Protein labeling and tethering kit (ybbR)

Protein folding
Protocol

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LUMICKS

Protocol introduction

Protein labeling and tethering kit (ybbR)

The Protein labeling and tethering kit (ybbR) is a highly efficient kit to study protein dynamics at a single-molecule level through optical tweezers. The methodology is divided into two main procedures:

1. Protein labeling with functionalized DNA handles.

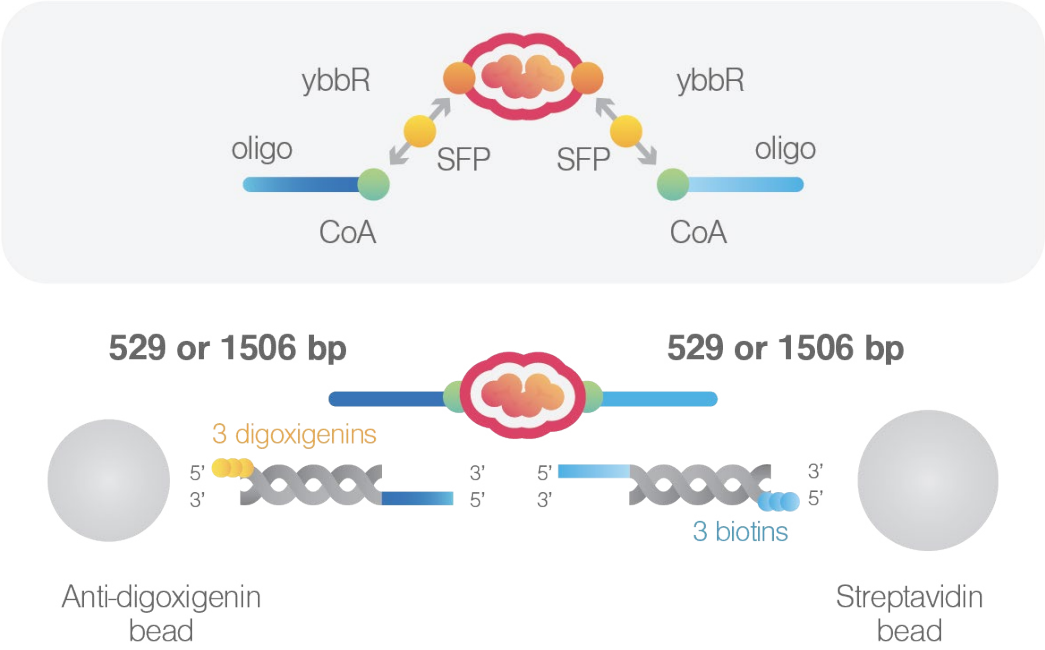
2. Protein tethering to beads for optical tweezers experiments.

The protein labeling procedure uses the Sfp phosphopantetheinyl transferase [1,2] to attach two DNA handles to the two ybbR peptides fused to the protein of interest (one handle per one ybbR peptides). The kit can be ordered with either 529 bp or 1506 bp DNA handles. The short handles will result in the highest force resolution, while the long handles allow an easier tethering procedure. The protein tethering procedure uses streptavidin and anti-digoxigenin-coated beads. The kit includes the adenylate kinase (AdK) protein [1] with two fused ybbR peptides, which can be used as a quality control protein for both the labeling and tethering procedures.

Important:

In addition to the two ybbR peptides, the protein of interest should also be fused to either 6xHis tag or GST tag to ensure optimal results.

Protein labeling and tethering procedure



If you have ordered your kit before July 2021, your kit contains streptavidin coated silica beads instead of the streptavidin coated polystyrene beads mentioned in this protocol. As the concentration of these beads is different, on page 9, add 2 µl of streptavidin-coated beads to 500 µl of buffer and load the entire volume into the syringe of channel 3.

Materials supplied in the kit

Components	Units	Volume	Storage temperature
CoA-modified oligos	2	20 µl	-80 ˚C
Sfp enzyme	2	5 µl	-80 ˚C
10x Sfp buffer	1	50 µ	-80 ˚C
TCEP (100 mM)	2	5 µl	-80 ˚C
DNA handles (529 bp or 1506 bp)	1	45 µl	-80 ˚C
6xHis AdK protein	1	15 µl	-80 ˚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

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 of tagged proteins, respectively
- Oxygen scavengers. For support with oxygen scavenging systems, please contact **store@lumicks.com**.
- Optional: for the electrophoretic mobility shift assay, use a 1% agarose DNA gel and non-denaturing gel loading dye without SDS (NEB, Cat. #B7025S)

Workflow

The protein labeling is a three-step procedure that assembles a protein of interest with two DNA handles (5' digoxigenin modification and 5' biotin modification). The kit can be ordered with either 529 bp or 1506 bp DNA handles.

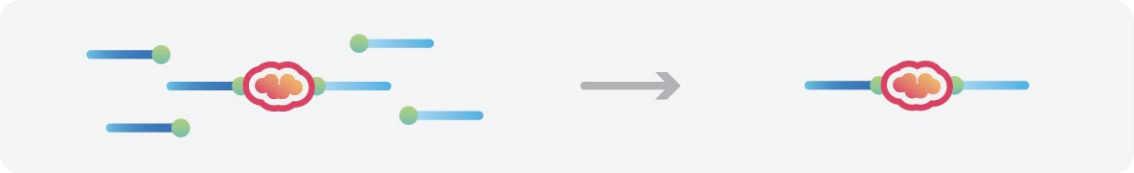
- In the **first step**, CoA-modified DNA oligos are attached to two 11-residue ybbR peptides fused to the protein of interest. The reaction is catalyzed by the Sfp phosphopantetheinyl transferase, which covalently transfers the 4′-phosphopantetheinyl (Ppant) group from CoA to the serine in the second position of the ybbR sequence (DSLEFIASKLA).
- In the **second step**, the protein labeled with 2 oligos (protein–oligo), is purified from the excess of unreacted CoA-modified oligos.
- In the **third and final step**, the oligos hybridize to the complementary overhangs on the DNA handles.

Step 1

Conjugation of the maleimide-modified oligos to protein

Step 2

Purification of protein-oligo chimera

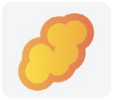


Step 3

Annealing to DNA handles

Important notes before labeling

- Briefly spin down the kit vials before use to avoid losing any reagents through the tube caps.
- To avoid thawing and freezing cycles of the DNA handles, make aliquots (4 µl) and store them at -80 °C. This will prevent the formation of nicks in the DNA handles.
- It is recommended to use 40–60 mM Phosphate or Tris buffer at pH 7.5 with 20–150 mM common salts (e.g., KCl or NaCl) for both the protein and CoA-modified oligo solutions.
- Do not use glycerol in the protein buffer as it inhibits Sfp activity.
- For optimal labeling conditions, the purity of the protein should be ≥ 90%.
- The optimal protein concentration to use is 50 µM in 15 µl buffer solution.
- Use aluminum foil to protect the reaction and stock solutions from light.



1. Protein labeling

Step 1

Conjugation of CoA-modified oligos to protein

- Add 4 µl of 10x Sfp reaction buffer and 1 µl of TCEP to 20 µl of CoA-modified oligos. Mix by gently pipetting 3–5 times while avoiding bubble formation.
- Bring the protein of interest to a concentration of 50 µM in 15 µl of recommended buffer solution (see **“Important notes before labeling”**) and mix it with the oligo solution containing Sfp reaction buffer and TCEP.
- Add 5 µl of Sfp enzyme. Mix by gently pipetting 3–5 times while avoiding bubble formation.
- Incubate for 2 h at room temperature to conjugate the oligos to the protein (oligo–protein).

Step 2

Purification of protein-oligo chimera

- Choose a small-scale affinity purification method suitable for the specific affinity tag fused to your protein. For GST and 6xHis tagged protein, GST SpinTrap™ (GE28-9523-59) and His SpinTrap™ (GE28-4013-53) are recommended (see below important notes applicable to protein–oligo purifications using GST SpinTrap™ and His SpinTrap™). For a detailed purification protocol please refer to ”**6xHis-AdK-oligo purification**” Upon completion of the protein–oligo purification, exchange the elution buffer with 20–50 mM Tris pH 7.5 and 50–150 mM NaCl. The buffer exchange can be performed by using ultrafiltration centrifugal concentrators, such as Vivaspin® Centrifugal Concentrators or Amicon® Ultra Centrifugal Filters.
- 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).
- The protein–oligo concentration should be at least 20 ng/µl for optimal results in the next step.
- Aliquot the protein–oligo to avoid thawing and freezing cycles, then snap freeze and store the aliquots at -80 ˚C.

Important notes applicable to protein–oligo purifications using GST SpinTrap™ (GE28-9523-59) and His SpinTrap™ (GE28-4013-53)

- Increasing the incubation time of both the binding and elution steps up to 5–10 min improves the yield of purified protein–oligo.
- Homogenous resuspension of the column resin during binding, washing, and elution steps is recommended.
- The protein–oligo purification requires up to four wash steps before a complete clean up from the excess of unreacted oligos.

Step 3

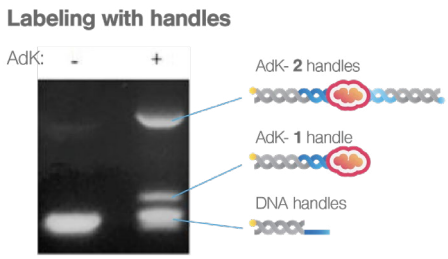
Oligo hybridization to DNA handles

The two conjugated oligos on the protein are hybridized to complementary overhangs of two DNA handles.

- Dilute the protein–oligo complex to a final concentration of 20 ng/µl using a buffer with 20–50 mM Tris pH 7.5, 50–150 mM NaCl.
- Spin down one aliquot of DNA handles (4 µl) to avoid loss of sample in the cap of the vial.
- Add 1 µl (20 ng) of protein–oligo to 4µl of DNA handles. Mix by gently pipetting 3–5 times while avoiding bubble formation. Incubate at room temperature for 10 min.
- The protein is now attached to two DNA handles and is ready for the tethering in the C-Trap®. Keep the sample on ice until you are ready for the tethering procedure.

Optional: Testing protein labeling by electrophoretic mobility shift assay

Upon completion of step 3, it is possible to verify the attachment of the DNA handles to proteins by an electrophoretic mobility shift assay (EMSA). Here, we show an example of an EMSA performed with AdK protein in non-denaturing conditions using a 1% agarose DNA gel. In addition to avoid denaturation, it is recommended to run the gel at low voltage (80-100 Volts) and fresh TBE buffer to avoid both overheating and change of pH during the gel run. The entire amount of AdK labeled with 529 bp DNA handles (5 µl) was loaded onto the gel after premixing with non-denaturing gel loading dye without SDS (NEB, Cat. #B7025S). As a control, one aliquot of DNA handles (4 µl) was used. EMSA of AdK labeled with DNA handles showed one small shift caused by one handle attached to AdK and an additional shift corresponding to the size of the second handle attached to AdK, as is illustrated in the image below. While the AdK gel below does not show a DNA marker, it is recommended to use a DNA marker to determine the size of the second shift that should correspond to the size of one handle.



Important note: if the labeling procedure is performed correctly, AdK labeling always shows a visible DNA band corresponding to the two handles attached to AdK. However, the protein of interest might show a weak or not visible band onto the gel, nonetheless, tethering on the C-Trap might be successful. In the latter case, it is suggested to attempt the tethering procedure without performing the 1 to 15 dilution of the protein-DNA handle solution (see preparation of channel 1 in the protein tethering procedure below). It is also strongly recommended to test tethering in the C-Trap using the AdK-handle complex as positive control.

2. Protein tethering in C-Trap®

Tethering is achieved by using optimized experimental conditions, specially developed for the DNA handles supplied with the Protein labeling and tethering kit (Cysteine). The final result is the protein of interest connected to two DNA handles (protein–DNA handles). Two sizes of DNA handles can be chosen to include in the kit, either 529 bp (0.175 µm) or 1506 bp (0.497 µm). The protein-oligo in complex with the two handles is then tethered to a 0.7-0.9 µm anti-digoxigenin coated bead and to a 1.0-1.4 µm streptavidin-coated bead. Such short tethers enable the detection of protein unfolding and conformational changes at the nanoscale [3].

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.
- Once the protein is attached to the DNA handles, it is stable for up to 2 h on ice.
- 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 below.

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

Channel 1

- Following Step 3 of Section 1 (Protein labeling), add 1 µl of the protein–DNA handle solution to 15 µl of a buffer of choice (1:15 dilution). For optimal results, use 40–60 mM Phosphate or Tris buffer at pH 7.5 containing 50–150 mM of common salts (e.g., KCl or NaCl).
- Vortex the anti-digoxigenin coated beads for 10 s and sonicate for an additional 5 s in a water bath sonicator. Transfer 5 µl of the anti-digoxigenin coated beads into a new tube. If a water bath sonicator is not available, vortex the beads for 20–30 s.
- Add 4 µl of the 1:15 protein–DNA handles dilution to 5 µl of anti-digoxigenin coated beads. Mix immediately by gently pipetting 3–5 times. Avoid bubble formation.

- Incubate at room temperature for 5 min to enable the binding of the protein–DNA handles to the beads.
- Dilute the bead-tethered proteins with 500 µl of the buffer of choice and load it into the syringe of channel 1.

Channel 2

- Prepare 500 µl of the buffer of choice supplemented with oxygen scavengers consisting.
- Load the buffer supplemented with the oxygen scavenger system into the syringe of channel 2.

Channel 3

- Vortex the streptavidin-coated beads for 10–20 s before use.
- Add 1 µl of streptavidin-coated beads into 1000 µl of buffer and load the entire volume into the syringe of channel 3.

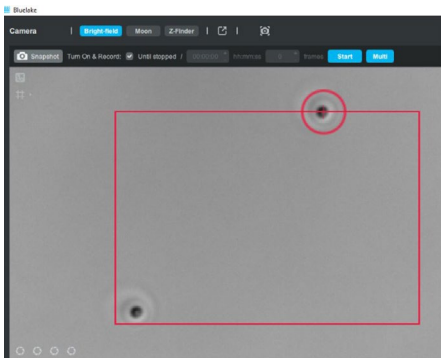
Catching beads and protein tethering

Here, we describe an optimized workflow to attach short protein–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**.

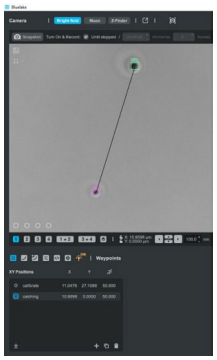
Low laserpowers are recommended. High overall laser power can damage DNA tethers and affect tethering efficiency.

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.
- 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).



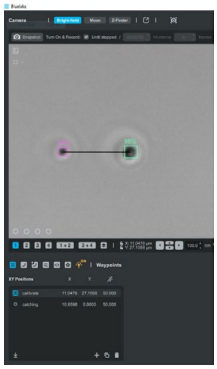
Above: Figure 1



Left: Figure 2

- Align Trap 1 at a 5–10 µm distance from Trap 2 at the same horizontal plane, as shown below. You can save the Trap 1 position as “tethering”. The saved “catching” and “tethering” positions of Trap 1 allow you to automatize and speed up the tethering procedure (Figure 3).

- Perform trap calibration according to the different sizes of the two trapped beads (Trap 1 bead = 1.28 µm; Trap 2 bead = 0.75 µm).



Left: Figure 3

Catching beads with the optical traps

- 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 protein between two beads to form the dumbbell configuration

For both handle lengths, 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 (1.06 kbp for the short handles or 3.01 kbp for the long handles, corresponding to the sum of the two DNA handles).
- 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.

- Increase the distance again and determine if a tether has formed. Repeat and tentatively reduce the initial distance between the two beads until 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 can sustain pulling forces of 45–50 pN. Therefore, if the force response of the tether is >60 pN, most likely, multiple tethers have been formed between the two beads.

- To decrease the number of tethers, reduce the amount of protein-handles complex incubated with anti-digoxigenin coated beads in section 2.1 (sample preparation of channel 1) before attempting a new tethering procedure.

- Once a tether has formed use the “Force Distance” feature to record FD curves between the tethering position and a user-defined FD end position.

References

[1] Yin J, Straight PD, McLoughlin SM, Zhou Z, Lin AJ, Golan DE, et al. Genetically encoded short peptide tag for versatile protein labeling by Sfp phosphopantetheinyl transferase. PNAS. 2005

[2] Pippig DA, Baumann F, Strackharn M, Aschenbrenner D, Gaub HE. Protein-DNA chimeras for nano assembly. ACS Nano. 2014

[3] 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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