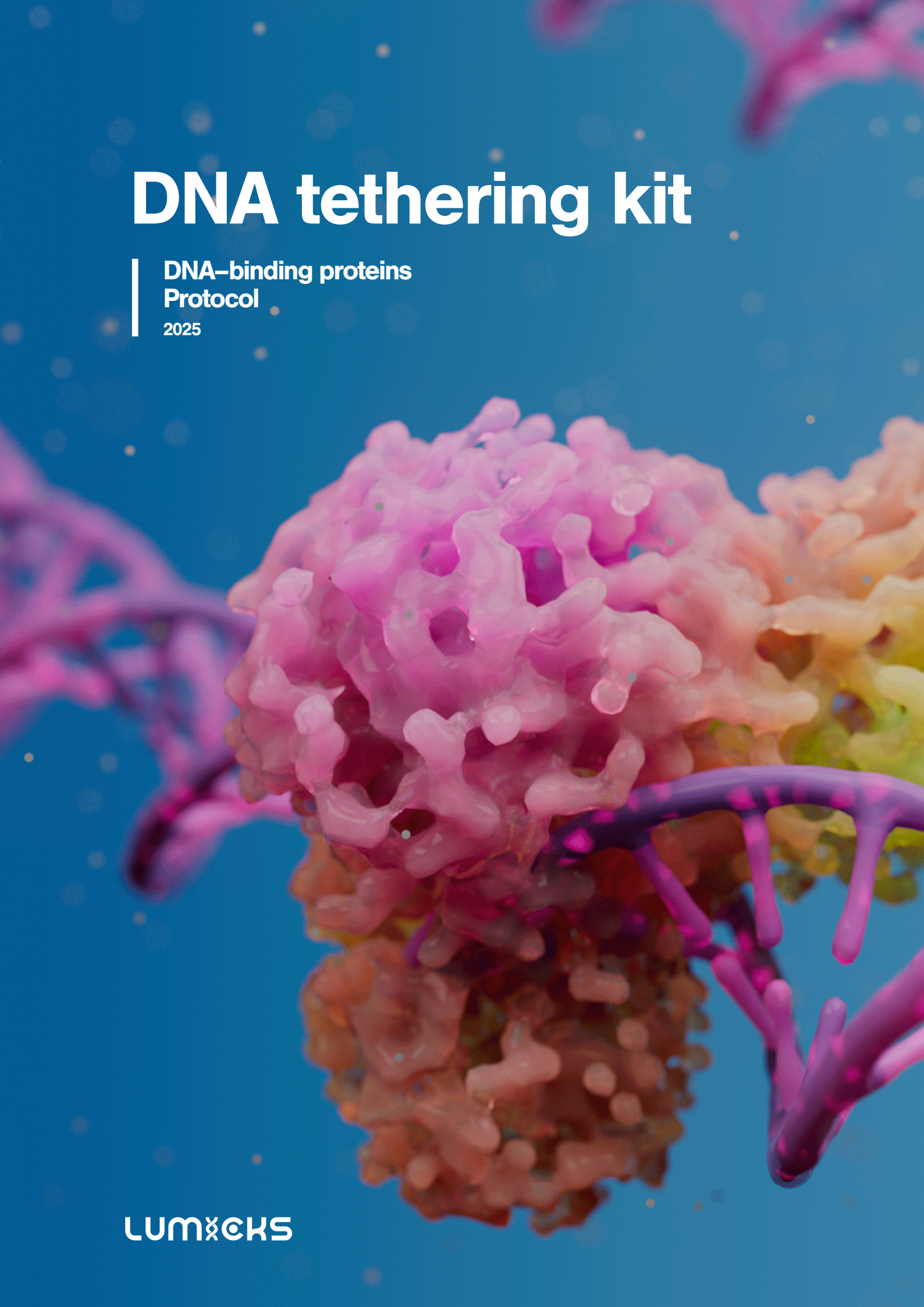
DNA tethering kit

DNA–binding proteins
Protocol

2025



LUMICKS

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DNA tethering kit

1. Description

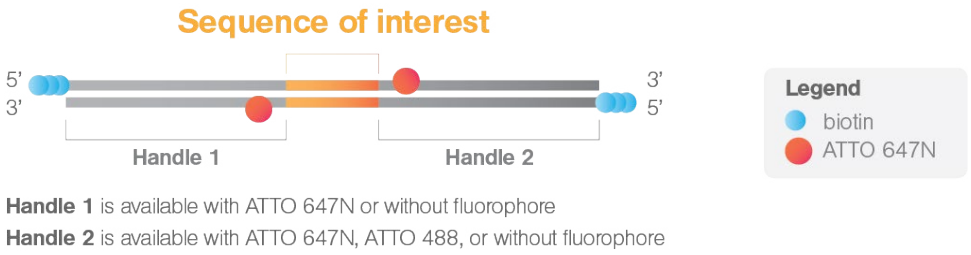
The DNA tethering kit is a highly efficient system for tethering a DNA sequence of interest (SOI) in optical tweezers and studying the dynamics of DNA-binding proteins. Using the kit, the SOI can be ligated to two DNA handles (Figure 1). Each DNA handle is 6.3 kb long (exact size: 6,298 bp), modified with 3 biotin moieties on one end and a 4-nucleotide single-stranded DNA overhang on the other end (Figure 2). To enable ligation of the SOI to the DNA handles, the SOI requires overhangs complementary to those of the DNA handles. The overhangs can be introduced into the SOI by digestion with a TypeIIS restriction enzyme, following the guidelines described below.

The ligation is designed so that DNA handle 1 will be ligated at the 5' end of the sequence of interest, and DNA handle 2 will be ligated at the 3' end. Additionally, the kit offers different versions of handles with or without ATTO fluorophores. The options for handle 1 include with ATTO 647N or without a fluorophore, while for handle 2, the options are with ATTO 647N, ATTO 488 or without a fluorophore.

Once the SOI is ligated to the two DNA handles, the biotin moieties of each DNA handle will allow tethering of the SOI between two streptavidin-coated beads trapped in optical tweezers (Figure 1). In this configuration, if handles with fluorophores are chosen, the fluorophores will flank the SOI (11 bps from the ligation sites) and can be used to position the SOI on the focal plane before incubation with fluorescent proteins. This enables the setup of optimal fluorescence imaging conditions before starting a DNA-protein interaction measurement, which allows capturing even the first interactions. By choosing handles labeled differently, an asymmetric configuration with the SOI can be achieved, and the 5'-3' directionality can be determined when tethered in the C-Trap. Additionally, in case both handles are labeled with ATTO fluorophores, the known distance between the two fluorophores can be used as a ruler on the tethered DNA to precisely determine the position on the DNA sequence of fluorescent proteins interacting with the SOI.

In addition to the DNA handles, the kit includes a DNA T4 Ligase enzyme, 10x T4 DNA Ligase buffer, a 3 kbp control DNA with overhangs (exact size: 3,065 bp), and a plasmid.

Figure 1.



Handle 1 is available with ATTO 647N or without fluorophore
Handle 2 is available with ATTO 647N, ATTO 488, or without fluorophore

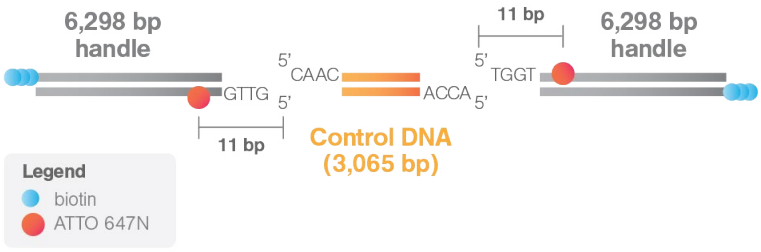
Here, we provide guidelines for producing the SOI with overhangs either by PCR or by cloning in the pUC-LUMICKS plasmid. The latter method can be useful for cloning multiple DNA fragments to form a composite SOI. Assembly of multiple DNA fragments using Golden Gate Assembly is also compatible with the DNA tethering kit. DNA fragments with a combined length of up to 24 kb have been ligated to 6.3 kb handles. For questions on using Golden Gate Assembly with the DNA tethering kit, please reach out to **store@lumicks.com**.

The kit provides material for 4 DNA Ligase reactions of the SOI to the DNA handles. Each DNA Ligase reaction can be used for at least 5 experimental sessions on the C-Trap.

1.1 Overhangs of the 6.3 kb DNA handles

The 6.3 kb DNA handles and the 3 kb control DNA provided with the kit already contain complementary overhangs. Ligation of the control DNA to the DNA handles is highly efficient and can be used as a positive control of the DNA Ligase reaction. To ligate the SOI to the DNA handles, the SOI requires the same overhangs of the control DNA (Figure 2).

Figure 2.



1.2 Important points before labeling

- The 10x T4 DNA Ligase buffer should be thawed at room temperature or in the palm of your hand. Do not incubate at 37 °C to avoid the breakdown of the ATP.
- To avoid thawing and freezing cycles of 10x T4 DNA Ligase buffer and DNA handles, make aliquots (e.g. 5 µl aliquots for the 10x buffer and 5 µl aliquots for the DNA Handles mix) and store them at -20 °C. This will prevent the breakdown of ATP in the 10x DNA Ligase buffer and formation of nicks in the DNA handles.
- Do not use EDTA or other chelating agents in the buffer of the SOI as they inhibit the Ligase reaction.
- Do not use salts (e.g. NaCl) in the buffer of the SOI as they inhibit the Ligase reaction.
- Avoid prolonged or repeated exposure to light during DNA Ligase reactions and storage.
- To ensure photostability during measurements in C-Trap, use oxygen scavenging systems.

2. Materials supplied in the kit

Table 1.

Components	Units	Volume	Storage temperature
Biotinylated DNA Handle 1 - no label / ATTO 647N	1	20 µl	-20 ˚C
Biotinylated DNA Handle 1 - no label / ATTO 488 / ATTO 647N	1	20 µl	-20 ˚C
T4 DNA Ligase	1	10 µl	-20 ˚C
10x T4 Ligase buffer	1	25 µl	-20 ˚C
Control DNA	1	5 µl	-20 ˚C
pUC-LUMICKS plasmid (50 ng/µl)	1	5 µl	-20 ˚C

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.
- Optional: Oxygen scavengers for imaging in the C-Trap.

3. Guidelines to obtain the sequence of interest with overhangs

Complementary overhangs at the two ends of the sequence of interest (SOI) can be introduced by digestion with TypeIIS restriction enzymes. These enzymes cleave outside of their recognition sequence allowing to choose the sequence of the overhangs formed upon digestion.

TypeIIS restriction sites can be introduced in the SOI either by PCR or by using the pUC-LUMICKS plasmid included in the kit.

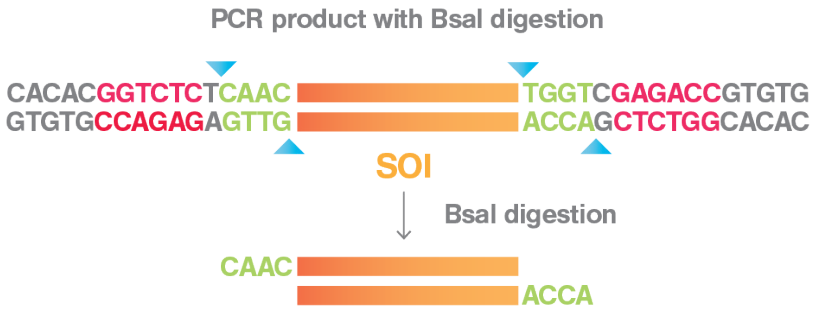
3.1 Using PCR

If PCR is the method of choice, the PCR primers – forward (Fw) and reverse (Rv) - can be designed using the flanking sequences that are listed in Table 2. The flanking sequences contain a 6-nucleotide sequence (red) that will form BsmBI, BbsI or BsaI restriction sites upon PCR amplification of the SOI (see Figure 3 for an example of PCR product using BsaI primers). The additional 5 nucleotides downstream of the restriction sites will form a sequence that, upon digestion of the PCR product, generates the overhangs (green) required for ligation to the DNA handles. Upstream of the restriction site, the additional 5 nucleotides will allow efficient cutting of the PCR product by the restriction enzymes. In Figure 3, there is an example of a PCR product of the SOI obtained using BsaI Fw and Rv primers.

Table 2.

BsmBI Fw	**5' CACACCGTCTCTCAAC——> 3'**
BsmBI Rv	**5' CACACCGTCTCGACCA——> 3'**
BbsI Fw	**5' CACACGAAGACCTCAAC——> 3'**
BbsI Rv	**5' CACACGAAGACAGACCA——> 3'**
BsaI Fw	**5' CACACGGTCTCTCAAC——> 3'**
BsaI Rv	**5' CACACGGTCTCGACCA——> 3'**

Figure 3.



Example of a PCR product of the SOI obtained using BsaI Fw and Rv primers. The arrows indicate the nicks introduced in the DNA sequence by digestion with BsaI with consequent formation of overhangs.

3.2 Using pUC-LUMICKS plasmid

The DNA tethering kit also provides a pUC-LUMICKS plasmid (link to the plasmid page and sequence). This plasmid has amulticloning site (MCS) within a LacZalpha gene, which is flanked by BsmBI, BbsI and BsaI restriction sites. The short DNAsequences downstream of each restriction site are designed to produce – upon digestion - overhangs that are compatiblefor the ligation to the DNA handles. The MCS can be used to insert the sequence of interest (SOI) in the plasmid. The consequent LacZalpha genedisruption can be used for white-blue screening assay based on beta-galactosidase activity. Once the SOI is inserted in theplasmid, BsmBI, BbsI or BsaI can be used to cut out the sequence of interest and ligate it to the DNA handles. In the finalproduct of ligation, the SOI will be within the disrupted LacZalpha gene and ligated to the DNA handles (Figure 4). The pUCLUMICKS plasmid is very useful to produce an SOI made of multiple DNA fragments, for example by sequential cloning or by Golden Gate Assembly.

Figure 4.

3.3 Purification of the sequence of interest

Independently from the method used to produce the sequence of interest (SOI) with overhangs (PCR or insertion in Plasmid), it is recommended to run the SOI on DNA agarose gel and purify it using a gel DNA extraction kit of choice. To ensure high efficiency of DNA Ligase reaction, it is also recommended to perform the extra steps usually described in DNA extraction kits to completely remove ethanol during the DNA purification.

4. Ligation of the sequences of interest to 6.3 kb DNA handles

A sequence of interest (SOI) with a length up to 5 kb have been tested and efficiently ligated to the 6.3 kb handles. As a control for ligation and tethering in the C-Trap, a parallel control reaction can be set up using 2 µl of the control DNA provided with the kit. This will correspond to 100 ng of control DNA (0.05 pmol).

- Quick spin down the kit vials before use to avoid losing any reagents through the tube caps.
- Prepare the following reaction mixtures, by first mixing 10x T4 DNA Ligase buffer with Milli-Q, then adding the DNA handles and DNA.
- Mix gently by pipetting up and down.
- Finally add T4 DNA Ligase and mix again by gently pipetting up and down avoiding bubble formation.

Components	SOI Ligation
10x T4 DNA Ligase buffer	2 µl
DNA handle 1	5 µl
DNA handle 2	5 µl
DNA	0.05 pmol
Milli-Q	to 20 µl
T4 DNA Ligase	1 µl

- Incubate at room temperature for 2 hours.
- Add EDTA (pH 7,5-8) to a final concentration of 30 mM and heat inactivate at 65 ˚C.

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 10 ng/µl to 30 ng/µl.

5. DNA tethering in the C-Trap

5.1 Microfluidics system set-up

Prepare the following solutions for the different channels of LUMICKS' u-Flux™ microfluidics system and load them into their corresponding syringes. For the preparation of channels 1-3, it is recommended to use a 40-60 mM phosphate or Tris buffer at pH 7.5, containing 20-150 mM of common salts. To enhance the efficiency of tethering and stability of the DNA tethers, it is advisable to use large beads (4.0-4.9 µm). Alternatively, smaller beads (1.5-1.9 µm, 1% (w/v)) also work efficiently.

Channel 1

- Add 10-15 µl of streptavidin beads (4-4.9 µm, 0.1% (w/v)) to 1 ml of buffer and load in the syringe of channel.

Channel 2

- Add 2-5 µl of the DNA ligase reaction mix, which contains the ligated DNA construct, to 500 µl of buffer and load it into the syringe of channel 2. Note: If multiple tethers are formed using this dilution, add an additional 500 µl of buffer to the syringe and mix.

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 or DNA-protein interactions are measured should be passivated, preventingproteins from non-specifically interacting with the surfaces of the flow cell.

5.2 DNA tethering

- Apply flow to channels 1, 2, and 3 with a pressure of 0.3–0.4 bar.
- Catch a single streptavidin-coated bead in each of the traps in channel 1, align the traps on the y-axis, and move them to channel 2.
- Keep the flow of channels 1-3 open and move Trap 1 to a distance approximately equal to half the contour length of your construct from Trap 2. Next, increase the distance and observe the force response to determine if a tether is formed.

- Once tethering is observed, move the traps to channel 3 and deactivate the flow for all channels. By examining how the force-distance (Fd) curve aligns with the worm-like chain model, you can differentiate between single and multiple tethers. Multiple tethers between the beads will manifest as a force increase at a shorter distance than predicted by the worm-like chain model. Additionally, the DNA overstretching plateau will occur at higher forces, proportional to the number of tethers between the beads (e.g., approximately 65 pN for one molecule, 120 pN for two, and so on)
- If multiple tethers are observed, they can be disrupted in channel 3 by gradually increasing the force until only a single tether remains. If multiple tethers continue to form consistently, it may be necessary to decrease the DNA concentration.
- Once the presence of a single tethered molecule is confirmed, it can be subsequently transferred to channels that contain the proteins of interest.
- At this stage, activate the corresponding excitation laser and begin recording either a sequence of 2D frames or a kymograph. To enhance the image quality, adjustments may be needed for the two traps along the z-axis to align the DNA with the imaging volume. This can be accomplished by moving the traps synchronously in the z-direction and utilizing the fluorophores of the DNA handles as a reference to determine the optimal height.

Important note: Collect the waste fluids from the experiment and dispose the waste in accordance with local/regional/national/international regulation. Do not dispose in drains to prevent the release of microbeads into the environment.

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