



**HAL**  
open science

## Robust, high-yield, rapid fabrication of DNA constructs for Magnetic Tweezers

Patryk Strzelecki, Anastasiia Shpiruk, Grzegorz Cech, Anna Kloska, Pascal Hébraud, Nicolas Beyer, Florent Busi, Wilfried Grange

► **To cite this version:**

Patryk Strzelecki, Anastasiia Shpiruk, Grzegorz Cech, Anna Kloska, Pascal Hébraud, et al.. Robust, high-yield, rapid fabrication of DNA constructs for Magnetic Tweezers. *Biochemical and Biophysical Research Communications*, 2024, 731, pp.150370. 10.1016/j.bbrc.2024.150370 . hal-04800066

**HAL Id: hal-04800066**

**<https://hal.science/hal-04800066v1>**

Submitted on 23 Nov 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Robust, high-yield, rapid fabrication of DNA constructs for Magnetic Tweezers

Patryk Strzelecki <sup>1,2</sup>, Anastasiia Shpiruk <sup>1</sup>, Grzegorz M. Cech <sup>2</sup>, Anna Kloska <sup>3</sup>, Pascal Hébraud <sup>1</sup>, Nicolas Beyer <sup>1</sup>, Florent Busi <sup>4,5</sup> and Wilfried Grange <sup>4,1,\*</sup>

<sup>1</sup>Institut de Physique et Chimie des Matériaux de Strasbourg, Université de Strasbourg, CNRS, UMR7504, 23 rue du Loess, F-67034 Strasbourg Cedex

<sup>2</sup>Department of Bacterial Molecular Genetics, Faculty of Biology, University of Gdańsk, Wita Stwosza 59, 80-308 Gdańsk, Poland

<sup>3</sup>Department of Medical Biology and Genetics, Faculty of Biology, University of Gdańsk, Wita Stwosza 59, 80-308 Gdańsk, Poland

<sup>4</sup>Université Paris Cité, UFR Sciences du vivant, F-75013 Paris and

<sup>5</sup>Université Paris Cité, CNRS, Unité de Biologie Fonctionnelle et Adaptative, F-75013 Paris

\*To whom correspondence should be addressed. WG: wilfried.grange@u-paris.fr. Correspondence can be also addressed to FB: florent.busi@u-paris.fr.

## Abstract

Single-molecule techniques are highly sensitive tools that can reveal reaction intermediates often obscured in experiments involving large ensembles of molecules. Therefore, they provide unprecedented information on the mechanisms that control biomolecular reactions. Currently, one of the most significant single-molecule assays is Magnetic Tweezers (MT), which probes enzymatic reactions at high spatio-temporal resolutions on tens, if not hundreds, of molecules simultaneously. For high-resolution MT experiments, a short double-stranded DNA molecule (less than 2,000 base pairs) is typically attached between a micron-sized superparamagnetic bead and a surface. The fabrication of such a substrate is key for successful single-molecule assays, and several papers have discussed the possibility of improving the fabrication of short DNA constructs. However, reported yields are usually low and require additional time-consuming purification steps (e.g., gel purification). In this paper, we propose the use of a Golden Gate Assembly assay that allows for the production of DNA constructs within minutes (starting from PCR products). We discuss how relevant parameters may affect the yield and offer single-molecule experimentalists a simple yet robust approach to fabricate DNA constructs.

## Introduction

Among force-based single molecule techniques, Magnetic Tweezers (MT) have emerged as a unique tool to study enzymatic reactions and provide new and valuable insights in major biological processes (e.g., gene regulation). The relative simplicity of MT as instruments contributes significantly to their effectiveness and popularity in this field. Today, MT benefits from the development of camera with high full well depths [1] as well as GPU-based parallel processing architectures [2]. This allows to probe hundreds of molecules in parallel at frequencies of a few tens of Hz [3] while maintaining nanometer accuracy [4].

In a typical Magnetic Tweezers experiment, a double-stranded DNA (ds-DNA) molecule is attached between a micron-sized magnetic bead and a surface coated with appropriate ligands (Figure 1). The most common strategy involves using streptavidin-coated beads and anti-digoxigenin antibodies on the surface [5]. In this setup, the ds-DNA molecule is modified at both ends with short handles decorated with either multiple biotin or digoxigenin-modified nucleotides on both strands. This increases the lifetime of bonds and allows for the supercoiling of DNA molecules in experiments involving enzymes that have a topological effect on DNA [6][7].

Several studies have discussed and proposed methods to optimize the fabrication of DNA tethers. In particular, a recent study has utilized a Golden Gate Assembly (GGA) [8][9][10] approach to fabricate long DNA tethers (10 kb) comprising several DNA fragments [11]. While this method improves the yield over standard ligation assays, this study does not provide guidelines or protocols for DNA tethers consisting of a single short DNA fragment (for example, 2 kb). However, these short tethers are important for the single-molecule community as they are needed in routine high-resolution MT experiments [4][12]. To the best of our knowledge, one of the most recent papers describing the fabrication of tethers comprising a single DNA fragment is the one from [13]. This study involves standard ligation assays that result in a poor yield of DNA tethers and often require additional steps of purification, such as gel excision.

In this paper, we present a robust method (based on GGA) to fabricate high yields of supercoiled DNA tethers within a timeframe that is half of standard approaches. Based on the results of high-throughput DNA sequencing assays available in the literature [14], we propose optimal overhangs for ligation and discuss how parameters (e.g., substrates concentration, time, presence of labelled dUTP) influence yield. These optimizations are rarely discussed in single-molecule papers and so we believe this work will be of benefit to the single-molecule community.

## Materials and Methods

Unless noted, enzymes and reactants were purchased from New England Biolabs (NEB).

### Polymerase Chain Reaction

For Magnetic Tweezers constructs, the fragment of interest (~1700 bp) was synthesized with Q5 Hot Start High-Fidelity DNA Polymerase (M0493). Handles (~550 bp) were synthesized with Hot Start Taq polymerase (M0495) using either biotin-16-dUTP (11093070910, Roche) or digoxigenin-11-dUTP (11093088910, Roche) at a 1:4 dUTP to dTTP ratio. PCR products were purified using a Monarch PCR & DNA Cleanup Kit (5 µg) from NEB and then visualized on a 0.8 to 1.2 % percent agarose gel resolved in Tris-acetate-EDTA electrophoresis buffer. Detailed protocols are provided in the Supplementary Text.

### Estimating fidelity of DNA polymerases

Given the averaged error rate  $e$  of specific polymerases [15], a straightforward method to determine the number of error-free fragments of lengths  $l$  after a given number of PCR cycles  $c$  would involve calculating:

This, however, overlooks the fact that some molecules are not replicated and still contribute to the overall number of error-free molecules. Additionally, it assumes that all PCR cycles have a constant efficiency of 1. Instead, we employ a more rigorous approach [16] that incorporates known efficiencies from [17].

### Golden Gate Assembly

Experiments were conducted using T4 DNA ligase (M0202) and BsaI-HFv2 (R3733). The final concentration of T4 DNA ligase was 12 U/ $\mu$ L, while BsaI-HFv2 had a final concentration of 0.6 U/ $\mu$ L. These were used in either CutSmart or rCutSmart Buffer, which was supplemented with 1 mM ATP (P0756, NEB) and 10 mM dithiothreitol (DTT, 43816). The total volume of the mixture was adjusted to 10  $\mu$ L. Detailed protocols are provided in the Supplementary Text.

### Agarose Gel Quantification

We utilized a custom routine, written in R, to measure band intensities with high precision. This routine automatically identifies lanes, eliminates the baseline, and projects the obtained sub-images perpendicular to the direction of migration for each lane. Finally, it fits the detected peaks using a Gaussian function. This method enables us to determine intensities even at a low signal-to-noise ratio and allows reproducible and quantitative measurements (see Supplementary Text for the R code and an example of the method, Figure S1).

### Magnetic Tweezers

We used a home-built Magnetic Tweezers instrument capable of tracking a few tens of beads in real-time at 400 Hz. For illumination, we utilize a Superluminescent Diode (SLD-mCS-261-MP2-SM, Superlum), which offers up to 2 mW at 670 nm with an approximate 0.43 mm ( $1/e^2$ ) beam waist and a  $\sim 2.5$  mrad divergence using a PAF-X-2-B fiberport (Thorlabs). The force exerted on the magnetic beads is regulated by a pair of magnets (W-05-N50-G, Supermagnete) arranged in a vertical configuration with a gap size of 1 mm. The position and rotation of the magnets are adjusted using two motors: M-126.PD1 (Physik Instrumente) and ST4118M1804-L (Nanotec). To image the beads, we use a UPLFL 100XOI objective (Olympus) mounted on a piezo-focusing system (P-726.1CD, Physik Instrumente) and a EF 300mm F/4 L USM telephoto lens (Canon) that offers a low distortion. Images are processed at high-speed using a HXC-13 camera (Baumer) operating at 400 Hz (1280 x 1024 pixels) and controlled by a microEnable IV AD4-CL frame grabber (Silicon Software). The position of the beads is determined using software based on the work of V. Croquette [18] and ported to CUDA by S.J. Tabrizi [2]. Our instrument operates on Linux and is written in Python.

## Results and Discussion

### Optimizing parameters

#### Golden Gate Assembly

Figure 2 shows the GGA-based approach to fabricate the DNA fragment of interest as well as the DNA handles that contain either multiple biotin or digoxigenin on both strands. This is achieved using  $\sim 40$  bases long primers that introduce the recognition site of a type IIS enzyme (which cleaves downstream the recognition sequence). Using a one-pot reaction in which both the endonuclease and the ligase are mixed together yields stable products that do not contain the recognition site of the type IIS enzyme [8].

In contrast, using a sequential approach (hydrolysis and then ligation) as suggested in most of MT studies would be detrimental [19][20]. Here, a type IIS allows to generate non palindromic overhangs, which then prevent the formation of concatamers (e.g., multiple fragments of interest) but this approach would greatly affect the overall yield as digested fragments could readily re-ligate (e.g., the digested biotin handle yields 2 products that can re-ligate to generate the original biotin handle). One option is to gel purify DNA fragments after hydrolysis, but this would be time-consuming and typically result in poor recovery, along with potential contamination with chaotropic agents (e.g., guanidine salt).

#### Choice of endonuclease

Limiting ourselves to enzymes that (i) generate 4-base overhangs (ii) recognize a 6-base sequence (iii) whose activity is compatible with the optimal conditions for ligation and (iv) require one recognition site on the substrate to cleave optimally, we identified the following type IIS enzymes at New England Biolabs (NEB): BsaI-HFv2, BbsI-HF, and Esp3I. For those enzymes, NEB has performed high-throughput DNA assays (studying possible ligations in a mixture containing all 256 possible overhangs) that evidence mismatch ligation in GGA assembly and so allow us to select optimal overhangs [14] (see below).

Another important point is that the recognition sequence of the endonuclease should not be present in the sequence of interest (the DNA fragment). Taking a sequence of  $n$  consecutive nucleotides, the probability of not having a given sequence is simply (assuming independence) :

$$1 - (1/4)^n$$

In a fragment of length  $l$  ( $l \gg n$ ), there are about  $l$  different locations and so the probability of not observing the recognition sequence is (again assuming independence):

$$(1 - 4^{-n})^l$$

Assuming a DNA fragment length of 2,000 bp (to ensure high resolution MT experiments) the probability of not observing a given sequence composed of six nucleotides is about 0.61. Considering the three restriction enzymes mentioned above, this means that there is a probability  $P^*$  of about  $(1-0.61)^3$  (or 0.06) that the sequence of interest contains the recognition sequence of either BsaI-HFv2, BbsI-HF or Esp3I. Given that ligation mismatch data are also available in the presence of T4 DNA ligase for SapI (which recognizes a 7-base sequence and generates 3-base overhangs, potentially making them less stable), one would lower  $P^*$  to about 0.02. Finally, there are other type IIS restriction enzymes for which mismatch ligations in the presence of both the endonuclease and T4 DNA Ligase are unknown. In these cases, one could potentially use the data obtained in the presence of T4 DNA ligase alone [21], yielding a  $P^*$  close to 0. In other words, it is virtually impossible not to find an appropriate type IIS restriction enzyme when amplifying a 2,000 bp sequence.

#### Choosing DNA Polymerase

As Taq polymerase is capable of incorporating modified nucleotides and because DNA handles can be of any sequence, it is best-suited for generating DNA handles. However, the situation is different for the fragment of interest. Using Taq polymerase to amplify a 2,000-base-pair long DNA fragment would generate errors in more than 95% of the molecules (assuming 25 amplification cycles). Using a DNA polymerase of higher fidelity (Phusion) would generate errors in about 7% of the molecules (2000 bp, 25 cycles). Therefore, we strongly suggest using a Q5 DNA polymerase, which has 280 times the fidelity of Taq), resulting in an error rate of about 1%.

#### Choosing overhangs

As the choice of the downstream sequence is arbitrary, one can then generate any non-palindromic overhangs that ensures successful GGA assembly (to prevent concatamers). One should however consider that ligases are capable of mismatch ligations, which would then potentially yield unwanted assemblies (e.g. a construct that contains multiple fragments of interest or where the fragment of interest

is ligated to two biotin handles). In addition, those overhangs should also have the capability to bind with their complementary pairs with high efficiencies.

Because the choice of the downstream sequence is arbitrary, non-palindromic overhangs can be generated to ensure successful GGA assembly and prevent concatemer formation. However, ligases may be capable of mismatch ligation, which would potentially yield unwanted assemblies (e.g., constructs that contain multiple fragments of interest or where the fragment of interest is ligated to two biotin handles). In addition, these overhangs should have the capability to bind with their complementary pairs with high efficiency. Intuitively, one would expect that stable overhangs (such as those with a high GC content) would yield high ligation frequencies when performing GGA. However, the situation is more complex. When the DNA is cleaved at the end, it produces two non-melted overhangs [22]. These overhangs may then readily re-ligate, which may slow down the overall process. Obviously, these effects depend on many factors such as the concentration of DNA and ligase, and the number of available DNA substrates that could potentially yield non-mismatched ligation. As such, the results of high-multiplexed overhangs assays performed in the presence of 256 substrates at sub-nM concentrations might not be directly applicable to experiments conducted at higher molarities in the absence of non-competitive partners [14].

### Choosing temperature

Standard GGA protocols suggest conducting experiments either at a stable temperature of 37°C (when the assembly comprises up to four fragments) or cycling the temperature between 37°C and 16°C (for a larger number of fragments) [10]. When cycling is used, the duration of each temperature step is either 1 minute (for assemblies with up to ten fragments) or 5 minutes (for assemblies with a larger number of fragments). Both the concentrations of the ligase and endonuclease and the overall duration depend on the number of fragments. To the best of our knowledge, there is no clear explanation for these differences. Given that the activity of T4 DNA Ligase is maximal at 37°C [23], one would expect that stable overhangs (those with high GC content) would also yield optimal GGA assemblies when performing experiments at 37°C. In contrast, using overhangs with a low GC content will be certainly deleterious. For this reason, we have chosen to use 5'-CCGC and 5'-CACC, which have overhang stabilities (free energies) below -4.5 kcal/mol and which do not yield mismatch ligations (and so prevent the formation of a full length fragment that would contain two handles with identical modifications). Furthermore, these overhangs are appropriate for use with modified dUTPs and generally do not impact the yields (see below).

### Choosing DNA molarities

GGA protocols suggest conducting experiments at concentrations at 5 nM or below. This is certainly reminiscent of cloning protocols, where both the insert and vectors are expected to be below 5 nM. However, the situation here is different because unwanted ligation yields unstable products (as they contain the restriction site of the endonuclease). Therefore, to maximize GGA efficiency, one can increase the molarities of reactants to virtually any value (as long as the concentrations of both the Ligase and the Endonuclease are not rate-limiting). Experimentally, however, molarities are limited by the amount of PCR products typically obtained at about 50 to 100 ng/μL after purification.

With regard to supercoilable DNA tethers (one main fragment and two handles), an optimal reaction would involve working in a 1:1:1 ratio and consuming all substrates (all three DNA fragments) to fabricate the DNA tether. This optimal reaction would certainly require large molarities (e.g.,  $\geq 100$  nM), which may be experimentally challenging. Here, we chose to keep the main fragment below 10 nM (7 nM) and use handles at a 3:1 molar ratio to facilitate the ligation of the fragment of interest to the handles. As demonstrated below, this allows us to yield large amounts of supercoilable DNA tethers.

### Gel assays and Magnetic Tweezers experiments

Figure 3.A shows a representative gel when performing GGA, conducted with optimized parameters (see the preceding discussion and Supplementary Text). The full-length construct, which is barely discernible on a gel when using traditional ligation methods (refer to Supplementary Figure S1 of [13]),

becomes distinctly visible within 10 minutes. By quantifying the intensity of the bands in the gel lanes (refer to Supplementary Text), it is possible to accurately calculate the product percentage for any form containing the fragment of interest. As depicted in Figure 3.B, approximately 50% of the fragment of interest is converted into a full-length fragment (comprising both handles) within 10 minutes, and this percentage steadily increases over time, reaching about 70% after 140 minutes. It is important to note that the final reaction involves an irreversible step (ligation); hence, we anticipate reaching 100% completion at infinite time. Nevertheless, those percentages (50% or 70%) significantly surpass what is typically achieved using standard ligation assays and are sufficient to generate a quantity of DNA tethers for several weeks of experiments.

Interestingly, the presence of biotin and digoxigenin labels (at least when using these DNA ends and a 1:4 dUTP to dTTP ratio ) does not impede the formation of the full-length fragment, as demonstrated in a control experiment using non-modified dUTPs for the PCR fabrication of DNA handles (Figure 2.B). The high yield characteristic of our approach allows to utilize the reaction mixture as is, thereby eliminating the need for an additional gel purification step. This also enables to determine the concentration of the full-length fragment with high precision, thereby facilitating control of the DNA to bead ratio.

Utilizing various fields of view, we estimate the proportion of supercoillable DNA. We identified a value of more than 0.5, which exceeds the values typically reported in the literature [13]. Importantly, the DNA molecules used in these experiments were ligated for 140 minutes, although shorter ligation durations, such as 30 or 70 minutes, could also be employed. Nevertheless, our approach enables the fabrication of molecules for MT in a reproducible manner within approximately three hours, starting from plasmid DNA. This duration is significantly less than that required by conventional methods, as discussed in [13].

## Conclusion

In this study, we have provided simple and robust guidelines for fabricating short DNA constructs suitable for single-molecule Magnetic Tweezers (MT) experiments. Specifically, we have discussed how relevant parameters can influence Golden Gate Assembly (GGA). By conducting experiments at 37°C with the fragment of interest at 7 nM (using an excess of DNA handles at 21 nM) and selecting 5'-CCGC and 5'-CACC overhangs, we have demonstrated the efficient fabrication of DNA constructs with exceptional yield within a few tens of minutes. We believe these guidelines are not only of interest to the MT community but also beneficial for other single-molecule techniques, such as optical tweezers and fluorescence assays, which may use similar DNA constructs.

## **Funding information**

This research was funded by the National Science Centre, Poland (grant SONATINA 2017/24/C/NZ1/00456 to Grzegorz M. Cech) and was supported by a French Government Scholarship (Campus France to Patryk Strzelecki). This study contributes to the IdEx Université Paris Cité (ANR-18-IDEX-0001).

## **Acknowledgments**

The authors thank Iryna Riabko and Nelson Gartiser (Unistra, Strasbourg) for assistance with Magnetic Tweezers experiments.

## References

- [1] V. Croquette, J.V. Orero, M. Rieu, J.-F. Allemand, Magnetic tweezers principles and promises, in: *Methods in Enzymology*, Elsevier, 2024: pp. 1–49. <https://doi.org/10.1016/bs.mie.2024.01.026>.
- [2] B.M. Lansdorp, S.J. Tabrizi, A. Dittmore, O.A. Saleh, A high-speed magnetic tweezer beyond 10,000 frames per second, *Review of Scientific Instruments* 84 (2013) 044301. <https://doi.org/10.1063/1.4802678>.
- [3] I. De Vlamincq, T. Henighan, M.T.J. Van Loenhout, I. Pfeiffer, J. Huijts, J.W.J. Kerssemakers, A.J. Katan, A. Van Langen-Suurling, E. Van Der Drift, C. Wyman, C. Dekker, Highly Parallel Magnetic Tweezers by Targeted DNA Tethering, *Nano Lett.* 11 (2011) 5489–5493. <https://doi.org/10.1021/nl203299e>.
- [4] D. Dulin, T.J. Cui, J. Cnossen, M.W. Docter, J. Lipfert, N.H. Dekker, High Spatiotemporal-Resolution Magnetic Tweezers: Calibration and Applications for DNA Dynamics, *Biophysical Journal* 109 (2015) 2113–2125. <https://doi.org/10.1016/j.bpj.2015.10.018>.
- [5] R. Janissen, B.A. Berghuis, D. Dulin, M. Wink, T. van Laar, N.H. Dekker, Invincible DNA tethers: covalent DNA anchoring for enhanced temporal and force stability in magnetic tweezers experiments, *Nucleic Acids Research* 42 (2014) e137–e137. <https://doi.org/10.1093/nar/gku677>.
- [6] T.R. Strick, V. Croquette, D. Bensimon, Single-molecule analysis of DNA uncoiling by a type II topoisomerase, *Nature* 404 (2000) 901–904. <https://doi.org/10.1038/35009144>.
- [7] A. Revyakin, R.H. Ebright, T.R. Strick, Promoter unwinding and promoter clearance by RNA polymerase: Detection by single-molecule DNA nanomanipulation, *Proc. Natl. Acad. Sci. U.S.A.* 101 (2004) 4776–4780. <https://doi.org/10.1073/pnas.0307241101>.
- [8] C. Engler, R. Kandzia, S. Marillonnet, A One Pot, One Step, Precision Cloning Method with High Throughput Capability, *PLoS ONE* 3 (2008) e3647. <https://doi.org/10.1371/journal.pone.0003647>.
- [9] S. Marillonnet, R. Grütznert, Synthetic DNA Assembly Using Golden Gate Cloning and the Hierarchical Modular Cloning Pipeline, *CP Molecular Biology* 130 (2020) e115. <https://doi.org/10.1002/cpmb.115>.
- [10] A.P. Sikkema, S.K. Tabatabaei, Y. Lee, S. Lund, G.J.S. Lohman, High-Complexity One-Pot Golden Gate Assembly, *Current Protocols* 3 (2023) e882. <https://doi.org/10.1002/cpz1.882>.
- [11] N.A.W. Bell, J.E. Molloy, Efficient golden gate assembly of DNA constructs for single molecule force spectroscopy and imaging, *Nucleic Acids Research* 50 (2022) e77–e77. <https://doi.org/10.1093/nar/gkac300>.
- [12] A. Revyakin, R.H. Ebright, T.R. Strick, Single-molecule DNA nanomanipulation: Improved resolution through use of shorter DNA fragments, *Nat Methods* 2 (2005) 127–138. <https://doi.org/10.1038/nmeth0205-127>.
- [13] F.S. Papini, M. Seifert, D. Dulin, High-yield fabrication of DNA and RNA constructs for single molecule force and torque spectroscopy experiments, *Nucleic Acids Research* 47 (2019) e144–e144. <https://doi.org/10.1093/nar/gkz851>.
- [14] J.M. Pryor, V. Potapov, R.B. Kucera, K. Bilotti, E.J. Cantor, G.J.S. Lohman, Enabling one-pot Golden Gate assemblies of unprecedented complexity using data-optimized assembly design, *PLoS ONE* 15 (2020) e0238592. <https://doi.org/10.1371/journal.pone.0238592>.
- [15] V. Potapov, J.L. Ong, Examining Sources of Error in PCR by Single-Molecule Sequencing, *PLoS ONE* 12 (2017) e0169774. <https://doi.org/10.1371/journal.pone.0169774>.
- [16] H. Sharifian, Errors induced during PCR amplification, (2010). <https://doi.org/10.3929/ETHZ-A-006088024>.
- [17] G. Weiss, A coalescent approach to the polymerase chain reaction, *Nucleic Acids Research* 25 (1997) 3082–3087. <https://doi.org/10.1093/nar/25.15.3082>.
- [18] C. Gosse, V. Croquette, Magnetic tweezers: micromanipulation and force measurement at the molecular level, *Biophys J* 82 (2002) 3314–3329. [https://doi.org/10.1016/S0006-3495\(02\)75672-5](https://doi.org/10.1016/S0006-3495(02)75672-5).
- [19] Y. Seol, K.C. Neuman, Combined Magnetic Tweezers and Micro-mirror Total Internal Reflection Fluorescence Microscope for Single-Molecule Manipulation and Visualization, in: E.J.G. Peterman (Ed.), *Single Molecule Analysis*, Springer New York, New York, NY, 2018: pp. 297–316. [https://doi.org/10.1007/978-1-4939-7271-5\\_16](https://doi.org/10.1007/978-1-4939-7271-5_16).
- [20] X.-C. Zhao, H.-L. Dong, X.-L. Li, H.-Y. Yang, X.-F. Chen, L. Dai, W.-Q. Wu, Z.-J. Tan, X.-H. Zhang, 5-Methyl-cytosine stabilizes DNA but hinders DNA hybridization revealed by magnetic tweezers and simulations, *Nucleic Acids Research* 50 (2022) 12344–12354.

<https://doi.org/10.1093/nar/gkac1122>.

[21] K. Bilotti, V. Potapov, J.M. Pryor, A.T. Duckworth, J.L. Keck, G.J.S. Lohman, Mismatch discrimination and sequence bias during end-joining by DNA ligases, *Nucleic Acids Research* 50 (2022) 4647–4658. <https://doi.org/10.1093/nar/gkac241>.

[22] V. Potapov, J.L. Ong, R.B. Kucera, B.W. Langhorst, K. Bilotti, J.M. Pryor, E.J. Cantor, B. Canton, T.F. Knight, T.C. Evans, G.J.S. Lohman, Comprehensive Profiling of Four Base Overhang Ligation Fidelity by T4 DNA Ligase and Application to DNA Assembly, *ACS Synth Biol* 7 (2018) 2665–2674. <https://doi.org/10.1021/acssynbio.8b00333>.

[23] B. Weiss, A. Jacquemin-Sablon, T.R. Live, G.C. Fareed, C.C. Richardson, Enzymatic breakage and joining of deoxyribonucleic acid. VI. Further purification and properties of polynucleotide ligase from *Escherichia coli* infected with bacteriophage T4, *J Biol Chem* 243 (1968) 4543–4555.

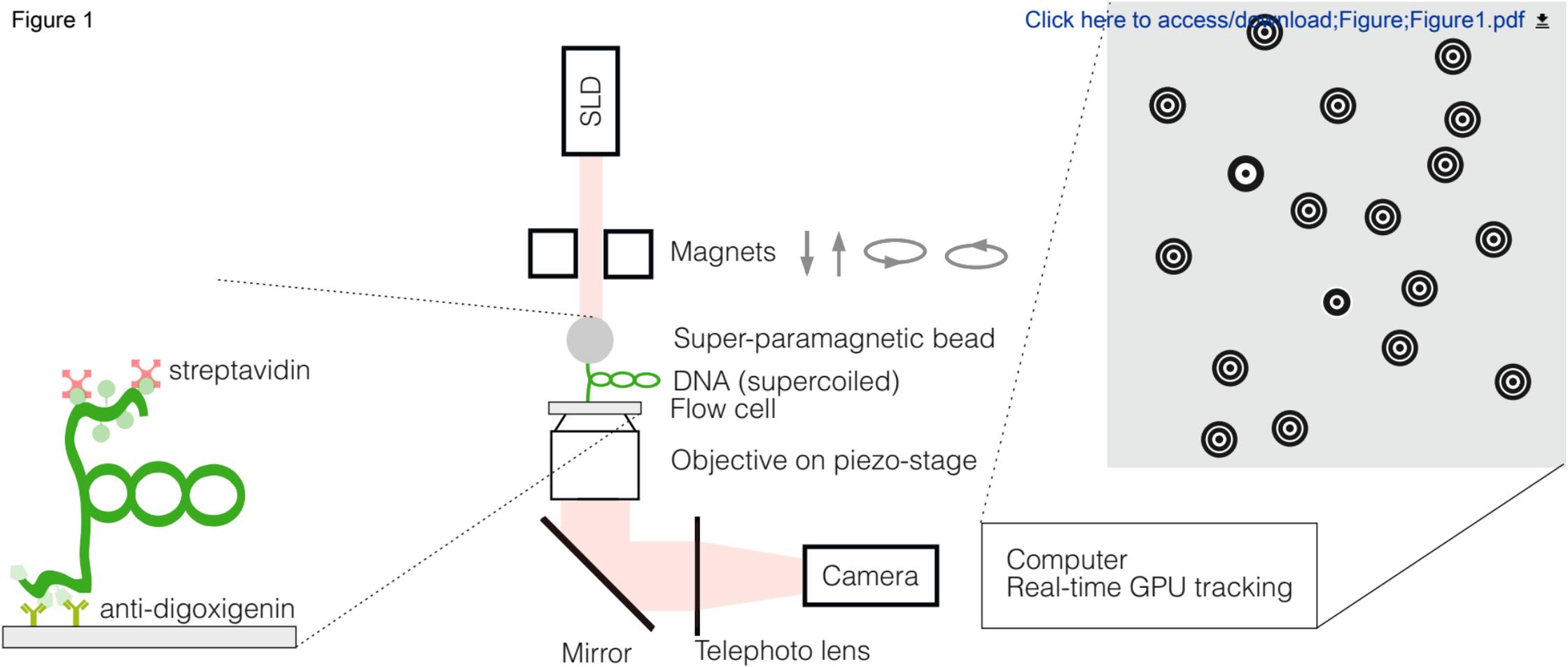
## Figure Captions

**Figure 1.** Schematic of the Magnetic Tweezers (MT) setup. Displayed here is a super-luminescent diode (SLD) operating at 670 nm that is collimated to generate a 0.43 mm ( $1/e^2$ ) beam. Magnets (cubes, 5 mm) are mounted on motors that allow control of the force as well as the torsion applied to the super-paramagnetic beads (500 nm in radius). The focus can be adjusted using a piezo-electric element that holds a high numerical aperture objective. Images are then focused on a fast CMOS camera using a telephoto lens. Tens of beads can be imaged in the field of view and tracked in real-time at 400 Hz, thus allowing highly parallel measurements at high spatio-temporal resolutions. Note that the DNA tether is modified at both ends with either biotin or digoxigenin. These modifications are present on both strands (not shown here for clarity) and allow for torsional constraint of the DNA.

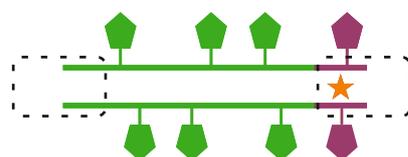
**Figure 2.** PCR reactions used to fabricate DNA tethers and Golden Gate Assembly (GGA) assay. PCR fragments are amplified using ~ 40-mer primers that contain a recognition site for BsaI-HFv2. Upon hydrolysis, this type IIS enzyme generates either 5'-CCGC (left-end of the fragment of interest and right-end of the biotin modified handle) or 5'-CACC (right-end of the fragment of interest and right-end of the digoxigenin modified handle) non-palindromic overhangs. In a conventional sequential digestion/ligation assay, a fragment containing a BsaI-HFv2 recognition site will be digested but may readily re-ligate, reforming the original fragment. However, in a GGA assay, where both the endonuclease and ligase exert their activity simultaneously, any re-ligated fragments containing a BsaI-HFv2 recognition site will be re-digested. This prevents the re-ligation from impairing the formation of the desired product. While the formation of the product of interest containing one handle occurs quickly (within a few minutes), the formation of a product with two handles requires either increasing the molarities of the handles (relative to the fragment of interest) or working in a 1:1:1 ratio at significantly higher molarities (substantially greater than a few tens of nanomolar).

**Figure 3. A.** Gel representation of the GGA assembly results, utilizing DNA handles (approximately 550 bp, 21 nM) decorated with multiple biotin or digoxigenin molecules on both strands, and a fragment of interest (~1700 bp, 7 nM). A full-length fragment becomes discernible on the gel within 10 minutes and represents a conversion of about 50% (see below), a quantity significantly greater than that achieved with standard ligation assays. **B.** Time-dependent percentage of product for forms containing the fragment of interest (with 0, 1, or 2 handles). The presence of biotin and digoxigenin labels on the DNA ends does not inhibit the formation of the full-length fragment or alter the product percentages. Raw images of gels are shown in Supplementary Figure S1.

Figure 1



digoxigenin-handle



★ BsaI-HFv2 recognition site

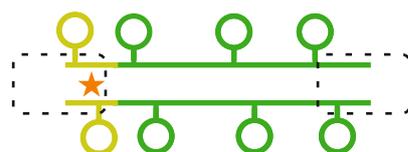
```

GATTGCACGCAGGTTCTCC
CAAGATGGATTGCACGCAGGTTCTCCGGCCGCT
GTTCTACCTAACGTGCGTCCAAGAGGCCGCGA
    
```

```

ACGGCGAGGATCTCGTCGTGACCCATGGCGATG
TGCCGCTCCTAGAGCAGCACTGGGTACCGCTAC
CCTAGAGCAGCACTGGGTA
GGCGGCTCTGGATGATA
    
```

biotin-handle



```

TACGCAGGTCAGGTGA
CGCTATCAGGACATAGCGTTG
GGCGGACCGCTATCAGGACATAGCGTTGGCTACCC
CCGCCTGGCGATAGTCCTGTATCGCAACCGATGGG
    
```

```

GGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTAC
CCCGACCGAATTGATACGCCGTAGTCTCGTCTAACATG
GAATTGATACGCCGTAGTCTCGTC
    
```

fragment of interest



```

ATGCGAGGTCGCGC
TTATCCAGGCAGCTAGAC
AATCTATTTATCCAGGCAGCTAGACTTCTAGG
TTAGATAAATAGGTCCGTCGATCTGAAGATCC
    
```

```

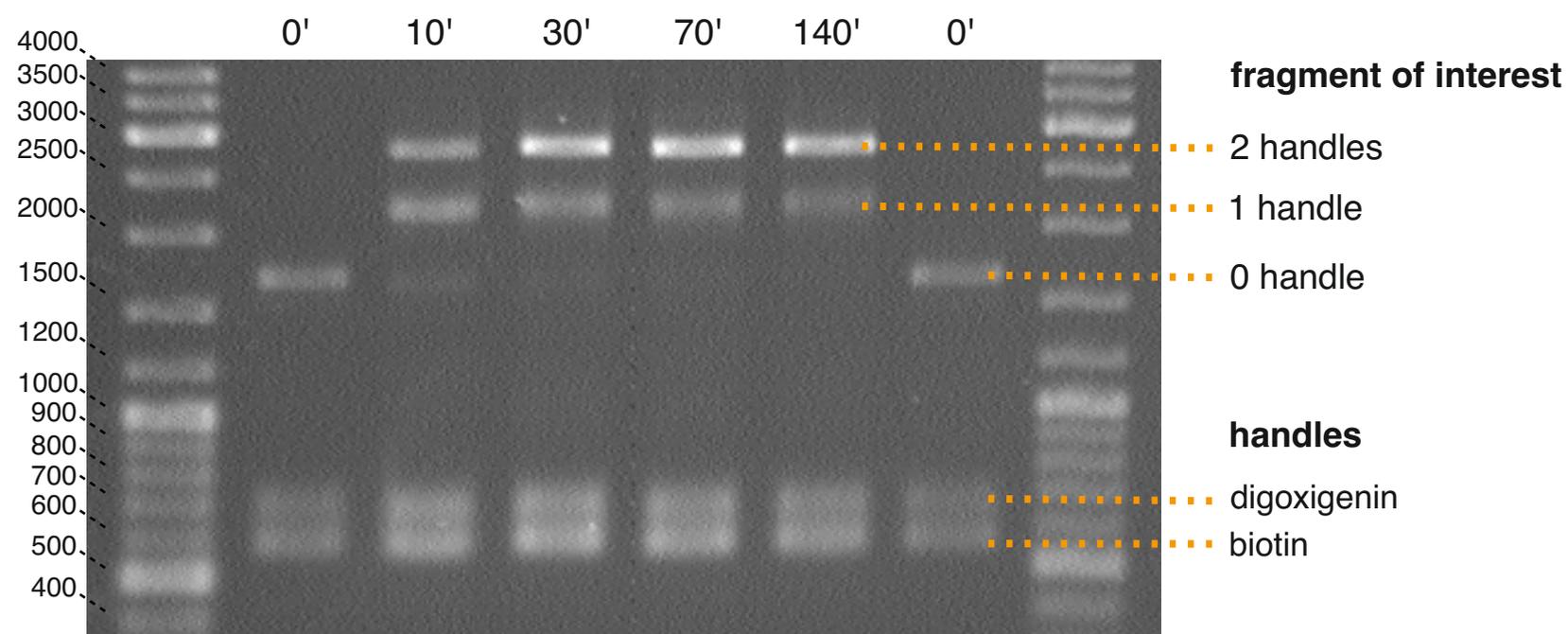
CCATGAAGTTATGGGAAGGCCATTGAATTCT
GGTACTTCAATACCCTTCCGGTAACTTAAGA
CAATACCCTTCCGGTAA
TCCACTCTCTGGATGCTG
    
```

## One-pot reaction

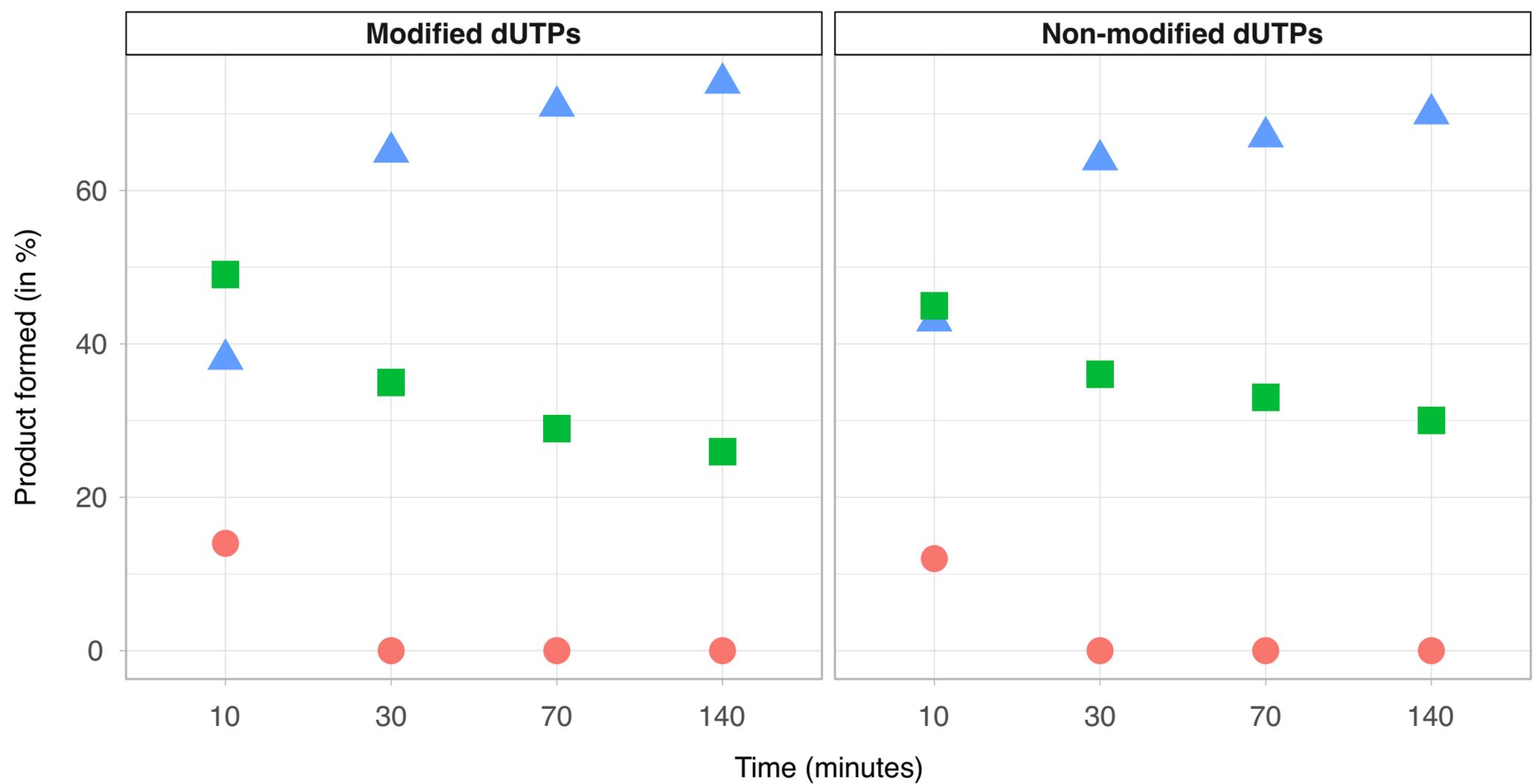
+ T4 DNA ligase  
+ BsaI-HFv2



A



B



● fragment of interest with 0 handle      ■ fragment of interest with 1 handle      ▲ fragment of interest with 2 handles