A Novel and Robust Real-time PCR System for DNA Polymorphism Analysis Directly from Crude Clinical Samples

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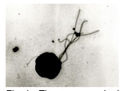
Introduction

Real-time quantitative PCR (qPCR) is a powerful technology that is used in many scientific disciplines, including analysis of clinical samples. However, conventional qPCR systems using Taq DNA polymerase have the following disadvantages:
(1) Target size limitations (< 200 bp),
(2) Poor amplification of GC-rich sequence, and
(3) PCR inhibition from the biogenic substances present in clinical samples.

To circumvent these problems, we developed a novel qPCR system (KOD-SYBR) that uses KOD exo(-) DNA polymerase and SYBR® Green I dye. Details of the KOD-SYBR system and examples of amplification from crude samples are shown.

What is KOD DNA polymerase?

KOD DNA polymerase was isolated from the hyperthermophilic archaebacteria *Thermococcus kodakaraensis* KOD1 strain in 1990.
The name of "KOD" originates from Kodakara Island in Japan where the KOD1 strain was isolated.



Growth temperature: 60~100 ℃ (optimum: 85℃)
Obligatory anaerobic
Heterotrophic nutrition

Fig. 1. *Thermococcus kodakaraensis* KOD1

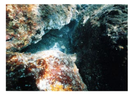


Fig. 2. Solfatara (Kodakara island, Japan)

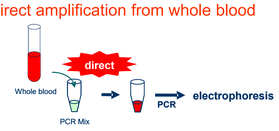
Fig. 3. Kodakara island (Kagoshima prefecture, Japan)

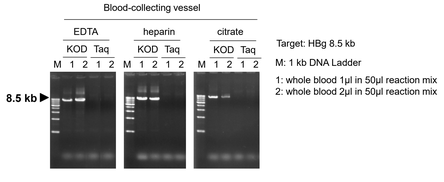
Compared with other polymerases, KOD DNA polymerase exhibits higher thermostability and higher efficiency of processivity and the elongation rate. Therefore, KOD DNA polymerase has excellent efficiency and robustness, especially for the amplification of GC-rich and long targets.

Property		Value for indicated DNA polymerase		
		KOD DNA polymerase	Pfu DNA polymerase	Taq DNA polymerase
Origin		Archaea	Archaea	Bacteria
Deduced molecular mass (kDa)		90.0	90.1	93.9
Optimum temperature (˚C)		75	75	75
Optimum pH at 75˚C		6.5	6.5	8.0-8.5
Thermostability (half-life)		95˚C, 12 h; 100˚C, 3.0 h	95˚C, 6 h; 100˚C, 2.9 h	95˚C, 1.6 h
5'→3' exonuclease activity		-	-	+
3'→5' exonuclease activity		+	+	-
Terminal transferase activity		-	-	+
Processivity (bases)		> 300	< 20	ND
Elongation rate (bases/s)		106-138	25	61
Basic PCR performances	Yield of amplicon	+++	+	++
	Detection sensitivity	+++	+	+++
	Error rates(×10^{-5})	3.4	12	141
Tolerance to GC-rich sequence		+++	++	+
Incorporation of nucleotide analog		+++	++	+
Capability to amplify long sequence		Up to 20 kb	Up to 2 kb	Up to 3 kb
Sensitivity to inhibitor		Resistant	Moderate	Sensitive

● Examples of PCR amplification using KOD DNA polymerase

Direct amplification from whole blood





Target: HBg 8.5 kb
M: 1 kb DNA Ladder
1: whole blood 1μl in 50μl reaction mix
2: whole blood 2μl in 50μl reaction mix

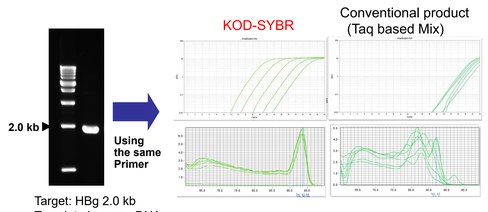
KOD DNA polymerase can amplify long targets from crude sample such as whole blood.

Novel qPCR system using KOD exo(-) DNA polymerase

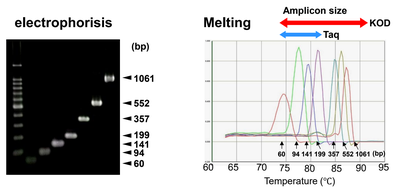
The novel qPCR system (KOD-SYBR) uses KOD exo(-) DNA polymerase, a mutant enzyme in which the 3'-5' exonuclease activity has been eliminated to reduce non-specific amplifications caused by this activity. KOD-SYBR can amplify large (< 2 kb) and GC-rich targets, and KOD-SYBR can be used with crude samples (e.g., whole blood, tissue lysates, hair roots, and oral mucosa) including clinical specimens.

	Conventional Method	Novel qPCR system KOD-SYBR
PCR enzyme	Taq DNA polymerase	KOD DNA polymerase
Amplifiable length of target	< 200 bp (Max : 300 bp)	< 2,000 bp
Amplifiable GC rate of target	Up to 70%	Up to 90%
Sensitivity for inhibitor	Sensitive (DNA purification is required)	Resistant

①Quantitative analysis using long targets (2.0 kb)



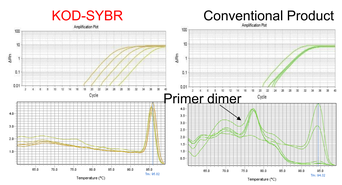
Target: HBg 2.0 kb
Template:human gDNA



KOD-SYBR enables the amplification and the quantitative analysis using long targets. This facilitates primer design.

②Quantitative analysis using GC-rich targets (83% GC rates)

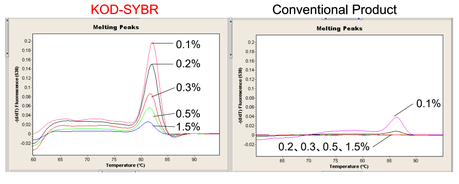
Target: IGF2R 189bp (83% GC rates)
Template:cDNA



KOD-SYBR enables the amplification and the quantitative analysis using GC-rich targets.
It is useful for analyzing GC-rich microorganisms or promoter regions that contain CpG islands.

③Direct amplification from whole blood

Target: Actin 200 bp
Template: whole blood



KOD-SYBR works better than conventional polymerases in whole blood.
In addition, KOD-SYBR works well in crude samples such as tissue lysates, hair roots or oral mucosa without purification.

Examples of applications using the KOD-SYBR system

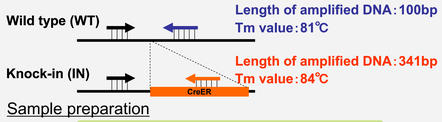
Detection of insertion/Deletion polymorphisms

Targeted loci of transgenic mice were analyzed.

Method:

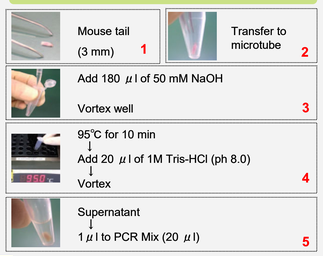
Primer design

Three primers were used to generate fragments of two different sizes: wild type (WT) allele of 100 bp; inserted allele (Knock-in; IN) of 341 bp.



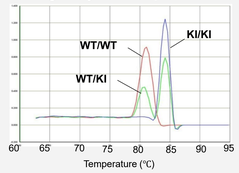
Sample preparation

Heat treatment of mouse tail



Result:

Melting analysis of amplicon



Both alleles (100 and 341 bp) from the mouse tail lysates were amplified by KOD-SYBR and the genotypes were analyzed successfully by a melting curve assay. The three variants tested (WT/WT, WT/IN, IN/IN) could be distinguished from each other. This method is applicable to the detection of indels in human genome.

Detection of single nucleotide polymorphisms (SNPs) in the human aldehyde dehydrogenase 2(*ALDH2*) gene

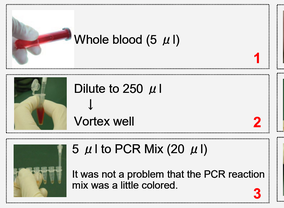
Method:

Primer design

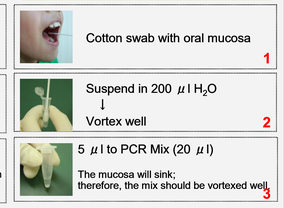
A G-specific primer-bearing tail sequence at the 5' end was used to obtain a fragment that was larger (57 bp) than of the fragment obtained by the other primer set (an A-specific primer and a common primer) that had no tail sequence (45 bp).

Sample preparation

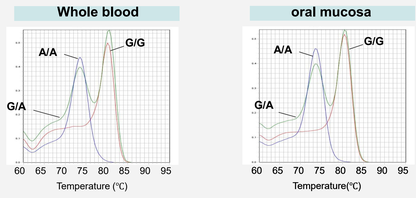
Preparation of whole blood



Preparation of oral mucosa



Result:



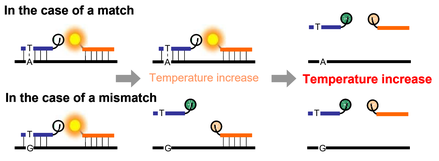
SNPs in *ALDH2* from the whole blood and oral mucosa specimens were detected using the allele-specific primers followed by melting curve analysis. The A/A, A/G, and G/G variants were detected in the crude specimen samples. As expected, peaks from both the 45 and 57 bp amplicons were detected by melting curve analysis at 73℃ and 80℃, respectively.

Further investigation for probe assay system

SNPs were detected in the human cytochrome CYP2C19 gene (CYP2C19*2 allele) from the whole blood sample (prepared as described above) using hybridization (Hyb) and Quenching (Q) Probe in the KOD exo(-) system without SYBR® Green I.

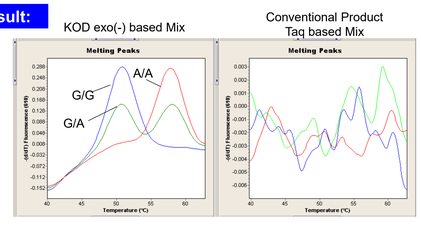
Hyb probe

SNPs were detected using donor and acceptor probes bearing a fluorophore and acceptor at the 3' and 5' ends of the probe, respectively. The polymorphic site was designed in the donor probe.



In the match case, the fluorescence can be at higher temperetures than in the mismatch.

Result:

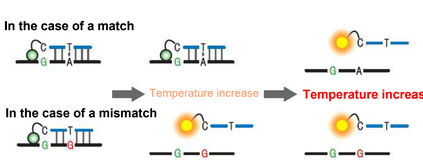


The two alleles were distinguished distinctly using the Hyb probe method.

Q probe

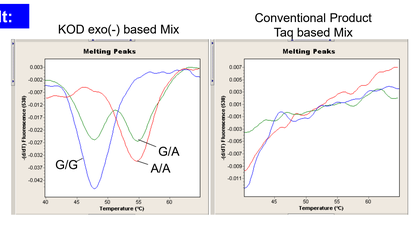
Q probe can bind to Guanine (G) and quenched in lower temperature. The fluorescence generated by the dissociated Q probe is analyzed at increasing temperatures.

* Reference: Nucleic Acids Res, 29(6): E34.



In the match case, the quenching can be retained at higher temperatures than in the mismatch.

Result:



The two alleles were distinguished clearly using the Q probe method.

We confirmed that a qPCR system based on KOD exo(-) DNA polymerase is useful not only in the SYBR® Green I system but also in the probe system.

Conclusions

KOD-SYBR is a robust system for polymorphism analysis of crude clinical samples. The flexibility of being able to use relatively large amplicon sizes (< 2 kb) for melting curve analysis is a distinct advantage of this system. Additionally, the qPCR system based on KOD exo(-) DNA polymerase can also be used in various probe assays. The KOD-SYBR system has the potential to become a powerful tool for high-throughput genotyping of clinical samples.