Experimental

Materials: A single strand M13mp18 (catalog number: N4040S) was purchased from a commercial source. All unmodified and biotin-labeled staple strands were purchased commercially, and used without further purification. The streptavidin modified Qdot solution and all other chemicals were purchased commercially. 1x TAE/Mg^{2+} buffer contains 40 mmol L^{-1} (mM) tris(hydroxymethyl)aminomethane (tris), 20 mmol L^{-1} (mM) acetic acid, 2 mmol L^{-1} (mM) ethylenediaminetetraaceticacid (EDTA), and 12.5 mmol L^{-1} (mM) magnesium acetate, pH 8.0 and 0.5 x TBE buffer has 45 mmol L^{-1} (mM) tris(hydroxymethyl)aminomethane (tris), 45 mmol L^{-1} (mM) Boric acid, and 1 mmol L^{-1} (mM) ethylenediaminetetraaceticacid (EDTA).

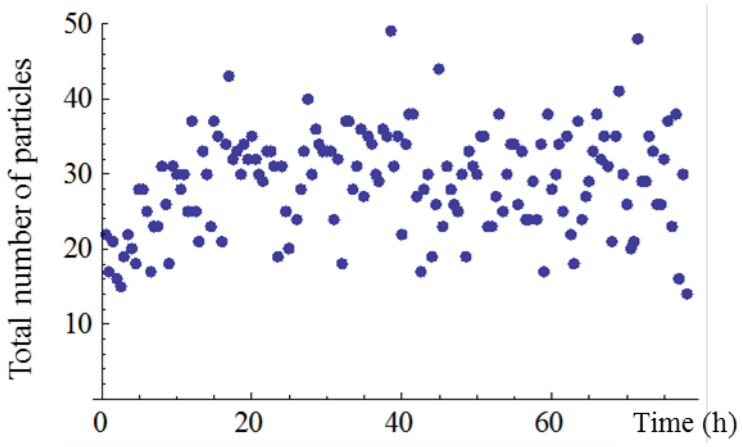
Self-assembly of DNA origami: Rectangular DNA origami was assembled according to the previous work.[1] A long single strand of M13mp18 and stapler strands were mixed at molar ratio of 1:5 in 1x TAE/Mg^{2+} buffer and slowly annealed at 1°C min^{-1} from 95°C to room temperature using a DNA thermal cycler. Excess staple strands were removed by washing four to five times with 1x TAE/Mg^{2+} buffer (400 μL) in a "100 000 molecular weight cutoff filter" (100 kDa MWCO) centrifuge filter at 3500 x g for 2 min in a microcentrifuge. To avoid stacking of origami along vertical edges, staple strands on vertical edges have been omitted.

Preparation and gel purification of Qdot-DNA origami conjugates as calibration samples: DNA origami solution (10 nmol L^{-1}, 20mL) was mixed with Qdot solution at 10 x molar ratio to the binding locations on an origami and incubated overnight at room temperature. Qdot-DNA origami conjugates were purified by 1 % agarose gel electrophoresis (running buffer 0.5 x TBE, loading buffer 50 % glycerol, and 9 V/cm). After the band of the conjugates was sliced, the conjugates were recovered by using either a dialysis membrane tube or a gel extraction spin column. In the dialysis tube case, gel pieces were sealed into a tube filled with 1 x TAE/Mg^{2+} buffer and the conjugates were electrically eluted from the gel piece into 1 x TAE/Mg^{2+} buffer. To use the gel in a spin column, the gel pieces were crushed, transferred into a column, and centrifuged for 3 min at 3000 g.

SEM characterization of gel-purified Qdot-DNA origami conjugates: A silicon wafer was treated with oxygen plasma before SEM imaging. The solution of Qdot-DNA origami (5 μL) was loaded on the wafer, left for 5 min, and washed with water. The sample was imaged by SEM using beam landing energies from 0.3 kV to1.0 kV.

Single molecule tracking measurements: The 3D real-time feedback tracking system is described in detail in our previous work.[2] The basic idea is that a focused excitation laser (532 nm) is scanned in a small (submicrometer) periodic pattern, while fluorescence photons are collected and demodulated in real-time to derive a feedback signal suitable for closed-loop tracking with a 3D piezoelectric stage. In order to measure the lifetime of Qdot, an additional modification of the system is made in the detection part, where the fluorescence signal is split by a 50/50 nonpolarized beamsplitter and detected by a pair of photon-counting avalanche photodiodes. The photon arrival times are recorded by a single photon-counting module with 4 ps timing resolution. Photon correlation curves are computed from photon arrival times after the measurements.

The optimum increment of time for tracking a particle to keep it localized at a particular position depends on the particles size and hence on its diffusion coefficient D. During a given tracking time increment δt the particle will diffuse, on average, a distance $(D\delta t)^{1/2}$ away from its initial position. On the other hand the precision with which the particle can be localized improves with increasing tracking time as $(\delta t)^{-1/2}$. It follows from this that a given tracking time increment δt is not optimum for all particles. In the current case the tracking time increment was optimized for the Qdot-Origami bound particles, not for the free Qdot particles which results in undercounting the number of free Qdots present. This effect can be seen in the total number of counted particles in the graph below. Fewer total particles are counted initially when they are predominantly free Qdots. This also has the effect of disconnecting the particle counts of the various species from the actual concentration of each species.



Graph showing total number of particles captured by the particle tracking system as a function of time over the course of a typical experiment. In the initial stages the number of tracked objects is lower than later, illustrating the under-counting that occurs for fast moving objects (free Qdots) versus slow moving objects (*n*Qdot-origami).

Errors in the estimate of the diffusion coefficient, D: There is currently no complete analysis of the error involved in estimating the diffusion coefficient in a particle tracking system. Here we follow the analysis of Michalet and Berglund,[4] who developed an error estimation method for camera-based particle tracking systems. This model takes into account both the static localization precision and the effect of diffusion occuring between particle position estimations. The static localization precision, σ, is given by the size of the beam spot, d (≈ 1 μm), divided by the standard deviation in the photon counts used to determine the position, that is the count rate (≈ 50 kHz) multiplied by the integration time of each sample, Δt (≈ 10 ms), to yield ≈ 45 nm. The dynamic localization precision is determined by the distance a particle moves during the sample integration time, and is given by $(2D\Delta t)^{1/2}$, which for D = 1 $\mu m^2 s^{-1}$ gives an uncertainty of ≈ 140 nm. The static and dynamic localization precisions are thus approximately equal and an estimate of S_D/D = 0.3 may be derived from the plot of S(D)/D versus $\sigma^2/D\Delta t$, Fig. 2 b in ref. [4], using the number of trajectory points, N = 100 (i.e. 1 s/10 ms).

Preparation of sample cell: The sample cell is made of two glass coverslips with approximately 100 μm spacing. Prior to use, the sample cell is pretreated with blocking solution to prevent the binding of Qdots to glass surface. The solution of DNA (glycerol/≈ 9 $mmoleL^{-1}$ (mM) Mg^{2+}) and Qdot (glycerol/≈ 9 $mmoleL^{-1}$ (mM) Mg^{2+}) are separately prepared and mixed together for the measurement. In order to make the mixed solution homogeneous, the solution was pipetted

vigorously before the measurement. After loading a small volume (20 μL) of origami solution, the sample cell is sealed with epoxy to prevent evaporation.

References

1. P.W. K. Rothemund, *Nature* **2006**, *440*, 297-302.
2. K. Du, J. A. Liddle, A. J. Berglund*, Langmuir* **2012**, *28*, 9181–9188.
3. B. Ding, Z. Deng, H. Yan, S. Cabrini, R. N. Zuckermann, J. Bokor, *J. Am. Chem. Soc.* **2010**, *132*, 3248.
4. X. Michalet, A. J. Berglund, Phys. Rev. E **2012**, *85*, 061916-1 - 061916-14

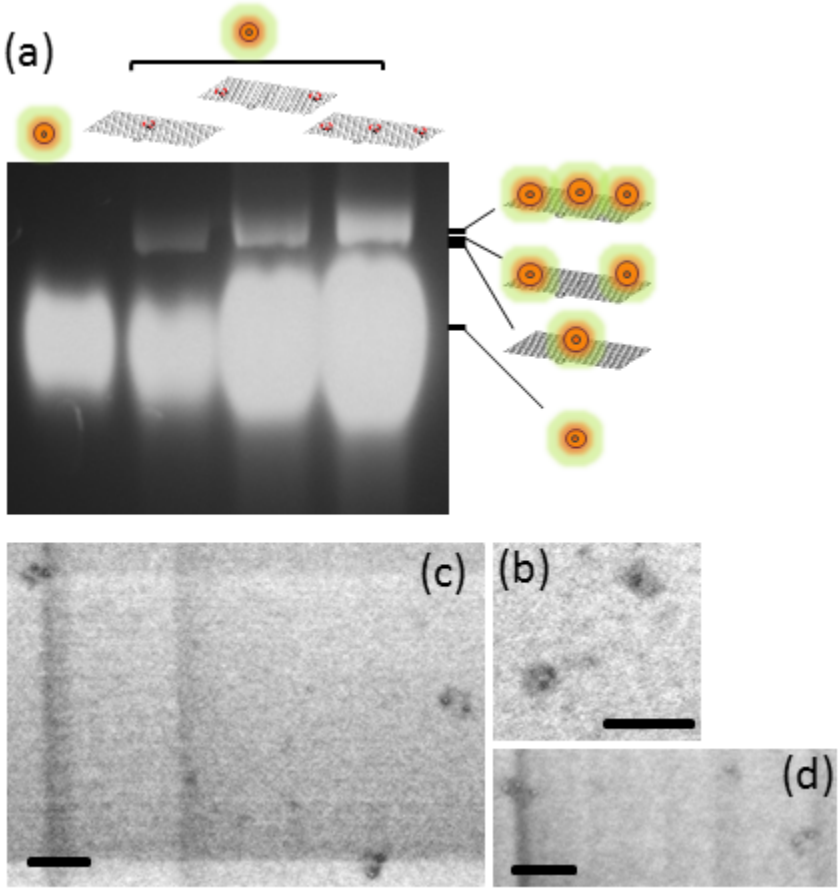


Figure S1. (a) Agarose gel analysis of various DNA origami-Qdot conjugates. The gel has not been stained but exposed to UV light showing only Qdots and Qdot conjugates. This gel without staining has been used for purification of origami-Qdot conjugates. (b)-(d) SEM images of various conjugates of DNA origami with one Qdot (b), two Qdots (c), and three Qdots (d). The conjugates has been separated and extracted from agaorse gel bands. The rectangular DNA origami is darker than background (Scale bar: 200 nm). Consistent with previous work,[3] we note that the gel does not enable the different conjugates to be separated, even though we use a higher concentration (1.5 %) of gel. However, with the multichannel detection, that the particle tracking enable we could clearly distinguish these different species. The SEM images show the typically low densities of the desired product obtained after extracing from gel pieces. The single molecular tracking system does not require high concetration, and the low overall yield of the gel-extracted product did not preclude measurement.

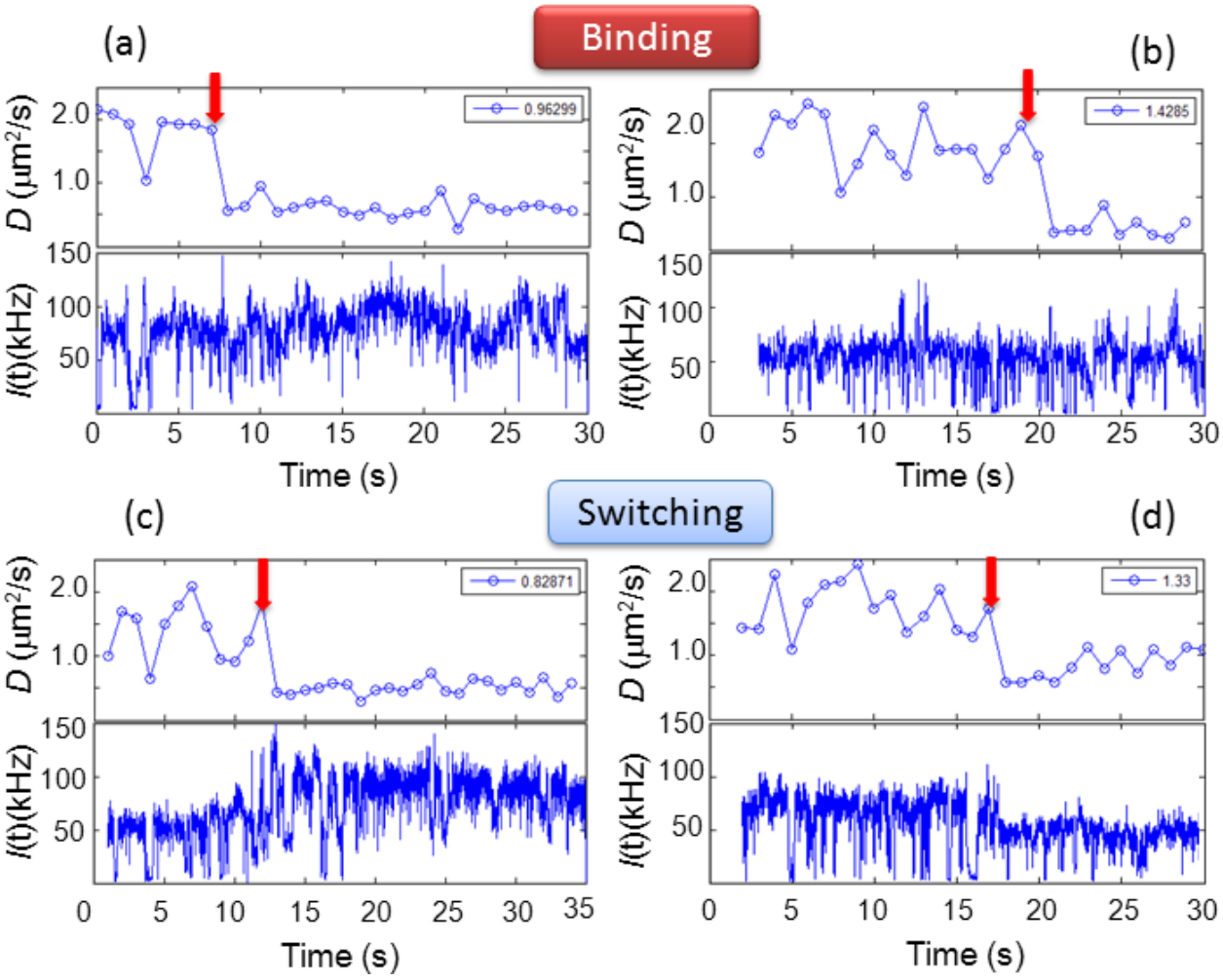


Figure S2. Representative data sets showing binding (a-b) and switching (c-d) events by measuring the diffusion coefficient and fluorescent intensity as a function of time for a tracked object. When a binding event occurs, the diffusion coefficient drops while the fluorescent intensity remains constant. In case of switching events (infrequently observed) the diffusion coefficient changes, but so does the average fluorescent intensity, indicating that a different emitter is being tracked.

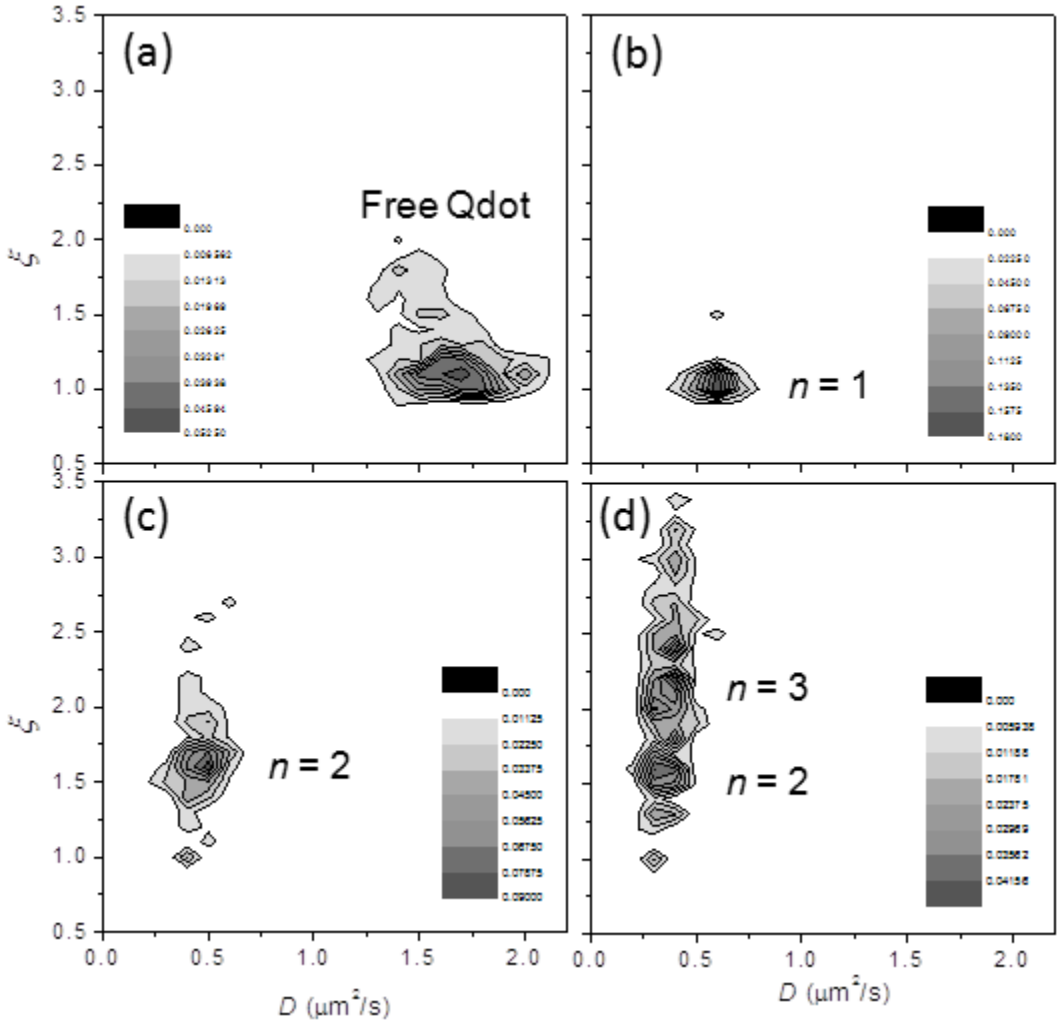


Figure S3. Combined distribution plot of ξ and D of agarose gel purified samples including (a) free Qdot, (b)1Qdot-DNA origami, (c) 2Qdot-origami and (d) 3Qdot-origami. A comparison of (c) and (d) shows that the nominal 3Qdot-origami sample contains a significant fraction of 2Qdot-origami, but identification of the difference species can be clearly made using the combined ξ and D plot.

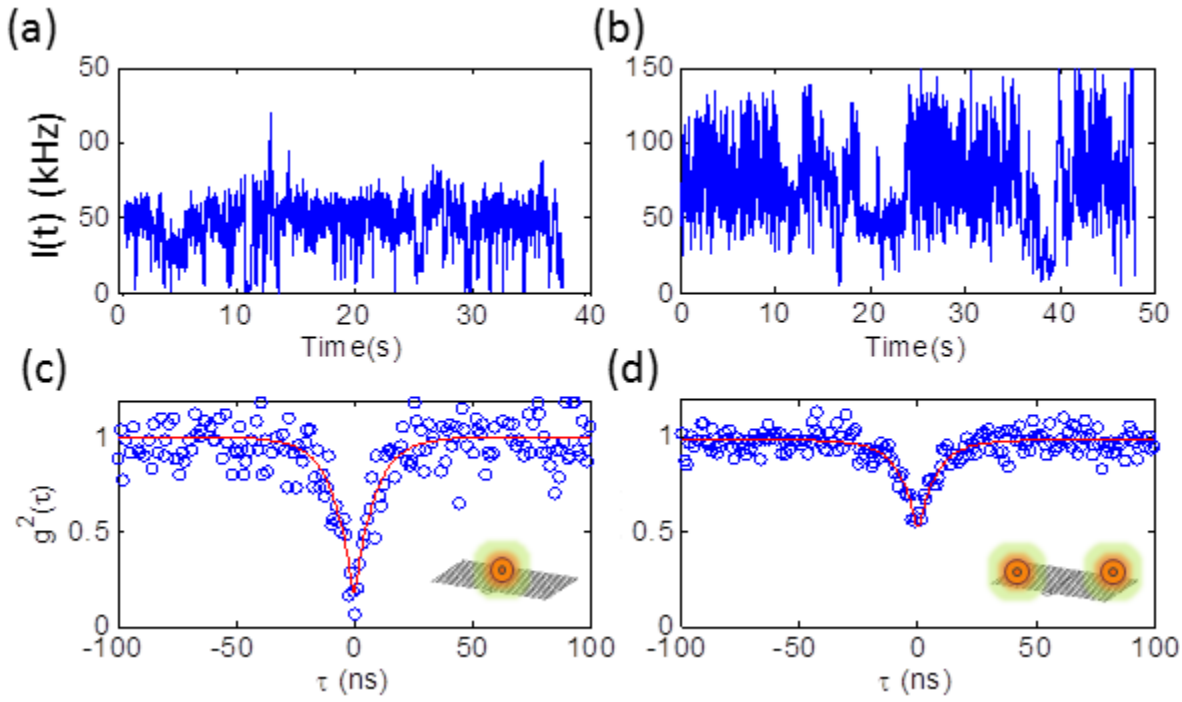


Figure S4. Typical fluorescence intensity measurements of (a) 1 Qdot-DNA origami and (b) 2 Qdot-DNA origami, which are tracked by the 3D real-time tracking apparatus. Second-order photon correlation function plots show (c) $g^2(\tau=0) \approx 0$ and (d) $g^2(\tau=0) \approx 0.5$, which clearly indicates the number of Qdots bound on the origami. These measurements provide an additional means of quantifying the number of bound species.

Sequence of single stranded M13mp18 can be found at the website of New England Biolab.
http://www.neb.com/nebecomm/tech_reference/restriction_enzyme/sequences/m13mp18.txt

Sequences of biotin labeled staple strands

For preparing biotinylated DNA origami, specific staple strands were replaced by 5'-biotin labeled strands. The sequences of biotin labeled strands have the same sequences as the corresponding unmodified staple strands and have an extra spacer of four thymines close to the 5'-biotin labeling. The following strands are for preparation of triple biotinylated DNA origami for different binding sites.

1. One binding site
   121, 122, 123

2. Two binding sites
   30, 31, 32, 160, 161, 162

3. Three binding sites
   30, 31, 32, 121, 122, 123, 160, 161, 162

Table S1. Sequences of the unmodified staple strands

Name	Sequence (5' → 3')
1	CAAGCCCAATAGGAAC CCATGTACAAACAGTT
2	AATGCCCCGTAACAGT GCCCGTATCTCCCTCA
3	TGCCTTGACTGCCTAT TTCGGAACAGGGATAG
4	GAGCCGCCCCACCACC GGAACCGCGACGGAAA
5	AACCAGAGACCCTCAG AACCGCCAGGGGTCAG
6	TTATTCATAGGGAAGG TAAATATT CATTCAGT
7	CATAACCCGAGGCATA GTAAGAGC TTTTTAAG
8	ATTGAGGGTAAAGGTG AATTATCAATCACCGG
9	AAAAGTAATATCTTAC CGAAGCCCTTCCAGAG
10	GCAATAGCGCAGATAG CCGAACAATTCAACCG
11	CCTAATTTACGCTAAC GAGCGTCTAATCAATA
12	TCTTACCAGCCAGTTA CAAAATAAATGAAATA
13	ATCGGCTGCGAGCATG TAGAAACCTATCATAT
14	CTAATTTATCTTTCCT TATCATTCATCCTGAA
15	GCGTTATAGAAAAAGC CTGTTTAG AAGGCCGG
16	GCTCATTTTCGCATTA AATTTTTG AGCTTAGA
17	AATTACTACAAATTCT TACCAGTAATCCCATC
18	TTAAGACGTTGAAAAC ATAGCGATAACAGTAC
19	TAGAATCCCTGAGAAG AGTCAATAGGAATCAT

20	CTTTTACACAGATGAA TATACAGTAAACAATT
21	TTTAACGTTCGGGAGA AACAATAATTTTCCCT
22	CGACAACTAAGTATTA GACTTTACAATACCGA
23	GGATTTAGCGTATTAA ATCCTTTGTTTTCAGG
24	ACGAACCAAAACATCG CCATTAAA TGGTGGTT
25	GAACGTGGCGAGAAAG GAAGGGAA CAAACTAT
26	TAGCCCTACCAGCAGA AGATAAAAACATTTGA
27	CGGCCTTGCTGGTAAT ATCCAGAACGAACTGA
28	CTCAGAGCCACCACCC TCATTTTCCTATTATT
29	CTGAAACAGGTAATAA GTTTTAACCCCTCAGA
30	AGTGTACTTGAAAGTA TTAAGAGGCCGCCACC
31	GCCACCACTCTTTTCA TAATCAAACCGTCACC
32	GTTTGCCACCTCAGAG CCGCCACCGATACAGG
33	GACTTGAGAGACAAAA GGGCGACAAGTTACCA
34	AGCGCCAACCATTTGG GAATTAGATTATTAGC
35	GAAGGAAAATAAGAGC AAGAAACAACAGCCAT
36	GCCCAATACCGAGGAA ACGCAATAGGTTTACC
37	ATTATTTAACCCAGCT ACAATTTTCAAGAACG
38	TATTTTGCTCCCAATC CAAATAAGTGAGTTAA
39	GGTATTAAGAACAAGA AAAATAATTAAAGCCA
40	TAAGTCCTACCAAGTA CCGCACTCTTAGTTGC
41	ACGCTCAAAATAAGAA TAAACACCGTGAATTT
42	AGGCGTTACAGTAGGG CTTAATTGACAATAGA
43	ATCAAAATCGTCGCTA TTAATTAACGGATTCG
44	CTGTAAATCATAGGTC TGAGAGACGATAAATA
45	CCTGATTGAAAGAAAT TGCGTAGACCCGAACG
46	ACAGAAATCTTTGAAT ACCAAGTTCCTTGCTT
47	TTATTAATGCCGTCAA TAGATAATCAGAGGTG
48	AGATTAGATTTAAAAG TTTGAGTACACGTAAA
49	AGGCGGTCATTAGTCT TTAATGCGCAATATTA
50	GAATGGCTAGTATTAA CACCGCCTCAACTAAT
51	CCGCCAGCCATTGCAA CAGGAAAAATATTTTT
52	CCCTCAGAACCGCCAC CCTCAGAACTGAGACT
53	CCTCAAGAATACATGG CTTTTGATAGAACCAC
54	TAAGCGTCGAAGGATT AGGATTAGTACCGCCA
55	CACCAGAGTTCGGTCA TAGCCCCCGCCAGCAA
56	TCGGCATTCCGCCGCC AGCATTGACGTTCCAG
57	AATCACCAAATAGAAA ATTCATATATAACGGA
58	TCACAATCGTAGCACC ATTACCATCGTTTTCA
59	ATACCCAAGATAACCC ACAAGAATAAACGATT
60	ATCAGAGAAAGAACTG GCATGATTTTATTTTG
61	TTTTGTTTAAGCCTTA AATCAAGAATCGAGAA
62	AGGTTTTGAACGTCAA AAATGAAAGCGCTAAT
63	CAAGCAAGACGCGCCT GTTTATCAAGAATCGC
64	AATGCAGACCGTTTTT ATTTTCATCTTGCGGG
65	CATATTTAGAAATACC GACCGTGTTACCTTTT
66	AATGGTTTACAACGCC AACATGTAGTTCAGCT

67	TAACCTCCATATGTGA GTGAATAAACAAAATC
68	AAATCAATGGCTTAGG TTGGGTTACTAAATTT
69	GCGCAGAGATATCAAA ATTATTTGACATTATC
70	AACCTACCGCGAATTA TTCATTTCCAGTACAT
71	ATTTTGCGTCTTTAGG AGCACTAAGCAACAGT
72	CTAAAATAGAACAAAG AAACCACCAGGGTTAG
73	GCCACGCTATACGTGG CACAGACAACGCTCAT
74	GCGTAAGAGAGAGCCA GCAGCAAAAAGGTTAT
75	GGAAATACCTACATTT TGACGCTCACCTGAAA
76	TATCACCGTACTCAGG AGGTTTAGCGGGGTTT
77	TGCTCAGTCAGTCTCT GAATTTACCAGGAGGT
78	GGAAAGCGACCAGGCG GATAAGTGAATAGGTG
79	TGAGGCAGGCGTCAGA CTGTAGCGTAGCAAGG
80	TGCCTTTAGTCAGACG ATTGGCCTGCCAGAAT
81	CCGGAAACACACCACG GAATAAGTAAGACTCC
82	ACGCAAAGGTCACCAA TGAAACCAATCAAGTT
83	TTATTACGGTCAGAGG GTAATTGAATAGCAGC
84	TGAACAAACAGTATGT TAGCAAACTAAAAGAA
85	CTTTACAGTTAGCGAA CCTCCCGACGTAGGAA
86	GAGGCGTTAGAGAATA ACATAAAAGAACACCC
87	TCATTACCCGACAATA AACAACATATTTAGGC
88	CCAGACGAGCGCCCAA TAGCAAGCAAGAACGC
89	AGAGGCATAATTTCAT CTTCTGACTATAACTA
90	TTTTAGTTTTTCGAGC CAGTAATAAATTCTGT
91	TATGTAAACCTTTTTT AATGGAAAAATTACCT
92	TTGAATTATGCTGATG CAAATCCACAAATATA
93	GAGCAAAAACTTCTGA ATAATGGAAGAAGGAG
94	TGGATTATGAAGATGA TGAAACAAAATTTCAT
95	CGGAATTATTGAAAGG AATTGAGGTGAAAAAT
96	ATCAACAGTCATCATA TTCCTGATTGATTGTT
97	CTAAAGCAAGATAGAA CCCTTCTGAATCGTCT
98	GCCAACAGTCACCTTG CTGAACCTGTTGGCAA
99	GAAATGGATTATTTAC ATTGGCAGACATTCTG
100	TTTT TATAAGTA TAGCCCGGCCGTCGAG
101	AGGGTTGA TTTT ATAAATCC TCATTAAATGATATTC
102	ACAAACAA TTTT AATCAGTA GCGACAGATCGATAGC
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104	TACATACA TTTT GACGGGAG AATTAACTACAGGGAA
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108	ACAAAGAA TTTT ATTAATTA CATTTAACACATCAAG
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110	GATGGCAA TTTT AATCAATA TCTGGTCACAAATATC
111	AAACCCTC TTTT ACCAGTAA TAAAAGGGATTCACCA GTCACACGTTTT
112	CCGAAATCCGAAAATC CTGTTTGAAGCCGGAA
113	CCAGCAGGGGCAAAAT CCCTTATAAAGCCGGC

114	GCATAAAGTTCCACAC AACATACGAAGCGCCA
115	GCTCACAATGTAAAGC CTGGGGTGGGTTTGCC
116	TTCGCCATTGCCGGAA ACCAGGCATTAAATCA
117	GCTTCTGGTCAGGCTG CGCAACTGTGTTATCC
118	GTTAAAATTTTAACCA ATAGGAACCCGGCACC
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122	TCGCAAATGGGGCGCG AGCTGAAATAATGTGT
123	TTTTAATTGCCCGAAA GACTTCAAAACACTAT
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125	GGAATTACTCGTTTAC CAGACGACAAAAGATT
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127	CCAAATCACTTGCCCT GACGAGAACGCCAAAA
128	CTCATCTTGAGGCAAA AGAATACAGTGAATTT
129	AAACGAAATGACCCCC AGCGATTATTCATTAC
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132	TGAGTTTCGTCACCAG TACAAACTTAATTGTA
133	CCCCGATTTAGAGCTT GACGGGGAAATCAAAA
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135	GAGTTGCACGAGATAG GGTTGAGTAAGGGAGC
136	GTGAGCTAGTTTCCTG TGTGAAATTTGGGAAG
137	TCATAGCTACTCACAT TAATTGCGCCCTGAGA
138	GGCGATCGCACTCCAG CCAGCTTTGCCATCAA
139	GAAGATCGGTGCGGGC CTCTTCGCAATCATGG
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216	CAGCGAAAA TTTT ACTTTCA ACAGTTTCTGGGATTT TGCTAAAC TTTT
Loop1	AACATCACTTGCCTGAGTAGAAGAACT
Loop2	TGTAGCAATACTTCTTTGATTAGTAAT
Loop3	AGTCTGTCCATCACGCAAATTAACCGT
Loop4	ATAATCAGTGAGGCCACCGAGTAAAAG
Loop5	ACGCCAGAATCCTGAGAAGTGTTTTT
Loop6	TTAAAGGGATTTTAGACAGGAACGGT
Loop7	AGAGCGGGAGCTAAACAGGAGGCCGA
Loop8	TATAACGTGCTTTCCTCGTTAGAATC
Loop9	GTACTATGGTTGCTTTGACGAGCACG
Loop10	GCGCTTAATGCGCCGCTACAGGGCGC