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PrimeSTAR® HS (Premix)

I. Description:
PrimeSTAR® HS (Premix) is an optimized mixture composed of PrimeSTAR® HS DNA Polymerase, which is a high-fidelity DNA polymerase developed by TaKaRa Bio Inc, reaction buffer and dNTP mixture as 2-fold concentration. As this product offers quick preparation of reaction mixture and reduction of contamination risk, it is also useful for high-throughput application.
PrimeSTAR® HS DNA Polymerase has a matchless proof reading activity due to very strong 3'-5' exonuclease activity, and besides its amplification efficiency is higher than that of Taq DNA Polymerase. Furthermore an antibody-mediated hot start formulation prevents false initiation events during the reaction assembly due to mispriming and primer digestion. When used with TaKaRa’s optimized reaction buffer, PrimeSTAR® HS achieves the high fidelity, high sensitivity, and high specificity required for applications such as DNA amplification from cDNA library.

II. Components (for 100 reactions):
PrimeSTAR® HS (Premix) ........................................500 μl X5
Content: PrimeSTAR® HS DNA Polymerase 1.25 U/25 μl
      dNTP Mixture 2 X conc.; 0.4 mM each
      PrimeSTAR® Buffer 2 X conc.; including 2 mM Mg²⁺

III. Storage:
— 20°C
Repeated freeze-thaw cycles may decrease the enzyme activity. Once it thawed, dispense into PCR tubes and store at — 20°C . (ex. For 50 μl PCR reaction, dispense by 25 μl each tube.)

IV. Features:
A: Accuracy
Mutation frequency of PrimeSTAR® HS was investigated by actually analyzing large number of bases of sequence data.

[Method]
Eight arbitrarily selected GC-rich regions were amplified with PrimeSTAR® HS and other enzymes, using the Thermus thermophilus HB8 genomic DNA as a template
Each PCR product (approx. 500 bp each) was coloned into a suitable plasmid. Multiple clones were picked up per region respectively, and were subjected to sequence analysis.

[Result]
Sequence analysis of DNA fragments amplified using PrimeSTAR® HS demonstrated only 12 mismatched bases per 249,941 total bases. This is higher fidelity than an alternative high fidelity enzyme from Company A, and 10x higher fidelity than Taq DNA polymerase.
PrimeSTAR® HS DNA Polymerase possesses extremely high priming efficiency. Thus by using a short annealing time, only 5 or 15 seconds, highly specific amplification can be achieved. Refer to "VI. PCR Conditions" (page 5) for determining reaction conditions.

The above method is the most realistic method to investigate the mutation frequency. Based on the above sequence analysis results, it is strongly recommended to use PrimeSTAR® HS DNA Polymerase for the PCR amplification that requires the extreme accuracy.

### B: High Priming efficiency - Short Annealing Time

PrimeSTAR® HS DNA Polymerase possesses extremely high priming efficiency. Thus by using a short annealing time, only 5 or 15 seconds, highly specific amplification can be achieved. Refer to "VI. PCR Conditions" (page 5) for determining reaction conditions.

<table>
<thead>
<tr>
<th>Template</th>
<th>M1: pHY Marker</th>
<th>M2: λ/Hind III digest</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1: Annealing at 55°C for 5 sec.</td>
<td>2: Annealing at 55°C for 30 sec.</td>
</tr>
</tbody>
</table>

**Template:** Human genomic DNA 100 ng  
50 μl PCR reaction  
3-step PCR Method, 30 cycles
C: Amplification ability using genomic DNA as a template

Amplification of various DNA fragment sizes, using human genomic and *E. coli* genomic DNA as the template was confirmed.

**Template: Human genomic DNA [50 ng/50 μl PCR reaction]**

**Thermal cycler: TaKaRa PCR Thermal Cycler Dice (Cat.# TP600)**

**Thermocycling conditions:**

<table>
<thead>
<tr>
<th>Fragment Size</th>
<th>Method</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 ~ 6 kbp</td>
<td>3-step PCR Method</td>
<td>98℃, 0 sec.</td>
<td>30 cycles</td>
<td></td>
</tr>
<tr>
<td>60℃, 5 sec.</td>
<td></td>
<td>72℃, 1 min./kb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5 ~ 8.5 kbp</td>
<td>2-step PCR Method</td>
<td>98℃, 10 sec.</td>
<td>30 cycles</td>
<td></td>
</tr>
<tr>
<td>68℃, 8 min.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**M**: λ -Hind III digest

**Template: E.coli genomic DNA [100 pg/50 μl PCR reaction]**

**Thermal cycler: TaKaRa PCR Thermal Cycler Dice (Cat.# TP600)**

**Thermocycling conditions:**

<table>
<thead>
<tr>
<th>Fragment Size</th>
<th>Method</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 kbp</td>
<td>3-step PCR Method</td>
<td>98℃, 10 sec.</td>
<td>30 cycles</td>
<td></td>
</tr>
<tr>
<td>4 kbp</td>
<td></td>
<td>60℃, 5 sec.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 kbp</td>
<td></td>
<td>72℃, 1 min./kb</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**M**: λ -Hind III digest

**C**: Amplification ability using genomic DNA as a template
V. General Composition of PCR Reaction Mixture:

- PrimeSTAR® HS (Premix) 25 μl
- Primer 1 0.2 ~ 0.3 μM (final conc.)
- Primer 2 0.2 ~ 0.3 μM (final conc.)
- Template < 200 ng
- Sterilized distilled water up to 50 μl

VI. PCR Conditions:

(A) 3-step PCR Method
98℃, 0 sec.
55℃, 5 sec. or 5 sec.
72℃, 1 min./kb
30 cycles

(B) 2-step PCR Method
98℃, 0 sec.
68℃, 1 min./kb
30 cycles

TaKaRa recommends first trying the 3-step PCR firstly with PrimeSTAR® HS (Premix).

- Denaturation conditions: 98℃, 5 ~ 10 sec.
  Alternatively, if a lower denaturation temperature 94℃ is used, set a denaturation time to be 10 ~ 15 sec must also be used.
- Annealing temperature: Initially, try 55℃ (optimization may be required.)
- Annealing time: Annealing time is dependent upon primer Tm values. Calculate primer Tm values using the following formula ( * );
  When Tm ≥ 55℃ Set it for 5 sec.
  When Tm < 55℃ Set it for 15 sec.

( * ) Tm value (℃) = 2(NA+NT) + 4(NG+NG) - 5

The above Tm value formula is valid for primers whose lengths are ≤ 25 mer. For primers longer than 25 mer, an annealing time of 5 sec. should be used.

[ Important Note ]
Because the priming efficiency of PrimeSTAR® HS is extremely high, an annealing time of 5 sec. or 15 sec. should be adopted. Longer annealing times may cause smearing of PCR products.

Please try the 2-step PCR when smeared DNA products are observed in following agarose gel electrophoresis of amplified DNA that has been obtained through the 3-step PCR or when using the primers with Tm values ≥ 70℃. Please refer to "VII. Optimization of parameters, IX. Troubleshooting" for further PCR condition recommendations.
VII. Optimization of Parameters:

In order to obtain the best PCR results, it is important to optimize your PrimeSTAR® HS reaction parameters to fully utilize the enzyme’s properties and advantages.

1) Template DNA amount

Recommended template DNA amounts (assuming a 50 μl reaction):
- Human genomic DNA: 5 ng ~ 200 ng (<200 ng)
- E.coli genomic DNA: 100 pg ~ 100 ng
- cDNA library: 1 ~ 200 ng
- λ DNA: 10 pg ~ 10 ng
- Plasmid DNA: 10 pg ~ 1 ng

Avoid using excess amounts of template DNA, which can lower enzyme reactivity.

2) Primer and PCR condition

The use of commercially available primer design software, such as OLIGO™ Primer Analysis Software (Mirai Bio Inc.) is recommended for obtaining appropriate primer sequences that follow general primer design guidelines yet are tailored specifically for your template DNA.

Guidelines for Primer Design:
- a) Primer length:
  i) For general amplification of DNA fragments, 20 ~ 25 mer primers are suitable.
  ii) Exact PCR conditions should be determined by referring to "VI. PCR condition".
- b) Modified bases:
  i) Never use primers containing inosine (I) with PrimeSTAR® HS (Premix)
- c) Degenerate primers:
  i) Degenerate primers may be used with PrimeSTAR® HS (Premix)

3) Annealing conditions

Annealing conditions should be determined by referring to "VI. PCR condition". When the amplified PCR product is of low yield, refer to the following troubleshooting recommendations:

<Smearing and/or extra bands appear on agarose gels.>
- i) Shorten the annealing time. For example, decrease time from 15 sec. to 5 sec.
- ii) If the annealing time is already 5 sec., then raise the annealing temperature to 58 ~ 65°C
- iii) Try 2-step PCR.
<Target product is not amplified.>

i) Extend the annealing time. For example, increase time from 5 sec. to 15 sec.

ii) Lower the annealing temperature to 50 ~ 53°C.

VIII. Electrophoresis, Cloning, and Sequencing of Amplified Products:

1) Electrophoresis of the amplified product

TAE Buffer is recommended for agarose gel electrophoresis of amplified products that are obtained using PrimeSTAR® HS (Premix).

**Note:** Use of TBE Buffer may result in DNA band patterns which are enlarged at the gel bottom.

2) Cloning

Most PCR products amplified with PrimeSTAR® HS (Premix) have blunt-end termini. Accordingly they can directly be cloned into blunt-end vectors (if necessary, phosphorylate before cloning), but are not clonable into T-vectors. Reagent Set for Mighty Cloning Kit (Blunt End) (Cat.#6027)* is recommended for cloning into a blunt-end vector.

* Reagent Set for Mighty Cloning Kit (Blunt End) should be used in combination with Micropure™-EZ (Millipore).

3) Restriction enzyme reaction

Prior to performing restriction enzyme digestion of amplified PCR products, remove all traces of PrimeSTAR® HS (Premix) from the reaction mix by phenol/chloroform extraction. Particularly for 3'-protruding restriction enzymes, such as Pst I, the 3'-protruding termini produced by these enzymes may be deleted during digestion by 3' → 5' exonuclease activity of residual amounts of PrimeSTAR® HS (Premix).

4) Direct sequencing

It is recommended to perform phenol/chloroform extraction of PCR products prior to direct sequencing and ensure inactivation of 3' → 5' exonuclease activity.
IX. Troubleshooting:

1) Problem: No amplified product, or low amplification efficiency.
Remedy:
   i) Extension time: Use on extension time > 1 min/kb.
   ii) Annealing time: 15 sec.
   iii) Annealing temperature: Lower temperature in decrements of 2°C.
       Alternatively, perform cycling using the 3-step PCR Method.
   iv) Purity and quality of template DNA: Use an appropriate amount of template
       DNA; refer to "VII. Optimization of Parameters". Use a more highly purified
       template DNA.
   v) Primer concentration: Test a final primer concentration in the range of
       0.2 ~ 0.5 μM.

2) Problem: Extra bands appear or DNA smearing is observed during agarose gel
   electrophoresis.
Remedy:
   i) Annealing time: 5 sec.
   ii) Annealing temperature: Raise the temperature in increments of 2°C.
       Alternatively, perform cycling using the 2-step PCR Method.
   iii) Extension time: 1 min/kb. Avoid excessive extension times.
   iv) Template DNA: Use an appropriate amount of template DNA;
       Avoid excessive amounts of template DNA.
   v) Cycle number: 25 ~ 30 cycles.
   vi) Primer concentration: Determine the optimal concentration that lies within
       a range of 0.2 ~ 0.3 μM (final conc.).
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