Supplementary Material for Liphardt *et al.*

Additional bias work term. Strictly, the energy $u_i(z=0)$ stored in the measurement apparatus at the beginning of the switching experiment, e.g. in the harmonic potential of an optical trap holding the bead, needs to be taken into account when calculating the work from force-extension curves: $w_i(z,r) = \int F_i(z',r)dz' - u_i(z=0)$ (*1*). We neglect this term in all our calculations since $u_i(z=0)$ is of order k_BT and thus $u_i(z=0) \ll \int F_i(z',r)dz'$.

Requirement for a microscopic system. Jarzynski's result states that in the limit of an infinite number of work trajectories, $e^{-\beta\Delta G} = \langle e^{-\beta w_i} \rangle$. However, only a finite number N of work trajectories are available, and so the practical usefulness of this result depends on how rapidly $W_{JE} = -\beta^{-1}\ln\langle e^{-\beta w_i}\rangle_N$ converges to ΔG as N is increased. That estimation error ΔG - W_{JE} depends on how well the distribution of works w_i can be sampled with N trials. We define the sampling efficiency s_{eff} as twice the fraction of works w_i in a sample that are smaller than W_{JE} (*2*). The dominant contribution to W_{JE} comes from values in the lower tail of the work distribution: s_{eff} is a (rough but useful) measure of how well this lower tail has been sampled given the standard deviation of works, σ. With a Gaussian distribution of works, w_i, and therefore in the near equilibrium regime (*3*), the sampling efficiency for large N is given by the integral of the left tail of the work distribution from $-\infty$ to W_{JE}:

$$s_{eff} = \frac{2}{\sigma\sqrt{2\pi}}\int_{-\infty}^{W_{JE}} e^{-(w-W_A)^2/2\sigma^2}dw = 1 - erf\left(\frac{\beta\sigma}{2\sqrt{2}}\right),$$

where $W_{JE} - W_A = -\beta\sigma^2/2$. Thus, the sampling efficiency s_{eff} decays rapidly as σ increases, which has an important consequence. If Jarzynski's equality is to be tested and used successfully with experimentally attainable N, then σ must not be much bigger than a few k_BT, at least until better sampling and averaging algorithms are found.

Statistical and systematic errors.

Statistical error. The statistical error due to low s_{eff} can be reduced by averaging m independent measurements of W_{JE}; $\langle W_{JE}\rangle = \frac{1}{m}\sum_{j=1}^{m} W_{JE_i}$.

Finite N systematic error of $\langle W_{JE}\rangle$. By averaging m independent measurements of W_{JE} one can reduce the statistical error but the systematic error remains. In particular, $\langle W_{JE}\rangle$ overestimates ΔG by ~ $\beta\sigma^2/2N$ (*2*). This estimate of the systematic error is valid for any distribution (and exact for a Gaussian distribution) but only for small N and $\beta\sigma$ ($N \le 4$ and $\beta\sigma \le 1$). For larger N and σ, estimates of the systematic error can be obtained numerically. In their simulations, Hendrix and Jarzynski (*3*) found that generally the systematic error is much smaller than the statistical error as $N\tau$ is kept constant (where τ denotes the switching time, see Fig. 3 of that reference). The systematic error can be reduced only by increasing the number of samples N used to compute each individual W_{JE}, and not by averaging over W_{JE}'s, as is the case for the statistical error.

Optical trap. A piezo-electric actuator controls the position of the chamber and the tip bead, connected to the chamber by a glass micropipette. The other bead is captured in an optical trap and the force is measured from the change in momentum of light that exits the dual-beam trap. The optical trap is described in (*4*). Position of the tip-bead was determined using a "light-lever" where a low-power diode laser coupled to a single-mode optical fiber (Thorlabs LPS-3224-635) was collimated by a positive lens (f = 1.45 mm) attached to the chamber. Deflection of the resulting beam, due to chamber movement, was detected by a distant (~ 1 m) position-sensitive photodiode (UDT Sensors DL-10). Position of the bead in the optical trap was inferred from the force (measured by light momentum change) and the trap stiffness. Both trap stiffness and the light-lever were calibrated using video measurements of bead positions. The end-to-end length of the molecule is obtained as the difference between the light-lever (tip bead) and the photon deflection (trap bead) measurements. The analog force and lever data were smoothed with an RC circuit (1 - 3 ms time constant), digitized with 12-bit resolution, and saved to disk at 200 - 1000 Hz. The laser tweezers apparatus was enclosed in a plexiglass box to reduce drift and acoustic noise as well as air currents. This modification reduced force zero drift by a factor of three (to < 2 pN/hour) compared to previous measurements (*5*).

Molecule synthesis. RNA was synthesized from a template obtained by PCR from bases 3821 to 628 of the pBR322 DNA plasmid (NEB), where the P5abc sequences (Operon) were cloned into the Eco RI and Hind III restriction sites and a T7 promoter was appended to the template in the course of the PCR reaction. The DNA components of the handles were prepared by PCR from pBR322. Handle A (pBR322 bases 3821 to 3) was biotinylated, and one of the primers used to amplify handle B (pBR322 bases 30 to 628) was purchased with a 5´ digoxigenin group. One bead was coated with streptavidin, and the other with anti-digoxigenin antibodies.

Experimental conditions. Experiments were performed in a buffer containing 250 mM NaCl, 10 mM EDTA, and 10 mM Tris-HCl pH 7.0. All stretching events were preceded by a low-force incubation at 2 pN lasting 1 s to ensure complete folding of the RNA prior to stretching.

Switching rate dependent noise and signal. A 30 nm switching reaction with two distinct relaxation timescales was analyzed to permit discrimination between switching rate-dependent responses of the instrument (such as piezo-electric actuator hysteresis) and true switching rate-dependent changes in the distribution of work values for P5abc RNA unfolding. The first ~ 5 nm of this switching process are dominated by stretching of the handles against their entropic elasticity, which is reversible at all three switching rates as demonstrated by superposition of forward and reverse curves. By contrast, the second regime ($5 < z < 25$ nm) is dominated by RNA unfolding, which is either reversible or irreversible depending on switching rate. The observation that W_A and σ increase with switching rate only in the second regime (Fig. 3, A and B), and subsequently no longer increase, suggests that those changes are of molecular origin: they represent true switching rate-dependent increases in work dissipation and initial state memory by the P5abc RNA, and not simply switching rate-dependent measurement artifacts.

Table S1. Energies ($k_B T$) at the three switching rates and at z = 5, 15, and 25 nm.

m datasets, each containing ~ N = 40 measurements	z (nm)	$\langle W_A - W_{A,rev} \rangle \pm \langle \sigma \rangle$ §	$\langle W_{FD} - W_{A,rev} \rangle$ †	$\langle W_{JE} - W_{A,rev} \rangle \pm \frac{\sigma_m}{\sqrt{m-1}}$ ‡
Slow switching (r ~ 2 to 5 pN/s)				
m = 7	5	0.00±0.30	-0.04	-0.04±0.01
	15	0.00±0.85	-0.36	-0.37±0.07
	25	0.00±1.33	-0.88	-0.91±0.18
Medium switching (r ~ 34 pN/s)				
m = 3	5	0.22±0.39	+0.14	+0.14±0.05
	15	1.19±1.46	+0.12	+0.09±0.23
	25	2.40±2.77	-1.43	-0.44±0.45
Fast switching (r ~ 52 pN/s)				
M = 4	5	0.22±0.47	+0.10	+0.11±0.01
	15	1.96±1.94	+0.09	+0.25±0.11
	25	3.30±3.30	-2.13	-0.61±0.14

§Mean dissipated work ± mean standard deviation of the dissipated work.
†Performance of W_{FD} compared to $W_{A,rev}$. ‡Performance of W_{JE} compared to $W_{A,rev}$ ± an estimate of the statistical error, namely the standard error of the mean.

References

1. G. Hummer, A. Szabo, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 3658 (2001).
2. R. H. Wood, W. C. Muhlbauer, P. T. Thompson, *J. Phys. Chem.* **95**, 6670 (1991).
3. D. A. Hendrix, C. Jarzynski, *J. Chem. Phys.* **114**, 5974 (2001).
4. S. B. Smith, Y. Cui, C. Bustamante, *Science* **271**, 795 (1996).
5. J. Liphardt, B. Onoa, S. B. Smith, I. Tinoco Jr., C. Bustamante, *Science* **292**, 5517 (2001).