repliQa® HiFi ToughMix®

Cat No.	95200-025	Size:	25 x 50 µL reactions (1 x 0.625 mL)	**Store at -25°C to - 15°C**
	95200-100		100 x 50 µL reactions (1 x 2.50 mL)	
	95200-500		500 x 50 µL reactions (1 x 12.50 mL)	

Description

The repliQa HiFi ToughMix is a unique, next generation 2x master mix that has 90x higher fidelity compared to *Taq* polymerase. The ToughMix has extreme speed, with extension times as fast as 1-10 sec/kb depending on target length. Additionally, the ToughMix has long range amplification properties as it can amplify fragments up to 24 kb from complex genomic DNA templates or fragments up to 40 kb from virus DNA templates such as *Escherichia virus Lambda* DNA.

The ToughMix is formulated with a genetically modified DNA polymerase coupled with hot start antibodies. It has 5′ → 3′ polymerase activity, 3′ → 5′ exonuclease activity, and generates blunt-ended products while providing the ability to amplify through uracils and primers containing inosine or uracil. It is *Tough Tested*, and is tolerant to multiple PCR inhibitors.

Components

repliQa HiFi ToughMix	2x reaction buffer containing optimized concentrations of $MgCl_2$, dNTPs and proprietarily formulated HiFi polymerase, hot start antibodies and ToughMix chemistry

Storage and Stability

Store kit components in a constant temperature freezer at -25°C to -15°C protected from light upon receipt.
For lot specific expiry date, refer to package label, Certificate of Analysis or Product Specification Form.

Guidelines for PCR

- The design of highly specific primers is a critical parameter for successful PCR. The use of computer aided primer design programs is encouraged in order to minimize the potential for internal secondary structure. For best results, primer size should be limited to 22 - 35 bp with a melting point of at least 63°C. Ideal GC-content of the primers is 45-60%. A final concentration of 300 nM each primer is effective for most applications. Primers with inosine (dI) and uracil (dU) are acceptable.
- We recommend checking the Tm of primer sequences using the 'Primer_Check' Task on Primer3Plus[1] on default settings. The resulting Tm can be utilized as a starting point to determine the annealing temperature. A temperature gradient is recommended to determine the proper annealing temperature.
- Preparation of a reaction cocktail is recommended to reduce pipetting errors and maximize assay precision. Assemble the reaction cocktail with all required components except sample template and dispense equal aliquots into each reaction tube. Add the DNA template to each reaction as the final step. Addition of samples as 2 to 5-µL volumes will improve assay precision.
- Suggested input quantities of template are: genomic DNA ≤ 200 ng; plasmid DNA ≤ 50 ng; cDNA ≤ 750 ng.
- After sealing each reaction, vortex gently to mix contents. Centrifuge briefly to collect components at the bottom of the reaction tube.
- Longer targets may require a higher primer melting temperature of at least 65°C, and a lower primer concentration of 150 nM.
- Maximum of 35 PCR cycles are recommended, regardless of input amount.

[1] Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., & Leunissen, J. A. M. (2007). Primer3Plus, an enhanced web interface to primer3. *Nucleic Acids Research*. https://doi.org/10.1093/nar/gkm306

Reaction Assembly

Component	Volume for 50 µL rxn	Final Concentration
repliQa HiFi ToughMix (2X)	25 µL	1x
Forward primer	variable	300 nM
Reverse primer	variable	300 nM
Nuclease-free water	variable	
Template	2 – 5 µL	variable
Final Volume (µL)	50 µL	

Note: For smaller or larger reaction volumes, scale all components proportionally.

For targets with high GC (>74%), it is recommended to add DMSO to achieve a 5% final concentration in the PCR reaction.

For further guidance, please refer to the Application Note on "Optimal PCR amplification for GC-rich templates using repliQa HiFi ToughMix® and enhancers".

PCR Cycling Protocol

Initial denaturation and final extension steps are typically not required with repliQa HiFi ToughMix.

The 10 s at 98°C during cycling are sufficient to fully activate the HotStart mechanism. For longer fragments, (>10 kb), an initial denaturation of 98°C for 30 s can be added to facilitate denaturation of the DNA template.

Maximum of 35 PCR cycles are recommended for amplification. Greater than 35 PCR cycles may result in non-specific amplification.

PCR cycling (25 - 35 cycles):

*We recommend using 2-step cycling for primers with Tm > 63°C as determined by Primer3Plus. For all other cases, we suggest using 3-step cycling.

2-Step Cycling*	3-Step Cycling
98°C, 10 s	98°C, 10 s
	Tm, 5 s
68°C ≤ 1 kb: 1 sec 1 ~ 10 kb: 5 sec/ kb ≥ 10 kb: 10 sec/ kb	68°C ≤ 1 kb: 1 sec 1 ~ 10 kb: 5 sec/ kb ≥ 10 kb: 10 sec/ kb

Quality Control

Kit components are free of contaminating DNase and RNase. 2x repliQa HiFi ToughMix is functionally tested for amplification of a 4-kb fragment from a single-copy gene in a human genomic DNA.

Nuclease Assay:

DNase: DNase activity must be below the detectable limits of 100 pg DNase I equivalent as assayed using a fluorogenic substrate following a 1 hour incubation at 37°C with each kit component at 1x concentration.

RNase: RNase activity must be below the detectable limits of 1 pg RNase A equivalent as assayed using a fluorogenic substrate following a 1 hour incubation at 37°C with each kit component at 1x concentration.

4.1 kb PCR Functional Assay: Negative control must be free of visible product with a single band at ~4.1 kb visible from 35 cycles of PCR using 20 ng human genomic DNA.

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Appendix A: Optimized PCR Product Cleanup

After PCR amplification, sample cleanup may be required to remove enzyme, dNTPs, and reaction buffer prior to sensitive downstream applications. The following reflects an optimized cleanup procedure using magnetic beads (i.e. sparQ PureMag Beads) to achieve high purity samples for further analysis.
When purifying highly concentrated or long DNA after amplification with repliQa HiFi ToughMix, abnormal pelleting of the beads may be observed, which can impede PCR product clean up. The following protocols can be used to improve bead clean up if this occurs.

Additional reagents and materials that are not supplied

Proteinase K (concentration 20 mg/ml) for Option 1 **ONLY**

10% Tween® 20 for Option 2 **ONLY**

Purifications Beads

Purification beads are not included with this kit and must be purchased separately. This protocol has been validated using the sparQ PureMag Beads (Cat No. 95196-005, 95196-060, or 95196-450) from Quantabio.

Reaction Cleanup with Proteinase K Digestion

Recommended for downstream ONT sequencing applications

1. Equilibrate the sparQ PureMag beads to RT.
2. If PCR reaction volume is less than 50 µL, adjust the volume to 50 µL using nuclease-free water or TE Buffer (10 mM Tris-HCl (pH 8.0, 0.1 mM EDTA).
3. Add 1 µL Proteinase K to the sample and mix well.
4. Thoroughly vortex the bead slurry and add 51 µL (1X) to the PCR reaction. Mix well by pipetting.
5. Incubate the mixture for 5 min at RT. Pellet the beads on a magnetic stand (e.g., DynaMag™) and carefully discard the supernatant.
6. Keep the tube on the magnetic stand and wash the beads with 200 µL of freshly prepared 80% ethanol.
7. Pellet the beads on the magnetic stand. Carefully discard the supernatant after the liquid is clear. Repeat the wash once.
8. Air-dry the beads on the magnetic stand for 5 min or until the beads are dry. Over-drying of beads may result in lower DNA recovery.
9. Resuspend the dried beads in 32.5 µL or desired volume of 10 mM Tris-HCl, pH 8.0 and incubate for 2 min at RT. Pellet the beads on the magnetic stand. Carefully transfer 30 µL of supernatant into a new tube. The sample can be stored at -20°C if not proceeding immediately to library quantification or other downstream processes.

Reaction Cleanup with Tween® 20

1. Equilibrate the sparQ PureMag beads to RT.
2. If PCR reaction volume is less than 50µL, adjust the volume to 50 µL using nuclease-free water or TE Buffer (10 mM Tris-HCl (pH 8.0, 0.1 mM EDTA).
3. Add 1 µL 10% Tween® 20 to the sample and mix well.
4. Thoroughly vortex the beads slurry and add 51 µL (1X) to the PCR reaction. Mix well by pipetting.
5. Incubate the mixture for 5 min at RT. Pellet the beads on a magnetic stand (e.g., DynaMag) and carefully discard the supernatant.
6. Keep the tube on the magnetic stand and wash the beads with 200 µL of freshly prepared 80% ethanol.
7. Pellet the beads on the magnetic stand. Carefully discard the supernatant after the liquid is clear. Repeat the wash once.
8. Air-dry the beads on the magnetic stand for 5 min or until the beads are dry. Over-drying of beads may result in lower DNA recovery.
9. Resuspend the dried beads in 32.5 µL or desired volume of 10 mM Tris-HCl, pH 8.0 and incubate for 2 min at RT. Pellet the beads on the magnetic stand. Carefully transfer 30 µL of supernatant into a new tube. The sample can be stored at -20°C if not proceeding immediately to library quantification or other downstream processes.