

PyroStart™ Fast PCR Master Mix (2X)

(#K0211 for 250 reactions of 20µl)

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1. COMPONENTS

PyroStart™ Fast PCR Master Mix (2X)	2 x 1.25ml
Water, nuclease-free	2 x 1.25ml

2. STORAGE

Store PyroStart™ Fast PCR Master Mix (2X) at -20°C. Repeated freeze-thaw cycles do not reduce performance of the product.

3. DESCRIPTION

The PyroStart™ Fast PCR Master Mix is designed for fast, specific, sensitive and reproducible PCR. It is a master mix containing a hot start *Taq* DNA polymerase, specifically designed to reduce the overall time required for PCR from approximately 2 hours to just 25 minutes. This results in considerable time savings and more efficient use of PCR instruments for high-throughput screening.

The PyroStart™ Fast PCR Master Mix is supplied as a 2X-concentrated solution containing hot start *Taq* DNA polymerase, reaction buffer, MgCl₂ and dNTPs. To perform PCR, only the addition of a template and primers is required. The PyroStart™ Fast PCR Master Mix can be used with conventional PCR machines.

PCR products are generated with single 3'-dA overhangs, and can be directly used for TA cloning (e.g. with InstAclone™ PCR Cloning Kit*, #K1213) or they can be blunted and cloned into blunt cloning vectors (e.g., GeneJET™ PCR Cloning Kit, #K1221)

*Available in certain countries only.

4. PROTOCOL FOR FAST PCR

4.1. General Considerations

During PCR, more than 10 million copies of a template DNA can be generated. Therefore, care must be taken to avoid contamination with other templates and amplicons that may be present in the laboratory environment. General recommendations to lower risk of such contamination are as follows:

- Prepare your DNA sample, set-up PCR mixtures, perform thermal cycling and analyze PCR products in separate areas.
- Set-up amplification reactions in a laminar flow cabinet equipped with a UV lamp.
- Wear fresh gloves for DNA purification and reaction set-up.
- Use containers and solutions dedicated for PCR. Use positive displacement pipettes or pipette tips with aerosol filters for preparation of DNA samples and setting up PCR.
- Use certified reagents and high quality water (e.g. Water, nuclease-free #R0581).
- Always perform control reactions without template DNA to confirm the absence of contamination.

4.2. Reaction Set-up

Reaction mixtures can be prepared at room temperature.

All reaction components should be thawed on ice, gently vortexed and briefly centrifuged. Keep components on ice while in use, and return to -20°C immediately following use.

- In a thin-wall PCR tube, add the following:

Component	Volume	Final concentration
PyroStart™ Fast PCR Master Mix (2X)	10µl	1X
Primer 1 (provided by user)	variable	0.2-1µM
Primer 2 (provided by user)	variable	0.2-1µM
Template DNA	variable	10pg-0.2µg
Water, nuclease-free	to 20µl	
Total volume: 20µl		

- Gently vortex and briefly centrifuge.
- Perform thermal cycling as described below.

4.3. Cycling Protocol

Optimal cycling parameters are crucial for successful amplification and depend on a number of factors, such as amplicon size, sequence and quality of template and primers, reaction volumes, PCR tubes and the parameters of the thermal cycler used in PCR. The cycling protocol may require optimization to achieve the best results on your PCR machine (see p. 8-9).

The PyroStart™ Fast PCR Master Mix (2X) can be used in both two-step and three-step PCR cycling protocols. However, the two-step PCR protocol is recommended for fast PCR cycling.

Two-step PCR Cycling Protocol

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	95	1min	1
Denaturation	94-96	0-1s	25-40
Annealing/Extension	50-72	25s/kb*	
Final extension	68-72	10s**	1

* If the primer annealing temperature is $\leq 50^{\circ}\text{C}$, prolong the incubation time to 30-35s/kb, or use the three-step PCR cycling protocol (see below). Under optimized conditions, when primer concentration is $0.5\mu\text{M}$ and amplicons are < 500 bp long, the annealing/extension time may be decreased to 0-1s.

** For amplicons longer than 1 kb, increase the extension time by 10s for each additional kb.

Three-step PCR Cycling Protocol

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	95	1min	1
Denaturation	94-96	0-1s	25-40
Annealing	37-70	5s	
Extension	68-72	25s/kb	
Final extension	68-72	10s*	1

* For amplicons longer than 1 kb, increase the extension time by 10s for each additional kb.

5. OPTIMIZATION OF PCR PARAMETERS

To optimize the Fast PCR protocol for specific templates, primers and thermal cyclers, follow the guidelines described below.

5.1. Primer Design

- **Primer length:** 17-30 bases.
- **Annealing temperature:** 60°C - 70°C . The higher annealing temperature, the faster cycling is achieved with greatly improved specificity and higher PCR yield. Primers with lower annealing temperatures can also successfully be used in fast PCR, but require a longer overall cycling time.
- **GC content:** 40-60%. Ideally, primers should be designed in such a way that their C and G bases are distributed uniformly.
- **Avoid** more than three G or C nucleotides at the 3'-end to lower the risk of nonspecific priming.
- **Avoid** primer self-complementarity, or complementarity between the primers to prevent hairpin formation and primer dimerization.
- **Avoid** possible additional sites of complementarity between the primers and template.
- **Difference** in melting temperatures (T_m) of the primers should not exceed 5°C .
- **Primer concentration:** the recommended concentration range is 0.2 - $1.0\mu\text{M}$. A primer concentration of $0.5\mu\text{M}$ should be used as a starting point for fast PCR. Lower primer concentrations may result in lower PCR yield, while higher concentrations increase the risk of nonspecific amplification.

5.2. Template DNA

- **Concentration:** In a total reaction volume of 20 μ l, the recommended amounts of template are 0.01-10ng of plasmid or phage DNA, or 5-200ng of genomic DNA. Generally, higher quantities of template DNA increase the risk of generation of nonspecific PCR products. Lower quantities of DNA may reduce the accuracy of the amplification.
- **Purity:** All routine DNA purification methods are suitable for template preparation. Trace contamination of agents used for DNA purification (phenol, EDTA, Proteinase K and other DNA purification reagents) can inhibit thermostable DNA polymerases. Ethanol precipitation with subsequent washes with 70% ethanol is usually sufficient to remove trace contaminants from DNA samples.

5.3. Reaction Volume

To ensure efficient thermal equilibration of the reaction mixture during shortened PCR cycles, reaction volumes of 20 μ l or lower are recommended. Larger (>20 μ l) volumes of PCR require longer incubation times, resulting in longer overall PCR cycling.

Use 0.2ml thin-wall PCR tubes or 96 (384)-well plates to ensure fast and efficient heat transfer during PCR cycling.

5.4. Cycling Parameters

Initial Denaturation and Polymerase Activation

PyroStart™ Fast PCR Master Mix (2X) contains a proprietary hot start *Taq* DNA polymerase which is activated in 1min at 95°C. This activation step also ensures complete denaturation of template DNA at the beginning of PCR and facilitates efficient utilization of the template during the first amplification cycle.

Denaturation During Cycling

The denaturation time depends on the type of PCR tubes, reaction volume and technical parameters of the thermal cycler. Therefore, thin-wall PCR tubes and a reaction volume of 20 μ l are highly recommended. Under these conditions, a denaturation time of 0-1s is generally sufficient for most thermal cyclers.

Denaturation is an important parameter and may require optimization for a specific thermal cycler. PCR yields may be reduced if the denaturation temperature is too low or if the denaturation time is too short. Prolonged denaturation times and/or excessively high temperatures may result in lower PCR specificity and yield.

Annealing/Extension

Calculate the primer melting temperatures (T_m) using a computer program, or the following equation for approximate T_m estimation (for primers less than 25 nucleotides long):

$$T_m = 4(G + C) + 2(A + T), \text{ where } G, C, A, T \text{ represent the number of respective nucleotides in the primer.}$$

- The annealing temperature should be approximately 5°C lower than the melting temperature. Annealing at higher temperatures may impair PCR, while the risk of nonspecific amplification during PCR may increase at lower annealing temperatures.
- Use the two-step PCR cycling protocol (denaturation and annealing/extension at primer annealing temperature) if the primer annealing temperature is $\geq 50^{\circ}\text{C}$.
- Annealing/extension time depends on the length of the amplicon. To optimize the annealing/extension time, start with 25 seconds per 1kb. If elongation time is insufficient, short nonspecific PCR products may be generated or the PCR may fail completely.
- Use the three-step PCR cycling protocol (denaturation, annealing and extension) if the primer annealing temperature is below 50°C or the template DNA is of lower purity, in cases such as colony PCR.

Number of Cycles

- 30-35 cycles for a single copy of a genomic target are recommended with 5ng-0.2 μg of template DNA
- 25-30 cycles are sufficient for plasmid or phage targets with 10pg-10ng of template DNA.

Final Extension

Incubation for 10 seconds at 72°C is generally sufficient to fill-in the protruding ends of PCR products and generation of 3'-dA overhangs. For amplicons longer than 1kb, increase the final extension time by 10 seconds per each additional kb.

6. TROUBLESHOOTING

Problem	Solution
Low yield or no PCR product	DNA polymerase was not activated Perform initial denaturation/enzyme activation step for 60 seconds at 95°C.
	Annealing/Extension temperature and/or time are suboptimal Optimize the annealing/elongation temperature in 2-4°C increments. Optimize the elongation time in 10 seconds increments.
	Low template purity Reprecipitate DNA template with ethanol and wash DNA pellet twice with 70% ethanol.
	Insufficient number of cycles Increase the number of cycles by 3-5.
	Insufficient amount of template Use more template or increase the number of cycles.
	Poor template quality Verify template integrity by agarose electrophoresis. Isolate fresh template using methods that minimize shearing and nicking, resuspend isolated template in TE buffer, pH 8.0, or in sterile water.
	Primer concentration is suboptimal, or the primers are degraded Optimize primer concentration by 0.1 μM increments. Check for possible degradation of the primers on a denaturing polyacrylamide gel.
Suboptimal primer design Confirm the accuracy of the sequence information. Redesign primers according to recommendations (<i>see p. 6</i>)	

Problem	Solution
Low yield or no PCR product	<p>Denaturation temperature and/or time are suboptimal Optimize the denaturation temperature in 1°C increments. Optimize the denaturation time in 1second increments.</p> <p>Difficult templates Increase annealing/extension time in 10 seconds increments or use the three-step PCR cycling protocol. If template has high GC content, and/or the template forms a complex secondary structure, the use of 1-5% dimethyl sulfoxide (DMSO) in the reaction mixture may improve PCR efficiency.</p>
Band smearing	<p>Insufficient heat transfer Use thin-wall PCR tubes and perform the reaction in 20µl volume to ensure efficient thermal equilibration of the reaction mixture during shortened PCR cycles.</p> <p>Too many cycles Reduce number of cycles in 3-5 increments.</p> <p>Too much template Reduce amount of template.</p> <p>Annealing/extension time too short Optimize annealing/extension time in 10 seconds increments. This can be especially important with longer amplicons (>1kb).</p> <p>Poor template quality Verify template integrity by agarose electrophoresis. If necessary, repurify template using methods that minimize shearing and nicking. Isolate a fresh template and resuspend it in TE buffer, pH 8, or in sterile water.</p> <p>Denaturation temperature is too low Optimize denaturation temperature in 1°C increments.</p> <p>Denaturation time is suboptimal Optimize denaturation time in 1second increments.</p>

Problem	Solution
Non-specific amplification products	<p>Annealing/extension temperature is too low Optimize annealing/extension temperature in 2°C increments.</p> <p>Primer concentration is suboptimal, or the primers are degraded Optimize primer concentration in 0.1µM increments. Check for possible degradation of the primers on a denaturing polyacrylamide gel.</p> <p>Number of PCR cycles too high Reduce the number of cycles to eliminate nonspecific PCR products.</p> <p>Template concentration too high When amplifying genomic DNA, the initial concentration of the template in the reaction mixture should not exceed 200ng per 20µl reaction volume.</p> <p>Suboptimal primer design Confirm the accuracy of the sequence information. Redesign primers according to the recommendations (see p. 6).</p>

7. QUALITY CONTROL

Functional Tests

PyroStart™ Fast PCR Master Mix has been functionally tested in:

- Fast amplification of 477 bp CAPN10 target from human genomic DNA in 22min on GeneAmp® 9700 system.
Cycling conditions: 95°C, 60 seconds; 95°C, 0 seconds, 67°C, 0 seconds, 30 cycles; 72°C, 10 seconds.
- Amplification of 4 kb tPA target from human genomic DNA in 73min on GeneAmp® 9700 system.
Cycling conditions: 95°C, 60 seconds; 95°C, 0 seconds, 65°C, 1min 40 seconds, 30 cycles; 72°C, 30 seconds.

Endodeoxyribonuclease Assay

No detectable conversion of the covalently closed circular DNA to a nicked DNA is observed after incubation of 25µl of the PyroStart™ Fast PCR Master Mix (2X) with 1µg of pBR322 DNA in a volume of 50µl for 4 hours both at 37°C and at 70°C.

Exodeoxyribonuclease Assay

No detectable degradation of linear double stranded DNA was observed after incubation of 25µl of the PyroStart™ Fast PCR Master Mix (2X) with 1µg of lambda DNA-HindIII in a volume of 50µl for 4 hours both at 37°C and at 70°C.

Ribonuclease Assay

No radioactivity is released into the trichloroacetic acid-soluble fraction after incubation of 25µl of the PyroStart™ Fast PCR Master Mix (2X) with 1µg of *E. coli* [³H]-RNA (40000cpm/µg) in 50µl for 4 hours at 37°C. Less than 0.5% of the total radioactivity is released into the trichloroacetic acid-soluble fraction after incubation of 25µl of the PyroStart™ Fast PCR Master Mix (2X) with 1µg of *E. coli* [³H]-RNA (40000cpm/µg) in 50µl for 4 hours at 70°C.

Quality authorized by:

 Jurgita Žilinskienė

8. RELATED PRODUCTS

GeneJET™ Plasmid Miniprep Kit	#K0501, #K0502, #K0503
DNA Extraction Kit	#K0513
Genomic DNA Purification Kit	#K0512
GeneJET™ PCR Cloning Kit	#K1221, #K1222
InsTAclone™ PCR Cloning Kit	#K1213, #K1214
FastRuler™ DNA Ladder, Ultra Low Range	#SM1233
FastRuler™ DNA Ladder, Low Range	#SM1103
FastRuler™ DNA Ladder, Middle Range	#SM1113
FastRuler™ DNA Ladder, High Range	#SM1123
GeneRuler™ Express DNA Ladder	#SM1551, #SM1552
GeneRuler™ Express DNA Ladder, ready-to-use	#SM1553
O'GeneRuler™ Express DNA Ladder, ready-to-use	#SM1563

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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.

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