

Droplet Digital Polymerase Chain Reaction (PCR) Outperforms Real-Time PCR in the Detection of Environmental DNA from an Invasive Fish Species

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Testing for PCR cycle number for ddPCR

We preliminary tested 3 PCR protocols for ddPCR; (1) 40 cycles: PCR conditions were 10 min at 95°C, 40 cycles of denaturation for 30 s at 95°C and extension for 60 s at 60°C with ramp rate of 2.5°C s⁻¹, followed by 10 min at 95°C and a hold at 4°C. (2) 45 cycles: PCR with 45 cycles of denaturation for 30 s at 95°C and extension for 60 s at 60°C in the same matter. (3) 55 cycles: PCR with 55 cycles of denaturation for 30 s at 95°C and extension for 60 s at 60°C in the same matter.

By our preliminary test using 8 pond samples (Table S1-2), the PCR with 40 cycles only detected DNA from 4 of 8 ponds, which was less than those by the PCRs with 45 and 55 cycles (6/8 ponds for both PCRs, Table S2). We performed Fisher's exact test for the detection rates and found no significant difference ($p = 0.14$) due to small sample size ($N = 24$). Although the detection rates at 45 and 55 cycles were similar, Bio-Rad recommended us to use the PCR cycle number less than 45 to avoid the decrease of droplets due to evaporation by heating and the non-specific amplification of non-target DNA. Takahara et al. (2013) and our study found that the maximum Ct values of real-time PCR that detected the amplification of target DNA was 40.8 using GEMM and EMM. Thus, in this study, we decided to perform PCR with 40 cycles.

Table S1 Detection rate (#/8 replicates) of bluegill eDNA from the pond samples using ddPCR with different PCR cycles.

Pond number	PCR cycles		
	40	45	55
10	0	1	0
15	0	2	1
19	0	0	0
22	0	0	1
24	3	3	3
25	1	2	1
49	1	2	1
65	3	3	3

Table S2 Number of detected/undetected ponds of bluegill eDNA using ddPCR with different PCR cycles.

	PCR cycles		
	40	45	55
Detected	4	6	6
Undetected	4	2	2

Table S3 Detection rate (#/8 replicates) of bluegill eDNA from the pond samples using real-time PCR with GEMM, EMM and ddPCR.

Pond number	real-time PCR with GEMM	real-time PCR with EMM	ddPCR
30	0	0	0
33	0	0	0
24	0	0	1
70	0	1	1
55	0	0	2
10	1	1	1
19	1	0	1
39	1	1	2
15	1	2	3
66	1	1	3
47	4	4	3
26	5	5	3
63	5	6	4
23	7	8	4
25	8	8	6
50	8	6	6
5	8	8	8
22	8	8	8
42	8	8	8
44	8	8	8
49	8	8	8
62	8	8	8
64	8	8	8
65	8	8	8

Table S4 All droplet data for all samples measured in this study using 45 PCR cycles. Lambda value was calculated by $-\ln(1-(k/n))$, where k and n is positive and total droplets, respectively.

Sample	Positives	Negatives	Accepted Droplets	Lambda
DNAspiking-5copies	2	12308	12310	0.000162483
DNAspiking-5copies	3	11412	11415	0.000263
DNAspiking-5copies	1	10634	10635	9.40336E-05
DNAspiking-5copies	0	12252	12252	0
DNAspiking-5copies	4	8814	8818	0.000453721
DNAspiking-5copies	2	8909	8911	0.000224467
DNAspiking-5copies	2	11206	11208	0.00017846
DNAspiking-5copies	1	10511	10512	9.51339E-05
DNAspiking-0copies	0	9150	9150	0
DNAspiking-0copies	0	9376	9376	0
DNAspiking-0copies	0	13013	13013	0
DNAspiking-0copies	0	13111	13111	0
DNAspiking-0copies	0	13214	13214	0
DNAspiking-0copies	0	14037	14037	0
DNAspiking-0copies	0	14731	14731	0
DNAspiking-0copies	0	15053	15053	0
DNAspiking-10copies	6	11065	11071	0.000542103
DNAspiking-10copies	2	11964	11966	0.000167154
DNAspiking-10copies	11	12078	12089	0.000910332
DNAspiking-10copies	3	12916	12919	0.000232243
DNAspiking-10copies	7	13139	13146	0.000532623
DNAspiking-10copies	2	13197	13199	0.000151538
DNAspiking-10copies	9	13487	13496	0.000667087
DNAspiking-10copies	6	13940	13946	0.000430323
DNAspiking-1copies	0	9880	9880	0
DNAspiking-1copies	0	10967	10967	0
DNAspiking-1copies	0	11295	11295	0
DNAspiking-1copies	0	11504	11504	0
DNAspiking-1copies	2	11678	11680	0.000171248
DNAspiking-1copies	0	11986	11986	0
DNAspiking-1copies	0	12386	12386	0
DNAspiking-1copies	0	13316	13316	0
DNAspiking-2copies	3	8895	8898	0.000337211
DNAspiking-2copies	1	9622	9623	0.000103923
DNAspiking-2copies	0	9851	9851	0
DNAspiking-2copies	0	10671	10671	0
DNAspiking-2copies	1	10729	10730	9.3201E-05
DNAspiking-2copies	1	11136	11137	8.97948E-05
DNAspiking-2copies	2	11758	11760	0.000170082
DNAspiking-2copies	0	12312	12312	0

LOD-0copies	0	13743	13743	0
LOD-0copies	0	12894	12894	0
LOD-0copies	0	12911	12911	0
LOD-0copies	0	13255	13255	0
LOD-0copies	0	11104	11104	0
LOD-0copies	0	8566	8566	0
LOD-0copies	0	8778	8778	0
LOD-0copies	0	9080	9080	0
LOD-10copies	2	9143	9145	0.000218723
LOD-10copies	0	9526	9526	0
LOD-10copies	2	9561	9563	0.000209161
LOD-10copies	4	10068	10072	0.000397219
LOD-10copies	2	10550	10552	0.000189555
LOD-10copies	1	10602	10603	9.43174E-05
LOD-10copies	0	10770	10770	0
LOD-10copies	1	10968	10969	9.11702E-05
LOD-2copies	0	9704	9704	0
LOD-2copies	0	10044	10044	0
LOD-2copies	0	10352	10352	0
LOD-2copies	0	10808	10808	0
LOD-2copies	0	11051	11051	0
LOD-2copies	1	11479	11480	8.71118E-05
LOD-2copies	1	11982	11983	8.3455E-05
LOD-2copies	0	13206	13206	0
LOD-5copies	1	9865	9866	0.000101363
LOD-5copies	0	10593	10593	0
LOD-5copies	0	11073	11073	0
LOD-5copies	0	11181	11181	0
LOD-5copies	0	11511	11511	0
LOD-5copies	1	12044	12045	8.30254E-05
LOD-5copies	1	12365	12366	8.08702E-05
LOD-5copies	0	12463	12463	0
LOD-1copies	0	11934	11934	0
LOD-1copies	2	12038	12038	0.000171248
LOD-1copies	0	12145	12145	0
LOD-1copies	0	12766	12766	0
LOD-1copies	0	13023	13023	0
LOD-1copies	0	13368	13368	0
LOD-1copies	0	13453	13453	0
LOD-1copies	0	13909	13909	0
P10	0	7653	7653	0
P10	0	7779	7779	0
P10	1	8432	8433	0.000118589
P10	0	9322	9322	0

P10	0	9610	9610	0
P10	0	9627	9627	0
P10	0	9848	9848	0
P10	0	10276	10276	0
P15	1	8998	8999	0.00011113
P15	0	9116	9116	0
P15	0	9455	9455	0
P15	0	10063	10063	0
P15	0	10219	10219	0
P15	0	10507	10507	0
P15	1	11386	11387	8.78233E-05
P15	1	11823	11824	8.45773E-05
P19	0	8317	8317	0
P19	0	8406	8406	0
P19	1	8717	8718	0.000114712
P19	0	8848	8848	0
P19	0	9142	9142	0
P19	0	9656	9656	0
P19	0	10436	10436	0
P19	0	10653	10653	0
P22	4	8360	8364	0.000478354
P22	1	9259	9260	0.000107997
P22	6	9404	9410	0.000637823
P22	2	9856	9858	0.000202901
P22	2	9945	9947	0.000201086
P22	7	9988	9995	0.000700596
P22	2	10019	10021	0.000199601
P22	6	10637	10643	0.00056391
P23	0	7377	7377	0
P23	4	7440	7444	0.00053749
P23	0	7528	7528	0
P23	0	7588	7588	0
P23	1	7607	7608	0.000131449
P23	3	7981	7984	0.000375822
P23	0	8649	8649	0
P23	1	8797	8798	0.000113669
P24	0	11240	11240	0
P24	0	11375	11375	0
P24	0	7430	7430	0
P24	0	7460	7460	0
P24	1	7701	7702	0.000129845
P24	0	7964	7964	0
P24	0	8256	8256	0
P24	0	8613	8613	0

P25	1	11181	11182	8.94334E-05
P25	7	7590	7597	0.000921841
P25	0	7674	7674	0
P25	2	7760	7762	0.000257699
P25	1	7948	7949	0.00012581
P25	2	8102	8104	0.000246822
P25	0	8502	8502	0
P25	2	9203	9205	0.000217297
P26	0	7658	7658	0
P26	0	8547	8547	0
P26	2	8775	8777	0.000227894
P26	0	8848	8848	0
P26	0	8921	8921	0
P26	2	9252	9254	0.000216146
P26	1	9633	9634	0.000103804
P26	0	9979	9979	0
P30	0	11884	11884	0
P30	1	12155	12156	8.22673E-05
P30	0	12256	12256	0
P30	0	12426	12426	0
P30	1	12873	12874	7.7679E-05
P30	1	12965	12966	7.71278E-05
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P30	0	13358	13358	0
P33	1	9683	9684	0.000103268
P33	0	10614	10614	0
P33	1	10993	10994	9.09628E-05
P33	0	11090	11090	0
P33	0	11277	11277	0
P33	0	11472	11472	0
P33	0	11695	11695	0
P33	1	11868	11869	8.42566E-05
P39	1	7789	7790	0.000128378
P39	1	8115	8116	0.000123221
P39	0	8504	8504	0
P39	0	8632	8632	0
P39	0	8852	8852	0
P39	0	9311	9311	0
P39	0	9992	9992	0
P39	0	10463	10463	0
P42	5	13476	13481	0.000370961
P42	11	10461	10472	0.001050972
P42	9	14249	14258	0.000631424
P42	7	10537	10544	0.000664105

P42	13	11225	11238	0.001157459
P42	11	10717	10728	0.00102588
P42	9	7335	7344	0.001226242
P42	7	8614	8621	0.000812301
P44	7	14830	14837	0.000471905
P44	10	11152	11162	0.000896298
P44	13	7825	7838	0.001659963
P44	18	9584	9602	0.001876369
P44	12	9554	9566	0.00125523
P44	22	7433	7455	0.002955402
P44	19	7503	7522	0.002529119
P44	8	7684	7692	0.001040583
P47	0	4689	4689	0
P47	11	7335	7346	0.001498536
P47	0	7580	7580	0
P47	1	7770	7771	0.000128692
P47	0	7816	7816	0
P47	1	7852	7853	0.000127348
P47	0	7986	7986	0
P47	0	8643	8643	0
P49	9	12437	12446	0.000723385
P49	12	14804	14816	0.000810263
P49	19	11951	11970	0.001588563
P49	10	11587	11597	0.000862664
P49	14	6559	6573	0.002132197
P49	16	7413	7429	0.002156044
P49	16	8319	8335	0.001921461
P49	26	10057	10083	0.002581928
P5	14	9019	9033	0.001551075
P5	7	9362	9369	0.000747424
P5	13	9641	9654	0.0013475
P5	15	9652	9667	0.001552876
P5	11	9673	9684	0.00113654
P5	9	9857	9866	0.00091264
P5	12	10331	10343	0.001160879
P5	12	11238	11250	0.001067236
P50	4	6116	6120	0.000653808
P50	0	7536	7536	0
P50	4	7610	7614	0.000525486
P50	1	7892	7893	0.000126703
P50	1	8256	8257	0.000121117
P50	2	8277	8279	0.000241604
P50	1	8439	8440	0.00011849
P50	0	8451	8451	0

P55	0	9990	9990	0
P55	0	11074	11074	0
P55	1	11081	11082	9.02405E-05
P55	0	11278	11278	0
P55	0	11601	11601	0
P55	0	11928	11928	0
P55	2	12170	12172	0.000164325
P55	3	13421	13424	0.000223505
P62	4	11977	11981	0.000333918
P62	6	4836	4842	0.001239926
P62	7	13662	13669	0.000512239
P62	49	8578	8627	0.005696034
P62	6	11018	11024	0.000544415
P62	7	7277	7284	0.000961473
P62	2	8029	8031	0.000249066
P62	8	8920	8928	0.000896459
P63	3	10558	10561	0.000284104
P63	31	4437	4468	0.006962409
P63	0	11672	11672	0
P63	4	7541	7545	0.000530293
P63	0	7311	7311	0
P63	0	7635	7635	0
P63	3	8476	8479	0.000353878
P63	0	9156	9156	0
P64	24	11247	11271	0.002131629
P64	20	13537	13557	0.001476342
P64	3	12005	12008	0.000249865
P64	8	7041	7049	0.001135557
P64	2	7462	7464	0.000267989
P64	7	7728	7735	0.000905387
P64	5	7871	7876	0.000635042
P64	6	9185	9191	0.000653026
P65	18	7384	7402	0.002434737
P65	7	7851	7858	0.000891209
P65	15	7894	7909	0.001898374
P65	11	8052	8063	0.001365188
P65	14	8184	8198	0.001709193
P65	1	8209	8210	0.00012181
P65	16	8252	8268	0.001937047
P65	12	8409	8421	0.001426025
P66	0	12285	12285	0
P66	0	11304	11304	0
P66	1	6198	6199	0.000161329
P66	14	10914	10928	0.001281934

P66	0	10819	10819	0
P66	0	12098	12098	0
P66	0	7297	7297	0
P66	1	7379	7380	0.000135511
P70	0	8121	8121	0
P70	0	9154	9154	0
P70	0	9750	9750	0
P70	0	10164	10164	0
P70	0	10336	10336	0
P70	1	10957	10958	9.12617E-05
P70	0	11105	11105	0
P70	0	12227	12227	0

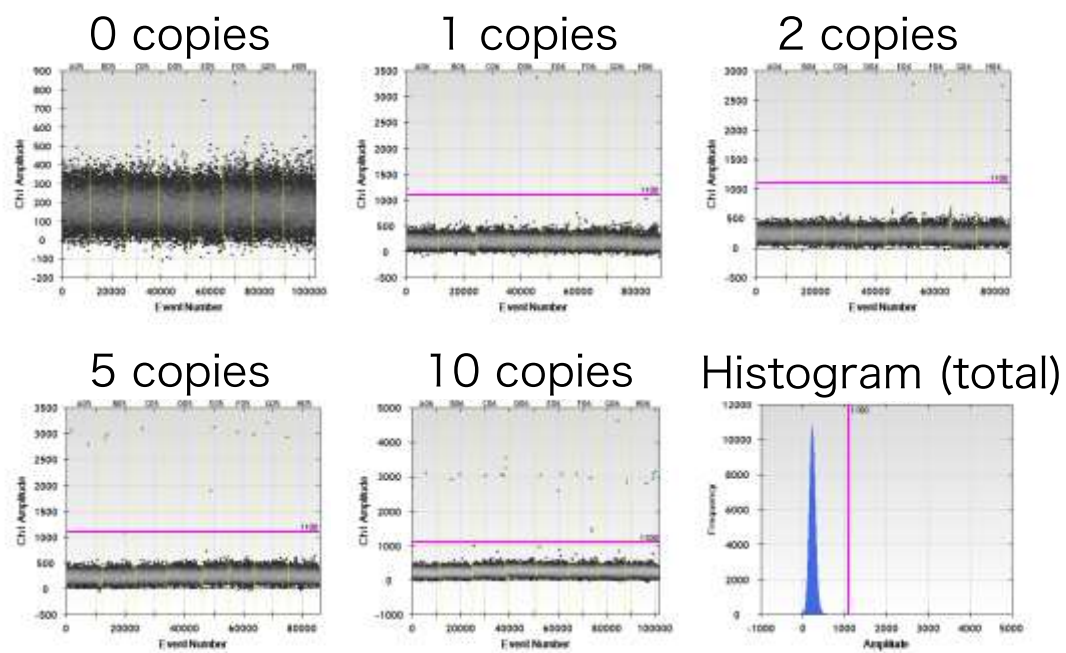


Figure S1 The amplitude (RFU) and histogram plots for DNA spiking experiment of LOD test. The pink line on 1100 RFU indicates the threshold.