

KOD Long PCR Master Mix

KML-101 1 mL x 5
Store at -20°C

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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 while using this kit. All trademarks, trade names, or company names referenced herein are used for identification only and are the property of their respective owners.

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[1] Introduction

Description

KOD Long PCR Master Mix is a 2 × PCR master mix formulated with genetically modified KOD DNA polymerase (UKOD). The combination of UKOD (known for its high fidelity and amplification efficiency) with an elongation accelerator enables rapid PCR at an elongation rate of 10 sec/kb. It maintains exceptional accuracy even with targets exceeding 10 kb. Thus, the master mix is ideally suited for amplifying long DNA fragments for long-read sequencing. It has a high success rate and also supports amplification from crude samples.

KOD Long PCR Master Mix contains two types of anti-KOD DNA polymerase antibodies that inhibit both polymerase and 3'→5' exonuclease activities, thus enabling Hot Start PCR. This master mix generates blunt-end PCR products because of the 3'→5' exonuclease (proof-reading) activity of KOD DNA polymerase.

Features

- Ideal for Long-Read Analysis: Efficient Amplification of Long DNA Targets

KOD Long PCR Master Mix enables the amplification of DNA fragments up to approximately 50 kb using human genomic DNA as template. It is optimized for long-read sequencing applications, allowing the rapid and accurate amplification of long targets.

- Fast PCR

Targets ranging 1–10 kb can be amplified with an extension time of 5 sec/kb. Even for targets exceeding 10 kb, high-speed PCR is achievable with an extension time of 10 sec/kb, significantly reducing overall reaction time. The cycling conditions can be flexibly set when various targets of different sizes are to be amplified.

- High Fidelity

KOD Long PCR Master Mix exhibits approximately 80-fold higher fidelity than the Taq DNA polymerase. This high accuracy is particularly advantageous for various applications, such as the preparation of long target amplicons for sequencing.

- Compatible with GC-rich and AT-rich Targets

Amplification is possible regardless of the target sequence composition. With low amplification bias and high speed, KOD Long PCR Master Mix is also ideal for long-read library preparation.

- Amplification from Crude Samples

KOD Long PCR Master Mix is effective for amplification from crude samples (e.g., biological samples). Various sample types or lysates can be directly used as templates.

[2] Components

KOD Long PCR master Mix includes the following components for 200 reactions (50 μ L total reaction volume):

<KML-101>

KOD Long PCR Master Mix (5 \times 1 mL)

Note:

The reagents can be stored at 4°C for up to 1 month. For longer storage, the reagents should be kept at -20°C.

[3] Primer Design

-Primers should be 25–35 bases in length, with a melting temperature (T_m) above 65°C.

-The optimal GC content of primers is 45%–60%. The ideal GC content in the 5' half and 3' half is 60%–70% and 45%–50%, respectively.

-Priming efficiency can be enhanced by anchoring the 3' end of primers with G or C.

-Primers should be designed to prevent the formation of intermolecular secondary structures or primer dimers.

-Primers containing inosine (dI) or uracil (dU) can be used for various purposes, including metagenomics analyses. Primer design can be facilitated using specific software that allows the substitution of thymine (dT) with dI or dU.

-The following online tool is recommended for amplification after bisulfate treatment:
MethPrimer, <http://www.urogene.org/methprimer/index.html>.

- T_m of the primers should be calculated using the Nearest Neighbor method. The T_m values in this manual were calculated using the following parameters:

Na⁺ concentration: 50 mM

Oligonucleotide concentration: 0.5 μ M

[4] Protocol

1. Standard reaction setup

Before preparing the reaction mixture, all components should be completely thawed and stir thoroughly.

| Component | Volume | Final Concentration |
|---------------------------|-------------|---|
| PCR grade water | X μ L | |
| KOD Long PCR Master Mix | 25 μ L | 1 \times |
| 5 pmol/ μ L Primer #1 | 1.5 μ L | 0.15 μ M |
| 5 pmol/ μ L Primer #2 | 1.5 μ L | 0.15 μ M |
| Template DNA | Y μ L | Genomic DNA \leq 200 ng/50 μ L Plasmid DNA \leq 50 ng/50 μ L cDNA \leq 750 ng (RNA equiv.)/50 μ L Crude sample \leq 5 μ L/50 μ L |
| Total reaction volume | 50 μ L | |

Notes:

-Thin-walled tubes are recommended For PCR analyses.

2. Cycling conditions

The following cycling program is recommended:

| 3-step cycle | Amplicon size < 10 kb | Amplicon size \geq 10 kb | |
|--------------------|------------------------------|-------------------------------|----------------|
| Pre-denaturation : | 94°C, 1 min | 94°C, 1 min | } 25–45 cycles |
| Denaturation : | 98°C, 10 sec | 98°C, 10 sec | |
| Annealing : | (T _m -5)°C, 5 sec | (T _m -5)°C, 5 sec | |
| Extension : | 68°C, 5 sec/kb | 68°C, 10 sec/kb | |

Notes:

-A longer extension time may enhance efficiency, especially for low-copy DNA or crude samples. In such cases, an extension time of 10 sec/kb is recommended.

-The extension temperature should be adjusted according to the T_m of the primers. If the primer T_m exceeds 73°C, the extension temperature should be set to 68°C.

-Poor amplification may be improved by changing the denaturation step to 94°C for 15 sec.

-To prevent incomplete double-strand formation, a final extension step (e.g., 68°C, 10 sec/kb) at the end of the cycle.

<Other cycles>

When non-specific or smeared bands are observed, the following cycles are recommended:

| 2-step cycle | Amplicon size < 10 kb | Amplicon size ≥ 10 kb | |
|---------------------|--------------------------|--------------------------|----------------|
| Predenaturation : | 94°C, 1 min | 94°C, 1 min | ← 25–45 cycles |
| Denaturation : | 98°C, 10 sec | 98°C, 10 sec | |
| Extension : | 68°C, 5 sec/kb | 68°C, 10 sec/kb | |

| Step-down cycle | Amplicon size < 10 kb | Amplicon size ≥ 10 kb | |
|------------------------|--------------------------|--------------------------|----------------|
| Predenaturation : | 94°C, 1 min | 94°C, 1 min | ← 5 cycles |
| Denaturation : | 98°C, 10 sec | 98°C, 10 sec | |
| Extension : | 74°C, 5 sec/kb | 74°C, 10 sec/kb | |
| Denaturation : | 98°C, 10 sec | 98°C, 10 sec | ← 5 cycles |
| Extension : | 72°C, 5 sec/kb | 72°C, 10 sec/kb | |
| Denaturation : | 98°C, 10 sec | 98°C, 10 sec | ← 5 cycles |
| Extension : | 70°C, 5 sec/kb | 70°C, 10 sec/kb | |
| Denaturation : | 98°C, 10 sec | 98°C, 10 sec | ← 15–30 cycles |
| Extension : | 68°C, 5 sec/kb | 68°C, 10 sec/kb | |

[5] Template

a. Purified DNA or cDNA

The appropriate template amounts for a 50 µL reaction are summarized in the following table:

| | | Recommended range | |
|------------------|-----------------|-----------------------|--------------------|
| Genomic DNA | Eukaryotic DNA | 1–200 ng | 50 ng |
| | Prokaryotic DNA | 0.1–200 ng | 10 ng |
| Plasmid DNA | | 1 pg–50 ng | 10 ng |
| cDNA | | < 750 ng (RNA equiv.) | 50 ng (RNA equiv.) |
| Lambda phage DNA | | 0.01–10 ng | 1 ng |

-For amplification of long DNA targets (> 10 kb), a higher amount of template DNA than the standard amount is recommended.

-Quality of the template DNA should be checked by electrophoresis. Both the length and purity of the template DNA affect amplification results.

-Contaminating RNA in cDNA can inhibit PCR. The reaction should contain < 750 ng of RNA equivalent per 50 µL reaction.

b. Tissues and cells

When adding biological samples directly to the PCR reaction mixture, the following sample types can be applied to a 50 μ L reaction.

| Sample | Appropriate template amount | Remarks |
|----------------|---|--|
| <i>E. coli</i> | Pick a small amount of cells from colonies | If reproducibility is poor, cells suspended in TE buffer should be added (2–5 μ L) |
| Yeast | Pick a small amount of cells from colonies | |
| Fungus | Pick a small amount of cells from colonies | |
| Cultured cells | 10^1 – 10^5 cells/2 μ L medium or PBS | |
| Whole Blood | 1–2 μ L | |
| Nail | 1 \times 1 mm | } As the concentration of extracted DNA is low, 35–40 cycles are required. |
| Hair root | 1–2 cm | |
| Leaf | 2 \times 2 mm | |
| Milled rice | 0.5 \times 0.5 mm | |
| Mouse tail | 1 \times 1 mm | On an agarose gel assay, a portion of amplicon may remain in the slots. |

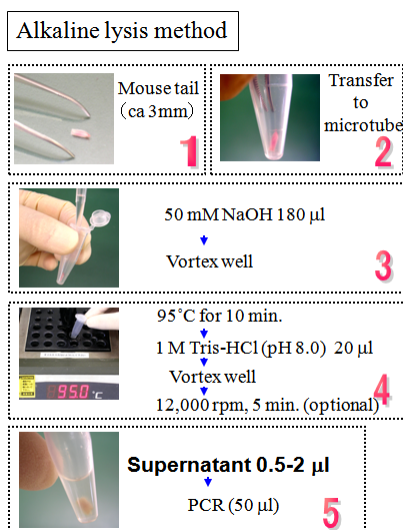
*In the case of direct amplification from animal tissues, such as mouse tail, a portion of the amplification product may remain in the gel slot during agarose gel electrophoresis. Adding 10 μ L of 20 mg/mL proteinase K to 50 μ L of PCR product prior to electrophoresis is effective for dissociating aggregates.

c. lysate

The following methods are recommended for preparing lysates for PCR. lysates can be stored at 4°C for several weeks. For long-term storage, the lysates should be kept at -20°C.

<Alkaline lysis method>

The “Alkaline lysis method” is recommended for rapid preparation of mouse tail or nail lysates suitable for PCR amplification.



*Mouse tails cannot be completely dissolved.

[96-well PCR plate protocol by alkaline lysis method]

The following protocol is suitable for preparing lysates from a large number of mouse tail samples:

1. Transfer mouse tails (approximately 3 mm) to a 96-well PCR plate.
2. Add 180 μ L of 50 mM NaOH and vortex.
3. Spin down.
4. Incubate the mixture at 95°C for 10 min in a thermal cycler.
5. Add 20 μ L of 1 M Tris-HCl (pH 8.0) and vortex.
6. Spin down.

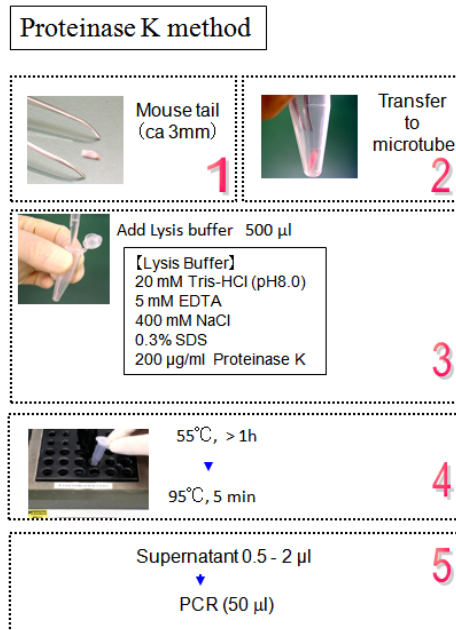
Supernatant (0.5–2 μ L) → PCR (50 μ L)

<Proteinase K method>

The “Proteinase K method” is recommended for efficient preparation of mouse tail or nail lysates suitable for PCR amplification. This protocol can also be applied to other sample types.

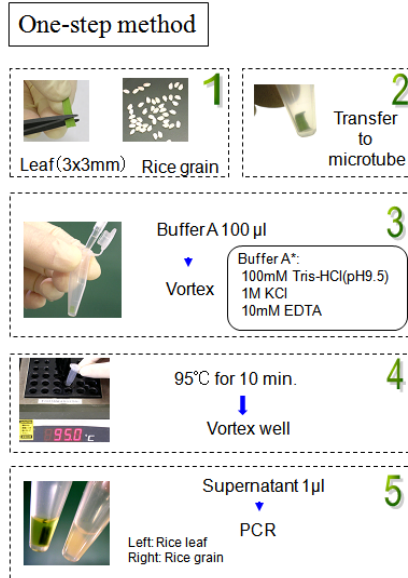
Sample sizes:

- Mouse tail: 3 mm
- Nail: 3 mm
- Leaf: 3 × 3 mm
- Rice grain: 1 grain



<One step method>

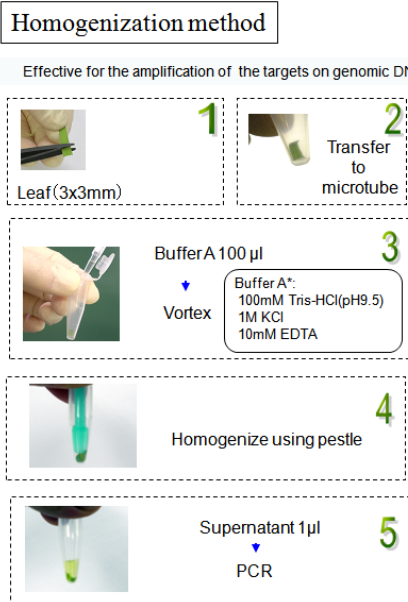
The “One-step method” is recommended for rapid preparation of a plant tissue lysate suitable for PCR amplification.



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<Homogenization method>

The “Homogenization method” is recommended for effective preparation of a plant tissue lysate suitable for PCR amplification. This method is effective in amplifying genomic DNA targets.



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[6] PCR product cleanup

-PCR product cleanup may be required to remove enzymes, dNTPs, and reaction buffers prior to sensitive downstream applications. When purifying PCR products generated with KOD Long PCR Master Mix using magnetic beads, abnormal bead pelleting may be observed. The following pre-treatment can be used to improve bead recovery if this occurs:

<Proteinase K treatment>

Add 1 μ L 10–20 mg/mL of Proteinase K to 50 μ L of PCR product, mix well, and incubate at room temperature for at least 1 min.

<Tween 20 treatment>

Add 1 μ L of 10% Tween 20 to 50 μ L of PCR product.

[7] Cloning of PCR products

-KOD Long PCR Master Mix generates blunt-end PCR products because of its 3'→5' exonuclease (proof-reading) activity. Therefore, the PCR products can be cloned using a blunt-end cloning method.

-The master mix should be purified prior to restriction enzyme treatment during cloning. The 3'→5' exonuclease activity of KOD DNA polymerase remains until the end of PCR.

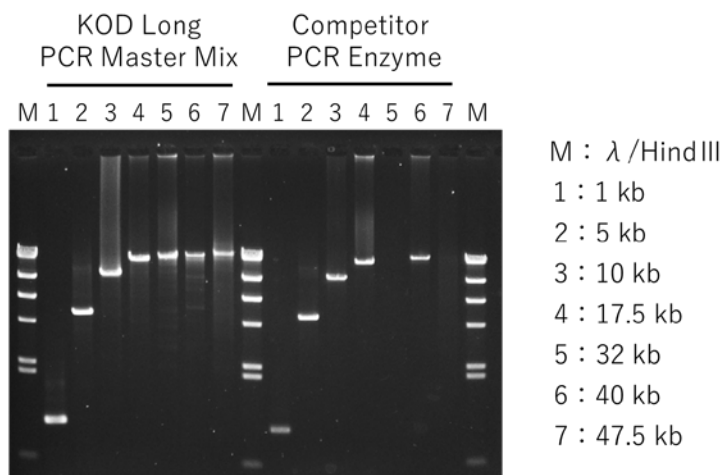
-The dedicated TA cloning kit “Target Clone™ -Plus- [Code No. TAK-201]” is recommended for cloning blunt-end PCR products generated by KOD DNA polymerase (see [10] Related products).

[8] Examples

Example 1. Amplification of long DNA targets (1–47.5 kb) using fast PCR.

Long DNA targets were amplified using KOD Long PCR Master Mix with human genomic DNA as template under fast PCR conditions.

Successful amplification of all long DNA targets was confirmed using this product.

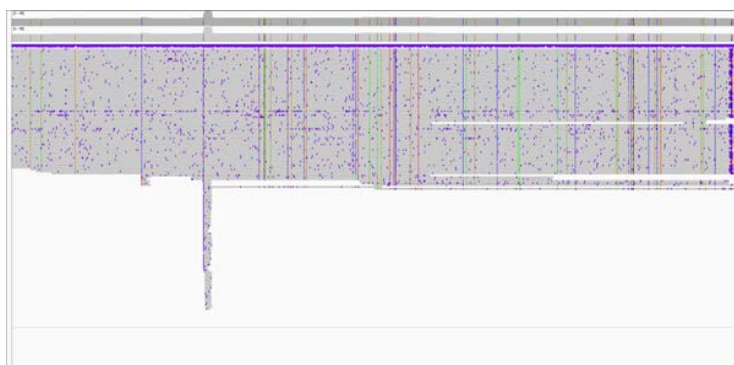


Example 2. Long-Read Analysis of PCR Products

The 47.5 kb PCR product, amplified as described above, was purified and used to prepare a long-read sequencing library following the recommended protocol of the Ligation Sequencing Kit DNA V14 (Code: SQK-LSK114; Oxford Nanopore Technologies). The resulting library was analyzed using a Flongle Flow Cell (R10.4.1) (Code: FLO-FLG114; Oxford Nanopore Technologies).

Reads covering the entire 47.5 kb length were obtained, confirming that the target sequence was successfully amplified.

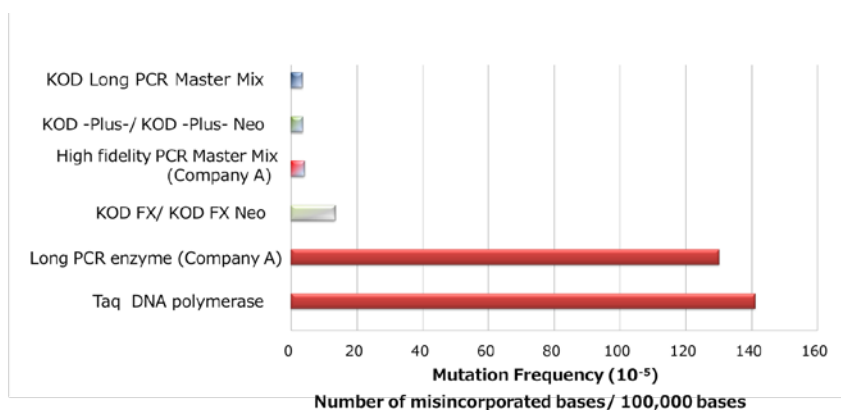
Confirmation of Full-Length Target Region Amplification Using Integrative Genomics Viewer (IGV)



Example 3. PCR Error Ratio

The error ratios of various PCR enzymes were compared by sequencing amplicons of the human β -globin gene. The amplicons were cloned into a vector using TArget clone™ -Plus- [Code No. TAK-201], and their sequences were determined.

KOD Long PCR Master Mix showed excellent fidelity, with a mutation frequency approximately 80 times lower than that of rTaq DNA polymerase.



[9] Troubleshooting

| Symptom | Cause | Solution |
|---|--|---|
| No PCR product/low yield | Cycling conditions are unsuitable. | Increase the extension time up to 10–30 sec/kb. |
| | | Increase the number of cycles by 2–5. |
| | | Lower annealing temperature in the 3-step cycle by up to $T_m - 7-10^{\circ}\text{C}$. |
| | Template DNA is of poor quality and/or quantity. | Increase the amount of template DNA. |
| | | Decrease the amount of template DNA to reduce the contaminated PCR inhibitors. |
| | | Use purified templates. |
| | | Degrade or eliminate RNA contamination in the DNA sample. |
| Primer is suboptimal. | Use freshly prepared primers. | |
| | Redesign primers. | |
| Smearing/Extra band | Cycling conditions are unsuitable. | Change from 3-step cycling to 2-step cycling. |
| | | Change from 2-step cycling to step-down cycling. |
| | | Decrease the number of cycles by 2–5. |
| | Excessive amount of template DNA. | Reduce the amount. |
| | Primer quality is insufficient. | Use fresh primers. |
| Redesign primers. (Longer primers, 25–35 mer, may eliminate smearing or extra bands) | | |
| Poor TA cloning efficiency | PCR products have blunt ends. | Follow blunt-end cloning guidelines. Use TArget Clone™ -Plus- [Code No. TAK-201] (see [10] Related products). |

[10] Related products

| Product name | Package | Code No. |
|-------------------------------|---------------------------|----------|
| TA rgent Clone™ -Plus- | 10 reactions | TAK-201 |
| 10 × A-attachment Mix | 25 reactions | TAK-301 |
| L igation high Ver.2 | 100 reactions (750 μL) | LGK-201 |

Target Clone™ -Plus- is a highly efficient TA cloning kit. The kit is suitable for TA cloning of blunt-ended PCR products amplified using products containing KOD DNA polymerase (KOD -Plus- [Code No. KOD-201]; KOD -Plus- Neo [Code No. KOD-401]; KOD FX [Code No. KFX-101]; KOD FX Neo [Code No. KFX-201]; KOD -Multi & Epi- [Code No. KME-101]; or KOD One™ PCR Master Mix [Code No. KMM-101, KMM-201]). The kit contains the pTA2 Vector, 2x Ligation Buffer, T4 DNA Ligase, and 10× A-attachment Mix.

The 10× A-attachment Mix is a reagent comprising an anti-KOD DNA polymerase antibody that specifically inhibits the 3'→5' exonuclease activity (proof-reading activity) of KOD, along with Taq DNA polymerase, which exhibits terminal transferase activity. PCR products generated using KOD DNA polymerase possess blunt ends due to its 3'→5' exonuclease activity. The 10× A-attachment Mix enables the addition of a single dA overhang at the 3' ends of these products. Products with 3'-dA overhangs can be directly cloned into arbitrary T-vectors using ligation reagents, such as Ligation high Ver.2 [Code No. LGK-201].

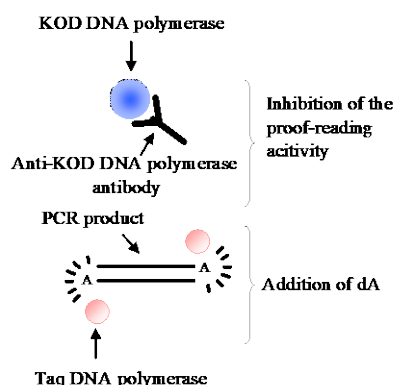


Figure. Principle of the 10× A-attachment Mix