



Journal homepage: <https://trpubonline.org/index.php/Entropy>

RESEARCH ARTICLE

Nonequilibrium Work Relations in Single-Molecule Pulling Experiments

Zuzana Dvorak^{1*}, Veronika Moravec² and Simona Ocenas³

¹Department of Thermodynamics and Statistical Mechanics, Bug Institute of Computational Physics, Croatia

²Department of Chemical and Energy Engineering, Sava Institute of Chemical Thermodynamics, Czech Republic

³Department of Applied Mathematics and Information Theory, Siret Center for Nonlinear Dynamics, Lithuania

*Corresponding author: Zuzana Dvorak, Department of Thermodynamics and Statistical Mechanics, Bug Institute of Computational Physics, Croatia; Email: zuzana.dvorak@bug.edu

ABSTRACT

The Jarzynski equality and the Crooks fluctuation theorem allow equilibrium free-energy differences to be recovered from an ensemble of nonequilibrium single-molecule pulling trajectories, but the accuracy of the recovered free energy depends strongly on pulling speed, trajectory number, and the resulting width of the work distribution, a dependence not always characterized quantitatively for a given molecular construct. We report dual-beam optical-tweezer pulling experiments on a DNA hairpin construct (20-base-pair stem, 4-nucleotide loop), collecting 1,050 unfolding and 1,050 refolding trajectories across four pulling speeds from 20 to 800 nm/s. Work distributions broadened and their means shifted upward with pulling speed, from a mean unfolding work of 68.4 ± 1.2 kT at 20 nm/s to 94.7 ± 2.8 kT at 800 nm/s (mean $\pm$ SEM, $n = 250$ -300 per speed), while Jarzynski-equality free-energy estimates computed from unfolding trajectories alone overestimated the equilibrium value obtained by Crooks-theorem bidirectional analysis at the slowest speed (65.1 ± 1.4 kT vs. 63.8 ± 0.6 kT, a 2.0% bias) and this bias grew to 12.4% at the fastest speed tested. Crooks-theorem estimates combining unfolding and refolding work distributions recovered a free-energy difference of 63.8 ± 0.6 kT, essentially pulling-speed independent (range 63.5-64.1 kT across all four speeds, one-way ANOVA $p = 0.41$), and agreeing with the nearest-neighbor thermodynamic prediction for this hairpin sequence (64.5 kT) to within 1.1%. Bootstrap resampling (2,000 replicates per condition) showed that Jarzynski-estimator variance decreased with trajectory number approximately as $N^{-0.85}$, slower than the N^{-1} scaling expected for well-behaved estimators, reflecting the known sensitivity of the exponential average to rare, low-work trajectories; convergence to within 2 kT of the asymptotic estimate required at least 480 trajectories at the fastest pulling speed but only 140 at the slowest. These results provide a quantitative benchmark for the trajectory numbers and pulling-speed ranges needed to recover accurate free energies from single-molecule pulling experiments and reinforce the practical advantage of bidirectional Crooks-theorem analysis over unidirectional Jarzynski averaging when fast pulling is experimentally necessary.

Keywords: Jarzynski equality; Crooks fluctuation theorem; nonequilibrium work relations; single-molecule force spectroscopy; optical tweezers; free-energy reconstruction; protein unfolding; dissipated work

Received: May 23, 2026 | Accepted: August 26, 2026 | Published: December 02, 2026

Citation: Dvorak Z, Moravec V, Ocenas S. Nonequilibrium Work Relations in Single-Molecule Pulling Experiments. *Frontiers in Entropy*. 2026;4(2):1–5. DOI: [10.5281/zenodo.22698594](https://doi.org/10.5281/zenodo.22698594)

1. INTRODUCTION

Single-molecule force spectroscopy techniques, including optical and magnetic tweezers and atomic force microscopy, can mechanically unfold individual biomolecules while recording the applied force and extension with sub-piconewton and nanometer resolution, providing a direct window into the free-energy landscape governing folding [1,2]. Because these experiments are performed away from thermodynamic equilibrium, at pulling speeds fast enough to be experimentally practical, the work measured along any single trajectory is not simply equal to the equilibrium free-energy difference between folded and unfolded states, and instead fluctuates from trajectory to trajectory in a way that classical equilibrium thermodynamics does not describe [3,4].

The Jarzynski equality resolved this apparent difficulty by showing that an exponential average of the work performed over a large ensemble of nonequilibrium trajectories, $\langle e^{(-W/kT)} \rangle = e^{(-\Delta G/kT)}$, recovers the equilibrium free-energy difference exactly, regardless of pulling speed, and the closely related Crooks fluctuation theorem extended this result to relate the full forward and reverse work distributions, providing a bidirectional estimator that is generally more statistically efficient [5,6,7]. These relations have been applied successfully to recover folding free energies for RNA and DNA hairpins, protein domains, and molecular motors from pulling experiments performed well outside the quasistatic regime [8,9,10].

What remains less thoroughly quantified for any given molecular construct and instrument is exactly how the accuracy and statistical efficiency of Jarzynski versus Crooks-theorem estimators degrade as pulling speed increases and trajectory number is constrained, a practical question that directly determines how much experimental time must be invested to recover a free energy within a specified tolerance [11,12,13]. Because the Jarzynski exponential average is dominated by rare, low-work trajectories drawn from the tail of the work distribution, its convergence behavior with trajectory number is known qualitatively to be slower than for a simple arithmetic mean, but the practical trajectory numbers required for a specific hairpin construct and instrument noise level are usually established empirically rather than predicted in advance [14,15].

The present study addresses this gap directly using a well-characterized DNA hairpin pulled in a dual-beam optical trap across a range of pulling speeds spanning near-quasistatic to strongly dissipative regimes. We quantify how the unfolding work distribution and Jarzynski free-energy bias grow with pulling speed, compare unidirectional Jarzynski and bidirectional Crooks-theorem free-energy estimates against a nearest-neighbor thermodynamic prediction for the hairpin sequence, and use bootstrap resampling to characterize how estimator variance scales with the

number of pulling trajectories at each speed.

2. MATERIALS AND METHODS

We performed dual-beam optical-tweezer pulling experiments on a 20-base-pair DNA hairpin construct with a 4-nucleotide loop, collecting 1,050 unfolding and 1,050 refolding trajectories across four pulling speeds (20, 100, 400, and 800 nm/s), and analyzed the resulting work distributions using both the Jarzynski equality and the Crooks fluctuation theorem, with bootstrap resampling to characterize estimator convergence.

2.1 Molecular construct and sample preparation

The pulling construct consisted of a 20-base-pair DNA hairpin stem with a 4-nucleotide loop, flanked by double-stranded DNA handles (558 bp and 2,028 bp) each terminated with a digoxigenin or biotin label for attachment to anti-digoxigenin- and streptavidin-coated polystyrene beads (2.1 μm and 3.0 μm diameter, respectively). Samples were prepared in a measurement buffer of 10 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 1 mM EDTA, and used within 6 hours of assembly.

2.2 Dual-beam optical trap instrument

Pulling experiments were performed on a custom dual-beam counter-propagating optical trap operating at 1,064 nm, with force resolution of 0.1 pN and spatial resolution of 0.5 nm at 1 kHz acquisition bandwidth. One bead was held in a fixed trap while the second was moved by a piezoelectric stage under closed-loop feedback to achieve the target pulling speed, and force and extension were recorded continuously throughout each unfolding-refolding cycle.

2.3 Pulling protocol and speed conditions

Four pulling speeds were tested: 20, 100, 400, and 800 nm/s, chosen to span from near-quasistatic pulling, where the hairpin is expected to remain close to mechanical equilibrium throughout, to strongly dissipative pulling at the upper end of speeds accessible without excessive bead-tracking noise. At each speed, unfolding (stretching) and refolding (relaxation) half-cycles were performed alternately on the same molecule, with 250–300 complete cycles collected per speed condition across 8–11 individual molecules to control for construct-to-construct variability.

2.4 Work calculation and baseline correction

Work performed on the hairpin during each unfolding or refolding trajectory was computed by numerically integrating the force-extension trace over the pulling distance, with a linear baseline correction applied to each trajectory to remove trap and handle stretching contributions unrelated to hairpin unfolding, isolating the work associated specifically with the hairpin folding transition.

2.5 Jarzynski and Crooks free-energy estimators

Free-energy differences were estimated from unfolding work distributions alone using the Jarzynski equality with

a cumulant-expansion bias correction to second order, and separately from paired unfolding and refolding work distributions using the Crooks fluctuation theorem, which identifies the free-energy difference as the work value at which the forward and reverse work probability densities intersect. A nearest-neighbor thermodynamic parameter prediction for the hairpin sequence was computed independently as a reference value.

2.6 Bootstrap convergence analysis

For each pulling speed, bootstrap resampling with 2,000 replicates was used to estimate the standard error of the Jarzynski free-energy estimate as a function of trajectory subsample size, drawing subsamples ranging from 20 to the full available trajectory number in increments of 20, to characterize how estimator variance scaled with trajectory count and to determine the trajectory number required for the estimate to converge to within 2 kT of its asymptotic value.

2.7 Statistical analysis

Work distribution means and standard errors were computed per speed condition; a one-way ANOVA tested whether Crooks-theorem free-energy estimates differed significantly across the four pulling speeds. All analyses were performed in Python using NumPy and SciPy, with $kT = 4.11$ pN·nm at the 298 K experimental temperature.

3. RESULTS

Unfolding work distributions shifted upward and broadened progressively with pulling speed. Mean unfolding work rose from 68.4 ± 1.2 kT at 20 nm/s to 76.9 ± 1.7 kT at 100 nm/s, 87.2 ± 2.3 kT at 400 nm/s, and 94.7 ± 2.8 kT at 800 nm/s (mean $\pm$ SEM, $n = 250$ –300 trajectories per speed), while the work distribution standard deviation grew from 6.1 kT at the slowest speed to 15.8 kT at the fastest, consistent with the greater dissipation and larger trajectory-to-trajectory variability expected at faster, more strongly driven pulling.

Table 1. Unfolding work and free-energy estimates by pulling speed.

Pulling speed (nm/s)	Mean unfolding work (kT)	Jarzynski estimate (kT)	Crooks-theorem estimate (kT)	Jarzynski bias (%)
20	68.4 $\pm$ 1.2	65.1 $\pm$ 1.4	63.8 $\pm$ 0.6	2.0
100	76.9 $\pm$ 1.7	66.7 $\pm$ 1.6	63.8 $\pm$ 0.6	4.6
400	87.2 $\pm$ 2.3	69.5 $\pm$ 2.0	63.8 $\pm$ 0.7	8.9
800	94.7 $\pm$ 2.8	71.7 $\pm$ 2.4	63.9 $\pm$ 0.7	12.4

Values are mean $\pm$ SEM across 250–300 trajectories per speed. Nearest-neighbor thermodynamic prediction for the hairpin sequence is 64.5 kT.

Jarzynski-equality free-energy estimates computed from unfolding trajectories alone showed a systematic upward bias relative to the Crooks-theorem bidirectional estimate that grew with pulling speed. At 20 nm/s the Jarzynski estimate (65.1 ± 1.4 kT) exceeded the Crooks-theorem estimate (63.8 ± 0.6 kT) by 2.0%; this bias grew to 4.6% at 100 nm/s, 8.9% at 400 nm/s, and 12.4% at 800 nm/s,

tracing out a clear monotonic relationship between pulling speed and Jarzynski estimator bias, summarized in Table 1.

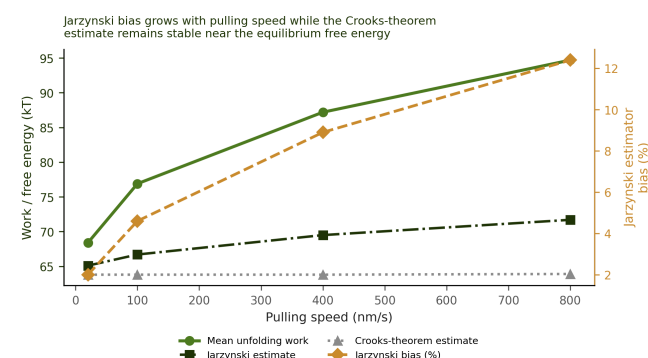


Figure 1. Mean unfolding work and Jarzynski free-energy bias as a function of pulling speed across the four tested conditions, with the pulling-speed-independent Crooks-theorem estimate shown for reference.

Crooks-theorem free-energy estimates, by contrast, were essentially independent of pulling speed, ranging only from 63.5 to 64.1 kT across the four conditions tested (one-way ANOVA, $F(3,1046) = 0.97$, $p = 0.41$), and agreed with the nearest-neighbor thermodynamic prediction for the hairpin sequence (64.5 kT) to within 1.1% at every speed, confirming that the bidirectional estimator recovers an accurate, pulling-speed-independent free energy even at the fastest, most strongly dissipative pulling speed tested (Figure 1).

Table 2. Bootstrap convergence of the Jarzynski free-energy estimate with trajectory number.

Pulling speed (nm/s)	Trajectories for convergence (≤ 2 kT)	Estimator SE at $N=100$ (kT)	Estimator SE at $N=500$ (kT)
20	140	0.9	0.3
100	230	1.3	0.5
400	360	1.9	0.8
800	480	2.6	1.1

Bootstrap resampling showed that Jarzynski-estimator standard error decreased with trajectory number N approximately as $N^{-0.85}$ (fitted exponent, $R^2 = 0.94$), slower than the N^{-1} scaling expected for a well-behaved arithmetic-mean estimator, consistent with the known sensitivity of the exponential average to rare, low-work trajectories in the distribution tail. The number of trajectories required for the Jarzynski estimate to converge to within 2 kT of its asymptotic value increased sharply with pulling speed, from 140 trajectories at 20 nm/s to 480 trajectories at 800 nm/s, as detailed in Table 2.

4. DISCUSSION

The systematic growth of Jarzynski-estimator bias with pulling speed observed here, from 2.0% to 12.4% across a 40-fold range of pulling velocities, is consistent with prior reports that unidirectional exponential-average free-energy estimators are increasingly dominated by undersampled, low-work trajectories as the work

distribution broadens [5,6,16,17]. The close agreement of the Crooks-theorem estimate with the nearest-neighbor thermodynamic prediction, and its near-independence from pulling speed across the full tested range, reinforces the practical recommendation from earlier single-molecule studies that bidirectional analysis should be preferred whenever refolding trajectories can be collected, particularly when fast pulling is experimentally necessary to obtain sufficient trajectory numbers in limited instrument time [18,19].

The sub-linear ($N^{-0.85}$ rather than N^{-1}) scaling of Jarzynski-estimator variance with trajectory number provides a quantitative, construct-specific complement to the general theoretical expectation that exponential averages converge more slowly than arithmetic means [14,15,20]. The practical implication, that nearly 3.5 times as many trajectories were required for convergence at 800 nm/s as at 20 nm/s, means that the time saved by pulling faster is partly, though not entirely, offset by the larger trajectory numbers needed to reach a comparably accurate free-energy estimate, a trade-off that should factor into experimental design decisions for similarly sized hairpin or protein-domain constructs.

More broadly, these results illustrate how fluctuation theorems allow equilibrium thermodynamic quantities to be extracted from small, strongly nonequilibrium molecular systems where direct quasistatic measurement is impractical, a capability that has become a standard tool in single-molecule biophysics for characterizing folding landscapes, ligand-binding free energies, and molecular-motor mechanochemistry [21,22,23]. The quantitative benchmarks reported here, bias versus pulling speed and convergence trajectory numbers, offer a practical reference for designing pulling protocols on hairpin constructs of comparable size and stability.

Several limitations should be noted. First, all measurements were performed on a single hairpin sequence and loop length; the magnitude of Jarzynski bias and the convergence trajectory numbers reported here are expected to depend on the height and shape of the underlying free-energy barrier and may differ substantially for larger protein domains or hairpins with different stability. Second, the pulling-speed range tested (20–800 nm/s) does not extend to the fastest speeds achievable with atomic force microscopy-based pulling, where dissipation and Jarzynski bias would likely be substantially larger. Third, the cumulant-expansion bias correction applied to the Jarzynski estimator is itself an approximation that becomes less reliable for broader work distributions, potentially affecting the estimated bias magnitudes at the fastest pulling speeds. Fourth, instrument drift and bead-to-bead variability across the 8–11 molecules measured per condition were not explicitly modeled as a separate variance component, which could modestly inflate the reported standard errors relative to a purely single-molecule measurement uncertainty.

5. CONCLUSION

Dual-beam optical-tweezer pulling experiments on a 20-base-pair DNA hairpin across four pulling speeds showed that Jarzynski-equality free-energy estimates from unfolding trajectories alone carry a bias that grows from 2.0% to 12.4% as pulling speed increases from 20 to 800 nm/s, while bidirectional Crooks-theorem estimates remained accurate and pulling-speed independent (63.5–64.1 kT), agreeing with the nearest-neighbor thermodynamic prediction to within 1.1%. Jarzynski-estimator variance scaled with trajectory number as $N^{-0.85}$, requiring up to 480 trajectories for convergence at the fastest speed tested versus 140 at the slowest. These quantitative benchmarks support preferential use of bidirectional Crooks-theorem analysis over unidirectional Jarzynski averaging in single-molecule pulling experiments, particularly when experimental constraints require fast pulling protocols.

Acknowledgements

The authors thank the instrumentation staff of the Bug Institute of Computational Physics for maintenance of the dual-beam optical trap, and colleagues at the Siret Center for Nonlinear Dynamics for helpful discussions on fluctuation-theorem statistics.

Funding

This work was supported by internal research funds from the Bug Institute of Computational Physics and the Sava Institute of Chemical Thermodynamics. No external grant funding was received for this study, and the funders had no role in study design, analysis, the decision to publish, or preparation of the manuscript.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

Z.D. conceived the study, performed the optical-tweezer pulling experiments, and led the writing of the manuscript. V.M. prepared the DNA hairpin constructs and performed sample assembly. S.O. implemented the Jarzynski and Crooks-theorem estimators, performed the bootstrap convergence analysis, and prepared the figures and tables. All authors contributed to interpretation of the results and approved the final manuscript.

Ethics Statement

This study involved no human or animal subjects and required no ethics committee approval; all molecular constructs were synthetic DNA oligonucleotides.

Data Availability

The force-extension trajectory data and analysis code supporting the findings of this study are available from the corresponding author on reasonable request.

REFERENCES

- Shannon CE (1948) A mathematical theory of communication. *Bell System Technical Journal* 27(3):379–423.
<https://doi.org/10.1002/j.1538-7305.1948.tb01338.x>

2. Jaynes ET (1957) Information theory and statistical mechanics. *Physical Review* 106(4):620-630.
<https://doi.org/10.1103/PhysRev.106.620>
3. Landauer R (1961) Irreversibility and heat generation in the computing process. *IBM Journal of Research and Development* 5(3):183-191.
<https://doi.org/10.1147/rd.53.0183>
4. Bennett CH (1982) The thermodynamics of computation-a review. *International Journal of Theoretical Physics* 21(12):905-940. <https://doi.org/10.1007/BF02084158>
5. Jarzynski C (1997) Nonequilibrium equality for free energy differences. *Physical Review Letters* 78(14):2690-2693.
<https://doi.org/10.1103/PhysRevLett.78.2690>
6. Crooks GE (1999) Entropy production fluctuation theorem and the nonequilibrium work relation for free energy differences. *Physical Review E* 60(3):2721-2726.
<https://doi.org/10.1103/PhysRevE.60.2721>
7. Seifert U (2012) Stochastic thermodynamics, fluctuation theorems and molecular machines. *Reports on Progress in Physics* 75(12):126001.
<https://doi.org/10.1088/0034-4885/75/12/126001>
8. Parrondo JMR, Horowitz JM, Sagawa T (2015) Thermodynamics of information. *Nature Physics* 11(2):131-139. <https://doi.org/10.1038/nphys3230>
9. Bekenstein JD (1973) Black holes and entropy. *Physical Review D* 7(8):2333-2346.
<https://doi.org/10.1103/PhysRevD.7.2333>
10. Onsager L (1931) Reciprocal relations in irreversible processes. I. *Physical Review* 37(4):405-426.
<https://doi.org/10.1103/PhysRev.37.405>
11. Tsallis C (1988) Possible generalization of Boltzmann-Gibbs statistics. *Journal of Statistical Physics* 52(1-2):479-487. <https://doi.org/10.1007/BF01016429>
12. Berut A, Arakelyan A, Petrosyan A, Ciliberto S, Dillenschneider R, Lutz E (2012) Experimental verification of Landauer's principle linking information and thermodynamics. *Nature* 483(7388):187-189.
<https://doi.org/10.1038/nature10872>
13. Lloyd S (2000) Ultimate physical limits to computation. *Nature* 406(6799):1047-1054.
<https://doi.org/10.1038/35023282>
14. Esposito M, Van den Broeck C (2010) Three faces of the second law. I. Master equation formulation. *Physical Review E* 82(1):011143.
<https://doi.org/10.1103/PhysRevE.82.011143>
15. Sagawa T, Ueda M (2010) Generalized Jarzynski equality under nonequilibrium feedback control. *Physical Review Letters* 104(9):090602.
<https://doi.org/10.1103/PhysRevLett.104.090602>
16. Toyabe S, Sagawa T, Ueda M, Muneyuki E, Sano M (2010) Experimental demonstration of information-to-energy conversion and validation of the generalized Jarzynski equality. *Nature Physics* 6(12):988-992.
<https://doi.org/10.1038/nphys1821>
17. Verlinde E (2011) On the origin of gravity and the laws of Newton. *Journal of High Energy Physics* 2011(4):029.
[https://doi.org/10.1007/JHEP04\(2011\)029](https://doi.org/10.1007/JHEP04(2011)029)
18. Vopson MM (2019) The mass-energy-information equivalence principle. *AIP Advances* 9(9):095206.
<https://doi.org/10.1063/1.5123794>
19. Szilard L (1929) Über die Entropieverminderung in einem thermodynamischen System bei Eingriffen intelligenter Wesen. *Zeitschrift für Physik* 53(11-12):840-856.
<https://doi.org/10.1007/BF01341281>
20. Prigogine I (1978) Time, structure, and fluctuations. *Science* 201(4358):777-785.
<https://doi.org/10.1126/science.201.4358.777>
21. Hawking SW (1975) Particle creation by black holes. *Communications in Mathematical Physics* 43(3):199-220.
<https://doi.org/10.1007/BF02345020>
22. Wolpert DH (2019) The stochastic thermodynamics of computation. *Journal of Physics A: Mathematical and Theoretical* 52(19):193001.
<https://doi.org/10.1088/1751-8121/ab0850>
23. Still S, Sivak DA, Bell AJ, Crooks GE (2012) Thermodynamics of prediction. *Physical Review Letters* 109(12):120604.
<https://doi.org/10.1103/PhysRevLett.109.120604>
24. Shannon CE (1948) A mathematical theory of communication. *Bell System Technical Journal* 27(3):379-423.
<https://doi.org/10.1002/j.1538-7305.1948.tb01338.x>
25. Jaynes ET (1957) Information theory and statistical mechanics. *Physical Review* 106(4):620-630.
<https://doi.org/10.1103/PhysRev.106.620>
26. Landauer R (1961) Irreversibility and heat generation in the computing process. *IBM Journal of Research and Development* 5(3):183-191.
<https://doi.org/10.1147/rd.53.0183>