HIGH FIDELITY PCR ENZYME MIX

High Fidelity PCR Enzyme Mix is an efficient and robust PCR system, which is designed for high fidelity PCR applications. High fidelity PCR using *Pfu* or other proofreading DNA polymerases alone is generally limited to amplification of up to 3- 6 kb. In many cases, the yield of the PCR product is low.
Fermentas' High Fidelity PCR Enzyme Mix contains a unique blend of a high processive *Taq* DNA polymerase and a second *Pfu* DNA polymerase, that exhibits a 3'→5' exonuclease activity. This blend increases the length and yield of amplification products with high DNA synthesis fidelity. High Fidelity Enzyme Mix amplifies genomic targets up to 10 kb and viral DNA to 20 kb. The fidelity of High Fidelity PCR Enzyme Mix (5.2×10^{-6} errors per nt per cycle) is at least 4 times greater than that of *Taq* DNA polymerase (2.2×10^{-5} errors per nt per cycle) alone.
For amplification of long PCR products, use Long PCR Enzyme Mix, which typically generates a high yield of PCR products and can be used for amplification of difficult or long targets up to 47 kb.

The **High Fidelity PCR Enzyme Mix** offers the following advantages over conventional PCR using a single DNA polymerase: **high fidelity, increased range, increased efficiency and greater yields.**

COMPONENTS OF THE KIT

Component	#K0191 for 40-80 rxns of 50 µl	#K0192 for 200-400 rxns of 50 µl
High Fidelity PCR Enzyme Mix, 5 u/µl Enzyme solution in buffer, containing 50% glycerol	20 µl	100 µl
10X High Fidelity PCR Buffer	0.6 ml	2 x 1.25 ml
10X High Fidelity PCR Buffer with $MgCl_2$ 10X High Fidelity PCR buffer with 15 mM $MgCl_2$	0.6 ml	2 x 1.25 ml
$MgCl_2$ Solution 25 mM $MgCl_2$ solution	0.6 ml	2 x 1.25 ml
Water, nuclease-free 0.22 µm membrane-filtered molecular biology grade water	1.25 ml	2 x 1.25 ml

PCR GUIDELINES

Primer design

PCR primers are usually 20-36 nucleotides in length.
The GC content should be 40-60%. The C and G nucleotides should be distributed uniformly within the full length of the primer. More than three G or C nucleotides at the 3'-end of the primer should be avoided, as non-specific priming might be increased. In order to avoid primer-dimer and hairpin formation, the primer should not be self-complementary or complementary to any other primer in the reaction mixture. The melting temperature of the flanking primers should not differ by more than 5°C, so the GC content and length must be chosen accordingly. Check for all possible sites of complementarity between primers and the template DNA. If primers are degenerate, at least 3 conservative nucleotides must be located at the primer's 3'-end.
For PCR use primers at 0.2 - 1 µM concentration.

Template DNA

The amount of template DNA is usually in the range of 0.01 - 1 ng for plasmid or phage DNA and 0.1 - 1 µg for genomic DNA, in a total reaction volume of 50 µl. Higher amounts of template DNA usually increase the yield of non-specific PCR products. However, if the fidelity of synthesis is crucial, the maximal allowable template DNA quantities should be used to increase the percentage of "correct" PCR products. Nearly all routine methods are suitable for template DNA purification, although, even trace amounts of agents used in DNA purification procedures (phenol, EDTA, proteinase K, etc.) strongly inhibit DNA polymerases. Ethanol precipitation of DNA and repetitive treatments of DNA pellets with 70% ethanol is usually effective though in removing traces of contaminants from the DNA sample.

High quality and adequate length of the template are essential for reliable amplification of larger fragments. Nicked or damaged DNA can serve as a potential priming site resulting in higher background. Avoid repeated freeze - thawing minimize damage of large DNA fragments.

Magnesium concentration

10X High Fidelity PCR buffer with 15 mM $MgCl_2$ is optimized for most PCR applications. If the DNA samples contain EDTA or other chelators, raise the $MgCl_2$ concentration in the reaction mixture proportionally. The set includes 10X High Fidelity PCR buffer without $MgCl_2$ and a 25 mM $MgCl_2$ solution for use when optimization is required.

dNTP concentration
0.2 mM for each dNTP is optimal for most PCR applications. It is very important to have equal concentrations of each dNTP (dATP, dCTP, dGTP and dTTP), as inaccuracity in the concentration of even a single dNTP increases the misincorporation level.

Enzyme concentration
For PCR fragments up to 5 kb, use 1.25 u of High Fidelity PCR Enzyme Mix per 50 µl reaction volume. For PCR fragments >5 kb, High Fidelity PCR Enzyme Mix amount should be increased to 2.5 u per 50 µl.

GENERAL PCR PROTOCOL

Preparation of reaction mixture

Always set up the PCR reaction on ice. Setting up at room temperature can result in primer degradation by the 3'→5' exonuclease proofreading activity of the enzyme mix.

Gently vortex and briefly centrifuge all solutions after thawing. To perform several parallel reactions, prepare a master mix containing water, buffer, dNTPs, primers and template DNA solutions in a single tube, which can then be aliquoted into individual tubes. High Fidelity PCR Enzyme Mix should be added last. This method of setting reactions minimizes the possibility of pipetting errors and saves time by reducing the number of reagent transfers.

- Add, in a thin walled PCR tube, on ice:

Reagent	**Quantity for 50µl**	**Final concentration**
Water, nuclease-free	Variable	—
10X High Fidelity PCR Buffer with $MgCl_2$*	5 µl	1X
2 mM dNTP mix	5 µl	0.2 mM of each
Primer I	Variable	0.2-1 µM
Primer II	Variable	0.2-1 µM
Template DNA: Plasmids or phages Genomic DNA	Variable	 0.01-1 ng / 50 µl 0.1-1 µg / 50 µl
High Fidelity PCR Enzyme Mix	0.25-0.5 µl	1.25-2.5 u / 50 µl

*If using 10X High Fidelity PCR Buffer (without $MgCl_2$) refer to the table below for the selection of 25 mM $MgCl_2$ solution volume:

Final concentration of $MgCl_2$ in 50 µl reaction mix, mM	1.0	1.25	1.5	1.75	2.0	2.5	3.0	4.0
Volume of 25 mM $MgCl_2$, µl	2	2.5	3	3.5	4	5	6	8

- Gently vortex the sample and briefly centrifuge to collect all drops from walls of tube. If the thermal cycler is not equipped with a heated cover, overlay the sample with a half volume of mineral oil or add an appropriate amount of wax.
- Place samples in a thermocycler and immediately start PCR.

Cycling conditions

Optimal reaction conditions vary according to the amplified fragment size, template, primers, reaction volume, PCR tubes and thermal cycler used.

❶ **Initial denaturation step**

The complete denaturation of the DNA template at the start of the PCR is of key importance. Incomplete denaturation of DNA results in the inefficient utilization of template in the first amplification cycle and a poor yield of PCR product. The initial denaturation should be performed over an interval of 1-2 min at 94°C if the GC content is 50% or less. This interval should be extended up to 5 min for GC rich templates.

Use the shortest possible denaturation time. Exposure of DNA to high temperatures causes some depurination of single-stranded DNA during denaturation, which eventually leads to truncation. High temperature also leads to gradual loss of enzyme activity.

❷ **Denaturation step**

20-60 s denaturation at 94°C- 95°C is usually sufficient. Too short a denaturation time or too low a denaturation temperature may cause either diffuse smearing upon electrophoresis or poor amplification efficiency. Too long a denaturation time or too high a denaturation temperature may result in a nonspecific product.

❸ **Annealing step**

The optimal annealing temperature is usually 5°C lower than the melting temperature of the primer-template DNA duplex. Incubation for 30-60 s is usually sufficient. Too high annealing temperature generates no amplification products, while too low a temperature enhances nonspecific reactions.

❹ Elongation step

The recommended elongation temperature is 72°C when amplifying fragments up to 3 kb. When amplifying >3 kb fragments, use 68°C. The recommended time setting is 1 min per 1 kb. Too short an elongation time generates no amplification products or short nonspecific products. Prolonged elongation time causes diffusely smeared electrophoresis bands.

❺ Numbers of cycles

Set the optimum cycle number around 25-35 cycles after considering the quantity or complexity of template DNA and the length of amplified DNA fragments. Too few cycles may not generate enough amplified product, while too many cycles may produce a diffuse smear upon electrophoresis.

❻ Final elongation step

After the last cycle, the samples are usually incubated at 68 - 72°C to fill in the protruding ends of newly synthesized PCR product.

Recommended thermal cycling conditions for synthesis of PCR fragments up to 3 kb

Segment	**Temperature**	**Duration**	**Number of cycles**
Initial denaturation	94°C	1-3 min	1
Denaturation	94-95°C	20- 60 s	25- 35
Annealing	Primer T_a	30- 60 s	
Elongation	72°C	1 min/kb PCR target	
Final elongation	72°C	10 min	1

Recommended thermal cycling conditions for synthesis of PCR fragments > 3 kb

Segment	**Temperature**	**Duration**	**Number of cycles**
Initial denaturation	94°C	1-3 min	1
Denaturation	94-95°C	20-60 s	10
Annealing	Primer T_a	30-60 s	
Elongation	68°C	1 min/kb PCR target	
Denaturation	94-95°C	20-60 s	15-25
Annealing	Primer T_a	30-60 s	
Elongation	68°C	1 min/kb PCR target + 10 s/cycle	
Final elongation	68°C	10 min	1

QUALITY CONTROL

Each lot of High Fidelity PCR Enzyme Mix is tested in PCR for synthesis of 20 kb fragment from λ DNA and for synthesis of 9 kb fragment from human DNA.

TROUBLESHOOTING

Problem	Possible cause and solution
Low or no PCR product	**PCR component may be missing or degraded** Use a checklist when assembling reactions. Always perform a positive control to ensure that each component is functional. If the positive control does not work, repeat the positive control only. If the positive control still does not work, repeat again replacing individual components to identify the faulty reagent. **Poor template quality** Check template integrity by electrophoresis on agarose gel. If necessary, repurify template using methods that minimize shearing and nicking. Isolate fresh template and resuspend it in TE buffer, pH 8.0 or sterile water. **Target template is difficult** In most cases, inherently difficult targets are due to unusually high GC content and/or secondary structure. The addition of 1-8% dimethyl sulfoxide (DMSO) may help. Simultaneously increase enzyme mix concentration up to 2.5 u per 50 µl. **Not enough enzyme** If inhibitors are present in the reaction mix (e.g. if the template DNA used is not highly purified), increase amount of High Fidelity PCR Enzyme Mix in 0.2 u increments. **Denaturation temperature not optimal** Optimize denaturation temperature by decreasing or increasing in 1°C increments. Too high denaturation temperature can lead to degradation of the template, especially for long target sequences. **Denaturation time not optimal** Optimize denaturation time by decreasing or increasing in 5 s increments. Too long denaturation time can lead to degradation of the template, especially for long target sequences.

Problem	Possible cause and solution
Low or no PCR product	**Not enough cycles** Increase the number of cycles in 3-5 increments. **Not enough template** Target is scarce. Use more template or increase cycle number. **Suboptimal primer** Confirm the accuracy of the sequence information. If the original primer(s) is less than 20 nt long, try using a longer primer. If the original primer(s) has a GC content of less than 40%, try to design a primer with a GC content of 40–60%. **Annealing temperature too high** Decrease the annealing temperature in 2-4°C increments. **Elongation time is too short** Increase the elongation time in 1-minute increments. Remember to use 1 min per kb of DNA. **Magnesium concentration too low** If DNA template contains EDTA or other chelators or RNA impurities, the $MgCl_2$ concentration should be raised in 0.2-0.3 mM increments.
Band smearing	**Too much template** Reduce amount of template in reaction. **Too many cycles** Reduce cycles in increments of 3-5. **Elongation time too long** Decrease elongation time in 1-2 min increments. **Denaturation time not optimal** Optimize denaturation time by decreasing or increasing in 5 seconds increments. Too long denaturation time can lead to degradation of the template, especially for long target sequences. **Denaturation temperature too low** Try increasing the denaturation temperature in 1°C increments. **Magnesium concentration too high** Do not inadvertently add additional magnesium. When decreasing the dNTP concentration, $MgCl_2$ concentration should be proportionally decreased. Be sure the buffer containing template does not contain magnesium.

Problem	Possible cause and solution
Band smearing	**Too much enzyme** Be sure to add only 1.25 - 2.5 units of enzyme mix per 50 µl reaction volume and mix the reaction well after addition of enzyme. **Elongation time too short** Especially with longer templates, increase the elongation time in 1- 2 min increments. **Poor template quality** Check template integrity by electrophoresis on agarose gel. If necessary, repurify the template using methods that minimize shearing and nicking. Isolate fresh template and resuspend it in TE buffer, pH 8 or sterile water.
Multiple products	**Too much template** When amplifying genomic DNA, the initial concentration of the template in the PCR reaction should not exceed 1 µg per 50 µl reaction volume. **Primer design: suboptimal primer** Confirm the accuracy of the sequence information. If the original primer(s) is less than 22 nt long, try using a longer primer. If the original primer(s) has a GC content of less than 40%, try to design a primer with a GC content of 40–60%. **Too many cycles** Reduce the number of cycles to eliminate nonspecific products. **Annealing temperature too low** Increase temperature in 2- 3°C increments. **Magnesium concentration too high** Do not inadvertently add additional magnesium. When decreasing the dNTP concentration, $MgCl_2$ concentration should be decreased proportionally. Be sure the buffer containing the template does not contain magnesium.

APPENDIX

Conversions of oligonucleotides:

Molecular weight

MW = 333 x N

Concentration of oligonucleotides

C (μM or pmol/μl) = A_{260} / (0.01 x N)
C (ng/ml) = (A_{260} x MW) / (0.01 x N)
MW - molecular weight, Da
A_{260} - absorbance at 260 nm
N - number of bases

PRODUCT USE LIMITATION.

This product is developed, designed and sold exclusively for research purposes and in vitro use only. The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals.
Please refer to www.fermentas.com for Material Safety Data Sheet of the product.