C–Trap Reference Kit Protocol

DNA–binding proteins Protocol

Version 1.0



LUMICKS

Table of Contents

1. Description 3

2. Materials Supplied in the Kit 4

2.1 Additional Materials (supplied by the user) 4

3. Protocol Workflow 5

4. C–Trap Reference Kit Protocol 6

4.1 Overnight Incubation with Alkaline Cleaning Reagent 6

4.2 Alkaline Cleaning Reagent Removal 6

4.3 Passivation with BSA 2% 7

4.4 Buffer Equilibration and Optics Setup 7

4.5 Microfluidics Setup 8

4.6 DNA Tethering 8

4.7 Confocal Setup 9

4.8 Flush in LacI–ATTO 565 9

4.9 Record Kymographs 10

4.10 Data Analysis with Lakeview 10

5. Trobleshooting Guide 11

5.1 Presence of Protein Aggregates 11

5.2 Low Efficiency of DNA Tethering 11

5.3 Binding of LacI-ATTO 565 is not Detected 11

5.4 Low Number of Binding Events 11

5.5 OFF Rate Deviates from the Expected Value 12

6. Supporting Information 13

7. References 13

C-Trap Reference Kit

1. Description

The C-Trap Reference Kit is designed to support user training, fast-track assay troubleshooting, and verify and monitor instrument performance. The kit provides standardized and validated reagents for a complete DNA-protein binding assay, including data analysis.

The C-Trap Reference Kit is best used in combination with a C-Trap with 561 nm and 638 nm lasers.

> **Note:** If your C-Trap has a 532 nm laser, please be aware that adjustments in confocal settings (laser power, pixel time) might be required to get a sufficient SNR (signal to noise ratio) for analysis.

The kit includes an ATTO 565-labeled version of the transcription factor lac repressor (LacI-ATTO 565) and a biotinylated DNA construct containing 12 repeats of the LacI DNA target sequence, known as the lac operator (LacO). The LacO DNA construct (37.8 kb) is flanked by two ATTO 647N fluorophores, which facilitate locating the optimal focal plane of the DNA tether prior to incubation with LacI-ATTO 565 (see Figure 1). This DNA construct was assembled using the **LUMICKS DNA Tethering Kit** following **LUMICKS Golden Gate Assembly Protocol**.

With this protocol, the LacO DNA construct is tethered between two streptavidin-coated beads in the C-Trap. The user can visualize and track the binding of LacI-ATTO 565 to the LacO DNA construct over time to create kymographs, which are then analyzed to determine the association (ON) and dissociation (OFF) rates of LacI (see Figure 1).

Figure 1

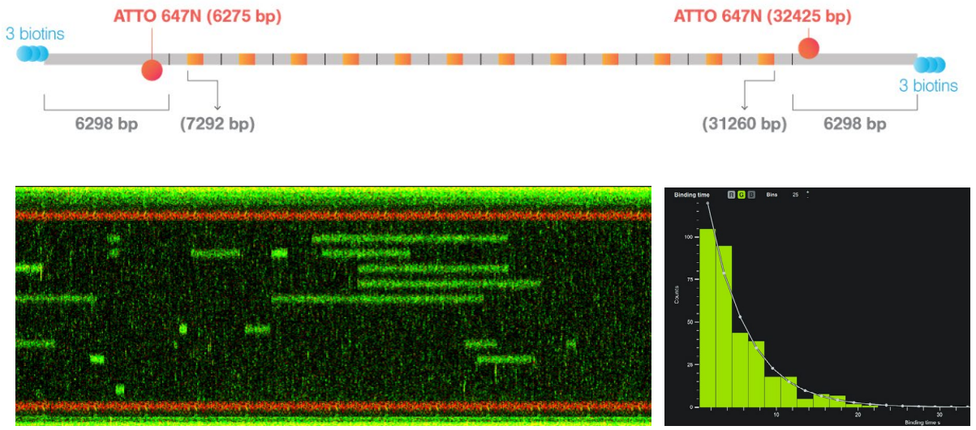


Figure 1. *Top panel:* schematic representation of biotinylated LacO DNA. The construct consists of 12 DNA fragments, each containing a LacO sequence (depicted in orange), ligated to the 6.3 kb handles of the DNA tethering kit. Positions of the ATTO 647N fluorophores are shown in parentheses. The first LacO repeat begins at position 7292 bp, and the last at 31260 bp, with ~2180 bp spacing between repeats. *Bottom Left:* Representative kymograph showing LacI–ATTO 565 binding events (green) and ATTO 647N fluorophores (red) stably bound to the DNA tether. *Bottom right***:** Histogram of binding lifetimes, analyzed using Lakeview. Exponential decay fitting yields the dissociation rate constant (k_{off}).

2. Materials Supplied in the Kit

Components	Units	Volume	Storage temperature
LacI–ATTO 565	1	20 µL	-20 ˚C
Biotinylated LacO DNA	1	25 µL	+4 ˚C
10x Binding Buffer	1	25 mL	RT
Streptavidin beads (□4–4.9 µm)	1	50 µL	+4 ˚C
Alkaline Cleaning Reagent	1	3 mL	RT

2.1 Additional Materials (supplied by the user)

Bovine Serum Albumin (BSA)

- We recommend BSA from Sigma-Aldrich (Cat. No. A7906), as the kit's performance was validated using this reagent.
- Prepare a 5x BSA solution (100 mg/ml) in PBS, aliquot 200 µL portions, and store at -20°C.
- Alternatively, prepare a fresh 20 mg/ml BSA solution in PBS and use it immediately after preparation.

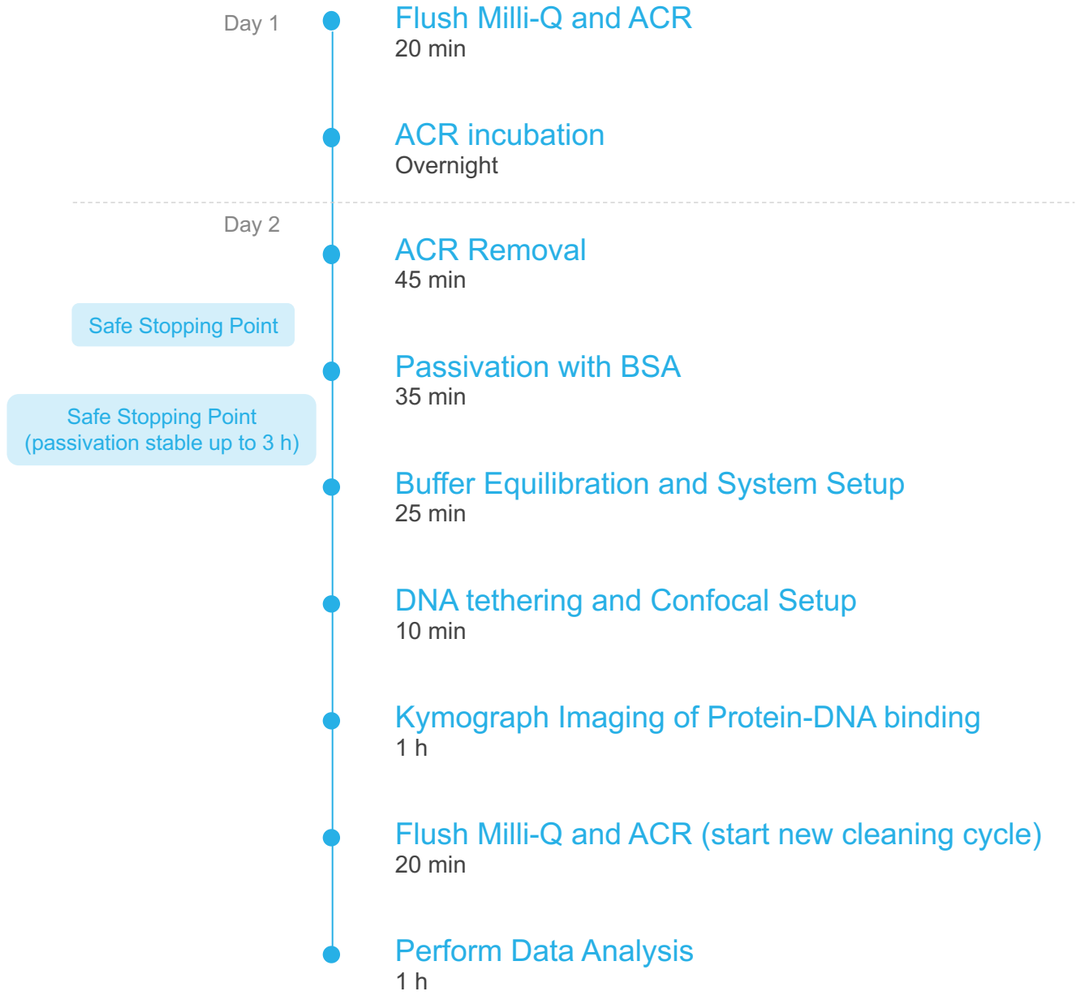
Buffer & Water

- Phosphate-Buffered Saline (PBS)
- Ultrapure water (e.g., Milli-Q)

Syringes

- The protocol requires replacing the syringes in the Microfluidics Module. Syringes can be purchased from the LUMICKS Store.

3. Protocol Workflow



4. C–Trap Reference Kit Protocol

This kit can be used with channel 4 or channel 5 as protein channel for LacI-ATTO 565.
For optimal results, we strongly recommend following the cleaning protocol outlined below, using the provided alkaline cleaning reagent (ACR).

Tip: During cleaning and equilibration of the Microfluidics Module, use a 10 mL serological pipette with a pipette controller to aspirate 5 or 10 mL of buffer (depending on the protocol section) and distribute 1 or 2 mL to each syringe, as precise microliter accuracy is not required. Replace the pipette after distributing ACR before proceeding with washing and equilibration procedures

Important: The recommended cleaning protocol includes an overnight incubation step with ACR.

4.1 Overnight Incubation with Alkaline Cleaning Reagent

1. Fill all syringes with 2 mL of Milli-Q water and flush all channels for 10 minutes at 2 bar.
2. Prepare 10 mL of 1x Alkaline Cleaning reagent (ACR) by diluting 200 µL of the 50x ACR solution with Milli-Q water.
3. Remove the remaining water from the syringes (~1.5 mL) and add 2 mL of 1x ACR to each syringe. Flush all channels for 30 minutes at 2 bar.
4. Stop the flow and incubate overnight.

Tip: Before leaving the system for overnight incubation, confirm that ACR is flowing uniformly in all channels by visually inspecting that the ACR level is decreasing evenly in all channels. If the ACR level is decreasing more slowly in one channel (i.e., it seems blocked), try to resolve the block by unmounting the syringe from the pressure box and manually pushing ACR through that channel using a plunger while the blocked channel and outlet valve (channel 6) are opened. This should resolve the blockage and ensure uniform flow in all channels.

Important: The ACR incubation step should not be longer than 60 hours (e.g., it should not extend over the weekend). Longer ACR incubation times can negatively impact experimental results.

4.2 Alkaline Cleaning Reagent Removal

Microfluidics Storage: After removing ACR and replacing it with Milli-Q water in step 3 of this section, the flow cell can be stored in Milli-Q water for up to one week. For longer storage, we recommend using Milli-Q water supplemented with sodium azide (0.1 mg/ml).

1. Remove the remaining Alkaline Cleaning Reagent (ACR) from the syringes (~500 µL).
2. Replace the syringes on all channels with new syringes.
3. To wash, add 2 mL of Milli-Q water to all syringes. Flush all channels for 10 minutes at 2 bar.
4. Stop the flow and remove the remaining water (~1.5 mL).
5. Repeat steps 3–4 two more times. After the final flush, skip step 4 and keep the Milli-Q water in the system.

Optional. Residual ACR can negatively impact experimental results. To maximize ACR removal, you can perform 3-5 rapid PBS washes before beginning passivation:

- Add 2 mL of PBS to all syringes and flush for 3 minutes at 2 bar.
- Remove the remaining 1.6-1.8 mL PBS from the syringes.
- Repeat this washing step 2-4 times.

4.3 Passivation with BSA 2%

1. Prepare 1 mL BSA solution in PBS at a final concentration of 20 mg/ml to use immediately in the next step.

Tip: To save time at the start of each experiment, prepare a 5x BSA solution (100 mg/ml) in PBS, make 200 µL aliquots and store at -20 ˚ C.

2. Remove the Milli-Q water from the syringe of the protein channel.
3. Add 1 mL of freshly prepared 20 mg/ml BSA solution to the protein channel. Leave Milli-Q water in the remaining channels.
4. Flush all channels for 2 minutes at 2 bar, followed by 10 minutes at 0.5 bar.
5. Ensure that at least 200 µL of BSA solution has flushed through the protein channel.
6. Stop the flow and incubate for 20 minutes.

Note: If the experiment cannot start immediately after passivation, the incubation in BSA can be extended to a maximum of 3 hours.

4.4 Buffer Equilibration and Optics Setup

To reduce the time between passivation and LacI binding measurements, we recommend starting the instrument and optics setup in Bluelake during the buffer equilibration step. Detailed instructions on positioning the objective, condenser and microstage are available in the C-Trap User Manual.

1. Prepare 20 mL of 1x binding buffer by diluting 2 mL of 10x binding buffer with Millli-Q water. The remaining 1x binding buffer can be used for following sample preparations (Sections 4 and 5).
2. Remove the remaining BSA solution from the syringes.
3. Add 1 mL of 1x binding buffer to all syringes. Flush all channels for 5 minutes at 0.5 bar. Stop the flow and remove the remaining binding buffer.
4. Repeat the washing step (step 3) two more times.
5. After the last wash, do not remove the remaining binding buffer.
6. Proceed immediately to the next step.

Note: Do not leave the Microfluidics Module in equilibration buffer for an extended period, as this may reduce the effectiveness of passivation.

4.5 Microfluidics Setup

Reagent preparation

1. Prepare bead solution: Add 5 µL of streptavidin beads (Ø 4–4.9 µm, 0.1% (w/v)) to 500 µL of 1x binding buffer. Mix by inverting the tube.

2. Prepare DNA solution: Add 2.5 µL of biotinylated LacO DNA to 500 µL of 1x binding buffer. Mix by inverting the tube.

3. Keep 500 µL of 1x binding buffer for channel 3.

Loading into Microfluidics channels

- Channel 1: Empty the syringe and load 500 µL of bead solution.
- Channel 2: Empty the syringe and load 500 µL of DNA solution.
- Channel 3: Empty the syringe and load 500 µL of 1x binding buffer.

4.6 DNA Tethering

During DNA tethering, keep the protein channels closed to avoid unnecessary flushing of passivated channels to avoid reduction the passivation layer.

1. Set the theoretical worm-like chain (WLC) model to 37.8 kb contour length. This corresponds to the total length of the biotinylated LacO DNA construct.

2. Flush channels 1-3 (beads, buffer, DNA, respectively) at 0.3 - 0.5 bar, while keeping the protein channels (4-5) closed.

3. Catch a single bead in each of the traps in channel 1, align the traps along the x-axis, then move them to channel 3 to perform a force calibration. The recommended trapping stiffness ranges between 0.2 and 0.3 pN/nm. Adjust the trapping laser power to stay within this range.

4. Move the beads to channel 2. Position Trap 1 at approximately half of the contour length of the DNA construct away from Trap 2 (5-7 µm bead-bead distance for this construct). Increase the distance and observe the force response to determine if a tether is formed .

5. Confirm that a single, intact tether of the expected length is formed by comparing the F,d-curve to the eWLC model.

Note: Due to the high sensitivity of the C-Trap in tethering biotinylated DNA at femtomolar concentrations, residual by-products from the Golden Gate reaction used to assemble the 37.8 kb biotinylated LacO DNA (see Section 1.3) may also be tethered. Following the optimized protocol minimizes unintended tethering and ensures the correct construct is selected.

4.7 Confocal Setup

The LacO DNA-construct includes two ATTO 647N fluorophores that can be used to optimize the position of the DNA tether in the focal plane.

1. Set the pixel size to 50 nm and the pixel time to 0.2 ms. This setting can also be used to image the binding of LacI-ATTO 565 to the DNA.

2. In channel 3, stretch the DNA by moving Trap 1 until the force is 10 pN.

3. Position a 2D scan region to include both beads. Take a single 2D scan with the red laser (638 nm) and confirm that both beads are within the scan region of the confocal scanner.

4. Ensure that the ATTO 647N fluorophores of the DNA construct are in the focal plane of the confocal scanner by moving “Trap1+2” along the z-axis.

Once buffer equilibration, tethering and confocal setup are completed, you are ready to record a kymograph of LacI-ATTO 565 binding to the LacO DNA tether.

4.8 Flush in LacI–ATTO 565

1. Empty the syringe of the protein channel.

2. Add 2 µL of LacI-ATTO 565 to 300 µL of 1x binding buffer, mix the solution by gently inverting the tube or by gently pipetting up and down.

3. Add the diluted LacI-ATTO 565 to the syringe of the protein channel.

4. Flush all in-use channels (beads, buffer, DNA, protein) at 0.5 bar for 5 minutes, while keeping any unused channels closed.

5. To confirm your fluorescent protein is effectively being flushed in the channel, start an “empty” kymograph (no beads or DNA) with the green laser (561 nm) ON in the protein channel to monitor the increase of fluorescence of LacI-ATTO 565 in the medium. The fluorescence signal should increase and reach a plateau within 3–5 minutes.

4.9 Record Kymographs

1. Trap two beads, calibrate and zero the forces, and catch a single DNA tether (see section 4.6).
2. Stretch the DNA by moving Trap 1 until the force is 10 pN.
3. Perform a 2D scan and verify that the tether is aligned with the confocal line scan using the ATTO 647N labels on the DNA tether.
4. Move the bead-DNA construct to the protein channel.
5. Start recording a kymograph. Record 10 kymographs of 5 minutes each, restarting a new recording with the same DNA tether every 5 minutes.
6. If a low binding rate is observed (fewer than 1 binding event per 10 seconds) or if the DNA tether breaks:

- Catch new beads and a new DNA tether.
- Flow channels 1-4 for 2 minutes at 0.5 bar before starting a new kymograph.

7. After recording is completed, either:

- Start a new cleaning cycle immediately after the experiment.
- Or refer to Section 5.4.1 of the troubleshooting guide to determine whether re-passivation and equilibration are needed before starting a new measurement of LacI binding.

4.10 Data Analysis with Lakeview

After recording the kymographs, we recommend using Lakeview for data analysis. Lakeview simplifies statistical analysis by enabling easy tracking of binding events without the need for coding. It automatically calculates the ON rate and dissociation (OFF) rate constant by aggregating data from all recorded kymographs, providing a streamlined workflow to quantifying binding kinetics. This ensures a robust assessment of experimental results.

By following this protocol, you can expect:

- 10 kymographs of 5 minutes each, resulting in more than 200 binding events in total.
- An average binding rate > 0.1 events/s (at least one binding event every 10 seconds on average).
- An expected dissociation rate (k_{off}) of 0.4 ± 0.06 s^{-1}.

5. Troubleshooting guide

5.1 Presence of Protein Aggregates

- If protein aggregates form, open all in-use channels (beads, buffer, DNA, protein) and flush at 0.5 bar for ≥1 min to refresh the protein channel with new LacI-ATTO 565. .
- ACR may contribute to aggregate formation if left in the Microfluidics Module for extended periods. Limit ACR incubation to ≤60 h (overnight preferred). For long-term storage, keep the flow cell in Milli-Q water with 1.5 mM sodium azide.
- If aggregates persist, improve ACR removal performing at least three additional PBS washing steps (or with Milli-Q water) before proceeding with passivation.

5.2 Low Efficiency of DNA tethering

- Remnant ACR may interfere with DNA tethering. To maximize ACR removal, we recommend at least three additional PBS washing steps (or Milli-Q water) after cleaning, before proceeding with passivation (between Sections 4.2 and 4.3).
- High trapping laser power may damage DNA or beads reducing tethering efficiency. To prevent this, reduce the overall trapping laser power and perform a force calibration. The recommended trapping stiffness is 0.2–0.3 pN/nm.

5.3 Binding of LacI-ATTO 565 is not Detected

- Check that the green (561 nm) confocal laser is ON. If your system has a 532 nm laser, note that a higher laser power might be required to achieve sufficient signal-to-noise ratio (SNR) for analysis.
- Ensure that the confocal scan line precisely overlaps with the DNA tether in xy and in z. If LacI-ATTO 565 binding and ATTO 647N DNA labels are observed in 2D but not in 3D (kymograph), the scan line is not positioned well in xy, and requires readjustment so that it overlaps with the DNA tether.
- Confirm that the tether is not broken by moving Trap 1 and applying force.

5.4 Low Number of Binding Events

- If you detect a low number of LacI-ATTO 565 binding events during the experiment, ensure that LacI-ATTO 565 is in the channel as described in Section 4.8.
- Ensure complete ACR removal: Residual ACR may interfere with binding efficiency. Always exchange syringes after cleaning (see Step 2, Section 4.2) and perform the required washes and buffer equilibration steps with the specified volumes and flow rates. As an additional precaution, perform at least three extra washing steps with PBS (or Milli-Q water) before proceeding with passivation (between Sections 4.2 and 4.3).
- Ensure optimal passivation. Prepare and use BSA (20 mg/ml) in PBS as described in Section 4.3. Begin measurements within 30 minutes of completing the equilibration.

5.4.1 Re-passivation (Without ACR Cleaning): If binding remains low despite the above steps, re-passivation without another ACR cleaning cycle may improve results. Tests suggest that re-passivation and re-equilibration within the same session can enhance LacI-ATTO 565 binding efficiency. Follow the following protocol to re-passivate:

- Prepare fresh BSA solution: After completing the last kymograph of the previous experiment, prepare a fresh 20 mg/mL BSA solution in PBS. You will need 1 mL per protein channel (channel 4 and/or 5).
- Remove all remaining samples from syringes in all in-use channels.
- Add 1 mL of Milli-Q water to syringes for Channels 1–3 (beads, buffer, DNA).
- Add 1 mL of BSA solution to the protein channel (channel 4/5).
- Ensure that any unused channel remains closed or filled with sufficient Milli-Q water.
- Flush the system: Flush all in-use channels for 2 minutes at 2 bar, followed by 10 minutes at 0.5 bar. Confirm that at least 200 µL of BSA solution has flushed through the protein channel.
- Incubate: Stop the flow and incubate for at least 20 minutes.
- Perform buffer equilibration: Proceed with buffer equilibration as described in Section 4.3.
- Continue with experiments: Once equilibration is complete, the flow cell is ready for a new experiment with LacI-ATTO 565, as outlined in Sections 4.6–4.10.

5.4.2 Switching the Protein Channel: If, after re-passivation, LacI-ATTO 565 binding remains low, consider switching to another protein channel:

- **Use a different protein channel:** For example, if Channel 4 was previously used, switch to Channel 5 and repeat the experiment. This can help determine whether the issue is specific to one protein channel.
- **Repeat cleaning and passivation for the new protein channel:** follow Sections 4.1–4.4 to clean, passivate, and equilibrate the new protein channel before starting the experiment.

5.5 OFF Rate Deviates From the Expected Value

- The binding of LacI-ATTO 565 to LacO is force-dependent and varies with the tension applied to the DNA tether. Ensure that the tether is under a tension of 10 pN during the measurement, as deviations in force can influence the OFF rate.
- Excessive laser power (green or red) can increase photobleaching, resulting in a shorter fluorescence lifetime than the actual binding time of LacI-ATTO 565, which can lead to underestimation of the binding time. To avoid this, reduce the confocal laser power to a minimum while maintaining a good signal-to-noise ratio (SNR).

6. Supporting Information

6.1 Fluorescently Labeled Protein LacI-ATTO 565

LacI-ATTO 565 is produced by incorporating a cysteine at amino-acid position 28 and labeling it with ATTO 565 maleimide. Additionally, two native cysteines are substituted with alanine (C107A and C140A) to prevent maleimide labeling at multiple sites, which could compromise the protein's functionality (Kipper et al., 2018; E. Marklund et al., 2020).

Furthermore, the C-terminal tetramerization helix (residues 339-360) was removed, resulting in the formation of protein dimers instead of tetramers. LacI dimers exhibit reduced dwell time when binding to LacO compared to tetramers, introducing faster ON and OFF binding dynamics (E. Marklund et al., 2020).

6.2 LacO DNA Construct

The C-Trap Reference Kit includes a 37.8 kb biotinylated DNA molecule with 12 repeats of the target DNA sequence LacO, as depicted in Figure 1. This DNA construct was assembled using the **LUMICKS DNA Tethering Kit** following **LUMICKS Golden Gate Assembly Protocol**.

The LacO DNA construct is flanked by two ATTO 647N fluorophores, which facilitate locating the optimal focal plane of the DNA tether prior to incubation with LacI-ATTO 565. Precise details regarding the positions of the fluorophores and LacO repeats can be found in the DNA sequence file,

7. References

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