repliQa™ HiFi Assembly Mix

Cat. No.	95190-010 95190-050	Size:	10 reactions 50 reactions	**Store at -25°C to -15°C**

Description

The repliQa™ HiFi Assembly Mix simplifies the construction of recombinant DNA through the simultaneous and seamless assembly of multiple DNA fragments possessing terminal regions of sequence overlap in a single, isothermal reaction. Similar in principle to the Gibson Assembly® Method[1], the high efficiency repliQa HiFi Assembly Mix is ideal for a range of genetic engineering applications including routine molecular cloning, site-directed mutagenesis, assembly of large constructs for synthetic biology applications, and the construction of diverse sequence libraries for directed evolution studies. The concentrated, two-component format allows flexibility in design of assembly reactions and compatibility with less concentrated DNA samples. The repliQa Mix has been optimized for use with a total input quantity of DNA fragments in the range of 0.03 to 0.5 pmols. The assembly of up to six DNA fragments is recommended, though the repliQa Mix has been successfully used for more complex assemblies.

Double stranded DNA fragments for assembly can be generated by PCR amplification, chemical synthesis, or isolation of restriction fragments. When working with fragments PCR amplified from plasmid vectors, the included DpnI restriction endonuclease can be used for selectively digesting methylated, residual plasmid DNA to reduce background transformants. The repliQa mix is directly compatible with most common *E. coli* cloning hosts and generally provides a high yield of accurately assembled product.

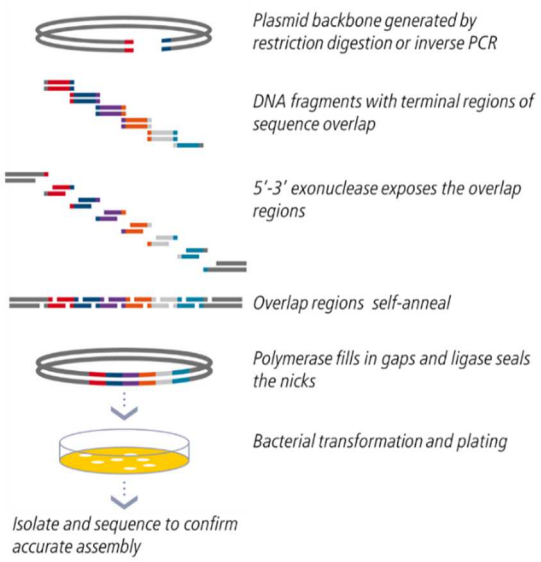
The DNA assembly occurs through the actions of three enzymes:

- A non-thermostable 5' to 3' exonuclease that partially eliminates one strand of a DNA duplex to expose complementary overlap regions for hybridization.
- A high-fidelity thermostable polymerase that fills the gaps remaining between the hybridized fragments of the overlapping regions.
- A thermostable DNA ligase that covalently seals the resulting nicks at fragment junctions, generating double-stranded, assembled DNA molecules suitable for transformation of cells.

Components

Reagent	Description	95190-010	95190-050
repliQa HiFi Assembly Enzyme Mix	Optimized formulation of enzymes for 5'-end resection, high fidelity 3'-end extension, and nick sealing.	1 x 0.02 mL	1 x 0.10 mL
repliQa 10X Assembly Reaction Buffer	10X reaction buffer containing dNTPs, magnesium, and cofactors.	1 x 0.1 mL	1 x 0.50 mL
DpnI (20 U/µl)	Restriction endonuclease for the (optional) post-PCR digestion of residual unamplified plasmid template.	1 x 0.05 ml	1 x 0.25 ml

A general diagram of assembly cloning is shown below:



Storage and Stability

Store kit components in a constant temperature freezer at -25°C to -15°C upon receipt. For long term buffer storage (> 30 days) store buffer at -70°C. Refer to the product label or lot-specific Product Specification Sheet (PSF) available at www.quantabio.com/resources for applicable expiration date.

Additional reagents and materials that are not supplied

- PCR-Grade, nuclease-free water (do not use DEPC-treated water)
- High Fidelity DNA Polymerase (Enzymatics VeraSeq™ 2.0, P7511L or equivalent)
- A heat block, thermocycler, or water bath capable of holding a temperature of 50 ± 2°C for one hour.
- PCR or microcentrifuge reaction tubes.
- PCR product purification kit (QIAGEN® QIAquick® PCR Purification Kit, 28104 or equivalent).
- Competent *E. coli* cells and accessories as recommended by manufacturer.

Before you begin

- Design the DNA fragment sequences and assembly strategy. Guidelines are given in Appendix 1.
- (Optional) Treat PCR reaction with DpnI if plasmid DNA was used as template for generating DNA fragments to be assembled. (Appendix 2).
- (Recommended) After determining PCR fragment or restriction endonuclease-digested fragment size and purity by agarose gel electrophoresis, purify using a spin column-based cleanup or other method. This step is not required but is highly recommended to achieve highest efficiency of fragment assembly.
- Measure the concentration of each isolated DNA fragment by absorbance at A_{260} or by using a fluorometric quantitation reagent. Agarose gel electrophoresis with mass-calibrated size standards can also be used to quantify fragment mass and quality simultaneously.
- Calculate the number of picomoles of each fragment using the following formula:
  pmols = (weight in ng) x 1000/(bp x 662).
- Determine the number of pmols of each fragment to add to the assembly reaction. For cloning, highest efficiencies are achieved with 0.02 to 0.04 pmols of linear vector fragment (50 to 100 ng of 4 kb vector) and 2 to 8-fold molar excess of inserts.
- Prepare outgrowth medium and culture plates with appropriate antibiotics for plasmid selection.
- Equilibrate the heat block, thermal cycler, or water bath to 50°C for incubation of the assembly reactions.

Protocol

1. Thaw the repliQa HiFi Assembly Kit components, briefly vortex to mix, and place on ice.
2. For each assembly, add reaction components in the order listed in the table below to chilled reaction tubes.

The optimal amount of enzyme mix to add per assembly reaction depends on the total quantity of DNA fragments present.

Component	Rxn. component volumes (µl) for varying amounts of total DNA		
	≤ 0.125 pmol	> 0.125 pmol but ≤ 0.25 pmol	> 0.25 pmol
Nuclease-free water	(17.5 – X) µl	(17.0 – X) µl	(16.0 – X) µl
repliQa 10X Assembly Reaction Buffer	2.0 µl	2.0 µl	2.0 µl
DNA fragments	X µl	X µl	X µl
repliQa HiFi Assembly Enzyme Mix	0.5 µl	1.0 µl	2.0 µl
Total volume	20 µl	20 µl	20 µl

3. Incubate reactions at 50°C in heat block, thermal cycler with heated lid (set to ~60-80°C), or covered water bath for 1 hr. Hold assembled product mix at 4°C until ready to proceed with transformations. If transformations cannot be performed on the same day, reactions can be stored at -20°C for up to one month.
4. Competent *E. coli* should be transformed, recovered, and plated as per manufacturer guidelines or standard lab practices. Note: If electroporation is to be used for transforming cells, we recommend first diluting the assembly reaction 1:5 in high purity water. There is no need to dilute the assembly reactions prior to transformation of chemically competent cells.
5. (Optional) Analyze a portion of the remaining assembly reaction by agarose gel electrophoresis. If DNA fragment assembly occurs properly, a ladder of higher molecular weight DNA bands would be generated.

Note: For reactions using three or fewer fragments the incubation time in step 3 can be shortened to 15 minutes.

Appendix 1 – Guidelines for Designing DNA Fragments for Assembly

1. When designing the DNA fragment sequences and assembly strategy, allow for a region of sequence homology between adjacent DNA fragments. Be sure to avoid regions of repeated bases or repeated short DNA motifs in the design of these overlaps where possible. Regions of secondary structure such as hairpins or stem loops should also be avoided.
2. The kit is optimized for the assembly of fragments with overlap regions between 15 – 60 bp. It is recommended that the overlaps be at least 20 bp with a minimum of 25% GC content, however overlaps of 30 bp or longer size will provide higher efficiency assembly reactions.
3. For generating PCR fragments to be assembled, design primers with a 5' segment of homology to the adjacent fragment or vector. If the adjacent fragment is also generated by PCR amplification, the overlap can be split between two primers if desired. The 3' segment of primers should contain sequence specific to the DNA target of interest. Amplify targets using a high-fidelity thermostable DNA polymerase such as VeraSeq 2.0 (Enzymatics, P7511L) or equivalent per manufacturer instructions.
4. When designing synthetic gene fragments for assembly, ensure that the 5' and 3' segments contain regions of homologous overlap sequence between adjacent gene blocks, PCR fragments, or isolated restriction fragments.
5. For site-directed mutagenesis applications, the assembly strategy should be designed such that the mutation of interest is centered between adjacent PCR fragments. Design the PCR primers as with the standard fragments above, except that the mutation (substitution, insertion, or deletion) should be included within the 5' segments for both of the adjacent fragments.
6. When designing DNA fragments to be assembled with isolated restriction fragments, be aware that any 5' overlaps from staggered restriction cuts will be eliminated because of the 5'-->3' nuclease present in the assembly mix, and so should not be included in the measurement of overlap size. If desired, design the 5' overlap segment of the adjacent fragment to either preserve or eliminate the restriction site.

Appendix 2 – DpnI treatment to remove residual plasmid DNA

When plasmid vector is used as PCR template to generate a fragment for assembly, it is recommended that the reaction be treated with DpnI to eliminate residual methylated plasmid prior to setting up the assembly reaction.

1. Add 1 µl DpnI (20U) directly to the PCR reaction (50 µl) following amplification of fragment.
2. Incubate at 37°C for 1 hr.
3. Heat inactivate DpnI by incubation at 80°C for 20 min.
4. (Recommended) Purify the fragment using a spin column-based PCR purification kit.

Quality Control

The repliQa HiFi Assembly Mix is functionally tested for assembly of three 1-kb PCR fragments into 2kb and 3 kb products.
The individual components of the repliQa HiFi Assembly Mix are tested to be free of contaminating DNase and RNase.

Limited Label Licenses

This product was developed, manufactured, and sold for in vitro use only. The product is not suitable for administration to humans or animals. SDS sheets relevant to this product are available upon request.

References

1. Gibson, D.G., et al. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods *6*, 343-5.