

# Rapid microbial gene detection and amplification techniques using colony-direct PCR

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*We demonstrate a method for rapid amplification of DNA directly from microbial colonies. KOD Hot Start DNA Polymerase permits multiplex PCR using primers for multiple targets. A critical factor for successful colony-direct PCR is limiting the amount of bacteria in the template.*

PCR has become an effective means for detecting infectious disease organisms, especially bacteria, fungi, and viruses that are difficult to handle or impossible to culture. PCR can also detect variations and mutations in genes. In some cases, PCR techniques offer a viable option for direct analysis of materials as opposed to indirect techniques based on antibody detection or differential culture.

In colony-direct PCR (CD-PCR), the polymerase chain reaction is performed by adding a template of intact bacteria directly from a colony into the reaction mixture. CD-PCR has been used successfully as a detection method for Gram-negative (Gram-) bacterial strains such as *E. coli*. Because reproducibility has been an issue with *Staphylococcus aureus* and other Gram-positive (Gram+) bacteria, CD-PCR had been considered inadequate for this type of detection analysis (1).

Here we present CD-PCR as a practical method for quick analysis of multiple antibiotic resistance in methicillin-resistant *S. aureus* (MRSA) and for detection of other Gram+ and Gram- bacterial strains. (For a description of multiplex CD-PCR for identification of Shiga toxin-producing *E. coli*, see page 11.) Severely limiting the amount of bacteria added to the reaction mixture and use of KOD Hot Start DNA Polymerase\* are key factors for successful and reproducible CD-PCR using MRSA or other Gram+ bacteria.

## Detect MRSA with multiplex CD-PCR

To perform multiplex CD-PCR, we identified five *S. aureus* drug-resistance genes as targets. Primers were designed to

**Table 1. MRSA PCR reaction setup**

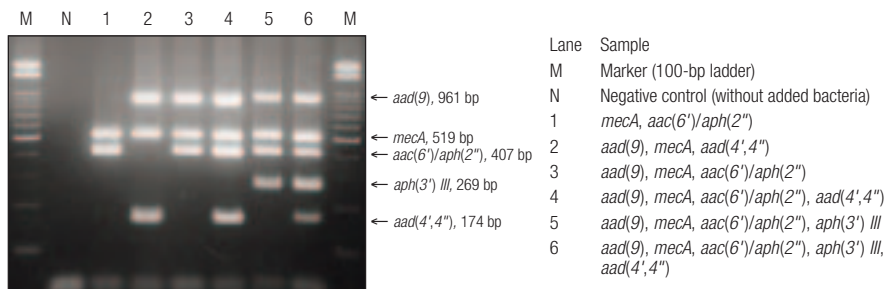
Component	Concentration
PCR buffer for KOD Hot Start	1X
dNTP Mix	0.2 mM each
MgSO <sub>4</sub>	1 mM
<i>aac(6'')/aph(2'')</i> primers	0.5 μM each
<i>aad(9)</i> primers	0.4 μM each
<i>mecA</i> primers	0.2 μM each
<i>aph(3'') III</i> primers	0.2 μM each
<i>aad(4', 4'')</i> primers	0.2 μM each
KOD Hot Start DNA Polymerase	0.4 U

produce amplification products of a unique size for each target gene. KOD Hot Start DNA Polymerase was used in all PCR reactions. To collect a small amount of bacteria, the tip of a sterilized toothpick was lightly touched to the surface of the test colony grown on agar medium. No sample was visible on the toothpick

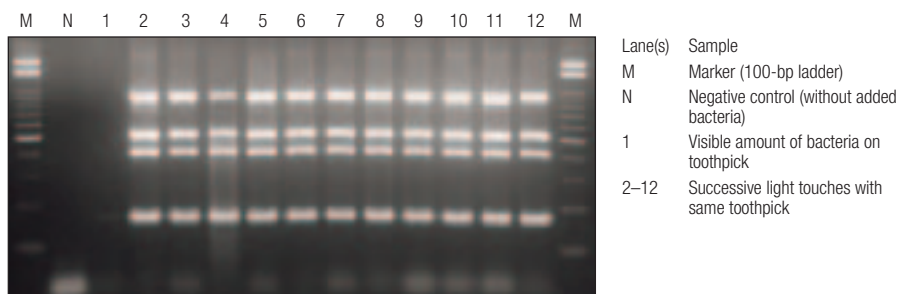
tip, which was immersed into the PCR reaction mixture and quickly withdrawn; stirring was carefully avoided. PCR reactions were performed using the following cycling conditions: initial denaturation at 95°C for 3 minutes; 30 cycles at 95°C for 30 seconds, 50°C for 30 seconds, 68°C for 1 minute; and final extension at 68°C for 3 minutes.

Figure 1 shows the results of the multiplex CD-PCR performed using the above primers for five target genes with a colony from a clinical MRSA isolate. Clear amplification was achieved for each of the target genes.

Next, contributing factors that could affect the CD-PCR were investigated. Results were not affected by the type of culture medium or by extended post-cultivation colony storage at 4°C (for up to six months, data not shown); the only observable effect was attributed to the amount of bacteria added (Figure 2). To study the effect of the amount of bacteria used for the PCR template, a dilution series was made. Each of 12 sample tubes was



**Figure 1. MRSA drug-resistant DNA detection using multiplex CD-PCR**



**Figure 2. Amplification using visible versus minute quantities of MRSA**

\* Manufactured by Toyobo and distributed by EMD Biosciences Inc. Novagen Brand. Not available through Novagen in Japan.

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**Table 2. Strains used for CD-PCR**

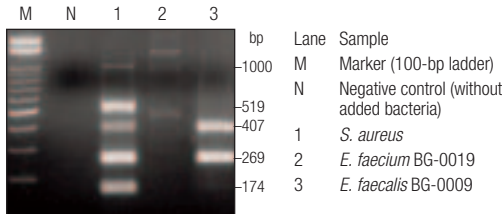
Organisms	Target Genes	PCR Products	Primers
<i>E. faecalis</i>	<i>aad(9)</i>	961 bp	
<i>E. faecium</i>	<i>mecA</i>	519 bp	
<i>S. aureus</i>	<i>aac(6'')/aph(2'')</i>	407 bp	multiplex
<i>S. aureus</i>	<i>aph(3'') III</i>	269 bp	
<i>S. aureus</i>	<i>aad(4',4'')</i>	174 bp	
<i>S. griseus</i>	<i>aac(3)</i>	930 bp	
<i>S. kasugaensis</i>	<i>aac(2'')</i>	810 bp	multiplex
<i>Streptomyces</i> strain 8	<i>aac(6')</i>	622 bp	
<i>L. pneumophila</i>	<i>mip</i>	168 bp	multiplex
<i>L. pneumophila</i>	5S rDNA	108 bp	
<i>S. marcescens</i>	<i>aac(6'')-Ib</i>	395 bp	
<i>C. albicans</i>	SAP6	265 bp	

**Table 3. PCR conditions for other strains**

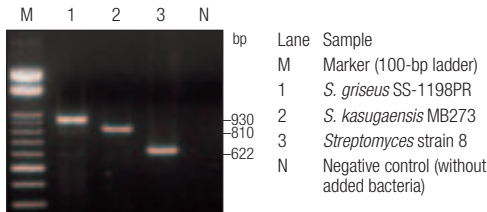
Organisms	PCR*	Primer	Product
	Anneal	size (mer)	%GG %GC
MSRA-Ent.	50°C	19–20	33–52 29–47
<i>Actinomycetes</i>	65°C	20	55–60 67–73
<i>Serratia</i>	55°C	17, 19	59, 37 54
<i>Legionella</i>	60°C	18–24	38–56 42–49
<i>Candida</i>	58°C	10, 22	50, 36 39

\* PCR Conditions: KOD Hot Start DNA Polymerase; extension at 68°C; denaturation at 95°C (except for *Actinomycetes*, at 98°C)

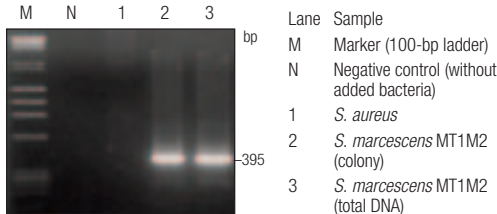
### A. Enterococci



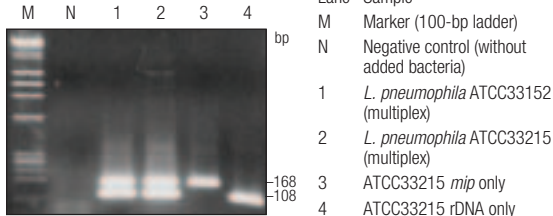
### B. Actinomycetes



### C. Serratia



### D. Legionella



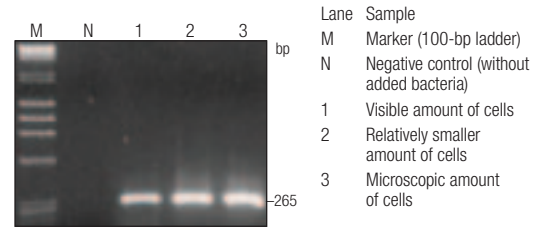
**Figure 3. CD-PCR for Enterococci, Actinomycetes, Serratia, and Legionella**

filled with reaction mixture, then a sterilized toothpick with a visible amount of bacteria on its tip was rinsed off into the first sample tube (Figure 2, lane 1, page 9). For samples 2–12 (Figure 2, lanes 2–12, page 9), this same toothpick was lightly touched to the solution in each successive tube. The inhibition of PCR amplification (Figure 2, lane 1, page 9) indicated that the addition of a visible amount of bacteria comparable to the amount of bacteria necessary for successful CD-PCR from *E. coli* did not produce favorable CD-PCR results with MRSA. Conversely, the positive results observed for less-concentrated samples confirmed that stable PCR amplification was possible when extremely small amounts of bacteria are used.

The amount of DNA template needed to yield PCR product sufficient for visualization through agarose gel analysis after a 30-cycle PCR reaction (approximately 10 ng) was calculated as several hundred femtograms. To meet this condition, theoretically between  $10^2$  and  $10^4$  colony-forming units (cfu) per 20- $\mu$ l reaction volume were required. The number of cells added to the PCR reaction is a key factor in achieving successful CD-PCR for MRSA (1). Furthermore, using KOD Hot Start DNA Polymerase is important for CD-PCR with reproducible results; this same order of consistency was not possible with *Taq* DNA polymerase (1).

### CD-PCR with other bacteria and with fungi

Aside from MRSA, we also confirmed positive results using CD-PCR for Gram+ and Gram- bacteria and for fungi. Gram+ bacteria used were *Enterococcus faecalis*, *E. faecium*, *S. aureus* (three strains), *Streptomyces griseus*, *S. kasugaensis*, and a rare *Streptomyces* strain (strain 8).



**Figure 4. CD-PCR for Candida**

Gram- bacteria used were two strains of clinically-derived *Legionella pneumophila* and one of *Serratia marcescens*. For fungi (yeast), one strain of *Candida albicans* was used (Table 2).

As shown in Table 2, primer pairs designed for the detection of multiple target genes were used in the multiplex CD-PCR with *E. faecalis*, *Streptomyces*, and *Legionella*. PCR cycling conditions are described in Table 3. Because the DNA in *Streptomyces* has a high GC content (roughly 70%), the denaturation temperature for *Actinomycetes* was raised to 98°C.

As shown in Figure 3, the targeted genes for each organism were amplified using CD-PCR. In each case, as with *S. aureus*, a favorable amplification was obtained by adding a microscopic amount of sample to the reaction. Adding a macroscopic (visible) amount of cells inhibited amplification in each case except *Candida* (Figure 4).

When a multiplex CD-PCR was performed (*E. faecalis*, *Streptomyces* and *Legionella*), a known gene profile and corresponding gene amplification pattern were confirmed, verifying that these PCR conditions offered high selectivity. The data from the target gene of *E. faecalis*, also common in *S. aureus*, further corroborated these results (data not shown).

For PCR using *Candida*, DNA obtained from a zymolase-treated (5 min at 37°C) colony can be used as a template (2). As described here, sufficient DNA amplification is possible by the CD-PCR method. In contrast to CD-PCR from bacterial strains, using a visible amount of yeast did not adversely affect amplification.

### Summary

In many procedures for colony PCR, template DNA is extracted by incubation of a bacterial colony in water for 3 minutes at 95°C. When colony-direct PCR (especially multiplex CD-PCR) is used, this

extraction step is no longer necessary, and the detection of multiple strains of bacteria becomes more efficient. A common concern with CD-PCR has been reproducibility. In these studies, KOD Hot Start DNA Polymerase and microscopic amounts of cells generated reproducible colony-direct PCR results.

#### REFERENCES

1. Tsuchizaki, N., Ishikawa, K., and Hotta, K. (2000) *Jpn. J. Antibiot.* 53, 422–429.
2. Ling, M., Merante F., and Robinson, B. H. (1995) *NAR* 23, 4924–4925.

Product	Size	Cat. No.
KOD Hot Start DNA Polymerase	200 U 5 × 200 U	71086-3 71086-4
<small>[includes KOD Hot Start DNA Polymerase (1.0 U/μl), 10X PCR Buffer for KOD Hot Start DNA Polymerase, 25 mM MgSO<sub>4</sub>, and dNTP Mix (2 mM each)]</small>		