DNA repeat assembly kit

DNA–binding proteins Protocol

2024

LUMICKS

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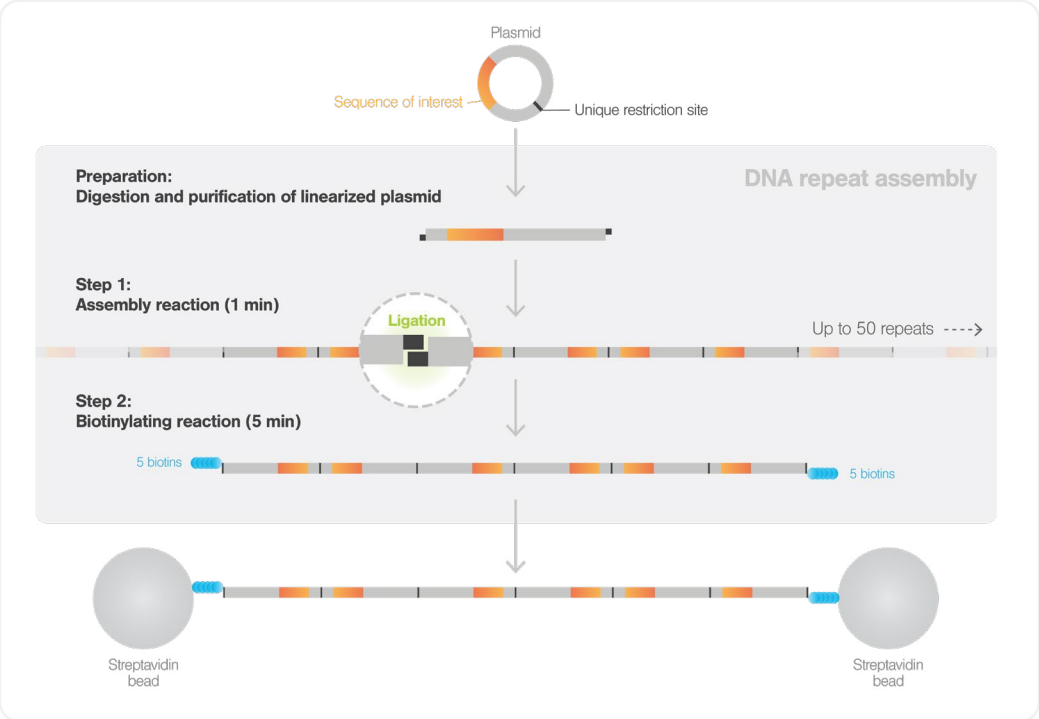
DNA repeat assembly kit

1. Description

DNA repeat assembly is a highly efficient system to quickly build DNA repeat arrays - up to 120 kb - from a linear DNA fragment with cohesive DNA overhangs. Using this kit, DNA fragments of interest are ligated to copies of themselves multiple times and then biotinylated by ligation to DNA adaptors with a compatible DNA overhang on one end five and biotin moieties on the other (Figure 1). The incorporation of five biotins in the adaptor is designed in a proprietary, optimized configuration to enhance the binding efficiency. This configuration enables fast tethering of the longest DNA repeat constructs. Furthermore, it ensures high stability of the formed tethers, enabling long data acquisition sessions. Binding events of fluorescent proteins to the DNA repeats of the array can then be measured in parallel with the C-Trap, greatly increasing the throughput of the experimental data acquisition.

Figure 1.



The number of repeats assembled into an array depends on the size of the DNA fragment and the type of overhangs used for the ligation (see section 3 Overhangs of biotinylated DNA adaptors). With a DNA fragment of 2-3 kb, the DNA repeat assembly kit typically produces arrays ranging from 20 to 50 repeats.

The kit includes one of three biotinylated DNA adaptors, a DNA Ligase enzyme, a 10x Assembly buffer and an EDTA buffer solution.

2. Materials supplied in the kit

Table 1.

Components	Units	Volume	Storage temperature
Biotinylated DNA adaptor	1	10 µl	-20 ˚C
DNA Ligase	1	10 µl	-20 ˚C
10x Assembly buffer	1	25 µl	-20 ˚C
EDTA buffer	1	10 µl	Room temperature

2.1 Additional Materials (supplied by the user)

- A TypeIIS restriction enzyme of choice among BsmBI, BbsI or BsaI (see protocol below).
- Amicon Ultra-0.5 mL Centrifugal Filters 30 kDa cutoff (Millipore UFC503096)
- EDTA buffer pH 7,5-8.

3. Overhangs of biotinylated DNA adaptors

The kit offers a choice between three different DNA adaptors, each adaptor compatible with one cohesive end. The cohesive ends can be produced by using the enzymes listed in Table 2.

Important note: see section 5.2 to estimate the efficiency of the assembly reaction based on the choice of cohesive ends.

Table 2.

Restriction endonuclease	Restriction site	5' overhang of the adaptor	Biotinylated DNA adaptor
HindIII	A/AGCTT	AGCT	Adaptor 1
SalI	G/TCGAC	TCGA	Adaptor 2
PspXI	VC/TCGAGB	TCGA	Adaptor 2
XhoI	C/TCGAG	TCGA	Adaptor 2
BamHI	G/GATCC	GATC	Adaptor 3
BclI-HF	T/GATCA	GATC	Adaptor 3
BglII	A/GATCT	GATC	Adaptor 3
BstYI	R/GATCY	GATC	Adaptor 3

4. Important notes before starting

- DNA repeat assembly generates DNA repeat arrays with different lengths in the same reaction tube. To catch the longest DNA arrays and maximize the throughput of the measurements on the C-Trap, follow the instruction in section 7 on DNA tethering.
- As the DNA repeat assembly uses palindromic DNA overhangs for DNA ligations, the DNA fragments are ligated in both orientations.
- To ensure high efficiency of assembly, follow the guidelines in section 6 to obtain high concentration of the fragment of interest and completely remove the ethanol from purified DNA.
- The efficiency of DNA repeat assembly depends on the DNA overhangs chosen for the reaction. For instructions on selecting the appropriate DNA overhangs and verifying the reaction on an agarose gel, please see section 5.2.
- To avoid thawing and freezing cycles of 10x Assembly buffer, make aliquots (e.g. 5 µl aliquots) and store them at -20 °C. This will prevent the breakdown of ATP in the buffer.

5. DNA repeat assembly protocol

The DNA repeat assembly kit provides material for a multi-step and time sensitive reaction (Figure 1). Therefore, it is recommended to prepare each tube corresponding to each reaction step before starting the experiment. As the volumes of some components are as small as 1 µl, we suggest using PCR tubes.

The protocol has been optimized using a 2.26 kb DNA fragment. DNA fragments ranging between 2 kb to 3.5 kb ensure the highest efficiency of the assembly reaction. Using longer DNA fragments might decrease efficiency. Recommended final concentration of DNA fragment in the pre-assembly mix is 300 ng/µl or higher. Concentrations as low as 50 ng/µl of a 2.26 kb DNA fragment have been tested, obtaining arrays containing up to 30 DNA repeats.

5.1 Tube preparation before assembly reaction

Prepare 3 different PCR tubes before starting the reaction.

- Prepare a 1st tube by mixing the following components (pre-assembly mix) and incubate for 5 minutes at room temperature.

Components	**20 µl pre-assembly mix**
10x Assembly Buffer	2 µl
DNA fragment (SOI)	Final concentration 50-300 ng/µl
Milli-Q	To 20 µl

- Prepare a 2nd tube with 1 µl of DNA Ligase (Assembly tube).
- Prepare a 3rd tube with 2 µl of biotinylated DNA adaptors (Biotinylating tube).

Step 1: Assembly Reaction

- Set a 1-minute timer.
- Add the pre-assembly mix to the Assembly tube containing 1 µl of DNA Ligase. Quickly pipette up and down 5 times to thoroughly mix the enzyme with the pre-assembly mix.
- Immediately start the timer to make sure to incubate the reaction for 1 minute.

Step 2: Biotinylating reaction

- After 1 minute incubation, quickly transfer the Assembly reaction mix to the Biotinylating tube containing 2 µl of biotinylated DNA adaptors. Slowly pipette up and down 2-3 times to avoid damage by shearing of the long DNA arrays.
- Incubate for 5 minutes.
- Add 1.5 µl of 500 mM EDTA solution to the reaction to stop ligation and mix by inverting the tubes or by gently pipetting up and down.
- To completely inhibit binding of DNA Ligase to the DNA, heat-inactivate the enzyme at 65°C for 10 minutes.

Step 3: Buffer exchange with Amicon Ultra-0.5 mL Centrifugal Filters 30 kDa cutoff (Millipore UFC503096)

Upon completion of the DNA Ligase reaction, perform a buffer exchange with Tris-HCl 10 mM, EDTA 1 mM, pH8 (TE) to allow long term storage (up to 3 months in +4 ˚C) of the final construct. Avoid thawing and freezing cycles of the ligation product. This will prevent formation of nicks in the DNA constructs

- Insert Amicon® Ultra-0.5 device into one of the provided microcentrifuge tubes.
- To wash the filter device: add 500 µl Milli-Q water to the filter device, invert few times to clean, discard the milli-Q.
- Repeat the previous step once
- To equilibrate the filter device: add 500 µl of TE buffer and centrifuge at 11,500 RCF for 3 minutes.
- Discard remaining TE buffer both from the filter device and the microcentrifuge tube

- Dilute DNA Ligase reaction up to 500 µl with TE buffer, add it to the filter device and centrifuge at 11,500 RCF for 5 minutes. You should obtain ~50 µl of DNA Ligase reaction in the filter device after 5 minutes of centrifugation.
- Discard the flowthrough from the microcentrifuge tube.
- Add 450 µl of TE to the filter device, centrifuge for 5 minutes at 11,500 RCF.
- As described by the manufacturer: to recover the concentrated solute, place the Amicon® Ultra filter device upside down in a clean provided microcentrifuge tube. Place in centrifuge, aligning open cap towards the center of the rotor; counterbalance with a similar device. Spin for 2 minutes at 1,000 RCF to transfer the concentrated sample from the device to the tube. The ultrafiltrate can be stored in the centrifuge tube.
- Measure concentration by nanodrop. You should obtain a concentration ranging from 30 ng/µl to 80 ng/µl.

5.2 Efficiency of DNA repeat assembly

A DNA repeat assembly reaction produces DNA arrays of different lengths in the same reaction tube as the self-ligation of the DNA fragments is stochastic (Figure 3). These arrays of different lengths can be visualized by agarose gel.

The efficiency of the reaction depends on the DNA overhang sequence. As a showcase, circular pUC-LUMICKS was linearized by digestion with HindIII, SalI or BamHI. Each fragment was purified (following the protocol in section 6), used in a DNA repeat assembly reaction at a final concentration of 300 ng/µl, and analyzed by agarose gel electrophoresis (Figure 2).

Agarose gel electrophoresis shows that arrays formed with HindIII cohesive ends form a smeared DNA band longer than 20 kb (DNA fragments longer than 20 kb are not resolved on gel). Repeat arrays with SalI also show discrete DNA bands running between 2 kb and above 20 kb indicating a less efficient assembly reaction than HindIII cohesive ends. Finally, BamHI cohesive ends showed the lowest assembly efficiency producing mostly DNA arrays running between 2 kb and 20 kb.

DNA tethering in the C-Trap confirmed that the arrays formed with HindIII overhangs were longer (~110 kb) than SalI (~90 kb) and BamHI (~45 kb) (see section 7, DNA tethering in the C-Trap).

Important note: while BamHI DNA arrays longer than 20 kb are typically not visible on agarose gel, they can still be tethered in the C-Trap with an average length of 45 kb – with the longest arrays of ~70 kb. This is possible due to high sensitivity of DNA tethering in C-Trap, which can catch DNA molecules that are not detectable by standard agarose gel electrophoresis.

Figure 2.

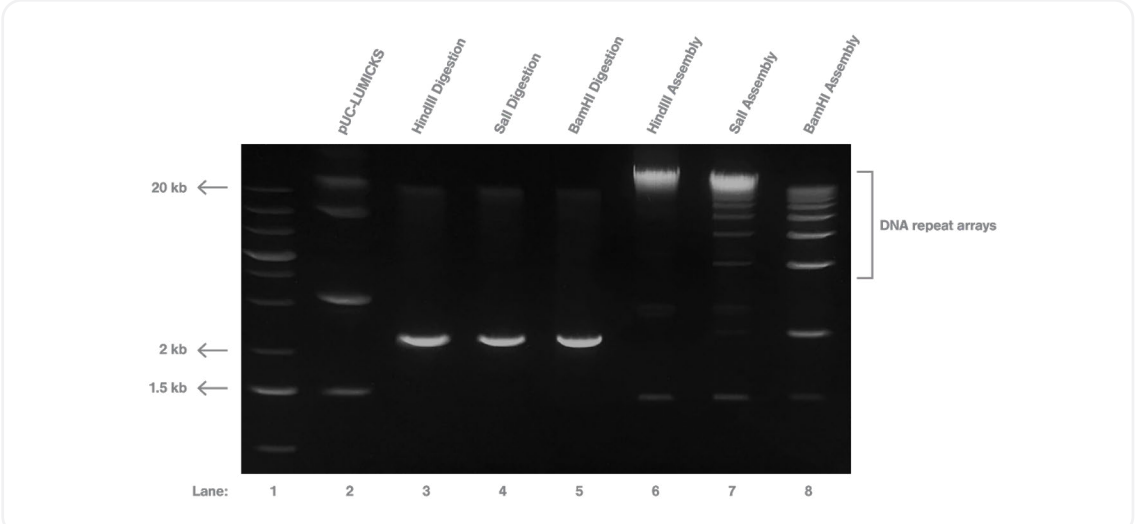


Figure 2. DNA gel electrophoresis was performed on a 0.75% agarose gel. 200 ng of circular plasmid and digested DNA fragments were loaded on gel. 600 ng of DNA repeat assembly reactions were diluted 1:10 with TE buffer before loading on the agarose gel. Circular pUC-LUMIKCS plasmid (2.26 kb; lane 2) was linearized by HindIII, SalI or BamHI digestion (lanes 3-5) producing a band corresponding to the size of 2.26 kb. DNA repeat assembly reaction were performed using the linearized fragments and analised by gel electrophoresis (lanes 6-8).

6. Guidelines to obtain the DNA fragment with overhangs

6.1 From a plasmid

The DNA repeat assembly reaction requires higher concentrations of DNA than standard DNA ligation reactions. If the sequence of interest is included in a plasmid containing a unique restriction site for the enzymes listed in Table 2, you can use the following protocol as a guideline to obtain high concentrations of the linearized plasmid. The protocol uses HindIII-HF from NEB (Cat. R3104) and a DNA purification kit from Promega (Cat. A9281). Enzymes or DNA purification kits from other suppliers can also be used.

Plasmid Digestion:

- Prepare the following reaction, adding the enzyme last.

Components	20 µl pre-assembly mix
Plasmid	25 µg
10x CutSmart buffer	25 µl
HindIII-HF	360 units (15 units per µg of DNA)
Milli-Q	To 250 µl

- Incubate 1-3 hours at 37°C.

DNA purification:

Perform purification using the DNA purification kit from Promega (Cat. A9281). Below, the purification steps are briefly described, including an additional air-dry step to completely remove ethanol and an additional elution step to improve DNA yield in this set up.

- Add 250 µl of DNA binding solution to 250 µl of HindIII-HF DNA digestion reaction.
- Transfer the mix to a Minicolumn (25 µg of DNA per Minicolumn).
- Incubate for 1 minute.
- Centrifuge at 16,000 x g for 1 minute.
- Wash with 700 µl Wash buffer centrifugating for 1 minute at 16,000 x g.
- Wash with 500 µl Wash buffer centrifugating for 5 minutes at 16,000 x g.

- Transfer the column into a new empty collection tube and centrifuge at 16,000 x g for 1 minute.
- Perform an extra air-dry step by leaving the column at room temperature on the bench for 10-30 minutes.
- Transfer the column into a new clean collection tube.
- For the first elution, add 30 µl of Tris-HCl 10 mM pH 8 to the Minicolumn. Incubate for 1 minute, then centrifuge at 16,000 x g
- Keeping the same collection tube, add 15 µl of Tris-HCl 10 mM pH 8 to the Minicolumn.
- Final elution volume should be ~35 µl.

6.2 From a PCR product

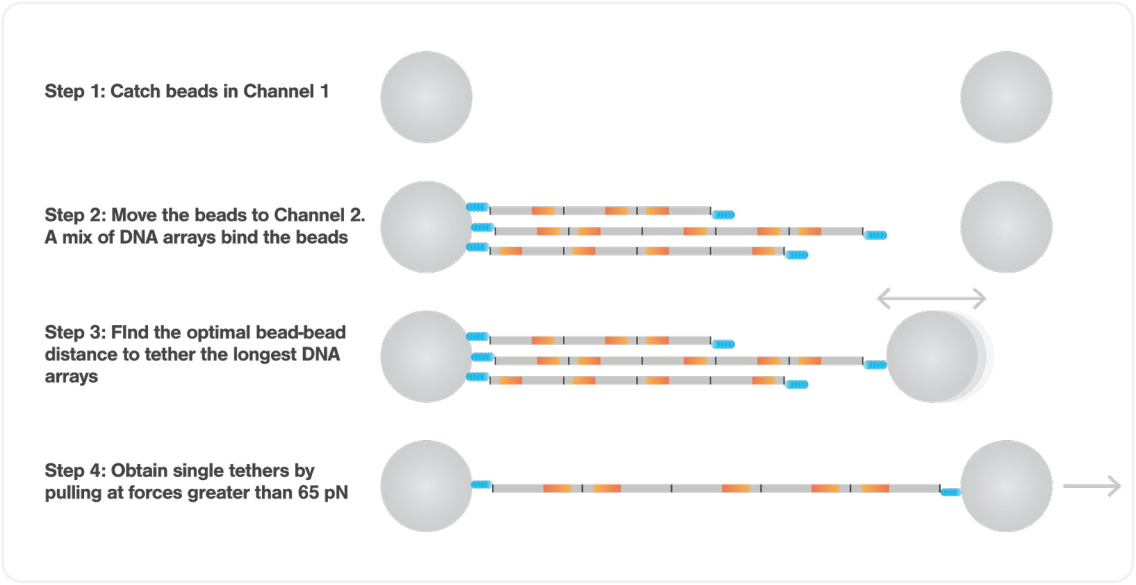
If a PCR is required to amplify the sequence of interest and obtain compatible cohesive ends, design primers with appropriate restriction sites. An additional 6 bases upstream of the restriction site is sufficient for digestion of DNA ends.

After completing PCR optimization, it is recommended to scale up the PCR reaction and purify the DNA band of the correct size from an agarose gel before proceeding with DNA digestion using the chosen restriction enzyme. Once the PCR product has been digested, you can follow the Promega purification protocol mentioned above as a guideline for DNA quantities, as well as the wash and elution steps.

7. DNA tethering in the C-Trap

The DNA repeat assembly produces DNA arrays of different lengths in the same reaction tube as the self-ligation of the DNA fragments is stochastic. Therefore, once the repeat assembly is completed and flowed into the LUMICKS' u-Flux™ microfluidics system, DNA arrays of different lengths and number of repeats are caught by the streptavidin beads (Figure 3). Below, we describe a procedure to selectively tether the longest DNA arrays produced in a DNA repeat assembly reaction.

Figure 3.



7.1 Microfluidics system set-up

Prepare the following solutions for the different channels of the LUMICKS' u-Flux™ microfluidics system and load them into the corresponding syringes. For the preparation of channels 1-3, a 40-60 mM Phosphate or Tris buffer at pH 7.5-8 containing 20 – 150 mM of common salts is recommended. Also, large beads (4.0-4.9 µm) are recommended to increase the efficiency of tethering and stability of the DNA tethers.

Channel 1

- Add 10 µl of streptavidin-coated polystyrene beads (4.0-4.9 µm, 0.1% (w/v)) to 1 ml of buffer and load in the syringe of channel 1.

Channel 2

- Add 300 ng of DNA to 250 µl buffer and add it to the syringe of channel 2. Increase or decrease the amount of DNA depending on the tethering efficiency.

Channel 3

- Load 1 ml of buffer in the syringe of channel 3.

Channel 4 (and 5)

- Every channel where proteins of interest are flowed in and DNA-protein interactions are measured should be passivated, preventing proteins from non-specifically interacting with the surfaces of the flow cell.

7.2 DNA tethering

Step 1

- Apply flow to channels 1, 2, and 3 with a pressure of 0.4 bar and keep the flow on until step 4.
- Catch a single streptavidin-coated bead in each of the traps in channel 1 and align the traps on the y-axis.
- Separate beads at a distance of 30 μm.

Step 2

- Move the beads to channel 2, which contains the mix of DNA arrays. Wait 20 seconds keeping the distance at 30 μm.
- **Note:** If multiple tethers are observed in the next steps, the incubation time of step 2 can be reduced until single tethers are obtained.

Step 3

- Move to channel 3 and move Trap 1 to increase the distance from Trap 2 until tether formation is detected by a force response.
- If no tethers are observed, decrease bead-bead distance by 5 μm, incubate for 5-20 seconds, and increase distance until tether formation is observed.
- Keep decreasing bead-bead distance in increments of 5 μm and incubating for 5-20 seconds until tether formation is observed.
- **Note:** If no tethers are observed even at 5 μm bead-bead distance, make sure that channel 2 is not clogged. Temporary clogging might be resolved by applying a flow of 2 bar for a couple of minutes

Step 4

- Once tether formation is verified, determine the number of tethers by pulling to forces greater than 65 pN. Single tethers show an overstretching plateau at ~65 pN. Instead, multiple tethers will show overstretching plateau at higher forces proportional to the number of tethers between the beads (i.e. around 65 pN for one molecule, ~120 pN for two, and so forth).
- In case of multiple tethers, they can be broken by increasing the forces until a single tether remains. Alternatively, you can optimize the incubation times of Step 2 to increase the probability to catch a single tether in channel 2.
- Once a single tether is obtained, tentatively fit the force distance (Fd) curve with the worm-like chain model to determine the contour length of the tether and therefore the number of repeats of the DNA array.

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