

[1] Introduction

Description

KOD SYBR® qPCR Mix is a highly efficient 2× Master Mix for real-time PCR using SYBR® Green I and based on the 3'-5' exonuclease deficient KOD DNA Polymerase[1)2)3)]. The master mix contains all the required components, except the ROX reference dye and primers (50× ROX reference dye is supplied separately with this kit). The master mix aids reaction setup, and improves the reproducibility of experiments.
This product was optimized to be highly efficient and robust in the SYBR® Green assay.

Features

-Effective for GC rich targets
Quantitative analysis can be achieved even at GC contents greater than 70%.

-Long target amplification (~2 kb)
Quantitative analysis can be achieved using long targets, up to 2kb. Therefore, primers for conventional PCR can be applied. This aids primer design.
This reagent enables PCR fragment length polymorphism analysis using a melting curve assay.

-High specificity
Optimization and hot start technology permit the highly specific amplification.

-Effective amplification from crude samples
Effective amplification can be achieved using crude samples, as shown in Table I. This reagent can be used for genotyping or SNP analysis using crude specimens.

Table I Applicable crude samples

whole blood	ca.1% (final)
nail	ca 1mm
hair root	1~2 mm
oral mucosa	Suspension*
cultured cells	~ 10^3 cells
animal tissue	lysate*
plant tissue	lysate *

*See p 5-6

-Compatibility with various real-time cyclers.
The reagent may be used in most real-time cyclers (i.e. Block type and glass capillary type). The 50× ROX reference dye is individually supplied with this kit; therefore, the kit can be applied to real-time cyclers that require a passive reference dye.

About the SYBR® Green I detection system

The SYBR® Green I assay system uses fluorescent emission when SYBR® Green is intercalated into double-stranded DNA. The signal depends on the amount of amplified DNA. However, this system cannot distinguish between target and non-specific amplicons. Therefore, melting curve analysis is necessary after amplification.

[2] Components

This kit includes the following components, sufficient for 40 reactions (QKD-201T) and 200 reactions (QKD-201), with a total of 50 µl per reaction. All reagents should be stored at -20°C.

<QKD-201T>

KOD SYBR® qPCR Mix	1 ml × 1
50xROX reference dye	50 µl × 1

<QKD-201>

KOD SYBR® qPCR Mix	1.67 ml × 3
50× ROX reference dye	250 µl × 1

Notes:

-KOD SYBR® qPCR Mix can be stored, protected from light, at 2-8°C for up to 3 months. For longer storage, this reagent should be kept at -20°C and protected from light. No negative effect was detected by 10 freeze-thaw cycles of KOD SYBR® qPCR Mix. This reagent does not contain the ROX reference dye.

-50× ROX reference dye can be stored, protected from light, at 2-8°C or -20°C. For real-time cyclers that require a passive reference dye, this reagent must be added to the reaction mixture at a concentration of 1× or 0.1×. The master mix solution with the ROX reference dye can be stored, protected from light, at 2-8°C for up to 3 months. For longer storage, this reagent should be kept at -20°C and protected from light. The pre-mixed reagents can be prepared according to the following ratios. [5] Table 3 shows the optimal concentration of the ROX dye.

1× Solution

KOD SYBR® qPCR Mix: 50× ROX reference dye = 1.67 ml: 66.8 µl (QKD-201)
KOD SYBR® qPCR Mix: 50× ROX reference dye = 1 ml: 40 µl (QKD-201T)

0.1× Solution

KOD SYBR® qPCR Mix: 50× ROX reference dye = 1.67 ml: 6.7 µl (QKD-201)
KOD SYBR® qPCR Mix: 50× ROX reference dye = 1 ml: 4 µl (QKD-201T)

For real-time cyclers that do not require a passive reference dye, KOD SYBR® qPCR Mix can be used without the ROX reference dye.

[3] Primer design

1. Primer conditions

Highly sensitive and quantitative data depend on good primer design. The primers should be designed according to the following suggestions:

-GC content of primer: 45-60%
-Target length: ≤ 2 kp (optimal)
-Melting temperature (Tm) of primers: 60-70°C
-Purification grade of primers: Cartridge (OPC) grade or HPLC grade

- Adding a G or C to the 3' end of the primers can enhance priming efficiency.

-The following primers should not be used.
- 3'-ends that have complementarity.
-High GC content in the 3' region.
-The primer has complementary regions.

-The Tm of primers should be calculated using the Nearest Neighbor method. The Tm values in this manual were calculated using this method with the following parameters.
Na^+ concentration: 50 mM
Oligonucleotide concentration: 0.5 μM

[4] Template DNA

The following DNA samples can be used as templates.

1. cDNA

Non-purified cDNA, generated by reverse transcription reactions, can be used directly for real-time PCR using KOD SYBR® qPCR Mix. Up to 10% of the volume of a cDNA solution can be used for a real-time PCR reaction. However, excess volume of the cDNA may inhibit the PCR. Up to 20% (v/v) of the cDNA solution from the ReverTra Ace® qPCR RT Kit (Code No. FSQ-101), the ReverTra Ace® qPCR RT Master Mix (Code No. FSQ-201) and the ReverTra Ace® qPCR RT Master Mix with gDNA remover (Code No. FSQ-301) can be used for real-time PCR.

2. Genomic DNA, Viral DNA

Genomic DNA and viral DNA can be used at up to 200 ng in 50 μl reactions.

Notes:

The background tends to be high at the high copy range for genomic DNA, because the template DNA intercalates SYBR® Green I.

3. Plasmid DNA

Although super-coiled plasmids can be used, linearized plasmid DNA produces more accurate assays. The copy number of the plasmid DNA can be calculated using the following formula.

Copy number of 1μg of plasmid DNA = 9.1×10^{11} / Size of plasmid DNA (kb)

Linearity tends to be lost at the low copy range because diluted plasmid DNA is easily adsorbed onto vessels. Linearity can be improved using solutions containing carriers (e.g. yeast RNA) as diluents of template DNA.

4. Crude samples

Crude samples can be used as templates. The samples should be added according to the following guidelines, because excessive crude samples may inhibit the reaction and detection.

Table 1 Applicable crude samples for 20 µl reaction

Whole blood	0.2 µl (2 µl: 1/10 diluted sample)
Nail (mouse toe)	1 mm
Hair	1–2 cm from a hair root
Oral mucosa	5 µl of 200 µl suspension of oral mucosa collected by a cotton swab.
Cultured cells	~ 10^3 cells
Animal tissue	0.5~2 µl of lysate (alkaline lysis method)
Plant tissue	0.5~2 µl of 1/10 diluted lysate (one step method)

<Alkaline lysis method>

The following alkaline lysis method is recommended for rapid preparation of animal tissue lysates (*e.g.* mouse tail lysate) suitable for amplification with KOD SYBR® qPCR Mix.

*Animal tissue cannot be dissolved completely.

Table 2 Starting materials for preparing lysates

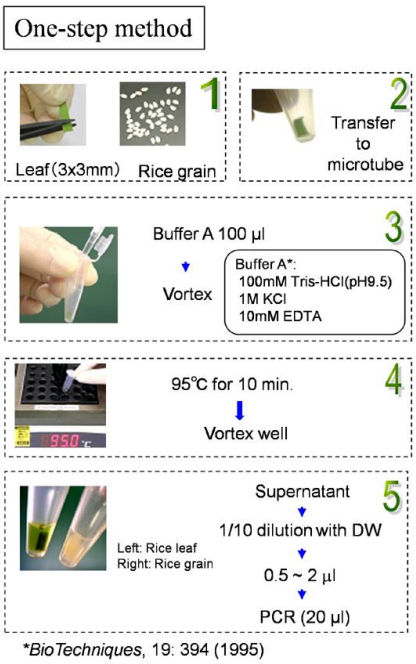
Mouse tail	3 mm
Pig muscle	20 mg
Cow muscle	20 mg
Nail (mouse toe)	5 mg

JAPAN
TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/e/bio
tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

<One step method>

The following one-step method is recommended for the rapid preparation of a plant tissue lysate suitable for amplification with KOD SYBR® qPCR Mix.



*BioTechniques, 19: 394 (1995)

Notes:

Homogenization of plant tissue with a pestle in Buffer A enhances the efficiency. In this case, heating is not necessary.

[5] Protocol

1. Reaction mixture setup

Reagent	Reaction volume 50µl	20µl	Final Concentration
DW	X µl	X µl	
KOD SYBR® qPCR Mix	25 µl	10 µl	1×
Forward Primer	10 pmol	4 pmol	0.2 µM[*1]
Reverse Primer	10 pmol	4 pmol	0.2 µM[*1]
50X ROX reference dye	1µl / 0.1µl	0.4µl / 0.04µl	1× / 0.1×[*2]
DNA solution	Y µl	Y µl	
Total	50 µl	20 µl	

Notes:

*1 Higher primer concentration tends to improve amplification efficiency; lower primer concentration tends to reduce non-specific amplification. The primer concentration should be between 0.05-1.0 μM.

*2 50× ROX reference dye must be added when using real-time cyclers that require a passive reference dye (i.e. Applied Biosystems, Agilent), according to Table 3. Table 3 shows the optimum concentration of the ROX reference dye. This dye is not necessary for real-time cyclers that do not require a passive reference dye.

Table 3 Recommended ROX dye concentration

Real-time cycler	Optimal dye concentration (dilution ratio)
Applied Biosystems 7000, 7300, 7700, 7900HT StepOne™, StepOnePlus™ etc.	1× (50:1)
Applied Biosystems 7500, 7500Fast, Agilent Technologies cyclers (Option) etc.	0.1× (500:1)
Roche cyclers, Bio-Rad cyclers, BioFlux cyclers etc.	Not required

Notes:

The pre-mixed reagents can be prepared according to the following ratios:

1× Solution

KOD SYBR® qPCR Mix: 50x ROX reference dye = 1.67 ml: 66.8 μl (QKD-201)
KOD SYBR® qPCR Mix: 50x ROX reference dye = 1 ml: 40 μl (QKD-201T)

0.1× Solution

KOD SYBR® qPCR Mix: 50x ROX reference dye = 1.67 ml: 6.7 μl (QKD-201)
KOD SYBR® qPCR Mix: 50x ROX reference dye = 1 ml: 4 μl (QKD-201T)

2. PCR cycling conditions

(A) Recommended cycling conditions.

The following table shows the recommended thermal conditions using primers designed according to the recommendations described in [3]. Almost all targets can also be amplified using the following conditions with other real-time PCR reagents.

If satisfactory results are not obtained, cycling condition should be altered according to the next section (B).

<3-step cycle>	Temperature	Time	Ramp
Pre-denaturation	98°C	2 min[*1]	Maximum
Denaturation:	98°C	10 sec	Maximum
Annealing	60°C[*2]	10 sec.	Maximum
Extension:	68°C	30 sec / 500 bp[*3]	Maximum
(40 cycles) [*4]		(Set 30 sec in the case of ≦ 500 bp)	
	<data collection should be set at the extension step>		
Melting / Dissociation Curve Analysis[*5]			

JAPAN
TOYOBO CO., LTD.
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*1 Pre-denaturation can be completed within 120 sec because of the anti-KOD antibody hot start PCR system.

*2 Insufficient amplification may be improved by decreasing the annealing temperature (to approx. 50°C), and non-specific amplification (e.g. abnormal shapes of the amplification curve at low template concentrations) may be reduced by increasing the annealing temperature (to approx. 68°C).

*3 When the target size is smaller than 500 bp, the extension time can be set at 30 sec on almost all real-time cyclers. Instability of the amplification curve or variation of data from each well may be improved by setting the extension time at 45–60 sec. Some real-time cyclers or software need over 30 sec for the extension step. In these cases, the time should be set according to each instruction manual (e.g. Applied Biosystems 7000/7300: ≥ 31 sec; Applied Biosystems 7500: ≥ 35 sec.).
When the target size is larger than 500 bp, the extension time should be calculated as 30 sec / 500 bp.

*4 When crude samples are used as templates, the Ct may be delayed. In such cases, the cycle number should be increased up to 50.

*5 Melting curve analysis is important to evaluate the specificity of the intercalation assay using SYBR® Green I. In the case of targets having >80% GC content, the upper limit of the melting temperature should be set at 99°C. When the melting curve analysis is incomplete, specificity should be confirmed by electrophoretic analysis on an agarose gel.

(B) Optimization of PCR cycling conditions

In cases of low specificity or poor amplification, the following optimization steps may be effective:

<Low specificity>

Higher annealing temperature or two-step cycling may improve the specificity. The annealing temperature should be determined to achieve a balance of specificity and amplification efficiency.

(Standard conditions) 98°C, 10 sec → 60°C, 10 sec → 68°C, 30 sec/500 bp (40 cycles)

↓

(Increase the annealing temperature) 98°C, 10 sec → ~68°C, 10 sec → 68°C, 30 sec/500 bp (40 cycles)

↓

(2 step cycling) 98°C, 10 sec → 68°C, 30 sec/500 bp (40 cycles)

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<Poor amplification (no amplification)>

Elongation of the extension time or a lower annealing temperature may improve amplification efficiency. Increasing the primer concentration may also improve efficiency.

(Standard conditions) 98°C, 10 sec → 60°C, 10 sec → 68°C, 30 sec/500 bp (40 cycles)

↓

(Elongation of the extension time) 98°C, 10 sec → 60°C, 10 sec → 68°C, ~ 1 min/500 bp (40 cycles)

↓

(Decreasing the annealing temperature[*1]) 98°C, 10 sec → 50°C~, 10 sec → 68°C, 30 sec/500 bp (40 cycles)

*1 The following functions are convenient for optimization of the conditions:
VeriFlex™: ABI StepOnePlus™, Thermal gradient: Bio-rad

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Tel(86)-21-58794900.4140

2-1. Real-time PCR conditions using Applied Biosystems StepOnePlus™

(Normal block type, software version 2.2.2)

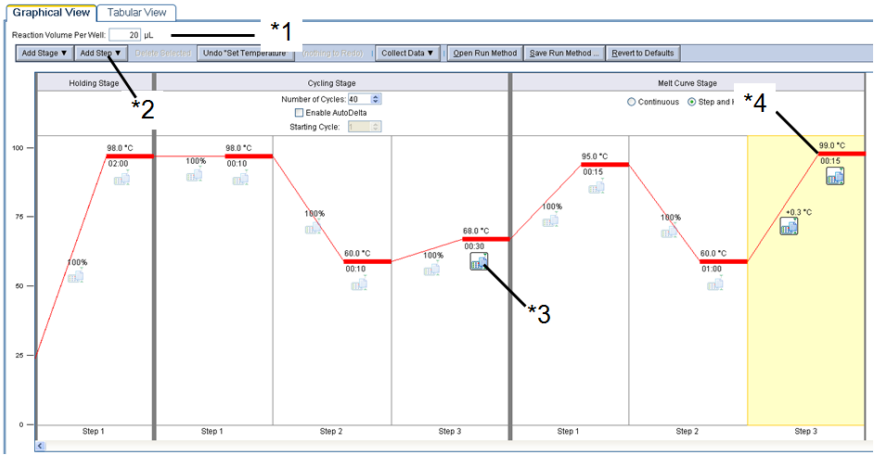
The following is an example of a SYBR® Green I assay using Real-time PCR conditions using Applied Biosystems StepOnePlus™.

(1) Select "Design Wizard", "Advanced Setup" or "QuickStart" after starting the software.

(2) Select "SYBR® Green Reagents" as reagents in the following tabs.

Design Wizard	Methods＆Materials
Advance Setup	Setup → Experiment Properties
QuickStart	Experiment Properties

(3) Select "Run Methods" and set the temperature conditions as follows:



*1 Input of actual reaction volume is important to achieve a successful analysis.
*2 Select "Add Step" and change the setting from a 2-step to a 3-step cycle.
*3 Set the data collection at the extension step.
*4 Add the condition for melting curve analysis. When the GC content of the target is high, the upper limit of the melting temperature can be set at 99℃.

(4) Insert the PCR tubes or plate

(5) Start the program

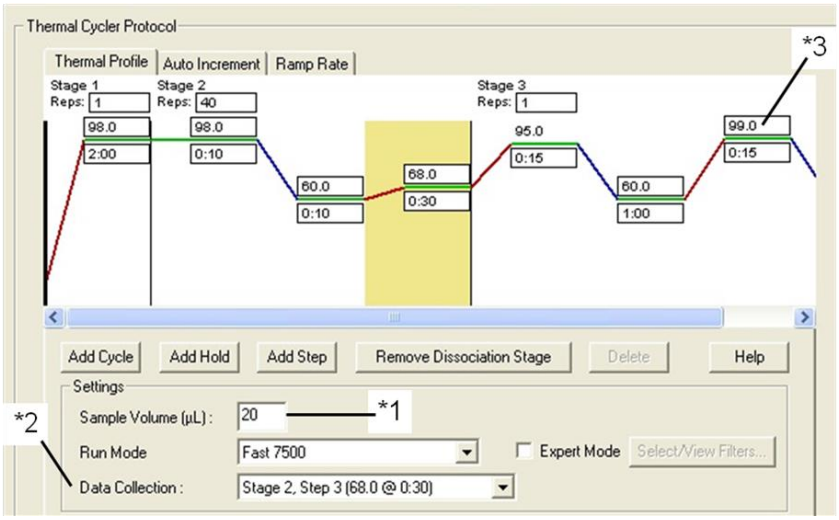
2-2. Real-time PCR conditions using Applied Biosystems 7500 Fast Real Time System

(Normal block type, software version 1.4)

The following is an example of a SYBR® Green I assay using Real-time PCR conditions using the Applied Biosystems 7500 Fast Real Time System.

(1) Select the "Instrument" tab after starting the software.

(2) Set the conditions as follows:



*1 Input the correct reaction volume.
*2 Set the data collection at the extension step.
*3 Add the condition for melting curve analysis. When the GC content of the target is high, the upper limit of the melting temperature can be set at 99°C. When the GC content of the target is >80%, the melting curve may be incomplete.

(3) Insert the PCR tubes or plate

(4) Start the program

2-3. Real-time PCR conditions using Roche LightCycler® Nano

(Software version 1.0)

The following is an example of a SYBR® Green I assay using the Roche LightCycler® Nano.

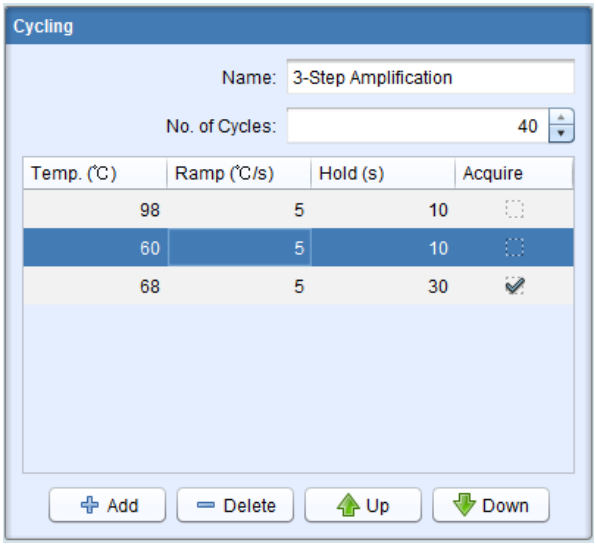
(1) Click “New” button, after starting the software.

(2) Input a title in the “Name” box in the “Experiment” tab.

(3) Select “Intercalating Dyes” in the “Run Settings” tab.

(4) Select “Profile” tab and set the cycling condition as follows:

a. Click “add” and select “Hold”.
b. Alter the temperature conditions to 98°C, 120 sec, 5°C/sec.
c. Click “add” and select “3-Step Amplification”.
d. Set the temperature conditions as follows:
e. Check box of “Acquire” at extension step.



f. Click “Add” and select “Melting”.
g. Confirm the settings as follows:

(5) Insert the PCR tubes

(6) Start the program

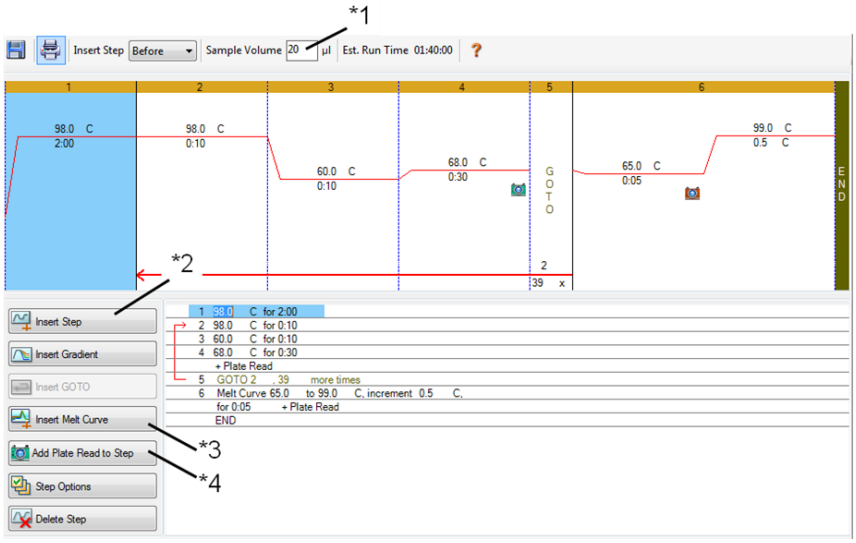
2-4. Real-time PCR conditions using Bio-Rad MiniOpticon

(Software version 2.0)

The following is an example of a SYBR® Green I assay using the Bio-Rad MiniOpticon

(1) Select “Create a new run”, after starting the software.

(2) Select “Create New…” and set the temperature conditions as follows:



*1 Input correct reaction volume.
*2 Select “Insert Step” and change the setting from “2-Step” to “3-Step”.
*3 Select “Insert Melt Curve” and add the cycle for the melting curve. When the GC content of the target is high, the upper limit of the melting temperature can be set at 99°C.
*4 Select “Add Plate Read to Step” and set the data collection point at the extension step.

(3) Insert the PCR tubes or plate

(4) Start the program

[6] Quantitation using long / GC rich targets

Application data 1: Long target amplification

<Method>

The efficiency was compared between KOD SYBR® qPCR Mix and conventional taq-based qPCR Master Mix for the amplification of the human TGFβ gene (1.9 kb) from human genomic DNA (10^n dilution) and no-template control (NTC).

PCR cycling condition of KOD SYBR® qPCR Mix:
(ABI StepOnePlus™)

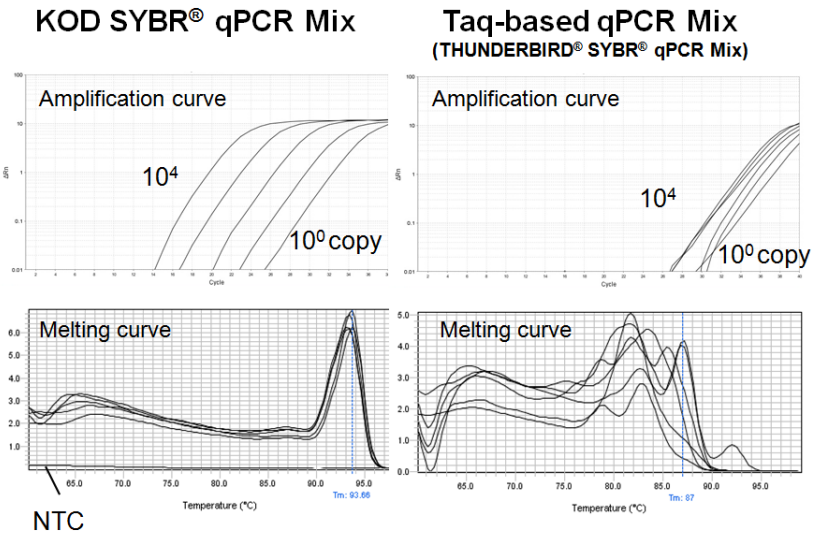
<3-step cycle>	Temperature	Time	Ramp
Pre-denaturation	98°C	2 min	Maximum
Denaturation:	98°C	10 sec	Maximum
Annealing	60°C	10 sec.	Maximum
Extension:	68°C	2 min*	Maximum
(40 cycles)			
	<Data collection was set at the extension step>		
Melting / Dissociation Curve Analysis			

*The extension time was set 30 sec/500bp.

Target: TGFβ (1.9 kb)

<Result>

KOD SYBR® qPCR Mix amplified the target (1.9 kb) successfully and quantitatively, whereas the conventional Taq-based master mix (THUNDERBIRD® SYBR® qPCR Mix) showed poor amplification.



Application data 2: Amplification of a GC-rich target

<Method>

The amplification efficiency was compared between KOD SYBR® qPCR Mix and high efficiency qPCR master mix compatible with high GC targets. Diluted cDNA (5^n) synthesized from HeLa cell total RNA using ReverTra Ace® qPCR RT Kit (Code: FSQ-101) was used as the template.

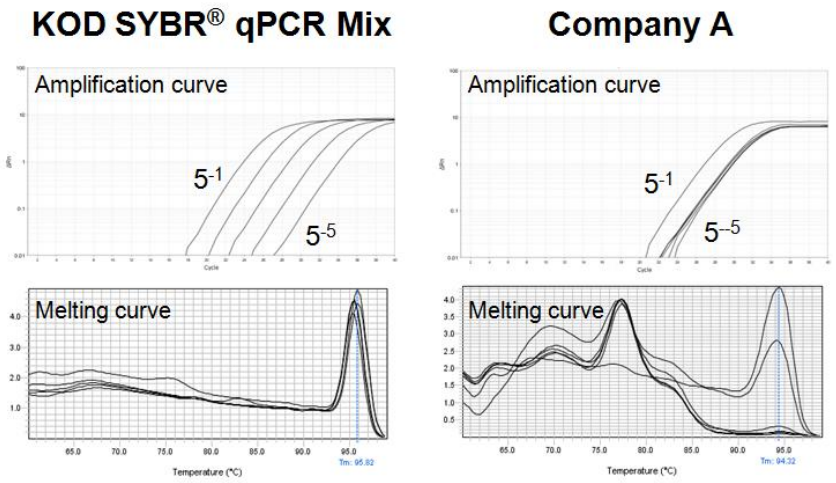
PCR cycling condition of KOD SYBR® qPCR Mix:
(ABI StepOnePlus™)

<3-step cycle>	Temperature	Time	Ramp
Pre-denaturation	98°C	2 min	Maximum
Denaturation:	98°C	10 sec	Maximum
Annealing	60°C	10 sec.	Maximum
Extension:	68°C	30 sec	Maximum
(40 cycles)			
	<Data collection was set at the extension step>		
Melting / Dissociation Curve Analysis			

Target: IGF2R gene (189 bp, GC content: 83%)

<Result>

KOD SYBR® qPCR Mix amplified the high GC target successfully and quantitatively, whereas the high efficiency qPCR master mix compatible with high GC targets (Company A) showed poor amplification and generated primer dimers.

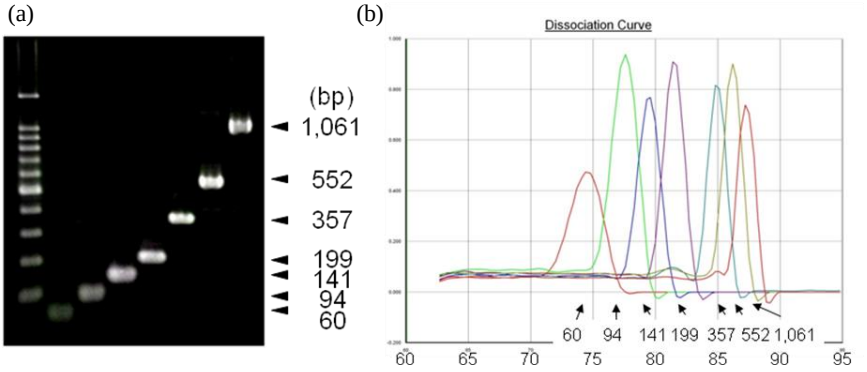


[7] PCR fragment length polymorphism analysis using a melting curve assay

KOD SYBR® qPCR Mix aids the design of amplicons having various Tm values because of its amplification ability up to 2 kb. Therefore, the reagent is able to realize multiplex PCR or a PCR fragment length polymorphism assay in one tube using melting curve analysis. In this analysis, the difference of Tm between the fragments should be > 3°C (optimally >5°C).

<Method>

(a) (b)



(c)

Fragment (bp)	Tm (°C)*1 <Predicted>	Tm (°C) <Measured>
60	72	74
94	76	77.5
141	79	79
199	82	82
357	84	85
552	86	86
1,061	88	87

Amplification of β-actin gene with various primers.

(a) Agarose gel electrophoresis analysis of amplicons.
(b) Melting curve analysis of amplicons.
(c) Comparison of the calculated and measured melting temperatures (Tm).

*1: The melting temperature (Tm) was calculated by the following formula.

$$Tm = 64.9 + 41 \times (yG+zC-16.4) / (wA+xT+yG+zG)$$

(W, x, y and z represent the number of A, T, G and C nucleotides in a fragment, respectively.)

Wallace RB *et al.* (1979) *Nucleic Acids Res* 6:3543-3557
Sambrook, J., and Russell, D.W. (2001) *Molecular Cloning*: *A Laboratory Manual*.

JAPAN
TOYOBO CO., LTD.
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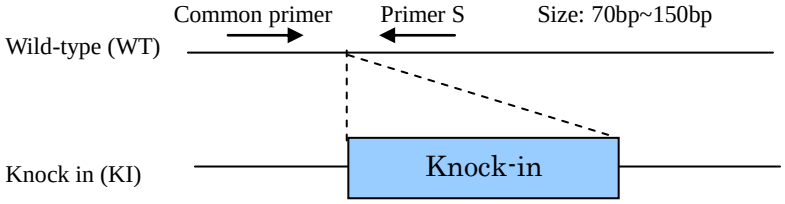
CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

<Construction of primers for genotyping using one common and two specific primers>

The following is a strategy for constructing primers for genotyping with PCR fragment length polymorphism using the KOD SYBR® qPCR Mix. This method is based on the difference in the melting temperatures between amplicons.

(1) Design a primer for a small fragment

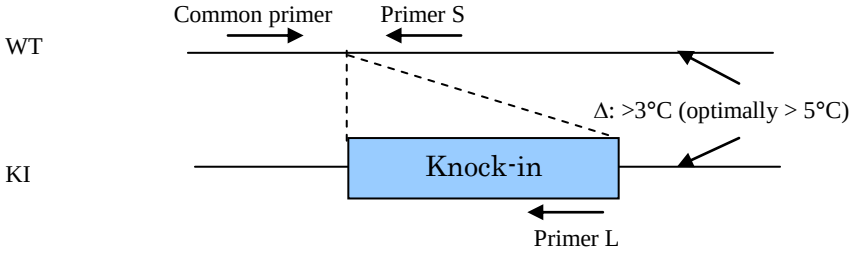
Primers should be designed such that the amplicon size is between 70 and 150 bp. Although the short target is set on the wild-type (WT) in the following example, the target can be set on both alleles.



(2) Calculate the Tm of the small fragment (refer to the formula on the previous page).

(3) Design a primer for a large fragment

Primers for large fragments (Primer L) should be designed such that the difference of Tm between small and large targets is greater than 3°C (optimally 5°C). The size of the long target should be less than 500 bp (optimally 300 bp) to prevent decreased PCR efficiency. If the difference of Tm is less than 3°C, the primer for the small fragment should be redesigned so that the difference of Tm is larger than 3°C.



(4) Preliminary experiment

The preliminary experiment should be done using the primers along with the above suggestions and appropriate templates (in this experiment, a heterozygous template is useful). The ratio of primer should be determined based on the following table, because the signal intensity is proportional to the amount (size) of the amplicon, not number of moles of it in SYBR® Green I assay.

	Primer concentration
Primer L	0.2μM x (small fragment [bp]) / (large fragment [bp])
Common primer Primer S	0.2μM

JAPAN
TOYOBO CO., LTD.
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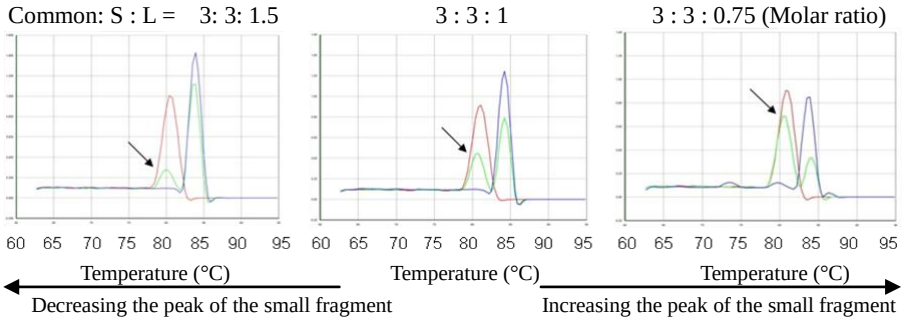
(5) Adjustment of peak intensities

When the intensities of the peaks are different, the concentration of the primer for the larger peak should be reduced. In the following experiment, the primer for the large fragment (Primer L) was changed. In this experiment, decreasing the concentration of Primer L increased the peak of the small fragment.

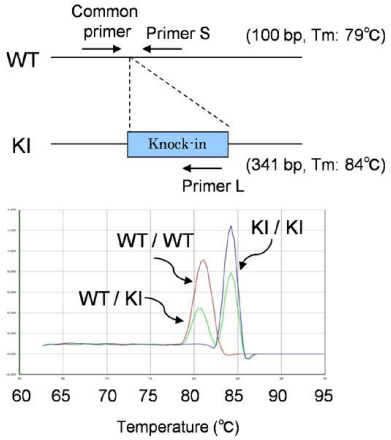


(Small fragment: 100 bp, Large fragment: 341 bp)

Application data 3: Genotyping using mouse-tail lysates

Genotyping was performed using mouse-tail lysates prepared by the alkaline lysis method. In this experiment, 100 bp (predicted Tm: 79°C) and 341 bp (predicted Tm: 84°C) fragments were amplified and analyzed. The primers were designed such that the difference in Tm was greater than 5°C.

Mouse-tail (ca.3mm)
| ←50mM NaOH 180 µl
| Vortex
| 95°C, 10 min
|← 1M Tris-HCl (pH8.0) 20 µl
| Vortex
| 12,000rpm, 5 min (optional)
Supernatant
0.5 ~ 2 µl
↓
20 μ l (reaction)



(ABI 7500 Fast Real Time System)

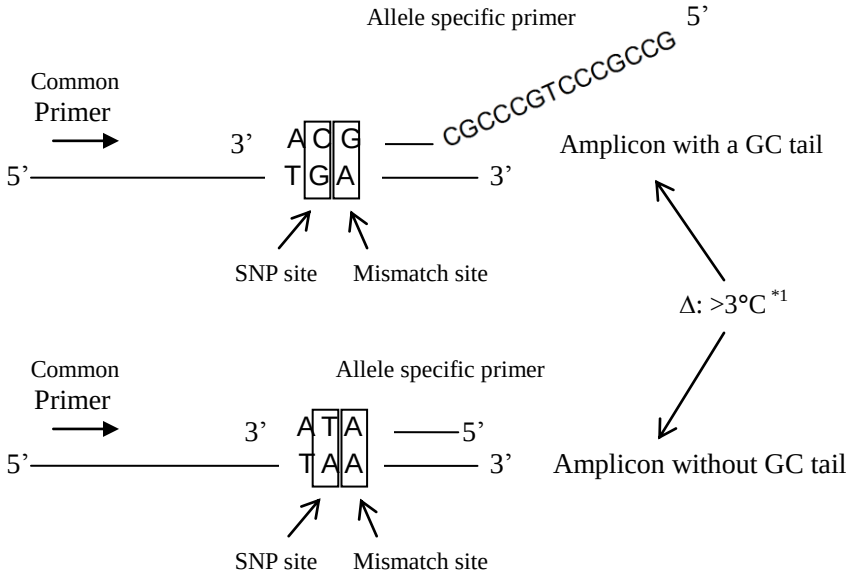
<3-step cycle>	Temperature	Time	Ramp
Pre-denaturation	98°C	2 min	Maximum
Denaturation:	98°C	10 sec	Maximum
Annealing	60°C	10 sec.	Maximum
Extension:	68°C	30 sec	Maximum
(40 cycles) *1			
	<data collection was set at the extension step>		
Melting / Dissociation Curve Analysis			

*1 Minimal cycling number should be chosen to prevent primer dimer generation.

[8] SNP analysis by ASP-PCR

The melting temperature of a PCR product can be increased by 3~5°C by adding a GC tail at the 5' end. This method uses one tube ASP (Allele specific primer)-PCR for SNP (Single nucleotide polymorphism) analysis. The primer design is the same as for ordinary ASP-PCR except for GC tailing. A SNP site should be located at the 3' region and is analyzed based on with or without amplification.

<Primer design>
-The target should be less than 100 bp to permit different Tms between amplicons.
-This method can be applied to various kinds of ASP-PCR. In Application data 4, SNP and mismatch sites were set at the second and third bases from the 3' end of the primer, respectively. In this case, the bases at the mismatch sites can be any bases except T, which corresponds to A. In this case, G and A were used.
-The difference in Tms between amplicons should be greater than 3°C[*1]. If the difference in the Tms is less than 3°C, the amplicon size should be decreased.
-The sequence of the GC tail can be flexible. Various kinds of GC tails have been reported. The following figure is an example of constructing primers for SNP analysis of ALDH2.
-If the intensities of the peaks are different, the primer concentration should be reconsidered according to the previous section "Construction of primers for genotyping using one common and two specific primers".



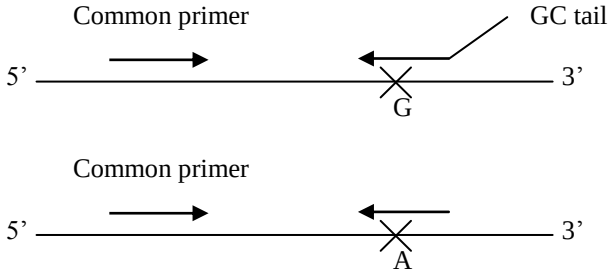
*1 The "Smoothing function" sometimes generates one broad peak from two adjacent peaks on the melting curve analysis when using several real-time cyclers. In such a case, the difference in Tms between two amplicons should be greater than 5°C, achieved by decreasing the amplicon size in decrements up to 50~60bp.

JAPAN
TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/e/bio
tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

Application data 4: Detection of ALDH2 SNP from whole blood and oral mucosa

<Method>
SNP of ALDH2 (aldehyde dehydrogenase 2) were detected directly from specimens of whole blood and oral mucosa, without purification. On this analysis, a primer with a GC tail was used to amplify the wild-type allele to make a difference in Tm between the amplicons.



(Sequences around the SNP site of ALDH2
487Glu 5’------ ctgcaggcatacact GAA gtgaaaactgtga ------3’
487Lys 5’------ ctgcaggcatacact AAA gtgaaaactgtga ------3’

(Primers for ASP-PCR analysis)
Common primer: 5'-GTACGGGCTGCAGGCATAC-3'
G specific primer: 5'-GCCGCCCTGCCCGCCACACTCACAGTTTTCACTGCA-3'
A specific primer: 5'-CCCACACTCACAGTTTTCACTATA-3'
: Mismatch site; : SNP site

(Amplification size and Tm)

Target	Size	Tm (Predicted)
Common primer – G specific primer	57 bp	78°C
Common primer – A specific primer	45 bp	72°C

Template DNAs was prepared by the following methods and added to the reaction mixtures directly.

(ABI 7500 Fast Real Time System)

<3-step cycle>	**Temperature**	**Time**	**Ramp**
Pre-denaturation	98°C	2 min	Maximum
Denaturation:	98°C	10 sec	Maximum
Annealing	60°C	10 sec.	Maximum
Extension:	68°C	30 sec	Maximum
(50 cycles)			
	<Data collection was set at the extension step>		
Melting / Dissociation Curve Analysis			

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TOYOBO CO., LTD.
Tel(81)-6-6348-3888
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tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
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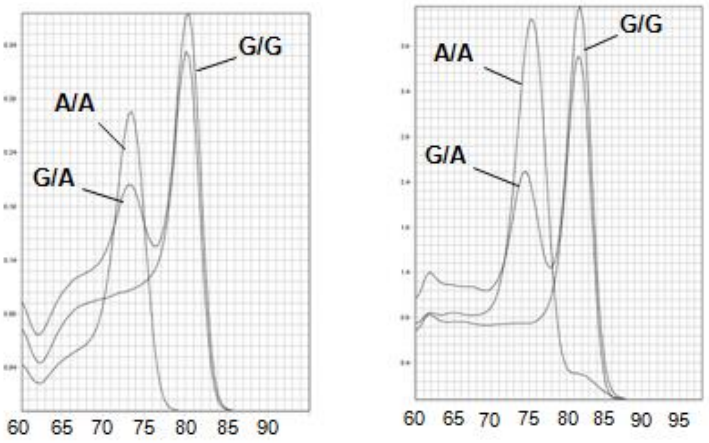
(Pretreatment of the specimens)

Whole blood 2 µl ↓ Suspend in 48 µl DW ↓ Suspension 5 µl ↓ 20 µl (reaction)	Cotton swab with oral mucosa ↓ Suspend in 200 µl DW ↓ Suspension 5 µl ↓ 20 µl (reaction)

<Result>



SNP typing was achieved successfully without purification of templates.

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TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/e/bio
tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

[9] Related protocol

1. cDNA synthesis

cDNA synthesized by various cDNA synthesis reagents can be used with KOD SYBR® qPCR Mix. However, cDNA synthesized by a reagent specialized for real-time PCR can increase sensitivity.

ReverTra Ace® qPCR RT Kit (Code No. FSQ-101) is a cDNA synthesis kit suitable for real-time PCR. Here, the protocol with ReverTra Ace® qPCR RT Kit is described. However, for the detailed protocol, please refer to the instruction manual of the kit.

(1) Denaturation of RNA

Incubate the RNA solution at 65°C for 5 min and then chill on ice.

Notes:

-This step can be omitted. However, this step may increase the efficiency of the reverse transcription of RNA, which forms secondary structures.

-Do not add 5× RT Buffer and/or enzyme solution at this step.

(2) Preparation of the reaction solution

Reagent	Volume (amount)
Nuclease-free Water	X µl
5× RT Buffer	2 µl
Primer Mix	0.5 µl
Enzyme Mix	0.5 µl
RNA solution	0.5 pg-1 µg
Total	10 µl

(3) Reverse transcription reaction

-Incubate at 37°C for 15 min. <Reverse transcription>

-Heat at 98°C for 5 min. <Inactivation of the reverse transcriptase>

-Store at 4°C or -20°C.*

*This solution can be used in the real-time PCR reaction directly or after dilution.

Notes:

The above temperature conditions are optimized for ReverTra Ace® qPCR RT Kit.

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tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

[10] Troubleshooting

Symptom	Cause	Solution
Loss of linearity in the high cDNA/DNA concentration region.	Intercalation of SYBR® Green I into the template DNA.	SYBR® Green I is also intercalated into the template DNA; therefore, the baseline tends to be higher when high concentration DNA samples are used. Diluted template should be used to obtain a correct Ct value.
	Inhibition of the reaction by PCR inhibitors in crude samples	Excessive crude specimen inhibits the PCR reaction. The amount of specimen should be decreased or the specimen should be purified.
	cDNA synthesis reagent is inappropriate (a certain component inhibits the reaction)	cDNA synthesis reagents designed for real-time PCR should be used.
Loss of linearity or lower signal in the low DNA/cDNA concentration region.	The template DNA is insufficient.	When the DNA/cDNA copy number is lower than 10 copies per reaction, the linearity of the reaction tends to be lost. The template concentration should be increased.
	Adsorption of the DNA to the tube wall.	The diluted DNA templates tend to be absorbed onto the tube wall. Dilution should be performed before the experiments.
	Competition with primer dimer formation.	Dimer formation may reduce the amplification efficiency of the target, especially for reactions at low template concentrations. The reaction condition should be optimized or the primer sequences should be changed.
Loss of linearity of the amplification carves.	Competition with non-specific amplification.	Non-specific amplification may reduce the amplification efficiency of the target. The reaction conditions should be optimized or the primer sequences should be changed.
	Circular plasmid DNA	Linearized plasmid DNA should be used because circular DNA tends to cause data spread.
The PCR efficiency is lower than 80% (slope: <-3.95)	Inappropriate cycling conditions.	Optimize the cycling conditions according to [5] 2. PCR cycling conditions.
	Tm of primers are low	Annealing is insufficient. Optimize the cycling conditions according to [5] 2. PCR cycling conditions.
	Degradation of the primers.	Fresh primer solution should be prepared.
	The calculation of the PCR efficiency is inappropriate.	The Ct value on the linear region should be used to calculate the PCR efficiency.
	Primer concentration is low	Increase the primer concentration.
The PCR efficiency is higher than 110% (slope > -3.1)	The calculation of the PCR efficiency is inappropriate.	The Ct value on the linear region should be used to calculate PCR efficiency.
	Non-specific amplification	Non-specific amplification may raise the apparent PCR efficiency over 110 %. The specificity should be confirmed by melting curve analysis.

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TOYOBO CO., LTD.
Tel(81)-6-6348-3888
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tech_osaka@toyobo.jp

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Symptom	Cause	Solution
Reproducibility is not good.	Poor purification of the template DNA	Low-purity DNA may contain PCR inhibitors. Re-purify the DNA samples.
	Absorption of the template DNA to the tube wall.	Diluted DNA templates tend to be absorbed onto the tube wall. Dilution of the template DNA/cDNA should be performed before the experiments.
	Plasmid DNA or PCR product is used as a template.	In general, plasmid DNA or PCR products are used at low concentrations. Diluted DNA templates tend to be absorbed onto the tube wall. Dilution of the template DNA/cDNA should be performed before the experiments. Dilution with a carrier nucleic acid solution (Yeast RNA) is also effective in improving linearity.
	Low purity of the primers	Different batches of primers may generate different results. When the batch is changed, prior testing of the primer should be performed.
Amplification from the non-template control (NTC).	Formation of primer dimers.	On the melting curve analysis, a peak at a temperature lower than that of the target peak suggests a primer dimer. The PCR cycle should be optimized according to [5] 2. PCR cycling conditions. If the result is not improved, the following should be performed: change the primer sequence and/or change the purification grade of the primer (HPLC grade).
	Contamination or carry over of the PCR products.	When the no-template control generates a peak at the same melting temperature as the target on the melting curve analysis, the amplification is caused by a carry-over or contamination. Use fresh reagents.
Low amplification curve signal / Unstable amplification curve signal.	Excessive amount of ROX reference dye.	Excessive amount of ROX reference dye may cause low signal. 50× ROX reference dye should be used according to [5] Table 3.
	Inappropriate settings of fluorescence measurement	Settings should be confirmed according to the instruction manual of each detector. Prolonged extension time (45~60 sec) may improve the instability of the amplification curve.
	Insufficient reaction volume.	Low reaction volume may cause an unstable signal. Increase the reaction volume.
Detection of multiple peaks on the melting curve analysis	Non-specific amplification.	Optimize the reaction conditions. If the result is not improved, the primer sequence should be changed.
	Formation of primer dimer.	On the melting curve analysis, a peak at a temperature lower than that of the target peak suggests a primer dimer. The PCR cycle should be optimized according to [5] 2. PCR cycling conditions. If the result is not improved, the following action should be performed: change the primer sequence and/or change the purification grade of the primer (HPLC grade)

Symptom	Cause	Solution
GC tail does not increase the Tm of amplicons	Target size is too large	The effect of the GC tail will be low when the amplicon size is long. Shorten the amplicon size to below 100 bp. Tms of DNA fragments can be calculated using the algorithm in [7] PCR fragment length polymorphism.
	GC content of the target is too high	When the GC content of the target is high, the effect of the GC tail will be low. Targets and primers should be determined in consideration of Tm.
Peaks from multiplex PCR cannot be separated on melting curve analysis	Target size is too large	Tms of targets can be increased by enlarging their sizes, to some extent (~ 1 kb). The fragment size should be determined in consideration of the difference in Tm of each PCR product using the algorithm in [7] PCR fragment length polymorphism.
	GC content of target is too high	When the GC content of the target is high, the effect of GC tail will be low. Targets and primers should be determined in consideration of Tm.
	Effect of “Smoothing function” of a real-time cycler.	“Smoothing function” sometimes generates one broad peak from two adjacent peaks on the melting curve analysis when using several real-time cyclers. In such cases, the difference in Tms between two amplicons should be greater than 5°C, achieved by decreasing the amplicon size in decrements up to 50~60bp.
Intensities of peaks from multiplex PCR are different on melting curve analysis	Sizes of the targets are too different	A signal intensity is in proportion to the amount (size) of an amplicon, not the number of moles of it in the SYBR® Green I assay. Primer concentration should be determined according to [7] PCR fragment length polymorphism.
	Annealing efficiency of the primers are different	The difference in Tms of primers may generate peak imbalances. In such cases, the primer concentration for the higher peak should be decreased according to [7] PCR fragment length polymorphism.

JAPAN
TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/e/bio
tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

[11] Related products

Product name	Package	Code No.
High efficient real-time PCR master mix for probe assay **THUNDERBIRD® Probe qPCR Mix**	1ml × 1 1.67ml × 3	QPS-101T QPS-101
High efficient real-time PCR master mix for SYBR® Green assay **THUNDERBIRD® SYBR® qPCR Mix**	1ml × 1 1.67ml × 3	QPS-201T QPS-201
High efficient cDNA synthesis kit for real-time PCR **ReverTra Ace® qPCR RT Kit**	200 rxns	FSQ-101
High efficient cDNA synthesis master mix for real-time PCR **ReverTra Ace® qPCR RT Master Mix**	200 rxns	FSQ-201
High efficient cDNA synthesis master mix for real-time PCR with genomic DNA remover **ReverTra Ace® qPCR RT Master Mix with gDNA remover**	200 rxns	FSQ-301
One-step Real-time PCR master mix for probe assay ***RNA-direct*™ Realtime PCR Master Mix**	0.5 ml × 5	QRT-101
One-step Real-time PCR master mix for SYBR® Green assay ***RNA-direct*™ SYBR® Realtime PCR Master Mix**	0.5 ml × 5	QRT-201

[12] References

1) Takagi M, Nishioka M, Kakihara H, Kitabayashi M, Inoue H, Kawakami B, Oka M, and Imanaka T., *Appl Environ Microbiol.*, 63: 4504-10 (1997)

2) Hashimoto H, Nishioka M, Fujiwara S, Takagi M, Imanaka T, Inoue T and Kai Y, *J Mol Biol*., 306: 469-77 (2001)

3) Mizuguchi H, Nakatsuji M, Fujiwara S, Takagi M and Imanaka T, *J Biochem*., 126: 762-8 (1999)

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TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/e/bio
tech_osaka@toyobo.jp

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TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

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www.toyobo.co.jp/e/bio
tech_osaka@toyobo.jp

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Tel(86)-21-58794900.4140

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