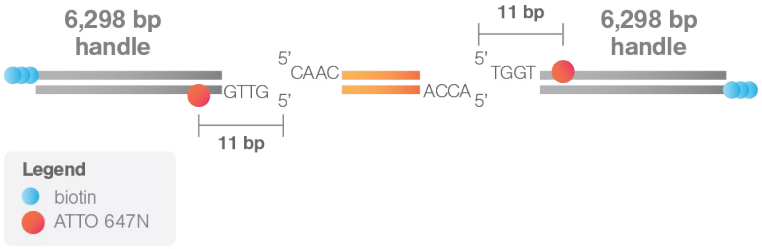
DNA Tethering Kit *Quick Protocol*

1. Preparation of DNA construct

To enable efficient ligation to biotinylated DNA handles, the sequence of interest (SOI, in orange) must carry complementary 4-nucleotide overhangs at its 5′′ and 3′′ ends. These overhangs can be introduced by digesting the SOI with Type IIS restriction enzymes (see Section 3 of the **full protocol** for details).



1.1 Ligation of the sequence of interest (SOI) to 6.3 kb DNA handles

1. Quick spin kit tubes before use to avoid reagent loss. The ligation can be performed in 20 µl or 10 µl reaction volume. The 10 µl format is useful for troubleshooting and gel analysis (see Section 4.1 of the **full protocol**,).
2. Prepare the reaction mixture in the table below, by first mixing 10x T4 DNA Ligase buffer, Milli-Q, and DNA components.
3. Finally, add T4 DNA Ligase and mix again by gently pipetting up and down to avoid bubble formation.
4. Incubate at room temperature for 2 hours.
5. Add EDTA (pH 7.5–8) to a final concentration of 30 mM and heat inactivate at 65˚C.

Components	20 µl Ligation	10 µl Ligation
10x T4 DNA Ligase buffer	2 µl	1 µl
DNA handle 1	5 µl	2.5 µl
DNA handle 2	5 µl	2.5 µl
DNA (SOI)	0.05 pmol*	0.025 pmol*
Milli-Q	to 20 µl	to 10 µl
T4 DNA Ligase	1 µl	0.5 µl

*Final concentration 2.5 nM

6. Optional: analyze ligation efficiency by agarose gel (see Section 4.1 of the full protocol).

1.2 Buffer exchange with Amicon Ultra-0.5 mL Centrifugal Filters (30 kDa cutoff)

1. Wash twice the filter with 500 µl TE (Tris-HCl 10 mM, EDTA 1 mM, pH 8).
2. Equilibrate the filter by adding 500 µl TE and centrifuging at 11,500 RCF for 3 min.
3. Dilute ligation reaction (from Section 1.1) up to 500 µl with TE buffer.
4. Load into filter and centrifuge at 11,500 RCF for 5 min.
5. Keep the remaining ~50 µl of concentrated sample in the filter.
6. Discard flowthrough. Add 450 µl TE, spin again (5 min, 11,500 RCF).
7. Recover sample by inverting filter into clean tube; spin at 1,000 RCF for 2 min.
8. Measure DNA concentration with a NanoDrop. Expected yield: 10–30 ng/µl.

2. DNA tethering in the C–Trap

2.1 Microfluidics Setup

Use buffer: 40–60 mM phosphate or Tris, pH 7.5, with 20–150 mM NaCl/KCl.

Channel 1: Add 10–15 µl of streptavidin beads (4-4.9 µm, 0.1% (w/v)) to 1 ml of buffer.

Channel 2: Add 2-5 µl of the T4 DNA ligase reaction mix to 500 µl of buffer.

Channel 3: Use 0.5–1 ml of buffer of choice.

Channel 4 (and 5): If using proteins, passivate to prevent non-specific surface interactions.

2.2 DNA Tethering

1. Set the eWLC using the contour length of the biotinylated DNA construct.
2. Flush channels 1, 2, and 3 at 0.3–0.5 bar, while keeping the protein channels (4 and 5) closed.
3. In channel 1, catch one streptavidin bead in each trap and align them on the y-axis.
4. In channel 2, with flow still active, move Trap 1 to a distance corresponding to ~50% of the contour length of the DNA construct.
5. Increase the distance by moving Trap 1 away from Trap 2 and observe the force response to determine if a tether is formed.
6. Single tethers follow the worm-like chain model with a force plateau of ~60 pN, while multiple tethers show a higher force plateau (e.g., ~120 pN for two tethers).
7. If multiple tethers are present, apply higher force to break them until only one remains. Alternatively, reduce DNA concentration to avoid formation of multiple tethers.
8. Once a single tether is confirmed, transfer the molecule to a protein-containing channel (e.g., channel 4).
9. Activate the appropriate laser and begin acquisition (2D frames or kymograph). For optimal focus, adjust the traps synchronously in z until the fluorophores on the DNA handles are in the focal plane.

Note: Collect waste fluids and dispose of them according to local environmental regulations. Do not release microbeads into drains.