May 16, 2020 Version 1

PCR Using Q5U Hot Start High-Fidelity DNA Polymerase (NEB #M0515): *Amplification of bisulfite-converted, deaminated, or damaged DNA (Including FFPE DNA)* V.1

In 1 collection

DOI
dx.doi.org/10.17504/protocols.io.7sdhna6

New England Biolabs[1]

[1]New England Biolabs

New England Biolabs (NEB)
Tech. support phone: **+1(800)632-7799** email: **info@neb.com**

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OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.7sdhna6

External link: https://www.neb.com/protocols/2019/07/02/pcr-using-q5u-hot-start-high-fidelity-dna-polymerase-neb-m0515

Protocol Citation: New England Biolabs 2020. PCR Using Q5U Hot Start High-Fidelity DNA Polymerase (NEB #M0515): Amplification of bisulfite-converted, deaminated, or damaged DNA (Including FFPE DNA). **protocols.io** **https://dx.doi.org/10.17504/protocols.io.7sdhna6**

Protocol status: Working
We use this protocol and it's working

Created: September 28, 2019

Last Modified: March 29, 2021

Protocol Integer ID: 28197

Keywords: Q5U, bisulfite-converted, deaminated, damaged DNA amplification

Abstract

Q5U Hot Start High-Fidelity DNA Polymerase is a modified version of Q5®High-Fidelity DNA Polymerase, a novel thermostable DNA polymerase that possesses 3′ to 5′ exonuclease activity, and is fused to a processivity-enhancing Sso7d domain. Q5U contains a mutation in the uracil-binding pocket that enables the ability to read and amplify templates containing uracil and inosine bases.

Guidelines

General Guidelines:

1. Template:
Purified DNA templates (where possible) greatly enhances the success of PCR.
Recommended amounts of DNA template for a 50 µl reaction are as follows:

	DNA	AMOUNT
	DNA Genomic	10 ng – 1 µg

2. Primers:
We recommend designing long (~26 – 35) oligonucleotide primers to amplify bisulfite treated/deaminated DNA. Because bisulfite DNA is often damaged, it is helpful to design targets between 150-500bp. Note that since the DNA strands are no longer complementary after bisulfite-treatment/deamination, an individual primer set will only amplify one strand of the target sequence. The first primer should be designed to anneal to the converted target sequence. The second primer should be designed to anneal to the extension product of the first primer, not the opposite template strand, as would be the case in traditional PCR. Primers with higher annealing temperatures (> 60 °C as determined by the **NEB T_m Calculator**) are recommended for optimal performance. If needed, include as many guanines as possible in the priming region or add additional bases to increase the Tm. Longer primers will also increase specificity. Ideally, CpG sites should be avoided; if essential, include them on the 5′-end of the primer and have them synthesized with a mixed base (Y = C/T, R = G/A) at the cytosine position. Computer programs such as MethPrimer, methBLAST or BiSearch can be used to design or analyze primers. The best results are typically seen when using each primer at a final concentration of 0.5 µM in the reaction.

3. Mg++ and additives:
Typically, the Mg^{++}concentration for Q5U Hot Start High-Fidelity DNA Polymerase should be 2.0 mM. When used at a final concentration of 1X, the Q5U Reaction Buffer provides this optimal Mg^{++}concentration. The addition of common PCR additives such as DMSO may improve amplification of certain difficult or long targets. In these cases, we recommend the addition of up to 2 % DMSO.

4. Deoxynucleotides:
The final concentration of dNTPs is typically 200 µM of each deoxynucleotide.

5. Q5U Hot Start High-Fidelity DNA Polymerase concentration:
We generally recommend using Q5U Hot Start High-Fidelity DNA Polymerase at a final concentration of 20 units/ml (1.0 unit/50 µl reaction). However, the optimal concentration of Q5U Hot Start High-Fidelity DNA Polymerase may vary from 10 – 40 units/ml (0.5 – 2 units/50 µl reaction) depending on amplicon length and difficulty. It is rarely helpful to exceed 2 units/50 µl reaction, especially for amplicons longer than 5 kb.

6. Buffers:
The 5X Q5U Reaction Buffer provided with the enzyme is recommended as the first-choice buffer for robust, high-fidelity amplification. The 5X Q5U Reaction Buffer contains 2.0 mM Mg^{++}at a final (1X) concentration.

7. Denaturation:
Q5U Hot Start High-Fidelity DNA Polymerase does not require a separate activation step.

An initial denaturation of 30 seconds at 98 °C is sufficient for most targets being amplified from pure DNA templates. Longer initial denaturation times can be used (up to 3 minutes) for templates that require it. During thermocycling, the denaturation step should be kept to a minimum. Typically, a 10 second denaturation at 98 °C is recommended for most templates.

8. Annealing:
Optimal annealing temperatures for Q5U Hot Start High-Fidelity DNA Polymerase tend to be higher than for other PCR polymerases. The **NEB T_m Calculator** should be used to determine the annealing temperature when using this enzyme. A temperature gradient can also be used to optimize the annealing temperature for each primer pair.

For high $T_m$primer pairs, two-step cycling without a separate annealing step can be used (see note 9).

9. Extension:
The preferred extension temperature for bisulfite-converted DNA, deaminated DNA, or damaged DNA is 68 °C. Extension times of 1 minute per kb for complex, genomic samples is beneficial when amplifying from challenging, converted and/or damaged substrates. If no amplification occurs, first verify the annealing temperature prior to changing the extension temperature.
A final extension of 5 minutes at 68 °C is recommended.

10. Cycle number:
Generally, 35 cycles yield sufficient product. Additional cycles can be added but other optimizations (particularly annealing temperature) should be attempted first.

11. 2-step PCR:
When primers with annealing temperatures ≥ 68 °C are used, a 2-step thermocycling protocol (combining annealing and extension into one step) is possible.

12. PCR product:
The PCR products generated using Q5U Hot Start High-Fidelity DNA Polymerase have blunt ends. If cloning is the next step, then blunt-end cloning is recommended. If T/A-cloning is preferred, the DNA should be purified prior to A-addition, as Q5U Hot Start High-Fidelity DNA Polymerase will degrade any overhangs generated.

Materials

MATERIALS

Q5U® Hot Start High-Fidelity DNA Polymerase **New England Biolabs** **Catalog #**M0515

Safety warnings

Please see SDS (Safety Data Sheet) for hazards and safety warnings.

Before start

Please note that protocols with *Q5U Hot Start High-Fidelity DNA Polymerase* may differ from protocols with other polymerases. Conditions recommended below should be used for optimal performance.

Reaction Setup:
Q5U Hot Start High-Fidelity DNA Polymerase is inhibited at room temperature, allowing flexible reaction setup (room temperature or ice).

All components should be mixed prior to use.

Amplification of bisulfite-converted, deaminated, or damaged DNA (Including FFPE DNA)

1 Set up the reaction using the following table:

Component	25 µl Reaction	50 µl Reaction	Final Concentration
5X Q5U Reaction Buffer	5 µl	10 µl	1X
10 mM dNTPs	0.5 µl	1 µl	200 µM
10 µM Forward Primer	1.25 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	< 1,000 ng
Q5U Hot Start High-Fidelity DNA Polymerase	0.25 µl	0.5 µl	0.02 U/µl
Nuclease-Free Water	to 25 µl	to 50 µl	

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 and begin thermocycling. The recommended cycle setup and temperatures are shown below.

Note

Q5U Hot Start High-Fidelity DNA Polymerase does not require a separate activation step.

STEP	TEMP	TIME
Initial Denaturation	98 °C	30 seconds
35 Cycles	98 °C	10 seconds

		*60 – 68 °C	20 seconds
		68 °C	1 minute/kb
	Final Extension	68 °C	5 minutes
	Hold	4 – 10 °C	

*Use of the NEB T_m Calculator is highly recommended to determine annealing temperatures. Other Tm's can be used, but for bisulfite-converted, deaminated, or damaged DNA substrates, this narrower range has been shown to support optimal performance.