Hairpin labeling and tethering kit (short handles)

DNA–binding proteins
Protocol
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LUMICKS

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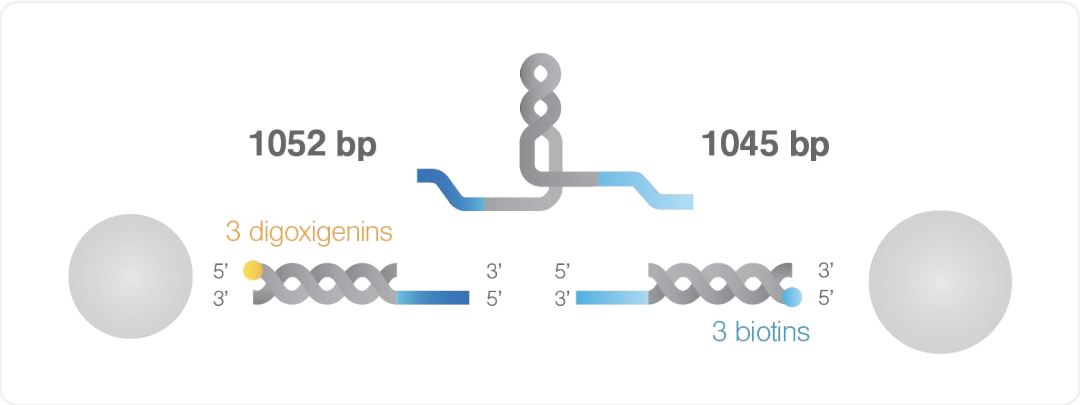
The hairpin tethering kit is a highly efficient system to characterize secondary DNA/RNA structures using high-resolution single-molecule optical tweezers. The methodology is divided into two main procedures:

1. DNA/RNA hairpin ligation to functionalized DNA handles.
2. DNA/RNA hairpin tethering to beads for optical tweezers experiments.

The ligation to functionalized DNA handles requires that the hairpin of interest is flanked by 15 nt DNA sequences at the 5' and 3' ends using a spacer of 3 thymidine nucleotides between the hairpin and the flanking sequences. It is necessary that the 5' of the flanking sequence is phosphorylated to allow the ligation to the handle with the digoxigenin. The tethering procedure uses streptavidin and anti-digoxigenin coated beads. The kit includes a DNA construct that can be used as a positive control for the ligation and tethering procedures.

DNA sequences flanking the hairpin (in brackets a spacer of 3 thymidine nucleotides)

5' CATTGCGCATTGCGT(TTT)-[hairpin]-(TTT)GGTATAGCATTCTAG 3'



Materials supplied in the kit

Components	Units	Volume	Storage temperature
DNA ligase	1	10 µl	-20 ˚C
10x Ligase buffer	1	25 µl	-20 ˚C
DNA handles (1045/1052 bp)	1	85 µl	-20 ˚C
Control DNA (5 µM)	1	5 µl	-20 ˚C
Streptavidin-coated polystyrene beads (⌀ 1.0-1.4 µm)	1	12 µl	+4 ˚C
Anti-digoxigenin-coated polystyrene beads (⌀ 0.7-0.9 µm)	1	60 µl	+4 ˚C

10x ligase buffer composition: 400 mM Tris-HCl, 100 mM MgCl2 , 100 mM DTT, 5 mM ATP (pH 7.8 at 25 °C).

Additional materials (supplied by the user)

- Oxygen scavengers. For support with oxygen scavenging systems, please contact **store@lumicks.com**.
- RNase inhibitors for RNA hairpins: 40 U/µl **Recombinant RNase inhibitor MoloX** is recommended.

Important points before labelling

- The 10x 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 ligase buffer and DNA handles, make aliquots (8 µl) and store them at -20 °C. This will prevent the breakdown of ATP in the 10x ligase buffer and formation of nicks in the DNA handles.

1. DNA/RNA hairpin ligation

- Quick spin down the kit vials before use to avoid losing any reagents through the tube caps.
- For RNA hairpins, add 0.5 µl of MoloX RNase inhibitor to 8 µl of DNA handles or RNase inhibitor of choice.
- Bring the hairpin of interest to a concentration of 0.1 µM using 10 mM Tris pH 8.0.
- Add 1 µl of 0.1 µM hairpin to 8 µl of DNA handles.
- Using a thermal cycler and PCR quality tubes, incubate the hairpin-handles mix at 68 ˚C for 2 min, then cool to 22 ˚C using a ramp rate of 0.1 ˚C/s. Note: if the ramp rate cannot be set, program the thermal cycler to have a gradual cooling (~20 min) from 68 ˚C to 22 ˚C.
- In the meantime, pre-mix 2 µl of 10x ligase buffer with 9 µl Milli-Q water and add it to the annealed hairpin-handles mix. Mix gently by pipetting 5 times. Avoid bubble formation.
- Add 1 µl of DNA ligase. Mix gently by pipetting 5 times. Avoid bubble formation.
- Incubate at 22 ˚C for 30 min, heat inactivate the DNA ligase at 65 ˚C for 10 min, finally cool to 4˚C for temporary storage.
- Optionally, EDTA to a final concentration of 1 mM can be added to the ligase reaction for storage at 4˚C.

2. Hairpin tethering in C-Trap®

Tethering is achieved by using optimized experimental conditions, specifically developed for the DNA handles supplied with DNA/RNA hairpin tethering kit. The final result is the hairpin of interest connected to 1052 bp and 1045 bp handles (hairpin–DNA handles). Each handle is tethered between the DNA/RNA hairpin and an anti-digoxigenin coated bead or a streptavidin-coated bead.

Important points before tethering

- Buffers should be kept at room temperature during the tethering. Cold buffers can reduce both tethering efficiency and the activity of oxygen scavengers.
- For optimal results, use 40–60 mM Phosphate or Tris buffer at pH 7.5-8.0 containing salt concentration of choice and 0.5 mM EDTA to inhibit DNase activity.
- For RNA hairpins, use 1.6 U/ml of RNase inhibitor MoloX or RNase inhibitor of choice in each channel of the flow cell employed during the experiment (i.e., channel 1,2, and 3 in the procedure described below).
- For overall detailed instructions, consult the C-Trap manual or watch the tutorial video at the following **link**.
- Controls and functions of the C-Trap, which are particularly critical for the trapping of short tethers, are described in the next page.

Experimental set-up for the microfluidics system with laminar flow cell

Channel 1

- Vortex the anti-digoxigenin coated beads for 10 s and sonicate for an additional 5 s in a water bath sonicator. If a water bath sonicator is not available, vortex the beads for 20-30 s. Add 2 µl of the DNA ligase reaction to 5 µl of anti-digoxigenin coated beads. Mix immediately by gently pipetting 5 times. Avoid bubble formation. **Important:** the amount of DNA to incubate with the anti-digoxigenin beads might require adjustment depending on the efficiency of tethering in the C-Trap. See page 8 of this protocol for details.

- Incubate at room temperature for 10 min to enable the binding of the hairpin–DNA handles to the beads.

- Dilute the bead-tethered hairpins with 500 µl of the buffer of choice, mix gently by pipetting 5 times, and load the mix into the syringe of channel 1.

Channel 2

- Prepare 500 µl of the buffer of choice supplemented with oxygen scavengers.

- Load the buffer supplemented with the oxygen scavenger system into the syringe of channel 2.

Channel 3

- Vortex the streptavidin-coated beads for 10 s.

- Add 1 µl of streptavidin-coated beads into 1000 µl of buffer, mix, and load the entire volume into the syringe of channel 3.

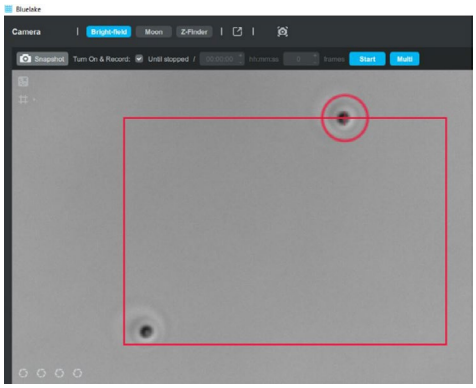
Catching beads and hairpin tethering

Here, we describe an optimized workflow to attach short (1.714 µm) hairpin–DNA handles between an anti-digoxigenin bead and a streptavidin-coated bead. An automation script to speed up the tethering workflow can also be used, as shown in the video at the following **link**.

Low laser powers are recommended. High overall laser power can damage DNA tethers and affect tethering efficiency.

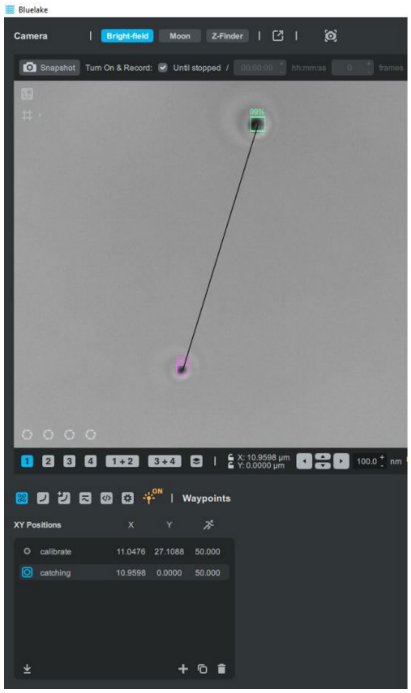
Trap positioning

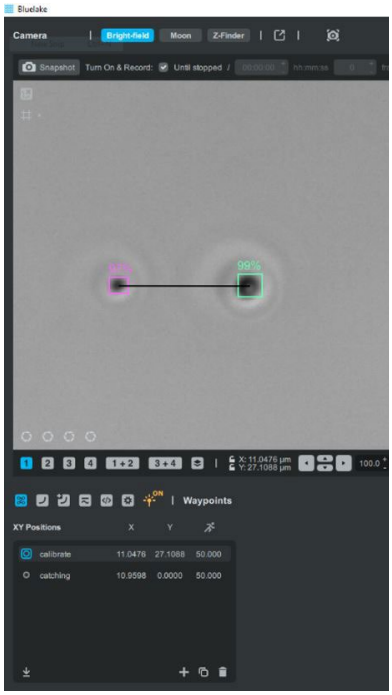
- Open channels 1–3 and apply a pressure of 0.3-0.5 bar.
- Determine the positions of Trap 1 and Trap 2 by catching 1 bead per trap.
- Bring both trapped beads into channel 2 by moving the microstage and stop the flow once aligned.
- Visualize Trap 1 and align Trap 1 with the red circle, either by moving the “Trap 1 +2” control or press “shift” and left-click on the bead in trap 1.



Above: Figure 1

- Move Trap 1 vertically to the top edge of the rectangle and Trap 2 to the bottom part of the rectangle to maximize the y-axis distance between the two beads, as shown below. You can now save the position of Trap 1 as “catching”.
- Define a template for each of the two beads on the brightfield camera window (Figure 2).
- Align Trap 1 at a ~5 µm distance from Trap 2 at the same horizontal plane, as shown below. You can save the Trap 1 position as “calibrating”. The saved “catching” and ”calibrating” positions of Trap 1 allow you to automatize and speed up the tethering procedure (Figure 3).





Left: Figure 2, Right: Figure 3

Catching beads with the optical traps and calibrating

- Release the beads from the previous step by applying flow to channels 1, 2, and 3 with a pressure of 0.3–0.5 bar.
- Set the position of Trap 1 to “catching”.
- Fine-tune the position of the microstage to move Trap 2 to channel 1 and catch a single anti-digoxigenin bead.
- Move the microstage until Trap 1 localizes at the edge between channels 2 and 3.
- Fine-tune the position of the microstage until Trap 1 is in channel 3 and catches a single streptavidin-coated bead. Keep Trap 2 inside channel 2.
- Move the two traps to channel 2 by moving the microstage and stop the flow.
- Set the position of Trap 1 to “calibrating”
- Perform trap calibration according to the different sizes of the two trapped beads (e.g. Trap 1 bead = 1.36 µm; Trap 2 bead = 0.86 µm). Zero the force. Perform a calibration for every new bead pair. This will also help assessing if the trapped beads are single beads.

Tethering of the hairpin between two beads to form the dumbbell configuration

It is recommended to use piezotracking.

- Switch on piezotracking, select the piezo distance, and add the theoretical worm-like chain (WLC) model with the total contour length of the DNA handles (2,1 kbp), corresponding to the sum of the two DNA handles of 1,045 and 1,052 bp).
- Use the “Force Distance” feature in Bluelake to record a “baseline” FD curve from 5 µm (the “calibrating” distance) to the distance where tethering is expected to happen (at 60% of the contour length), which is further referred to as “tethering” distance. The baseline can be used in post-processing to correct for the force interaction of the beads at short distances. Alternatively, if the baseline feature is enabled in Bluelake, apply the baseline correction. In the Fd-window it is then possible to visualize the corrected force.
- To avoid abrupt movements of Trap 1, move Trap 1 to a distance of 1 µm from Trap 2 and lock the speed of Trap 1 to 0.1 µm/s.
- Move Trap 1 as close as possible to Trap 2, allowing the beads to almost touch each other. Increase the distance again and determine if there is a tether formed by observing the force response. Repeat and tentatively reduce the initial distance between the two beads until tether a force response is observed. **Important**: The close distance between the two beads will hide the bead templates. However, the PT mode will stay active.
- A single tether will show an overstretching regime at pulling forces >60 pN. In case of multiple tethers, they can be broken by increasing the force until a single tether remains. In case multiple tethers are regularly being formed, reduce the amount of hairpin incubated with antidigoxigenin coated beads during the sample preparation of channel 1.
- In case multiple tethers are regularly being formed, the amount of DNA to incubate with the anti-digoxigenin beads can be lowered. In case of low tethering efficiency, the amount of DNA to incubate with the anti-digoxigenin beads can be increased.

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