Protein labeling and tethering kit (cysteine)

Protocol

Protein Folding

Version 2.0

LUMICKS

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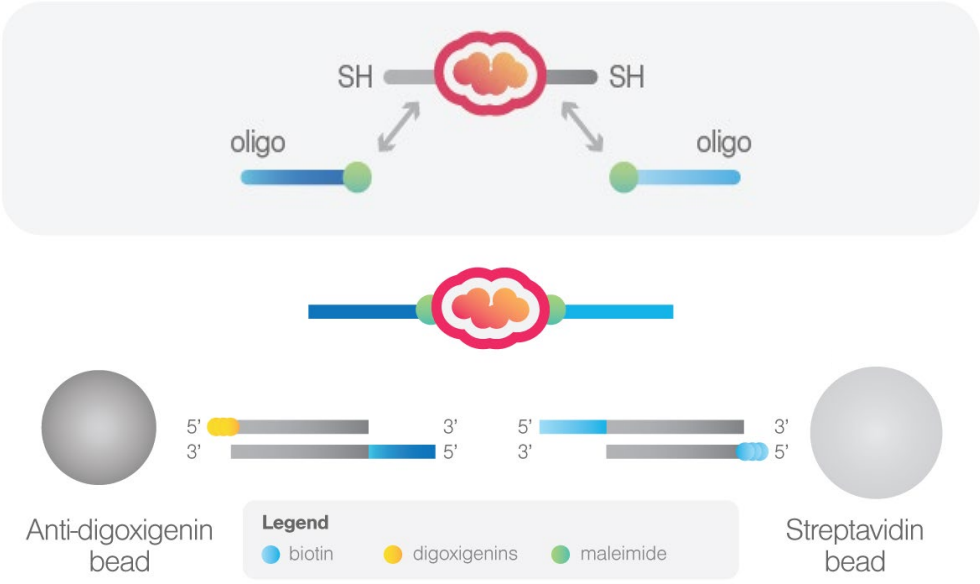
Protein Labeling and Tethering kit (cysteine)

1. Description

The Protein Labeling and Tethering Kit (cysteine) is a highly efficient kit for studying protein dynamics at the single-molecule level through optical tweezers. The methodology is divided into two main procedures:

1. Protein labeling with functionalized DNA handles.
2. Protein tethering to beads for optical tweezers experiments.

The protein labeling procedure uses maleimide–cysteine chemistry to attach two DNA handles to two cysteines of the protein of interest. The kit can be ordered with 517 bp, 1506 bp or 5039 bp DNA handles (referred to as 0.5 kb, 1.5 kb, and 5 kb handles, respectively). The protein tethering procedure uses streptavidin- and anti-digoxigenin-coated beads. The kit includes the adenylate kinase (AdK) protein with two cysteines [1], which can be used as a quality control protein for both the labeling and tethering procedures.



Protein labeling and tethering schematics. The top panel illustrates the conjugation of maleimide–modified DNA oligos to the two cysteines of the protein of interest.

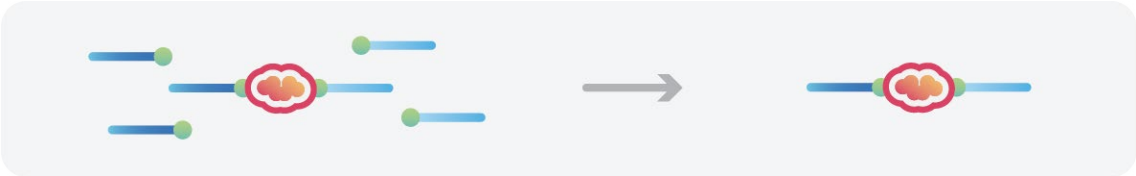
The bottom panel shows the labeled protein–oligo chimera, ready for annealing with the biotin- and digoxigenin-labeled DNA handles (Step 3 of protein labeling). Purification of the chimera from excess oligo (Step 2 of protein labeling) is not shown. Each DNA handle carries 3x biotin or 3x digoxigenin moieties at the 5' end, and both share the same 5' overhang sequence, complementary to the oligo.

1.1 Protein labeling overview

This protocol assembles a protein of interest with two DNA handles — one carrying 3x digoxigenin at the 5’ end, the other 3x biotin. The labeling process consists of three steps:

Step 1: Conjugation of maleimide–modified oligo to a protein with two cysteines
Maleimide–modified DNA oligo is attached to two cysteines thiol groups (–SH) on the protein of interest, forming covalent thioether bonds. The result is a protein–oligo chimera.

Step 2: Purification of protein-oligo chimera
The protein labeled with two oligos (protein–oligo chimera), is purified from excess unreacted maleimide-modified oligos. **Note:** Protein purification requires a tag such as GST or 6×His to enable small-scale cleanup using affinity-based methods (e.g., glutathione or Ni-NTA resin).



Step 3: Annealing of DNA handles
The purified chimera is annealed to the complementary overhangs of the DNA handles.

Note: The kit provides a tube containing a single maleimide-modified oligo, labeled at the 3’ end with maleimide and carrying the sequence 5’-cgt tgt tgc tat tgg tag gcg gtg acc cgt ccc aac -3’. This oligo binds to the two cysteines of the protein of interest. The DNA handles are provided as a 50/50 pre-mix, with 50% of the handles labeled at the 5’ end with 3x Biotin and the remaining 50% labeled with 3x Digoxigenin moieties. Both handles share the same 5’ overhang sequence: 3’ - /ab/ gcaacaacgataaccatccgccactgggcagggttg -5’, where /ab/ indicates an abasic site. These handles anneal with the oligos attached to the protein.

1.2 Important points before protein labeling and tethering

- The protein of interest requires two exposed cysteines and a tag such as GST or 6xHis to allow a small-scale purification from excess unreacted oligos.
- Recommended buffers for all the steps of protein labeling are 40–60 mM phosphate or Tris buffer at pH 7.5 with 20–150 mM common salts (e.g., KCl or NaCl).
- TCEP must be used as a reducing agent as it does not react with maleimide. Do not use dithiothreitol (DTT) since it reacts with maleimide groups and inhibits the maleimide–cysteine conjugation.
- For step 1 of protein labeling (Section 1.1 and 3.1), the optimal protein concentration for conjugation to maleimide–modified oligo is at least 20 µM, in a final volume not exceeding 40 µl.
- For step 2 of protein labeling (Section 1.1 and 3.2), refer to the following protocol as an example for purifying protein–oligo chimera from excess unreacted oligos, see "**6xHis-AdK-oligo purification**".
- For cleaning of the microfluidics, refer to Beads and Cleaning Kit - Recommended protocol. Passivation is not required for protein tethering with DNA handles.
- The DNA handles in the kit feature a 5' single-stranded overhang with an abasic site at the junction with the double-stranded region. This abasic site prevents ligation of the annealed protein-bound oligos. Despite this, the high melting temperature of the annealed complex ensures the formation of stable tethers that typically break at 45–50 pN forces in the C-Trap.

2. Materials Supplied in the Kit

2x reactions: conjugation with CoA-modified oligonucleotide

Component	Units	Volume	Storage Temperature
Malemide Oligo (250 µM)	2	40 µl	-80 ˚C
TCEP (10 mM)	2	5 µl	-80 ˚C

10x reactions: annealing with DNA handles and tethering in the C-Trap

Component	Units	Volume	Storage Temperature
DNA Handles	1	See table below	-80 ˚C
Streptavidin beads (⌀1.0-1.4 µm)	1	12 µl	+4 ˚C
Anti-digoxigenin beads (⌀0.7-0.9 µm)	1	60 µl	+4 ˚C

AdK-6xHis (cysteine) for 1x control reaction

Component	Units	Volume	Storage Temperature
AdK - 6xHis (cysteine)	1	40 µl	-80 ˚C

Note: The components listed above are included in the complete kit. Materials for 2x conjugation reactions with oligos (Oligonucleotides for Protein Labeling Kit (Cysteine)) or 10x annealing reactions with DNA handles (DNA Handles for Protein Tethering Kit) can be purchased separately. The Oligonucleotides for Protein Labeling Kit (Cysteine) includes maleimide–modified oligonucleotides and a control reaction with AdK, while the DNA Handles for Protein Tethering Kit includes DNA handles and beads for 10x annealing and tethering in the C-Trap. Both kits are available on our webstore.

2.1 DNA handle selection table

The kit can be ordered with 517 bp, 1506 bp, or 5039 bp DNA handles (referred to as 0.5 kb, 1.5 kb, and 5 kb handles, respectively). DNA handles are supplied in one tube per kit as a 50/50 pre-mix, with 50% of the handles labeled at the 5' end with 3x biotin and 50% labeled with 3x digoxigenin.

The concentration, total volume per tube, and volume per 1x annealing reaction vary depending on the selected DNA length, providing enough material to perform 10 annealing reactions (see Step 3 of protein labeling).

DNA Length	Concentration	Volume per tube	Volume per annealing
0.5 kb (exact size 517 bp)	50 ng/µl	45 µl	4 µl
1.5 kb (exact size 1506 bp)	150 ng/µl	45 µl	4 µl
5 kb (exact size 5039 bp)	75 ng/µl	65 µl	6 µl

2.2 Additional materials (supplied by the user)

- Centrifugal protein concentrators: Vivaspin® Centrifugal Concentrators or Amicon® Ultra Centrifugal Filters.
- Small-scale affinity purification kits: His SpinTrap™ (GE Healthcare, 28-9523-59) and GST SpinTrap™ (GE Healthcare, 28-9523-59) are recommended for 6xHis– and GST–tagged proteins, respectively.
- Oxygen scavengers are required for tethering with 0.5 kb and 1.5 kb handles, but are not needed for longer tethers with 5 kb handles. For support with oxygen scavenging systems, please contact **store@lumicks.com**.
- Optional: For the electrophoretic mobility shift assay, use an agarose DNA gel (1% for 0.5 and 1.5 kb handles, and 0.5–0.75% for 5 kb handles) and a non–denaturing gel loading dye without SDS (NEB, Cat. #B7025S).

3. Protein Labeling with DNA Handles Protocol

3.1 Conjugation of maleimide–modified oligo to protein (Step 1)

The protein of interest containing two accessible cysteine residues is conjugated with maleimide–modified oligonucleotide via thiol–maleimide chemistry, which forms a covalent thioether bond between the maleimide group and the thiol (–SH) of each cysteine.

- Quickly spin down the kit tubes before use to avoid losing any reagents through the tube caps.
- Adjust the protein of interest solution to a concentration of at least 20 µM in the buffer of choice, ensuring the final volume does not exceed 40 µl.
- Slowly add 2 µl of TCEP (10 mM) to the protein solution while gently swirling. This helps prevent abrupt local changes in concentration or pH that could affect protein stability.
- Add 40 µl of maleimide-modified oligo solution to the 40 µl of protein solution (total volume of the reaction mixture is ≤ 80 µl). **Important:** Add the oligo slowly (dropwise) while swirling the tube containing the protein solution. Avoid the formation of bubbles.
- Incubate at room temperature for 2 hours, or overnight at +4 ˚C.

Components	Volume
Protein (≥ 20 µM)	≤ 40 µl
TCEP (10 mM)	2 µl
Maleimide–modified oligo (250 µM)	40 µl

3.2 Purification of protein-oligo chimera (Step 2)

1. Use a small-scale affinity purification method suitable for the specific affinity tag fused to your protein. For 6xHis– and GST– tagged protein, His SpinTrap™ (GE28-4013-53) and GST SpinTrap™ (GE28-9523-59) are recommended. For a detailed purification protocol, please refer to ”**6xHis-AdK-oligo purification**”.

2. Measure the concentration of the purified protein–oligo by reading the absorbance at 260 nm with a NanoDrop and using the optical density (OD) conversion factor to measure single-stranded DNA (33 ng·cm/µl).

3. Ensure the protein–oligo concentration is at least 20 ng/µl for optimal results in the next step.

4. Aliquot the protein–oligo to avoid repeated freeze–thaw cycles, then snap-freeze and store the aliquots at –80 °C.

3.3 Annealing to DNA handles (Step 3)

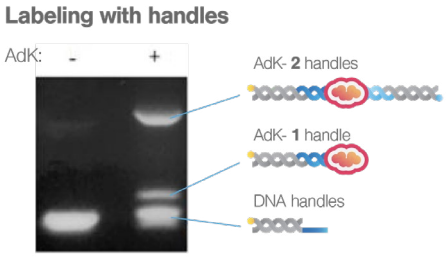
The oligos of the protein–oligo chimera are annealed to the overhangs of the DNA handles. Volume used in the annealing reaction varies depending on the selected DNA handle length.
Use 4 µl for the 0.5 and 1.5 kb handles, and 6 µl for the 5 kb handles.

- Quickly spin down sample tubes before use to avoid losing any reagents through the tube caps.
- Dilute the protein–oligo chimera to a final concentration of 20 ng/µl using a buffer containing 20–50 mM Tris pH 7.5 and 50–150 mM NaCl.
- Prepare the following mixture adding the protein–oligo chimera to the DNA handles. Mix by gently pipetting 3–5 times, avoiding bubble formation.
- Incubate at room temperature for 10 min to allow annealing to DNA handles.
- Keep the sample on ice until ready for tethering in the C-Trap.

Components	Volume
Protein–oligo chimera (20 ng/µl)	1 µl
DNA handles (0.5, 1.5 or 5 kb)	4 µl or 6 µl

3.4 Optional: Testing annealing by electrophoretic mobility shift assay

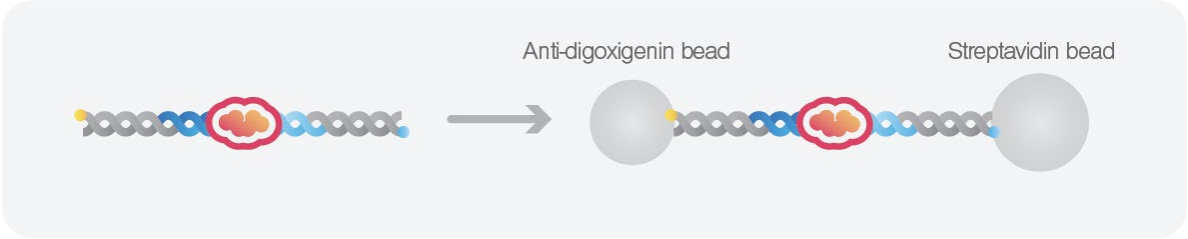
Annealing of DNA handles to the protein–oligo chimera can be verified by electrophoretic mobility shift assay (EMSA). Below is an example using AdK–oligo chimera annealed to ~0.5 kb DNA handles. Run a 1% agarose gel at low voltage (80–100 V) with fresh TBE buffer and non-denaturing loading dye (NEB, Cat. #B7025S) for best results.



EMSA of AdK–oligo annealed to 0.5 kb DNA handles. 4 µl of DNA handles (control, left lane) and 5 µl of AdK–oligo annealed to DNA handles (right lane) were compared on gel. AdK attached to one handle showed a small first upper shift, while the second big shift corresponds to the size of a second handle attached to AdK (~ 0.5 kb, DNA marker not shown).
Important: The band corresponding to the second shift (i.e., two handles bound) may appear faint or be absent with protein of interest, but tethering on the C-Trap can still be successful by skipping the 1:15 dilution of the protein–DNA handle mix during the preparation of channel 1 (see Section 4.1).

4. Protein Tethering in the C-Trap

Annealing of the protein–oligo chimera results in a mixed population of protein–DNA handle complexes (see Section 3.4). By placing an anti-digoxigenin bead in Trap 2 and a streptavidin bead in Trap 1, the tethering procedure below enables selective tethering of complexes with one 3xDigoxigenin and one 3xBiotin DNA handle. For detailed instructions on setting up optics and tethering, refer to the C-Trap user guide.



4.1 Microfluidics setup

- Ensure proper cleaning of the microfluidics according to Beads and Cleaning Kit - Recommended protocol. **Note:** Passivation is not required for protein tethering with DNA handles.
- Use a 40–60 mM phosphate or Tris buffer at pH 7.5, supplemented with 50–150 mM of common salts (e.g., KCl or NaCl). This buffer can be used for all dilution steps in Channels 1, 2, and 3.
- Vortex beads for 10–20 s before use.

Channel 1

- Following annealing (Section 3.3):
    - For **0.5 and 1.5 kb handles,** dilute the protein–DNA handle complex 1:15 (1 µl complex + 14 µl buffer).
    - For **5 kb handles,** no dilution is required.
- Add 5 µl of the (diluted or undiluted) protein–DNA handle complex to 5 µl anti-digoxigenin beads. Mix gently by pipetting 3–5 times, avoiding bubbles.
- Incubate at room temperature for 5 minutes.
- Dilute the bead–protein complex in 500 µl of buffer and load into the syringe of channel 1.

Channel 2

- Load 500 µl buffer supplemented with oxygen scavengers in the syringe of channel 2 if using 0.5 kb or 1.5 kb handles. Oxygen scavengers are not needed when working with 5 kb handles. For support with oxygen scavenging systems, please contact **store@lumicks.com**.

Channel 3

- Add 1 µl of streptavidin beads to 1000 µl of buffer. Mix gently and load the full volume into the syringe of channel 3.

4.2 Positioning Trap 1 and Trap 2

Saving the position of Trap 1 and Trap 2 in pre-set “catching” and “tethering” configurations allows you to automate and speed up the tethering procedure.

- Flow samples through channels 1, 2, and 3, setting the pressure to 0.3–0.5 bar.
- Catch one bead in Trap 2 and one in Trap 1. At this stage, it is not important which specific beads are in the optical traps. Then stop the flow.
- Select “Trap 1” and activate “Show Trap 1 overlay” option to display its range of motion of Trap 1 (red rectangle in Figure 1).
- Ensure that the bead in Trap 1 is positioned within the red circle (Trap 1 overlay). To align it, press Shift and left-click on the bead in Trap 1.
- Move Trap 1 to the top edge of the red rectangle and Trap 2 to the bottom edge, maximizing the y-axis distance between the beads.
- Save the position of Trap 1 as “catching” in the software.

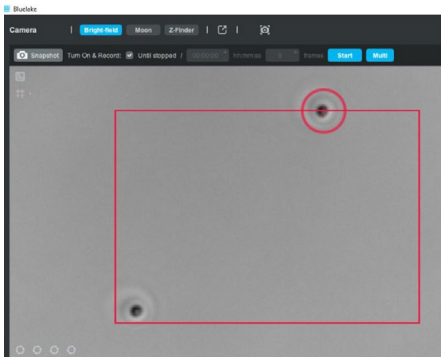


Figure 1

- Keeping the optical traps in “catching”, flow samples in channels 1, 2, and 3 at 0.3–0.5 bar and release the beads from the traps.
- Move the microstage to locate Trap 2 in channel 1 and catch a single anti-digoxigenin bead.
- Then move the microstage until Trap 1 reaches the edge between channels 2 and 3.
- Adjust the stage until Trap 1 is in channel 3 and captures a single streptavidin–coated bead. The “catching” configuration allows to keep Trap 2 in channel 2.

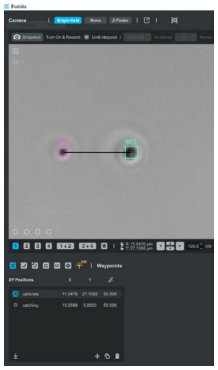


Figure 2

- Define a template for each bead and align Trap 1 at a 5 µm horizontal distance from Trap 2, keeping both beads on the same y-axis (Figure 2). Save this position as “tethering.”
- Move both traps to channel 2 and set Trap 1 to the “tethering” position.
- Perform trap calibration according to the different sizes of the two beads.

4.3 Tethering

Set the theoretical worm-like chain (WLC) model to the total contour length of two DNA handles (1.034, 3.012, or 10.078 kb for 0.5, 1.5, or 5 kb, respectively).

Important note: A single tether typically sustains 45–50 pN. Forces >60 pN suggest multiple tethers. If multiple tethers persist despite adjusting tethering distance or wait time at tethering distance, consider lowering the concentration of protein–DNA complex during incubation with anti-digoxigenin beads (see Section 2).

For 5 kb handles:

- Use default bead tracking mode and bead templates.
- Apply 0.3–0.5 bar flow and move Trap 1 with the trapped streptavidin bead to 50–60% of the contour length (~1.5–1.7 µm). Increase distance to test for tether formation. If no response, reduce initial distance and/or wait a few seconds before retrying.

For 0.5 kb and 1.5 kb handles:

- Enable piezotracking mode. Tethering occurs at short distances between the beads (about 0.15 and 0.5 µm, respectively), which may cause bead templates to disappear.
  Note: Flow is not required for tethering with 0.5 and 1.5 kb handles.
- At short distances (<0.5 µm), bead interactions affect the baseline of Force–Distance (FD) measurements. Record an FD baseline by moving Trap 1 from a 5 µm distance (calibrating distance) to 60% of the contour length. Use this baseline to correct for short-distance bead interaction (in post-processing or during measurements, if baseline correction is enabled).
- Proceed with tethering by positioning Trap 1 at 50–60% of the contour length, as done for 5 kb handles.

5. References

[1] Benjamin Pelz, Gabriel Žoldák, Fabian Zeller, Martin Zacharias & Matthias Rief. Subnanometre enzyme mechanics probed by single-molecule force spectroscopy. Nature Communication 2016

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