High-Fidelity DNA Polymerase Chain Reaction

Materials

2 × Hieff Canace® Plus PCR Master Mix (With Dye), including Hieff Canace® Plus High-Fidelity DNA Polymerase, dNTPs, and an optimized buffering system, was obtained from YEASEN (Shanghai, China).
Forward primer and reverse primer were obtained from Genscript (Jiangsu, China).
DNA template was obtained from Genscript (Jiangsu, China).
RNase-free ddH_2O

Procedures

1. Prepare the PCR mix in a single microcentrifuge tube on ice by adding the components in the order indicated below:

Reagents	Volume
2×Hieff Canace® Plus PCR Master Mix (with dye)	25μl
RNase-free ddH_2O	18.5μl
DNA template (1ng/μl)	2.5μl
Forward primer (10μM)	2μl
Reverse primer (10μM)	2μl

2. Thoroughly mix the above reagents and briefly centrifuge them. Perform PCR using the following cycling parameters:

Step	Temperature	Duration	Cycles
Initial Denaturation	98 **ºC**	3 min	1
Denaturation	98 **ºC**	10s	30
Extension	68 **ºC**	13s	
Final extension	72 **ºC**	5 min	1

In Vitro Transcription

Materials

5 × TranscriptMax Reaction Buffer, NTP mix, TranscriptMax Enzyme Mix, and Nuclease-free water were all supplied by TranscriptMax T7 High Yield RNA Synthesis Kit from Tolobio (Shanghai, China).
DNA template was obtained from Genscript (Jiangsu, China).

Procedures

1. Prepare the reaction system in a single microcentrifuge tube on ice by adding the components in the order indicated below:

Reagents	Volume
5 × TranscriptMax Reaction Buffer	4μl
NTP mix	8μl
TranscriptMax Enzyme Mix	2.1μl
Nuclease-free water	0.9μl
DNA template	5μl

(The 5 × TranscriptMax Reaction Buffer contains spermidine, and spermidine could form complex precipitates with nucleic acids. The DNA transcription template should be added to the reaction system as late as possible.)

2. Thoroughly mix the reagents and briefly centrifuge them. Incubate at 37°C for 4 hours to perform in vitro transcription.

Transcription product purification

Materials

2 × DNase Ⅰ Buffer, DNase Ⅰ, DEPC-treated Water, and Magnetic Beads were supplied by TOLO RNAclean Kit from Tolobio (Shanghai, China).
Transcription product

Procedures

Remove the DNA template

1. Prepare 30 μL of DNase Ⅰ reaction solution in a single microcentrifuge tube on ice by adding the components according to the table below, and add it to 20 μL of the transcription product to remove the DNA template in the transcription system.

Reagents	Volume
2 × DNase Ⅰ Buffer	25μl
DNase Ⅰ	4μl
DEPC-treated Water	1μl

2. Incubate the reaction system at 37°C for 30 minutes.

Transcription product purification

1. First, take the magnetic beads out of the 4℃ fridge and leave them at room temperature (RT) for about 30 minutes to allow their temperature to reach RT. Use a vortex to mix the magnetic beads thoroughly. Pipette 25 μL of magnetic beads and 50 μL of isopropyl alcohol into the 50 μL RNA sample to be purified, and mix thoroughly by pipetting up and down.
2. Incubate at RT for 5 minutes to make RNA bind with magnetic beads.
3. Put the sample on the magnetic rack for 5 minutes. When the solution becomes clear, carefully remove the supernatant.
4. Keep the sample on the magnetic rack, then add 200 μL freshly prepared 80% (v/v) alcohol to wash the magnetic beads. Incubate at RT for 30 seconds, then carefully remove the supernatant.
   Note: the 80% (v/v) alcohol needs to be freshly prepared using Nuclease-free water.
5. Repeat step 4 to rewash the magnetic beads.
6. Always keep the sample on the magnetic rack and open the cover to air-dry the magnetic beads for 5 minutes.
   Note: When opening the cover to dry the magnetic beads, it is necessary to avoid excessive drying.
7. Take the sample out of the magnetic rack. Add 50 μL Nuclease-free water and mix thoroughly by pipetting up and down. Leave the sample at RT for 5 minutes.
8. Put the sample on the magnetic rack for 5 minutes. When the solution becomes clear, carefully transfer the supernatant to a new Nuclease-free PCR tube.
9. Incubate the purified RNA at 70 °C for 10 minutes to inactivate residual DNase Ⅰ.
10. The purified RNA is preserved at -20 °C.

Colony PCR

Materials

2 × Hieff Canace® Plus PCR Master Mix (With Dye), including Hieff Canace® Plus High-Fidelity DNA Polymerase, dNTPs, and an optimized buffering system, was obtained from YEASEN (Shanghai, China).
Forward Primer and Reverse Primer were obtained from Genscript (Jiangsu, China).
E. coli MG1655 was obtained from D&B (Shanghai, China).
ddH_2O
LB

Procedures

shake-flask culture

1. Prepare LB liquid medium and dispense into conical flasks. Fill the conical

flask to no more than one-third of its volume to allow headspace for oxygen. Sterilise using an autoclave at 121°C for 20 minutes, then cool and set aside for later use.

2. Use a sterilized pipette to pick a single colony from the solid LB medium and transfer it to the sterilized liquid LB medium. Place the conical flasks in a shaker, set the temperature to 37°C, and the rotation speed to 220 revolutions per minute. Incubate for 12-16 hours.

Colony PCR

1. Prepare the PCR mix and bacteria solution in a single microcentrifuge tube on ice by adding the components in the order indicated below:

Reagents	Volume
2×Hieff Canace® Plus PCR Master Mix (with dye)	25μl
ddH_2O	16μl
MG1655 solution	5μl
Forward primer (10μM)	2μl
Reverse primer (10μM)	2μl

2. Thoroughly mix the above reagents and briefly centrifuge them. Perform PCR using the following cycling parameters:

Step	Temperature	Duration	Cycles
Initial Denaturation	98 **ºC**	3 min	1
Denaturation	98 **ºC**	10s	30
Extension	68 **ºC**	13s	
Final extension	72 **ºC**	5 min	1

PCR product purification

Do as mentioned above.

sgRNA/Cas9 Incubation

Do as mentioned above.

Agarose Gel Electrophoresis

Prepare a 1.5% agarose gel, then load 5 μL DNA marker and 10 μL product. Start the electrophoresis at 120V for 25min.