Supporting Information

High-Fidelity Nanopore Sequencing of Ultra-Short DNA Targets

Brandon D. Wilson[1], Michael Eisenstein[2,3], H. Tom Soh[2,3,4*]

Affiliations:

[1]Department of Chemical Engineering, Stanford University, Stanford, CA 94305, USA.

[2]Department of Electrical Engineering, Stanford University, Stanford, CA 94305, USA.

[3]Department of Radiology, Stanford University, Stanford, CA 94305, USA.

[4]Chan Zuckerberg Biohub, San Francisco, CA 94158, USA.

* Correspondence to tsoh@stanford.edu

Supplemental Calculation 1: Determination of threshold thermodynamics for design of MIP secondary structure
Figure S1: Gel electrophoresis of target DNA before and after circularization
Table S1: List of barcodes used for multiplexing
Table S2: Experimental conditions for data presented in Figure 5

Supplemental Calculation 1: Determining the threshold for efficient circularization. We took care to design MIPs that are predicted to exhibit minimal secondary structure – especially in the hybridization regions – in order to ensure highly efficient circularization. Our threshold was based on the secondary structure thermodynamics that allowed for >90% conversion from linear to circular DNA over five temperature cycles:

$$\Delta G_{folding} = -RTln\left(\frac{1-f_{unfold}}{f_{unfold}}\right) \tag{1}$$

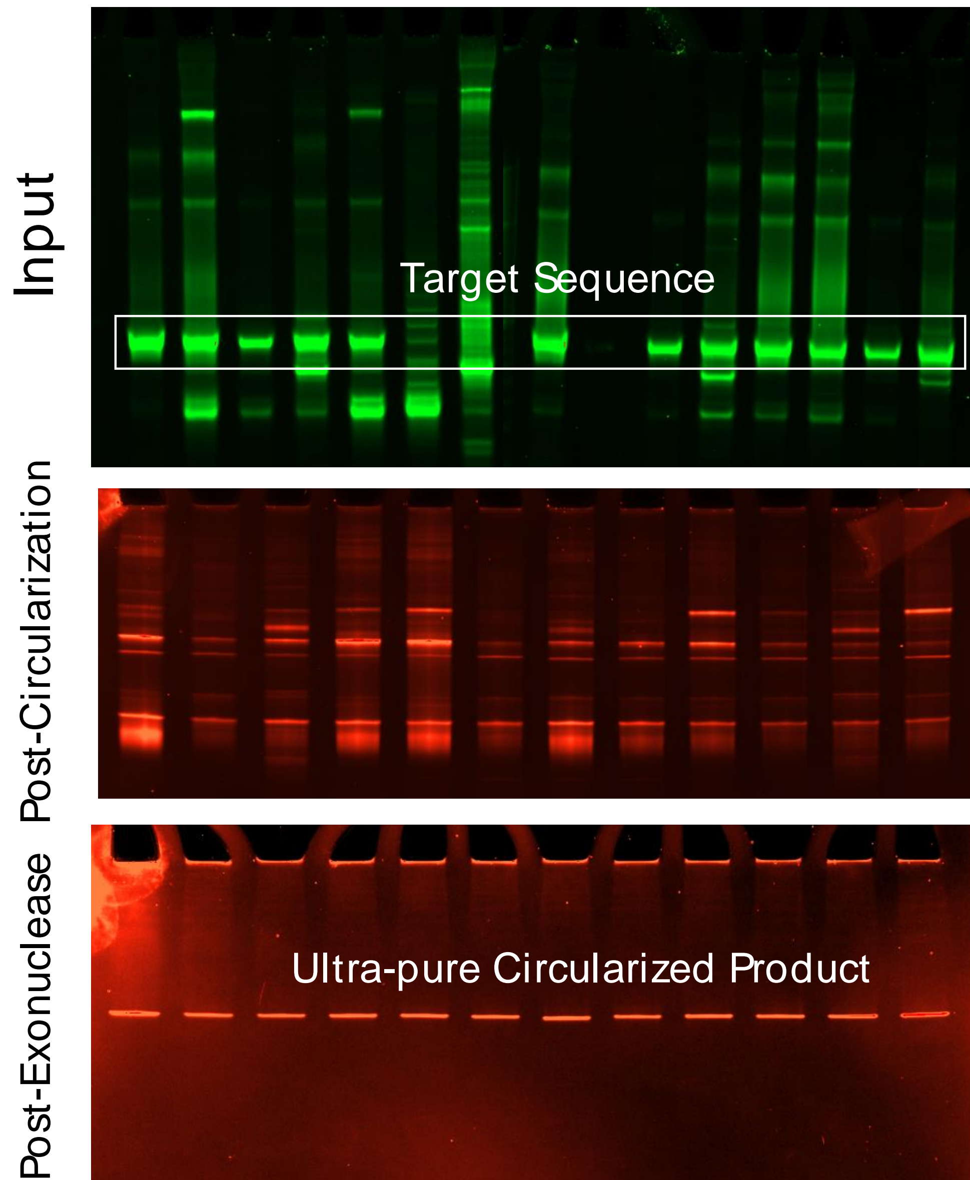
Total conversion, ε, is related to the fraction of unfolded MIPs, f_{unfold}, and the cycle number, n:

$$\varepsilon = 1 - \left(1 - f_{unfold}\right)^{n} \tag{2}$$

we can thus calculate the minimum $\Delta G_{folding}$ of any individual motif::

$$\Delta G_{folding} \geq -RTln\left(\frac{(1-\varepsilon)^{1/n}}{1-(1-\varepsilon)^{1/n}}\right) \tag{3}$$

To achieve >90% circularization in 5 cycles, we must have $f_{unfold} > 0.37$. Therefore, $\Delta G_{folding}$ must be greater than $-0.33\,\frac{kcal}{mol}$ for any secondary structure motif involving one of the anchor sites. $\Delta G_{folding}$ was calculated on the *mfold* DNA folding form[14] at 37 °C and 10 mM Mg^{2+}. A secondary check was performed on NUPACK[13] to determine that $f_{unfold} > 0.37$ for all bases in both anchor sites.



Supplemental Figure 1: MIPs can confer ultra-high specificity from a background of non-specific product. (top) PCR amplification can result in a large background of non-specific product, whereas (middle) circularization alone produces multiple bands. (bottom) Treatment with exonucleases I & III leaves only a single band of pure circular product. Top panel is a native TBE gel, whereas the other two panels are TBE-urea denaturing gels.

Barcode	Sequence
b1	5'-TAGTCATCTCTA-3'
b2	5'-TACCAGGTCCTA-3'
b3	5'-TATCCATCCTTA-3'
b4	5'-TAAGCTCGCATA-3'
b5	5'-ATCCTCTCCTCA-3'
b6	5'-CGTCTACGATGC-3'
b7	5'-CACGAAGTGGAA-3'
b8	5'-GTTCCCTGTCCC-3'
b9	5'-ACGTTAAGGCCA-3'
b10	5'-TCCTCGTGAGGT-3'
b11	5'-CATCTAACCTAG-3'
b12	5'-TCGGAATTGGCT-3'

Table S1: List of barcodes used for demultiplexing. Barcodes are located in the MIPs at the site indicated in **Table S2**

Barcode		Input concentration to PCR rxn		Fraction of Target 1
Rep. 1	Rep. 2	Target #1	Target #2	
b1	b7	4.5 fM	500 aM	0.90
b2	b8	1.5 fM	500 aM	0.75
b3	b9	500 aM	500 aM	0.50
b4	b10	167 aM	500 aM	0.25
b5	b11	56 aM	500 aM	0.10
b6	b12	0 aM	500 aM	0.00

Barcode		Input concentration to PCR rxn		Fraction w/ Three SNVs
Rep. 1	Rep. 2	w.t.	w/ SNVs	
b1	b7	500 aM	0 aM	0.00
b2	b8	472 aM	28 aM	0.06
b3	b9	450 aM	50 aM	0.10
b4	b10	400 aM	100 aM	0.20
b5	b11	300 aM	200 aM	0.40
b6	b12	250 aM	250 aM	0.50

Table S2: Experimental conditions for a) ratiometric and b) SNV experiments. The barcode sequence was used to demultiplex pooled samples (**Table S1**). Values shown represent the concentrations in the mixture added to the PCR reaction.