Multiplex PCR 5X Master Mix

1-800-632-7799
info@neb.com
www.neb.com

M0284S 01615031703 1

M0284S

100 reactions (50 µl vol) **Lot: 0161503**

RECOMBINANT Store at –20°C Exp: 3/17

Description: Multiplex PCR can simultaneously detect two or more products in a single reaction. There is an increasing demand for multiplex PCR techniques in assays conducted in research laboratories and forensic/diagnostic genotyping assays (1,2). Multiplex PCR can also be used for semi-quantitative gene expression analysis using cDNA templates.

The NEB Multiplex PCR 5X Master Mix is an easy-to-use solution featuring high quality recombinant *Taq* DNA Polymerase. The mix is optimized for high yield and robust performance. Its performance is illustrated in a 15-plex PCR reaction using human genomic DNA (Figure 1) and an 8-plex PCR reaction using cDNA products as templates (Figure 2). The 5X formulation allows the user maximal input of primers, template DNAs and additional components.

Source: An *E. coli* strain that carries the *Taq* DNA Polymerase gene from *Thermus aquaticus* YT-1.

Reaction Conditions: 1X Multiplex PCR Master Mix, DNA template and primers in a total reaction volume of 25 or 50 µl.

1X Multiplex PCR Master Mix:
20 mM Tris-HCl (pH 8.9 @ 25°C)
50 mM KCl
30 mM NH_4Cl
2.5 mM $MgCl_2$
100 units/ml *Taq* DNA Polymerase
0.3 mM each dNTP
3.2% glycerol
0.08% IGEPAL® CA-630
0.07% Tween® 20

Unit Definition: One unit is defined as the amount of enzyme that will incorporate 10 nmol of dNTP into acid-insoluble material in 30 minutes at 75°C.

Unit Assay Conditions: 1X ThermoPol® Reaction Buffer, 200 µM dNTPs including [^{3}H]-dTTP and 15 nM primed M13 DNA.

Heat Inactivation: No

Quality Control Assays

15-plex PCR: The Multiplex PCR 5X Master Mix is able to amplify all bands in a 15-plex PCR using 10 ng human genomic DNA in 35 PCR cycles.

Quick Protocol:
Assemble reactions on ice. A manual hot-start practice is recommended to improve specificity. Add the master mix last and start PCR cycling immediately.

1. Add the following components to a thin-walled PCR tube on ice:

COMPONENT	25 µl REACTION	50 µl REACTION	FINAL CONCENTRATION
Multiplex PCR 5X Master Mix	5 µl	10 µl	1X
1 µM Primer Stock	3.75 µl	7.5 µl	0.15 µM (0.05–0.4 µM)
Template DNA	variable	variable	<1,000 ng
Nuclease-Free Water	to 25 µl	to 50 µl	

Notes: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

2. Transfer PCR tubes to a PCR machine with the block preheated to 95°C and begin the programmed cycling:

STEP	TEMP	TIME
Initial Denaturation	95°C	1 minute
30–40 Cycles	95°C	20 seconds
	55–68°C	1 minute
	68°C	1–2 minutes/kb*
Final Extension	68°C	5 minutes
Hold	4–10°C	

* The specified extension time is based on the longest amplicon in the reaction. For example, in a 4-plex reaction of 200, 300, 400 and 500 base pairs we recommend an extension time of 30 seconds to 1 minute at 68°C.

Usage Notes:

- Use high quality primers (desalted or HPLC purified).
- Accurately quantify and adjust primer stock concentrations to 50 µM in 0.5X TE Buffer.
- Individually test the PCR primer pairs, preferably in a temperature-gradient PCR machine.
- Mix all primers equally at 1 µM in 0.5X TE buffer.
- Test multiplex PCR with equal molar concentration of all primer pairs, preferably in a temperature-gradient PCR machine.

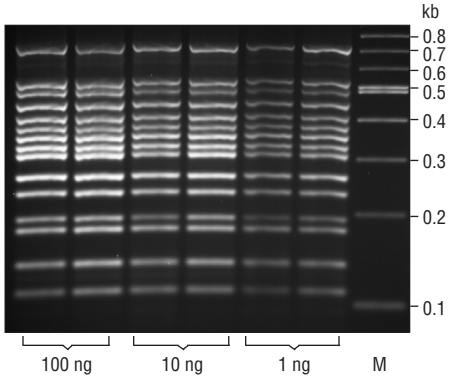

Figure 1: *15-plex PCR using varying amounts of human genomic DNA. 1X Multiplex PCR 5X Master Mix was used with 0.15 µM of each primer. The cycling conditions were 95°C for 1 minute, 35 cycles of 95°C for 20 seconds, 60°C for 1 minute and 68°C for 2 minutes. Marker M is the 2-Log DNA Ladder (NEB #N3200).*

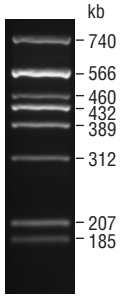

Figure 2*: 8-plex PCR using cDNA products from 1 ng human spleen total RNA. Cycling conditions were 95°C for 1 minute, 30 cycles of 95°C for 20 seconds, 60°C for 30 seconds and 68°C for 2 minutes.*

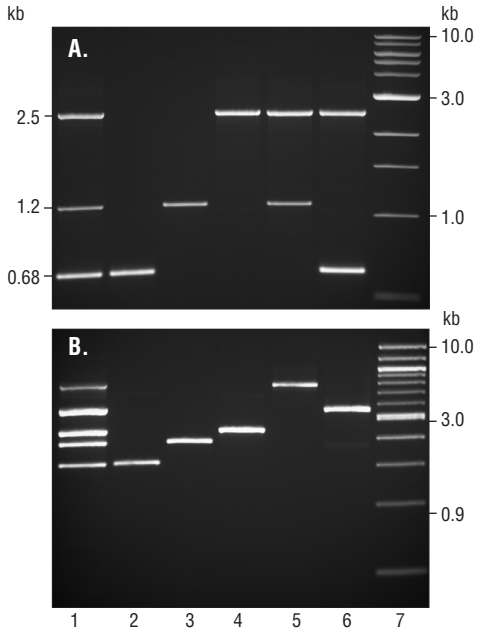

Figure 3*: Comparison of PCR product yields between single-plex and multiplex PCR reactions.*

Figure A shows amplification of human genomic DNA fragments (sizes are listed to the left of the gel). Lane 1 is a 3-plex reaction, Lanes 2–4 are single-plex reactions, and Lanes 5–6 are 2-plex reactions; Lane 7 is the 1 kb DNA Ladder (NEB #N3232).

Figure B shows the results of expression analysis of five mRNAs using cDNA templates (first strand synthesis was carried out using the ProtoScript Kit with human spleen total RNA); Lane 1 is a 5-plex PCR from cDNA reactions, while Lanes 2–6 are single-plex PCR reactions; Lane 7 is the 2-Log DNA Ladder (NEB #N3200).

Multiplex PCR Guidelines

Reaction Setup: Nonspecific primed synthesis during reaction setup and first heating cycle have been identified as a source of undesired products in some PCR reactions. This can often be avoided by doing a manual "hot-start". To carry out a manual hot-start, assemble reaction components except for the master mix and heat up to 95°C for 2 minutes, pause, add the master mix to the tube and then immediately start PCR cycling.

An alternative convenient manual "hot-start" can be carried out as follows: assemble all reaction components on ice, add the PCR Master Mix last and immediately transfer the reactions to a thermocycler preheated to the denaturation temperature (95°C).

(see other side)

CERTIFICATE OF ANALYSIS

Template: The quality of the DNA template has a great impact on the PCR amplification. For DNA samples of high complexity such as human genomic DNA, we recommend 10 ng to 1 µg template DNA in a 50 µl reaction. For DNA of low complexity, such as lambda DNA or plasmid DNA, we recommend 10 pg to 10 ng template DNA in a 50 µl reaction.

Primers:

1. Primer Design
   Primer design is critical to successful multiplex PCR reactions. Primers are generally 24–35 nucleotides in length and ideally have a GC content of 40–60% (preferably 50–60%). Try to avoid complementary sequences at the 3´ end of all primers, runs of three or more G/C at the 3´ end, and secondary structures within primers. Computer programs such as PrimerSelect™ (DNAStar Inc., Madison, MI) and Primer3 (http://frodo.wi.mit.edu/primer3) can be used to design or analyze single primer pairs.

   The melting temperature (T_m) of all primers for mPCR should be greater than 60°C according to the formula T_m (°C) = 2 x (nA+nT) + 4 x (nG + nC). Primers with T_m higher than 68°C are preferred because the difference in T_m of primer pairs does not affect performance as much.

2. Primer Quality and Molar Concentration
   Primers should be purchased desalted or HPLC-purified as the primer quality is a critical factor for good multiplex PCR reactions. Primers should be dissolved in 0.5X TE buffer (5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0) and the concentration should be accurately measured with a spectrophotometer. The molar concentration should be calculated using the molar extinction coefficient (ε_{260}) and absorbance at 260 nm.

   Molar conc. of primers (M) = $A_{260}/\varepsilon_{260}$

   ε_{260} = 0.89 x (nA x 15480 + nC x 7340 + nG x 11760 + nT x 8850) where n is the number of respective bases.

   For example, if a primer with 6A, 7C, 8G, and 9T, then its ε_{260} is 283,011 [0.89 x (6 x 15480 + 7 x 7340 + 8 x 11760 + 9 x 8850)].

Adjust the concentration of the primer stock to 50 µM (store at –20°C to –80°C). Mix all primers at equimolar concentration to 1 µM in 0.5X TE buffer and store in small aliquots at –20°C or –80°C. Repeated freeze-thaw cycles should be avoided since they may lead to primer degradation.

The final concentration of each primer in a typical mPCR reaction is between 0.05–0.4 µM. In most cases, a final concentration of 0.15 µM gives satisfactory results. Increasing the primer concentration up to 0.4 µM may increase the yield.

The Multiplex PCR 5X Master Mix is used at a final concentration of 1X in most cases; however, in some cases, the Multiplex PCR 5X Master Mix can be used as low as 0.8X, or up to final 1.5X to increase product yields.

3. Annealing temperature
   Single-plex PCR should first be performed for each pair of primers, testing a gradient of annealing temperature to determine the optimal conditions. If the single-plex PCR gives non-specific PCR bands or very low yield, the primers should be re-designed since they are unlikely to perform well in mPCR. In multiplex PCR reactions, choose an annealing temperature that allows all the single-plex PCR reactions to give specific products.

   If you cannot perform temperature-gradient PCR reactions, 60°C is a good starting point. If some bands are missing, lower annealing temperature in 1.5°C steps. If non-specific bands exist, increase annealing temperature in 1.5°C steps.

Reaction Parameters:

1. Mg^{++} and additives
   The Mg^{++} concentration in Multiplex PCR 5X Master Mix is 12.5 mM, which gives a final 2.5 mM at 1X in PCR reactions. This gives satisfactory amplification of most amplicons. However, Mg^{++} can be further optimized in 0.5–1.0 mM increments using $MgCl_2$.

   For some difficult targets such as GC-rich sequences, additives such as DMSO (3), may be included to improve amplification.

2. Denaturation
   An initial 1–2 minute denaturation at 95°C is recommended prior to PCR cycling to fully denature the DNA.

   Subsequent denaturation cycles should be 5–30 seconds.

3. Annealing
   The annealing step is typically 30 seconds to 1 minute. Annealing temperature can be optimized by doing a temperature gradient PCR starting 5°C below the calculated T_m. We recommend using NEB's T_m Calculator, available at www.neb.com/TmCalculator to determine appropriate annealing temperatures for PCR.

4. Extension
   The recommended extension temperature is 68°C. Extension time is based on the size of the largest amplicon in the mPCR reaction. An extension rate of 1 minute per kb is recommended for multiplex PCR reactions with five pairs of primers or less and 2 minutes per kb for multiplex PCR reactions with more than six pairs of primers. A final extension of 5 minutes at 68°C is recommended.

5. Cycle number
   Generally, 30–35 cycles yield sufficient product. Up to 40 cycles may be required to detect low-copy-number.

6. 2-step PCR
   When primers with annealing temperatures above 60°C are used, a 2-step PCR protocol is possible.

STEP	TEMP	TIME
Initial Denaturation	95°C	1 minute
30–40 Cycles	95°C 60–68°C	20 seconds 1–2 minutes/kb
Final Extension	68°C	5 minutes
Hold	4–10°C	

Notes: The Multiplex PCR 5X Master Mix is stable for fifteen freeze-thaw cycles when stored at –20°C. Multiplex PCR 5X Master Mix is also stable for three months at 4°C, so for frequent use, an aliquot may be kept at 4°C.

Troubleshooting Guide:

Problem: Little or no product

- Insufficient amount of template DNA
- Poor quality template DNA
- Insufficient number of cycles
- Annealing temperature too high

Problem: Missing bands

- Poor quality primers
- Poor quality template DNA
- Non-equal molar concentration of primers
- Poor primer design or performance
- Annealing temperature too high
- Increase extension time to 2 minutes per kb

Problem: Non-specific bands

- Poor quality primers
- Annealing temperature too low
- Too many PCR cycles
- Amplification of pseudogene sequences

References:

1. Beggs, A.H. et al. (1990) *Hum. Genet.,* 86, 45–48.
2. Krenke, B.E. et al. (2002) *J. Forensic Sci.,* 47, 773–785
3. Sun, Y., Hegamyer, G. and Colburn, N. (1993) *Biotechniques,* 15, 372–374.

Tm 5 Annealing temperature