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PRODUCT INFORMATION

Thermo Scientific Phusion U Multiplex PCR Master Mix

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Lot __ Expiry Date __

Store at -20°C

Ordering information

Component	2X Phusion U Multiplex PCR Master Mix	2X Phusion U Green Multiplex PCR Master Mix	Water, nuclease-free
#F-562S 100 rxns	2 × 1.25 mL	-	2 × 1.25 mL
#F-562L 500 rxns	10 × 1.25 mL	-	10 × 1.25 mL
#F-564S 100 rxns	-	2 × 1.25 mL	2 × 1.25 mL
#F-564L 500 rxns	-	10 × 1.25 mL	10 × 1.25 mL

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For Research Use Only. Not for use in diagnostic procedures.

Rev.3

1. Introduction

Thermo Scientific™ Phusion™ U PCR Master Mix is a ready-to-use, 2X end-point PCR master mix designed for simultaneous amplification of multiple targets in a single tube. Over 20 primer pairs may be combined into a single reaction for highly specific and efficient multiplexing over a broad range of primer and template concentrations.

The master mix is based on Phusion U Hot Start DNA Polymerase, a high performance enzyme developed using fusion technology. Similarly to other enzymes from Phusion family, Phusion U DNA Polymerase incorporates more nucleotides per binding event than any other DNA polymerase. This allows achieving high yields of PCR products with shorter extension times and consequently reduced total protocol times. Significantly enhanced processivity also enables successful multiplex PCR on difficult targets such as GC-rich templates or templates of suboptimal purity. Being highly tolerant of many PCR inhibitors, Phusion U Multiplex PCR Master Mix can even be used with unpurified samples such as blood or serum.

Specificity of multiplex PCR is increased by Affibody®-mediated hot start mechanism.[1,2] Reversibly bound Affibody ligand inhibits the polymerase at ambient temperatures preventing amplification of nonspecific products and formation of primer-dimers.

The Phusion U Multiplex PCR Master Mix contains a proprietary buffer with balanced concentrations of all PCR components eliminating the need for tedious optimization.

The Phusion U Green Multiplex PCR Master Mix further simplifies the workflow - it includes a density reagent and two electrophoresis tracking dyes for direct loading of PCR products on gels. The dyes do not interfere with PCR performance and are compatible with downstream applications such as DNA sequencing, ligation and restriction digestion. For applications that require PCR product analysis by absorbance or fluorescence excitation, we recommend using the colorless Phusion U Multiplex PCR Master Mix or purifying the PCR product prior to analysis.

2. Storage conditions

Phusion U Multiplex PCR Master Mix should be stored at -20 °C immediately upon arrival. For short term use, the master mix can be stored at 2-8 °C for up to 3 months without compromising performance.

3. Important Notes

- Use 98 °C for denaturation (see 6.1 & 6.2).
- The annealing rules are different from many common DNA polymerases (such as *Taq* DNA polymerases). Read Sections 5.2 and 6.3 carefully for instructions.
- Use 15 -30 s/kb for extension (see 6.4).
- Phusion U Hot Start DNA Polymerase produces DNA products with blunt ends.

4. Guidelines for using Phusion U Multiplex PCR Master Mix

Carefully mix and centrifuge all tubes before opening to ensure homogeneity and improve recovery. When using Phusion U Multiplex PCR Master Mix, it is not necessary to perform the PCR setup on ice.

Due to the unique nature of Phusion U DNA Polymerase, optimal reaction conditions may differ from standard enzyme protocols. Phusion DNA Polymerases tend to work better at elevated denaturation and annealing temperatures due to higher salt concentrations in its buffer. Please pay special attention to the cycling conditions listed in section 6 when running PCR reactions. Following the guidelines will ensure optimal enzyme performance.

Table 1. Pipetting instructions (add items in this order)

Component	20 µL rxn	50 µL rxn	Final conc.
H_2O	add to 20 µL	add to 50 µL	
2X Phusion U Multiplex PCR Master Mix *or* 2X Phusion U Green Multiplex PCR Master Mix	10 µL	25 µL	1 X
Primer Mix	X µL	X µL	0.3 µM each*
Template DNA	X µL	X µL	See section 5.3 for guidelines

*Recommended final primer concentration is 0.3 µM, but it can be varied in a range of 0.2-0.4 µM.

Table 2. Cycling instructions

Cycle step	Temp.	Time	Cycles
Initial denaturation	98 °C	30 s	1
Denaturation (see 6.2)	98 °C	10 s	30 – 40**
Annealing (see 6.3)	X °C	30 s	
Extension (see 6.4)	72 °C	15-30 s/kb*	
Final Extension	72 °C 4 °C	5 - 10 min hold	1

*Calculate extension time based on the size of the largest amplicon and the level of multiplexing (see 6.4)

**30 cycles give sufficient yield of PCR product in most cases. Larger number of cycles may be required to increase assay sensitivity when low amounts of template DNA are used.

5. Notes about reaction components

5.1. 2X Phusion U Multplex PCR Master Mix

Phusion U Multiplex PCR Master Mix contains all the necessary reaction components for PCR except for template DNA and primers. The master mix composition is designed to give optimal results in simultaneous amplification of multiple targets ranging in size from 70 to 2,500 bp. It contains a proprietary buffer and balanced ratio of all components, eliminating the need for tedious optimization. The master mix provides 1.5 mM $MgCl_2$ and 200 µM of each dNTP in the final reaction concentration.

5.2. Primers

The recommended final primer concentration is 0.3 µM of each primer. If required, the primer concentration may be optimized in the range between 0.2 µM and 0.4 µM.

Special attention to primer design parameters is critical for a successful multiplex PCR. Usually, primers of 21-34 nt length are used. Optimal GC content of the primer is 40-60%. Ideally, G and C nucleotides should be distributed uniformly along the primer. Avoid significant homology between the primers and self-complementary primer regions, as well as three or more G/C nucleotides at the 3'end. If possible, the primer should terminate with a G or C nucleotide at the 3'end. When designing degenerate primers, place at least 3 conservative nucleotides at the 3'end.

The results from primer Tm calculations can vary significantly depending on the method used. Always use the Tm calculator and instructions on website: www.thermofisher.com/tmcalculator to determine the Tm values of primers and optimal annealing temperature. The recommended Tm of all primers should be around 60 °C and should not differ by more than 5-6 °C.

Before starting multiplex PCR reactions, validate each primer pair in singleplex PCR. Use only those primers which produce a single product of correct size.

Ensure that product sizes differ sufficiently for separation from adjacent amplicons.

Table 3. Recommended differences between adjacent amplicons

Size of amplicons	Minimum difference
50 – 200 bp	25 bp
200 – 700 bp	50 bp
700 – 1000 bp	300 bp
1000 – 2500 bp	500 bp

Note that primer quality is also an essential factor for good multiplex PCR results. Primers should be purchased desalted or purified, from a reliable oligonucleotide manufacturer. Dissolve the primers in TE buffer and check the concentration by spectrophotometry. For easier pipetting we recommend to pool all the primer pairs at equimolar concentrations. To avoid repeated freeze/thawing, store the primer mix in small aliquots at -20 °C.

5.3. Template

The recommended amount of DNA template depends on the range of amplicon sizes. To simultaneously amplify DNA fragments up to 1 kb, it is recommended to use 0.1 ng – 1 µg of template DNA. If the size of the largest amplicon is longer than 1 kb, the amount of template DNA should not exceed 250 ng. Alternatively, add up to 4% of whole blood or up to 8% of serum directly to final PCR reaction mix for amplification of targets up to 1 kb.

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6. Notes about cycling conditions

6.1. Initial denaturation

Denaturation should be performed at 98 °C. Due to the high thermostability of Phusion U Hot Start DNA Polymerase even higher than 98 °C denaturation temperatures can be used. A 30-second initial denaturation at 98 °C is recommended for most templates. Some templates may require longer initial denaturation time, and the length of the initial denaturation time can be extended up to 3 minutes.

6.2. Denaturation

Keep the denaturation as short as possible. Usually 5-10 seconds at 98 °C is enough for most templates. Note: the denaturation time and temperature may vary depending on the ramp rate and temperature control mode of the cycler.

6.3. Primer annealing

The optimal annealing temperature for Phusion U Hot Start DNA Polymerase may be significantly different from the annealing temperature with other DNA polymerases. **Always use the Tm calculator and instructions on website: www.thermofisher.com/tmcalculator to determine the Tm values of your primers and optimal annealing temperature.**

If necessary, use a temperature gradient PCR to find the optimal annealing temperature for each template-primer mix combination.

6.4. Extension

The extension should be performed at 72 °C. The recommended extension time is 15-30 s/kb and depends on the amplicon length and the level of multiplexing. Calculate the extention time based on the size of the largest amplicon. Increase extension time to 30 s/kb for longer amplicons or higher level of multiplexing.

7. References

1. Nord K. *et al.* (1997) *Nature Biotechnol.* 15: 772–777.
2. Wikman M. *et al.* (2004) *Protein Eng. Des. Sel.* 17: 455–462.

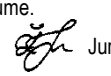
8. Troubleshooting

No product at all or low yield	
• Repeat and make sure that there are no pipetting errors. • Make sure that the cycling protocol was performed as recommended. • Titrate template amount. As a starting point we recommend using 20-50 ng of genomic DNA per 50 µL reaction. • Increase extension time. • Increase the number of cycles.	• Check the concentration and quality of template DNA. Process the sample carefully to minimize DNA damage. • Check the purity and concentration of the primers. Check primer design. • Optimize annealing temperature using temperature gradient PCR.
Non-specific products	
• Make sure that the extension time used was not too long. (Recommended extension time is 15-30 s/kb). • Increase annealing temperature or perform a temperature gradient PCR.	• Titrate template amount. • Reduce the total number of cycles. • Decrease primer concentration. • Check primer design. • Validate each primer pair in singleplex PCR. Use only those primers which produce a single product of correct size.
High amount of primer dimers	
• Check primer design. • Ensure that each primer pair produces a single band without primer dimers in singleplex PCR reactions.	• Reduce the number of cycles. • Reduce primer concentration in final reaction mix.
Uneven amplification	
• Annealing temperature is too low or too high. Test annealing temperatures in increments of 2 °C or perform temperature gradient PCR. • Do not exceed 250 ng of template DNA to achieve efficient amplification of amplicons longer than 1 kb. • Increase primer concentration for lower yield amplicons.	• Decrease primer concentration for too abundant amplicons. • Make sure that the thermal cycler has been used according manufacturer's recommendations. Appropriate instrument maintenance might be critical for successful and reproducible multiplex PCR assays. Use a high quality instrument with excellent thermal uniformity and accuracy.

CERTIFICATE OF ANALYSIS

Multiplex PCR assay

Performance in multiplex PCR is tested by the amplification of 15 fragments (99 - 1606 bp) from 50 ng human genomic DNA in 50 µL reaction volume.

Quality authorized by:  Jurgita Zilinskiene