

## 6.3 kb Handle Kit – User Guide

### 1. Kit content and use

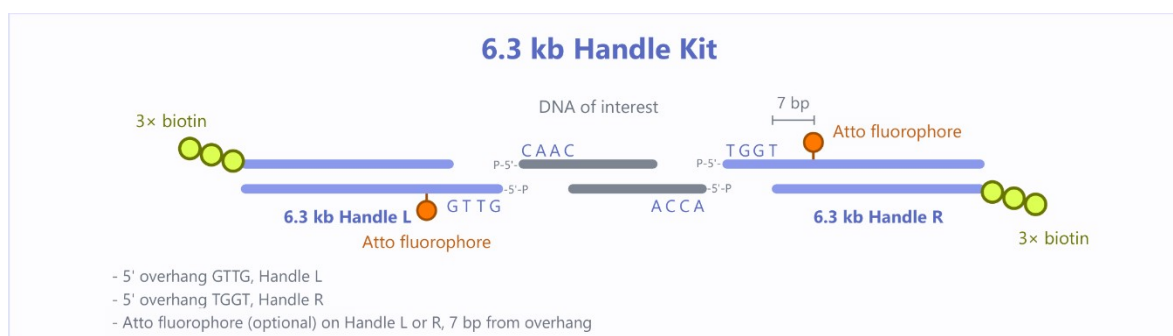
The kit includes two biotinylated 6.3 kb double-stranded DNA handles, Handle L and Handle R (fluorophore options on the webstore), a 5.3 kb Control DNA and a ligation STOP solution. The kit is optimized for fast, efficient ligation with no purification steps before tethering in the C-Trap: once your DNA of interest is ready, single-molecule measurements can start about 1 hour later. The handles are sufficient for 8 ligase reactions. Each reaction gives 5 runs on the C-Trap with standard C-Trap workflows, for a total of up to 40 experiments.

| SUPPLIED WITH THE KIT (8 RXN) | CONCENTRATION | VOLUME | STORAGE    |
|-------------------------------|---------------|--------|------------|
| 6.3 kb Handle L               | 40 ng/μl      | 20 μl  | –20 °C     |
| 6.3 kb Handle R               | 40 ng/μl      | 20 μl  | –20 °C     |
| Control DNA 5.3 kb            | 25 ng/μl      | 8 μl   | –20 °C     |
| STOP solution                 | 5×            | 350 μl | Room temp. |

**SUPPLIED BY THE USER** T4 DNA ligase. Tested with Thermo Scientific EL0011 (5 Weiss U/μl) and NEB M0202 (400 U/μl in NEB cohesive-end units, about 6 Weiss U/μl). Both are used at the same volume, 0.5 μl per 10 μl ligation.

Each handle has three biotins at one end for capture on streptavidin beads and a 5'-phosphorylated 4-nucleotide overhang on the other end, ready for ligation.

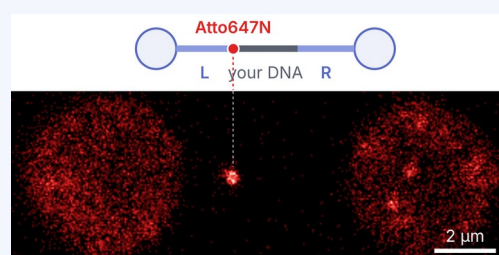
Your sequence of interest must carry 5'-phosphorylated **CAAC** and **ACCA** overhangs, complementary to the overhangs of the handles as shown in the schematic below.



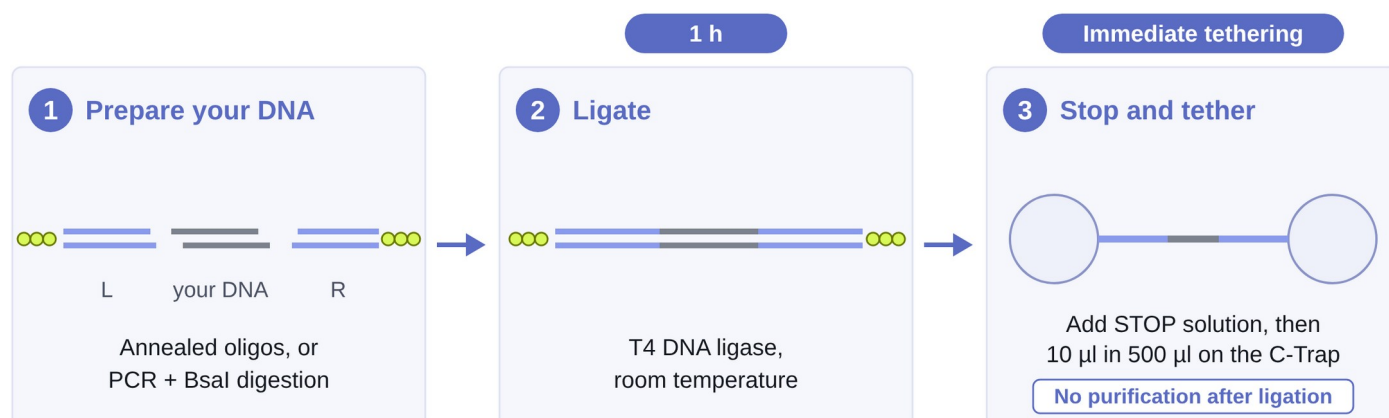
#### FLUORESCENT HANDLES

Handles labeled with Atto dyes help to find the focal plane of the tether. For DNA of interest longer than about 2 kb, the off-center position of the dye also shows the orientation of each tethered molecule.

**Available dyes:** Atto488, Atto565 or Atto647N on either handle.



## A simple, efficient workflow: from your DNA to the C-Trap in about 1 hour

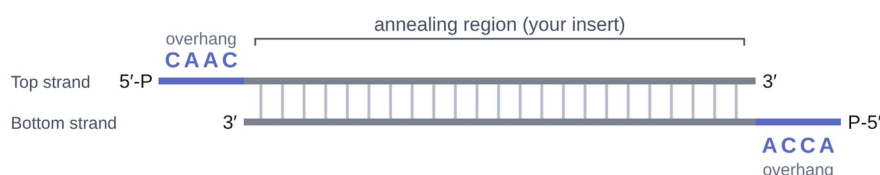


## 2. Making your DNA of interest

Two routes give your DNA the 5'-phosphorylated **CAAC** and **ACCA** overhangs shown in the schematic in section 1. There is no size limit: choose the route that fits the length of your sequence.

### Short inserts: annealed oligonucleotides

Order two oligonucleotides with a 5' phosphate: the top strand starts with **CAAC** followed by your sequence, the bottom strand starts with **ACCA** followed by its reverse complement. Anneal them (95 °C for 2 min, then cool slowly to room temperature) and use the duplex directly in the ligation.



### Longer inserts: PCR and Type IIS digestion

Add the 5' flanks from the table to your forward and reverse primers and amplify your sequence by PCR. Digest the PCR product with the matching Type IIS enzyme and purify it. Choose an enzyme whose recognition site (both orientations listed) is not present in your sequence; Bsal is our first choice. The cut leaves 5'-phosphorylated **CAAC** and **ACCA** overhangs, so no kinase step is needed.

| ENZYME (NEB)              | SITE, BOTH STRANDS     | FW PRIMER, 5' FLANK       | RV PRIMER, 5' FLANK       |
|---------------------------|------------------------|---------------------------|---------------------------|
| <b>Bsal</b> -HF v2, R3733 | <b>GGTCTC</b> / GAGACC | CACAC <b>GGTCTCTCAAC</b>  | CACAC <b>GGTCTCGACCA</b>  |
| BsmBI-v2, R0739           | <b>CGTCTC</b> / GAGACG | CACAC <b>CGTCTCTCAAC</b>  | CACAC <b>CGTCTCGACCA</b>  |
| BbsI-HF, R3539            | <b>GAAGAC</b> / GTCTTC | CACAC <b>GAAGACCTCAAC</b> | CACAC <b>GAAGACAGACCA</b> |

Red: recognition site. Underlined: overhang. Keep the 5 extra bases (**CACAC**) so the enzyme cuts efficiently at the end of the PCR product.

**Contact us if you would like help designing either approach.**

### 3. Ligation

Use **0.025 pmol of the DNA of interest** per 10 µl ligation, giving a final concentration of 2.5 nM in the ligase reaction. As a reference, 0.025 pmol corresponds to ~80 ng of a 5 kb double-stranded DNA.

**Control reaction.** Use 3.5 µl of Control DNA 5.3 kb (0.025 pmol) per 10 µl ligation in place of your DNA of interest.

| COMPONENT                        | 10 µl    |
|----------------------------------|----------|
| Nuclease-free water              | to 10 µl |
| 10× T4 DNA Ligase buffer         | 1.0 µl   |
| Handle L                         | 2.5 µl   |
| Handle R                         | 2.5 µl   |
| Your DNA (0.025 pmol per 10 µl)* | X µl     |
| T4 DNA Ligase                    | 0.5 µl   |

\* X (µl) = length of your DNA (bp) × 0.01625 ÷ its concentration (ng/µl), maximum 3.5 µl. Example: a 2,000 bp fragment at 10 ng/µl gives X = 3.3 µl.

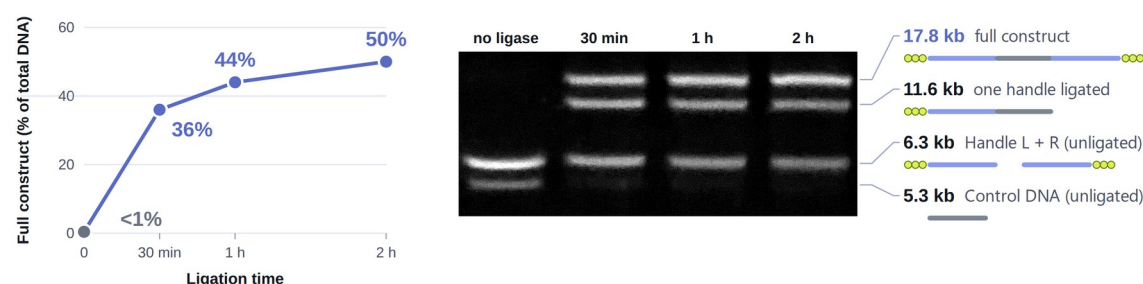
1. **Spin the handle tubes briefly before opening**, to recover liquid from the caps.
2. Combine water, ligase buffer, both handles and your DNA. Mix gently.
3. Add the ligase last. Mix by slow pipetting — do not vortex, avoid bubbles.
4. Incubate 1 h at room temperature.
5. Stop the reaction: add 4 volumes of STOP solution (40 µl per 10 µl) and mix gently. No heat inactivation is required.
6. Use 10 µl of the stopped reaction directly for tethering, or store it at 4 °C. See section 5 for tethering.

### 4. Control of ligation on agarose gel (optional)

Ligation efficiency can be checked on an agarose gel. Set up a 10 µl control ligation and stop 5 µl at each of two time points (e.g. 30 min and 1 h) in 20 µl STOP solution. Load the full 25 µl with loading dye.

Example below: Control DNA 5.3 kb, 5 µl of ligation + 20 µl STOP solution per time point, 0.5 % agarose gel. After 1 h, 44 % of the DNA is full construct, and 10 µl of stopped reaction gives fast tethering on the C-Trap. One-handle products and unligated handles do not interfere with trapping.

#### Ligation efficiency with Control DNA 5.3 kb



### 5. Tethering

Add **10 µl of the stopped reaction to 500 µl of tethering buffer**, then capture the construct between two streptavidin-coated beads under flow.

**Suggested tethering buffer:** 10 mM Tris-HCl pH 8.0, 50 mM NaCl (0.1 mM EDTA optional). With ligation efficiency above 40 %, this buffer gives tethers within 5–10 seconds. Keep NaCl between 50 and 150 mM during tethering. On the C-Trap, use this buffer in channels 1 and 2 (beads and DNA) and your buffer of choice in the experiment channels: once formed, tethers are stable in a wide range of salt and buffer conditions.