

Supporting Information

Multiplexed assembly and annotation of synthetic biology constructs using long-read Nanopore sequencing

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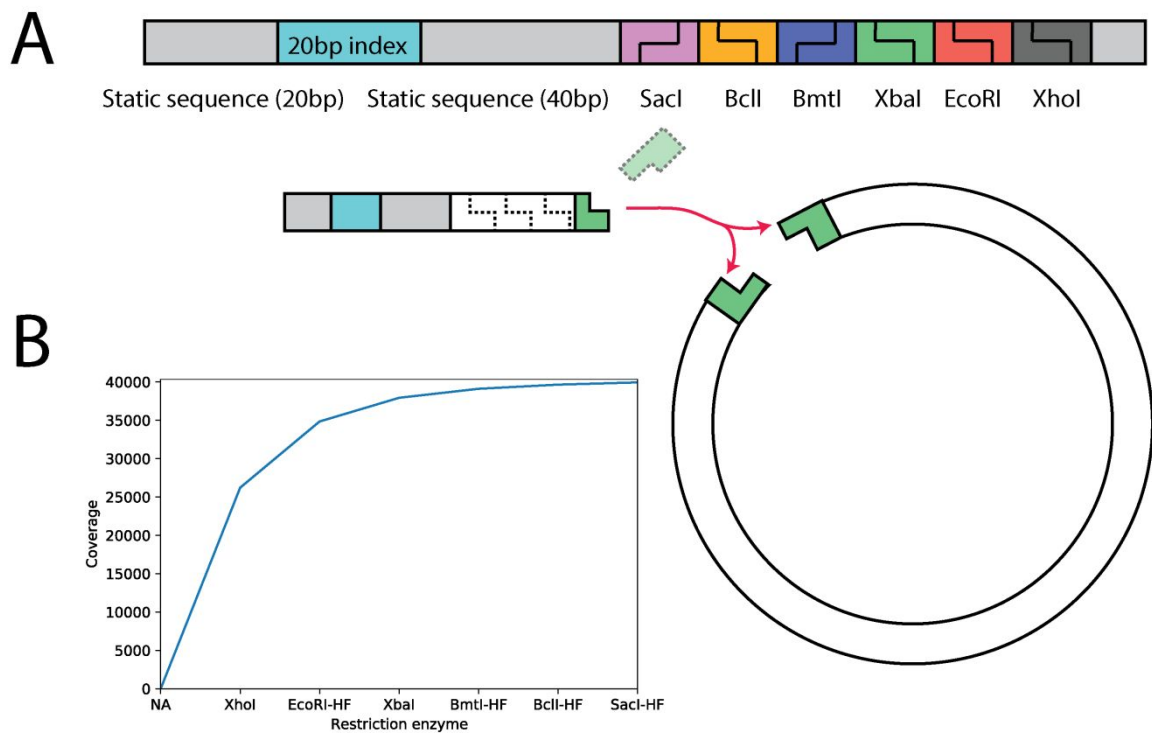


Figure S1. (A). Barcode ligation requires a single, known restriction site (**green**) for each plasmid that is shared with the ligation adapters. Using a set-cover approach, we searched all known plasmids in the Addgene database to establish a candidate list of restriction enzymes that cut once and only once per plasmid (**see methods**). From this search, we selected a set of 6 restriction enzymes and incorporated their restriction sites into individual barcodes for ligation. **(B)** These restriction enzymes covered a total of 62% of the 63,017 Addgene plasmids with a single restriction site. The resulting barcode sequences can then be ligated onto plasmids that are cut with the matching enzyme (**methods**).

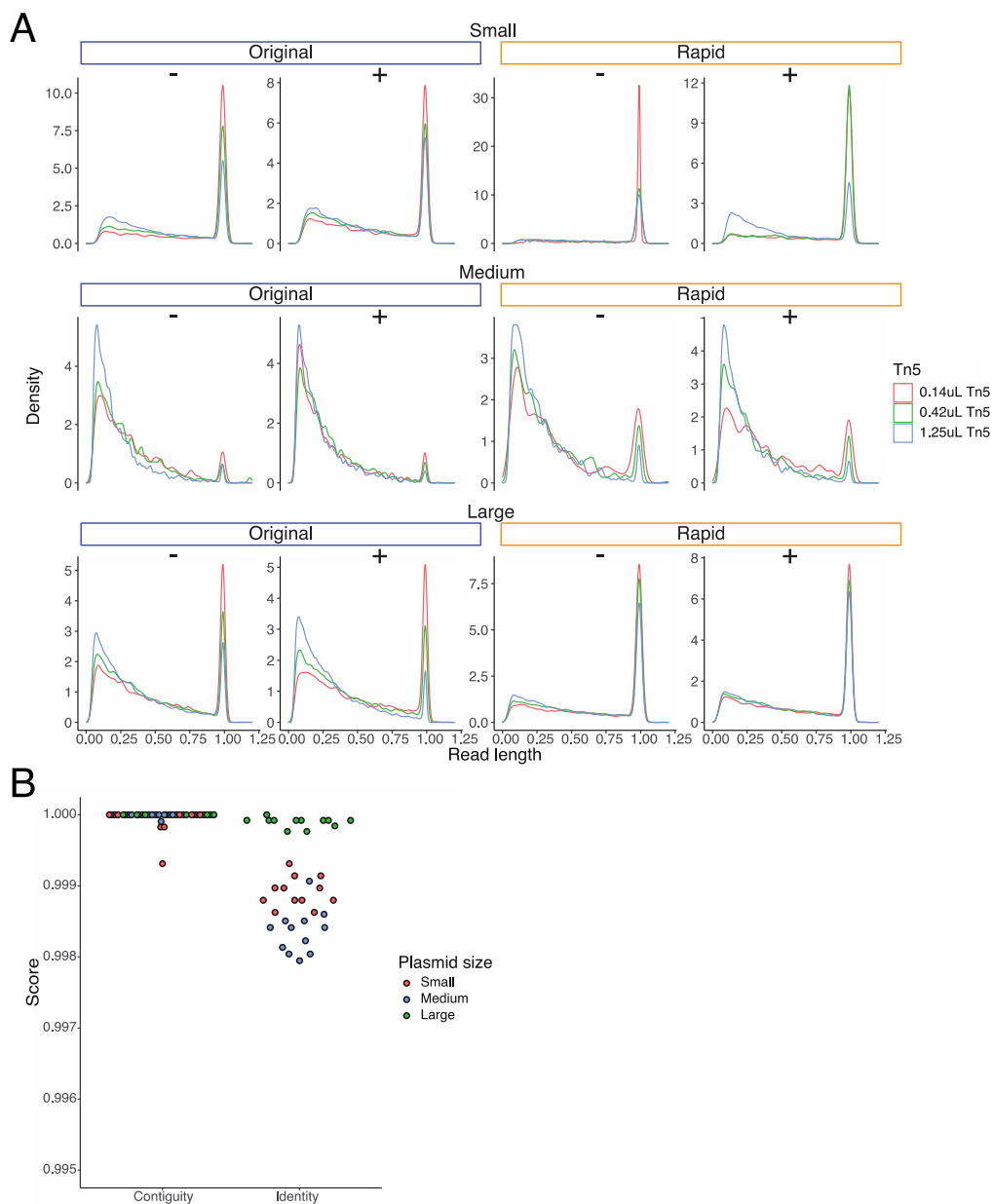


Figure S2. (A). To determine conditions that would maximize read length, we processed three plasmids of various lengths (6kbp to 13kbp) using a modified pipeline. Density plots of read lengths, normalized to the plasmid length, are shown for small, medium, and large plasmids (top to bottom rows), amount of Tn5 (colors), the duration of tagmentation (left and right), as well as method for Tn5 inactivation (- for SDS only, + for SDS with heat inactivation). **(B).** Once the reads from each of the conditions were assembled, we calculated contiguity and identity scores for each assembly. Identity scores cluster by plasmid type due to sequence complexity and are unrelated to reaction conditions.

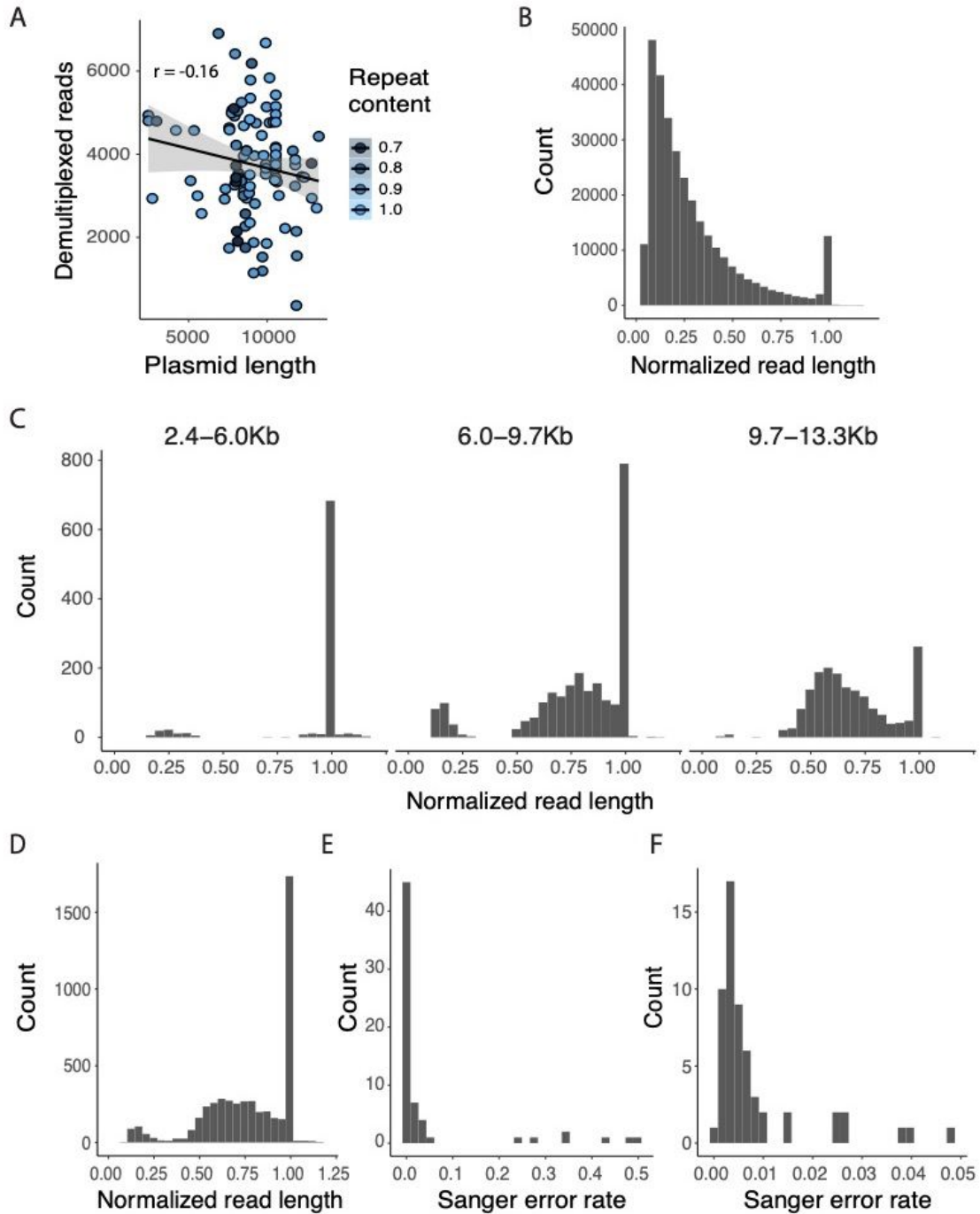


Figure S3. (A). The correlation of recovered Nanopore sequencing read counts to input plasmid length, where point color indicates the relative repeat content of the input plasmid. (B) Histogram of unfiltered Nanopore read lengths normalized to the originating plasmid sequence length. (C) Post-filtering and normalized read lengths across ranges of plasmid lengths and (D) all plasmid lengths. (E) Error rates for all 64 Sanger sequencing trace files after quality control and (F) after removing 7 traces with error rates over 10%.

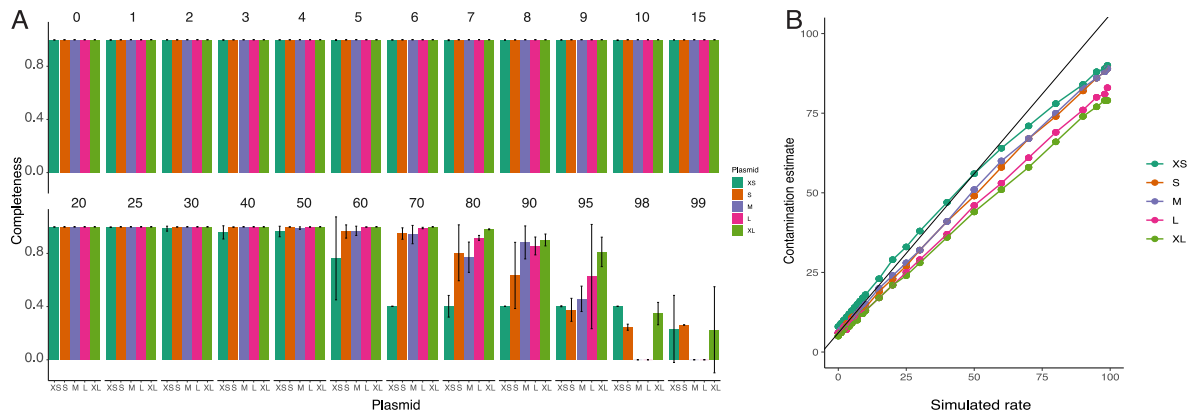


Figure S4. (A) Plasmid assembly completeness from simulated reads. Each of five plasmids (XS=extra small, S=small, M=medium, L=large, XL=extra-large) were mixed with randomly sampled reads from the remaining 91 plasmids at contamination levels from 0% to 99%. Assembly issues in the smaller plasmids started at 30-40% contaminated, with most assemblies having serious issues at contamination levels greater than 80%. **(B)** Contamination estimates when reads are mapped against the known plasmid map instead of the assembled reference as in figure 3. Here contamination estimates better track simulated rates to the upper limits of contamination.

Table S1. Primer and barcode sequences for the Tn5 V1 approach.

Name	Sequence
NanP_tn5_v1_001	ATCGTAGCAACAACAACAACAACCGAGATGTGTATAAGAGACAG
NanP_tn5_v1_002	ATCGTAGCAACAGAACAATGGATGGAGATGTGTATAAGAGACAG
NanP_tn5_v1_003	ATCGTAGCAACCGACTACGTGATTCAGATGTGTATAAGAGACAG
NanP_tn5_v1_004	ATCGTAGCAACGCCAATTATCGAGCAGATGTGTATAAGAGACAG
NanP_tn5_v1_005	ATCGTAGCAACTCTGCCAAGGACAAAGATGTGTATAAGAGACAG
NanP_tn5_v1_006	ATCGTAGCAAGACGAATTGGACGAGAGATGTGTATAAGAGACAG
NanP_tn5_v1_007	ATCGTAGCAAGCCTAACTAATCCGGAGATGTGTATAAGAGACAG
NanP_tn5_v1_008	ATCGTAGCAAGGTTGACACTCTGGAAGATGTGTATAAGAGACAG
NanP_tn5_v1_009	ATCGTAGCAATAAGTCTCCTAGTCGAGATGTGTATAAGAGACAG
NanP_tn5_v1_010	ATCGTAGCAATCCTTGACCTCGCCAAGATGTGTATAAGAGACAG
NanP_tn5_v1_011	ATCGTAGCAATTAGTGCTCTCATGGAGATGTGTATAAGAGACAG
NanP_tn5_v1_012	ATCGTAGCACAAGCTAAGGATACCAAGATGTGTATAAGAGACAG
NanP_tn5_v1_013	ATCGTAGCACACTCGCCGGTCTAAAGATGTGTATAAGAGACAG
NanP_tn5_v1_014	ATCGTAGCACATGAATCTTACGGAGATGTGTATAAGAGACAG
NanP_tn5_v1_015	ATCGTAGCACCATTACGTCATTCCAGATGTGTATAAGAGACAG
NanP_tn5_v1_016	ATCGTAGCACCTAATAAACACTCTCCAGATGTGTATAAGAGACAG
NanP_tn5_v1_017	ATCGTAGCACGACGAGCTTGGAGGTAGATGTGTATAAGAGACAG
NanP_tn5_v1_018	ATCGTAGCACGCTCCTTATCGAACCCAGATGTGTATAAGAGACAG
NanP_tn5_v1_019	ATCGTAGCACGTGCGACAAGCCTCTAGATGTGTATAAGAGACAG
NanP_tn5_v1_020	ATCGTAGCACTCCAGGTATGTATTCAGATGTGTATAAGAGACAG
NanP_tn5_v1_021	ATCGTAGCACTTATACGACCACTTCAGATGTGTATAAGAGACAG
NanP_tn5_v1_022	ATCGTAGCAGAATAACCTCGGATACAGATGTGTATAAGAGACAG
NanP_tn5_v1_023	ATCGTAGCAGAGGAACGAGAATGTGAGATGTGTATAAGAGACAG
NanP_tn5_v1_024	ATCGTAGCAGCATATTGTAGACTGGAGATGTGTATAAGAGACAG
NanP_tn5_v1_025	ATCGTAGCAGCGGTTCTCATATAAGAGATGTGTATAAGAGACAG
NanP_tn5_v1_026	ATCGTAGCAGGAGCTTATATGTGGTAGATGTGTATAAGAGACAG
NanP_tn5_v1_027	ATCGTAGCAGGTTGTAGGTGTAGTAAGATGTGTATAAGAGACAG
NanP_tn5_v1_028	ATCGTAGCAGTCTCTAGCTCAATACAGATGTGTATAAGAGACAG
NanP_tn5_v1_029	ATCGTAGCAGTTGGTATTCTTAAGCAGATGTGTATAAGAGACAG
NanP_tn5_v1_030	ATCGTAGCATACTTAGGATGTGCCGAGATGTGTATAAGAGACAG
NanP_tn5_v1_031	ATCGTAGCATCAGGTATCGCTCCGAGATGTGTATAAGAGACAG
NanP_tn5_v1_032	ATCGTAGCATCTATTCTTCGCTTGGAGATGTGTATAAGAGACAG
NanP_tn5_v1_033	ATCGTAGCATGCTAGTCGGAACGCCAGATGTGTATAAGAGACAG
NanP_tn5_v1_034	ATCGTAGCATTAGTGCCAACAGAGCAGATGTGTATAAGAGACAG
NanP_tn5_v1_035	ATCGTAGCATTGTGGAAGGTTGAGCAGATGTGTATAAGAGACAG
NanP_tn5_v1_036	ATCGTAGCCAAGATGGAGCCTAACTAGATGTGTATAAGAGACAG
NanP_tn5_v1_037	ATCGTAGCCAATGTCCAGCACTCCTAGATGTGTATAAGAGACAG
NanP_tn5_v1_038	ATCGTAGCCACCTCAATATAAGCCAAGATGTGTATAAGAGACAG
NanP_tn5_v1_039	ATCGTAGCCACTGTTAAGCGGTAGGAGATGTGTATAAGAGACAG
NanP_tn5_v1_040	ATCGTAGCCAGCGTTCACACTCGTAGATGTGTATAAGAGACAG
NanP_tn5_v1_041	ATCGTAGCCATAACGTCCTGTAGTAAGATGTGTATAAGAGACAG
NanP_tn5_v1_042	ATCGTAGCCATGCACCAATAGTAGCAGATGTGTATAAGAGACAG

NanP_tn5_v1_043 ATCGTAGCCCAAGAAGGTCGTGCTGAGATGTGTATAAGAGACAG
NanP_tn5_v1_044 ATCGTAGCCCCTGCTCACTAACGGAGATGTGTATAAGAGACAG
NanP_tn5_v1_045 ATCGTAGCCCATTAAGCAGGTGTGTAGATGTGTATAAGAGACAG
NanP_tn5_v1_046 ATCGTAGCCCGCACTTGGAGAGCTTAGATGTGTATAAGAGACAG
NanP_tn5_v1_047 ATCGTAGCCCGGTGGTATCAATTAAGATGTGTATAAGAGACAG
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NanP_tn5_v1_049 ATCGTAGCCCTCTTAGTCTCCGCTCAGATGTGTATAAGAGACAG
NanP_tn5_v1_050 ATCGTAGCCGAACCACTCAACAGCGAGATGTGTATAAGAGACAG
NanP_tn5_v1_051 ATCGTAGCCGAGACAACGACACTAAAGATGTGTATAAGAGACAG
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NanP_tn5_v1_094	ATCGTAGCTGCACCGTCGAGTTCTAAGATGTGTATAAGAGACAG
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NanP_tn5_v1_096	ATCGTAGCTGTAAGTCATAGCAACGAGATGTGTATAAGAGACAG
NanP_tn5_v1_097	ATCGTAGCTGTTGCATTGAGGTGGTAGATGTGTATAAGAGACAG
NanP_tn5_v1_098	ATCGTAGCTTAGCCTGCAACAAGAAAGATGTGTATAAGAGACAG
NanP_tn5_v1_099	ATCGTAGCTTCCGCATTAGCTGACCAGATGTGTATAAGAGACAG
NanP_tn5_v1_100	ATCGTAGCTTGACAGGTGAACGTGCAGATGTGTATAAGAGACAG

Table S2. Primer and barcode sequences for the Tn5 V2 approach.

Name	Sequence
NanP_tn5_v2_001	ACTGAAACAACAACAACCCTACGCCGGTGAATAGATGTGTATAAGAGACAG
NanP_tn5_v2_002	ACTGACCACCACTTGTTCACCTAGTAACCGGAGATGTGTATAAGAGACAG
NanP_tn5_v2_003	ACTGAGGACAAGTGGTGGCGGTAGGTGTTCATAGATGTGTATAAGAGACAG
NanP_tn5_v2_004	ACTGACCTGAGAGATGCTATCGCCGGAAGTTGAGATGTGTATAAGAGACAG
NanP_tn5_v2_005	ACTGAGAGTAGTAACCAAGATTGATGTTGTGCAGATGTGTATAAGAGACAG
NanP_tn5_v2_006	ACTGACAGTCCTAGATGAACCAGTGTGTGTAGATGTGTATAAGAGACAG
NanP_tn5_v2_007	ACTGATTGATTGAACCGCAGTCTACGTGCCTAGATGTGTATAAGAGACAG
NanP_tn5_v2_008	ACTGAAAGTGCCATTAGAAGTCTATAACGAAGGAGATGTGTATAAGAGACAG
NanP_tn5_v2_009	ACTGAGAAGCATCAATCGCTAATAACCGTACCAGATGTGTATAAGAGACAG
NanP_tn5_v2_010	ACTGAAACCAATTAGTCCACGTGAAGCATACCAGATGTGTATAAGAGACAG
NanP_tn5_v2_011	ACTGAGCTACCACCTTCCCCTCTGACAAGCGTAGATGTGTATAAGAGACAG
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NanP_tn5_v2_013	ACTGAGCCAACCGGACTAAATCGTTCATGTGCAGATGTGTATAAGAGACAG
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NanP_tn5_v2_015	ACTGAACGCGCTTATATTCCTACCTCCATTAAAGATGTGTATAAGAGACAG
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NanP_tn5_v2_017	ACTGAACAGCCAACGTAACATACCGGAATCTTAGATGTGTATAAGAGACAG
NanP_tn5_v2_018	ACTGAATCCTTCCAACAACCAGAACCACCAATAGATGTGTATAAGAGACAG
NanP_tn5_v2_019	ACTGACCAGTAGCGCCTTTCCAAGCAACCAGTAGATGTGTATAAGAGACAG
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NanP_tn5_v2_033	ACTGAATGCCGCCGAAGAACAATACTCACTCACGAAGATGTGTATAAGAGACAG
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NanP_tn5_v2_037	ACTGATTAGCCTTGTCTTCAATGTGCGCACGGAAGATGTGTATAAGAGACAG
NanP_tn5_v2_038	ACTGAAGTTCGTTCTGCGAATAGTCATACCGGAGATGTGTATAAGAGACAG
NanP_tn5_v2_039	ACTGAACTGTTGCGTTAAGCCTCGATCAGAAGAGATGTGTATAAGAGACAG
NanP_tn5_v2_040	ACTGAGATGGATTGATTCAGTTCCGTTGCCTAGATGTGTATAAGAGACAG
NanP_tn5_v2_041	ACTGATGCCGACACTCTTCCATATTCTAGCTCAGATGTGTATAAGAGACAG
NanP_tn5_v2_042	ACTGACAGCTCGTGCGTATTGAGTCCATTAGGAGATGTGTATAAGAGACAG

NanP_tn5_v2_043 ACTGATCAGTTGGTAGCCGTGCAGGAAGTAAGAGATGTGTATAAGAGACAG
NanP_tn5_v2_044 ACTGAGTATTGCTAACTGAGCCATATTGAACCAGATGTGTATAAGAGACAG
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NanP_tn5_v2_053 ACTGACTTAATTGCCACATAAGATTGTCCTCGAGATGTGTATAAGAGACAG
NanP_tn5_v2_054 ACTGAAATCGAGTACCATGGTCTGTACTGCGAAGATGTGTATAAGAGACAG
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NanP_tn5_v2_061 ACTGAGCACAAGAACATTGAACCTTAGAATGGAGATGTGTATAAGAGACAG
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NanP_tn5_v2_063 ACTGACCATCTTGAGCTAATTATCCAGCGAGAGATGTGTATAAGAGACAG
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NanP_tn5_v2_066 ACTGACCTAATATACAGCACACAGTTACGTCAGATGTGTATAAGAGACAG
NanP_tn5_v2_067 ACTGAAAGGTCCGATGAGTGTTCCTCAAGATGAGATGTGTATAAGAGACAG
NanP_tn5_v2_068 ACTGAACCACAAGTTACCCATATAGGTGCATCAGATGTGTATAAGAGACAG
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NanP_tn5_v2_070 ACTGACCTCCATAGCAAGTACTGGACGAGCACAGATGTGTATAAGAGACAG
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NanP_tn5_v2_078 ACTGAAGAACATAACAGGGCACAACGAGCTATAGATGTGTATAAGAGACAG
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NanP_tn5_v2_080 ACTGAGCATGAGACCTGTCTATCTCGTTATGGAGATGTGTATAAGAGACAG
NanP_tn5_v2_081 ACTGAAATACGACAGCGGAAGAACAACACTGCGCTAGATGTGTATAAGAGACAG
NanP_tn5_v2_082 ACTGATATACCGCCGCTTCGGTCTTCCTAACAGATGTGTATAAGAGACAG
NanP_tn5_v2_083 ACTGATTACGGTGGAACACGACAGCGGTAATAGATGTGTATAAGAGACAG
NanP_tn5_v2_084 ACTGAGGTGGAACCTCATCTAGGAGGTGGAGATGTGTATAAGAGACAG
NanP_tn5_v2_085 ACTGAAGCCACCGTCGTATAAGGACGTTGCGGAGATGTGTATAAGAGACAG
NanP_tn5_v2_086 ACTGATGCTTATTACTGCAATAGCAAGTTCGCAGATGTGTATAAGAGACAG
NanP_tn5_v2_087 ACTGACCTTCAGATTACTGCTTGTAGTTCCGCAGATGTGTATAAGAGACAG
NanP_tn5_v2_088 ACTGAATGGTTCCTCCGCGTATTCGTGGTAAGAGATGTGTATAAGAGACAG
NanP_tn5_v2_089 ACTGATGAAGGACACACACAGGTGATCACAAGATGTGTATAAGAGACAG
NanP_tn5_v2_090 ACTGAGAATAATGTGCGGAGACAATTCCGTAACAGATGTGTATAAGAGACAG
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NanP_tn5_v2_093 ACTGACCAGAACGGTGAACGAAGGAGTTGTACAGATGTGTATAAGAGACAG

NanP_tn5_v2_094	ACTGAAAGCGCCTGTCCTAACACCGTACAGGAAGATGTGTATAAGAGACAG
NanP_tn5_v2_095	ACTGATGACCACGCATACACAACATACTGCTCAGATGTGTATAAGAGACAG
NanP_tn5_v2_096	ACTGACGCTTCTCGTCTAACAGTCGAAGAGCAGATGTGTATAAGAGACAG

Table S3. Cost analysis of Circuit-seq with Tn5 approach.

Cost of a 96-well run

Item	Bulk cost (\$)	# runs	Per run cost (\$)
Flongle	810	12	67.5
LSK110-kit	599	12	49.92
End Repair	250	48	5.21
FFPE	165	48	3.44
Quick Ligase	388	30	12.93
Tn5	2000	1333	1.5
Barcodes	440	1000	0.44
Total cost per run			140.94

Table S4. Primer sequences for Restriction Enzyme approach.

primer	barcode_sequence (reverse complement)	full sequence (5' -> 3')
common_fwd	AACAACAACCAACAAGGTGG	CAAGTGCACCTCGAGGAATTCTCTAGAGCTAGCTGATCA GAGCTCGTCCGATACTACATTCCGGAACGTAACACGAGC TGGAACGA
rev_1	AACCGATTCCAACCTCTCGCC	CCTAAGAAGGACAGCACGTCCCACCTTGTTGGTTGTTG TTTCGTTCCAGCTCGTGTTACG
rev_2	AAGACTGAGAAATGGTAACG	CCTAAGAAGGACAGCACGTCCGCGAGAGTTGGAATCGG TTTCGTTCCAGCTCGTGTTACG
rev_3	ACAGTTATGCACCGCAAGAC	CCTAAGAAGGACAGCACGTCCGTTACCATTTCTCAGTC TTTCGTTCCAGCTCGTGTTACG
rev_4	ACGCCTCTTAAGCTACCATG	CCTAAGAAGGACAGCACGTCTTGCAGGTCATAACT GTTTCGTTCCAGCTCGTGTTACG
rev_5	AGGAATTGCCAGTGTGGTCC	CCTAAGAAGGACAGCACGTCCATGGTAGCTTAAGAGGC GTTTCGTTCCAGCTCGTGTTACG
rev_6	CCACCTTGTTGGTTGTTGTT	CCTAAGAAGGACAGCACGTCCGACCACACTGGCAATTC CTTCGTTCCAGCTCGTGTTACG

Table S5. Addgene plasmid identifiers for the plasmids used in our simulated contamination experiments.

addgene_id	Used_as_standard
1015	-
27340	-
47504	-
52535	-
32751	-
12298	-
10953	-
71720	-
14080	-
64073	-
48140	-
18110	-
104991	-
11427	-
14723	-
32515	-
45161	-
21232	-
52925	-
8454	-
24595	-
12260	-
8381	-
50473	-
68122	-
37120	-
31485	-
45605	-
21915	-
47327	-
20737	-
11908	-
16542	-
57822	-
61463	-
10808	-
10878	-
29435	-
66801	-

31815	TRUE
47549	-
48139	-
16337	TRUE
36412	-
49172	-
12251	TRUE
35175	-
59946	-
12301	-
26722	-
43796	-
52963	-
62988	-
31367	-
40729	-
66810	-
47443	-
87360	-
14129	-
36325	-
73501	-
36939	-
12253	-
70219	-
60415	-
20960	-
52961	TRUE
26678	-
26646	-
81070	-
22945	-
39196	-
75127	-
51692	-
65974	-
12259	-
11181	-
27052	-
49792	TRUE
80925	-
110060	-
14873	-
14436	-
48138	-
8449	-
63890	-
35617	-
42230	-

1864	-
83467	-
40342	-
1654	-
11916	-
52213	-
30313	-
107177	-