Supplementary Information

Single-Molecule Detection of Deoxyribonucleoside Triphosphates in Microdroplets

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Materials and Equipment

Dye-labelled DNA oligos were purchased from ATDbio (Southampton, UK), All other oligos were purchased from Integrated DNA Technologies (Coralville, IA, USA). We use PAGE purified Probe oligos and double HPLC purified Capture and Ligation oligos.

Enzymes were purchased from New England Biolabs (Ipswich, MA, USA), Qiagen Beverly (Enzymatics) (Beverly, MA, USA) or Agilent (Santa Clara, CA, USA).

Spermine was purchased from Abcam (Cambridge, UK), Tetraethylenepentamine (TEPA; Acros, technical. grade) and Ambion nuclease-free water was purchased from Fisher Scientific (Waltham, MA, USA), and the latter was used throughout. All other reagents were purchased from Sigma (St. Louis, MO, USA) at the highest purity available, unless otherwise noted.

384-well PS Microplate were purchased from Greiner Bio-one (Frickenhausen, Germany; REF 784076).

Typical Detection Reaction and Time-Course – Bulk Solution

In a typical, four-color 70 µL (1x) or 175 µL (2.5x) detection reaction, the following reagents were combined in a 1.5 mL tube.

Supplementary Table 1: Composition of a typical 70 µL (1x) or 175 µL (2.5x) four-color detection reaction.

Component	Stock Concentration	Final Concentration	Volume (µL) 1x	Volume (µL) 2.5x
Water	-	-	39.695	99.238
Buffer[1]	5x	1x	14	35
TIPP	2,000 U/mL	66.6 U/mL	2.33	5.825
TEPA	0.3%	0.015%	3.5	8.75
Spermine	100 mM	1 mM	0.7	1.75
Spermidine	100 mM	1 mM	0.7	1.75
Oligo mix[2]	100x	1x	0.7	1.75
***Pfu*Ultra II Fusion HS**	100 Reactions/mL	1.43 Reaction/mL	1	2.5
***Bst* L.F. DNA pol.**	8,000 U/mL	28.57 U/mL	0.25	0.625
***E. coli* ligase**	10,000 U/mL	17.85 U/mL	0.125	0.3125
Total	**-**		63	157.5

[1] see Supplementary Table 8 for further information on the buffer composition
[2] see Supplementary Table 7 for further information on the Oligo Mix

From the 2.5x volume mix, two samples of 63 µL were taken and added to either 7 µL of 250 pM dNTP or 7 µL of water, to give the 25 pM and 0 pM samples, respectfully. Each sample (25 pM or 0 pM) was split into five reactions by adding 14 µL of each sample into 5 separate PCR tubes. The five tubes (for each sample), were incubated in a PCR thermocycler for the required amount of time (Supplementary Table 2).

Supplementary Table 2: Description of the time-course used in typical, four-color detection reaction. The thermocycler program is 10 min @ 37 ºC, followed by 90 min @ 69.5 ºC.

Tube	Sample	Time Point (min)	Temperature (ºC)
1	**25 pM & 0 pM**	**0**	RT
2	**25 pM & 0 pM**	**10**	37
3	**25 pM & 0 pM**	**40**	69.5
4	**25 pM & 0 pM**	**70**	69.5
5	**25 pM & 0 pM**	**100**	69.5

Fluorescence Measurements and Data Analysis – Bulk solution

a. Fluorescent Intensity Measurements of the four color detection reaction

Fluorescence measurements (Fluorescence Intensity = FI) were performed on a Clariostar Plate Reader (BMG LABTECH GmbH, Ortenberg, Germany). The four colors (532 nm, 594 nm, 655 nm, 700 nm) are defined below (Supplementary Table 3).

Supplementary Table 3: Definition of the four colors measured by the Clariostar fluorescent plate reader.

Color	Excitation (nm)	Dichroic (nm)	Emission (nm)	Gain
700 nm	705-10	722.5	785-100	2450
655 nm	643-20	665.2	690-25	2030
594 nm	580-15	603.0	647-57	1707
532 nm	530-12	548.5	570-18	1663

Prior to reading, samples of 10.5 µL were plated onto a 384-well PS Microplate (Greiner Bio-one, Frickenhausen, Germany; 784076). Water was used on the 384 well plate as a blank reference for background correction.

b. Calculation of the Fluorescent Intensity Signal

$$S = FI_{i(25\ pM)} - FI_{i(0\ pM)}$$

Where:-

S = Signal

$FI_{i(25\ pM)}$ = Fluorescent Intensity for the 25 pM sample at time i

$FI_{i(0\ pM)}$ = Fluorescent Intensity for the 0 pM sample at time i

c. Ratio of the Fluorescent Intensity Signal over background

$$R = \frac{FI_{i(25\ pM)} - FI_{i(0\ pM)}}{FI_{i(0\ pM)} - \frac{(FI_{0(25\ pM)} + FI_{0(0\ pM)})}{2}}$$

Where:-

R = Ratio

$FI_{i(25\ pM)}$ = Fluorescent Intensity for the 25 pM sample at time i

$FI_{i(0\ pM)}$ = Fluorescent Intensity for the 0 pM sample at time i

$FI_{0(25\ pM)}$ = Fluorescent Intensity for the 25 pM sample at 0 min

$FI_{0(0\ pM)}$ = Fluorescent Intensity for the 0 pM sample at 0 min

d. Digestion of the Oligo Mix with Exonuclease T and Exonuclease I

To ascertain the maximum amount of signal that can be generated from the labelled probes within a standard reaction mix, the Oligo Mix was digested using Exonuclease T and Exonuclease I (Supplementary Table 4 & 5).

Supplementary Table 4: Reaction mixture for the digestion of the Oligo Mix with Exonuclease T and Exonuclease I, reactions were incubated at 37 ºC for 30 mins.

Component	Stock Concentration	Volume (µL)
Water	-	53.3
Buffer[1]	5x	14
Oligo mix[2]	100x	0.7
Exonuclease T	5,000 U/mL	1
Exonuclease I	20,000 U/mL	1

[1] see Supplementary Table 8 for further information on the buffer composition
[2] see Supplementary Table 7 for further information on the Oligo Mix

Supplementary Table 5: The maximum fluorescent intensity that can be obtained from the four labelled Probe oligos (700 nm, 655 nm, 594 nm and 532 nm) after digestion of the Oligo Mix with Exonuclease T and Exonuclease I.

Digestion	700 nm	655 nm	594 nm	532 nm
Exo T/Exo I	166960	165572	111612	191448
PFu UF	70808	52271	38205	41887
% Digested	42.4	31.6	34.2	21.9

DNA Oligos - Design and Sequences

Nomenclature for non-standard bases and modifications: -

F = dT with corresponding fluorophore attached

Q = dT with corresponding fluorescence quencher attached

/3C6/ = 3'-terminal hexanediol spacer

* = phosphorothioate linker

/5Phos/ = 5'-terminal phosphate group

/3InvdT/ = 3'-terminal 3'-5'-inverted deoxythymidine base

Black Hole Quencher 1 (BHQ1) was incorporated into the oligos using BHQ-1-dT-CE Phosphoramidite. Black Hole Quencher 2 (BHQ2) was incorporated into the oligos using BHQ-2-dT-CE Phosphoramidite and ATTO dyes were incorporated onto an amino modifier C6 dT-phosphoramidite post-synthesis (Supplementary Table 6).

Supplementary Table 6: Description of the Probe, Ligation, Capture, Captured and Target oligos used.

Oligo	Color	Sequence	F/Q
Probe	**700**	5′-CAAGTGACTCGGTCCAGGGCCCAGFFQATTTCAGCACATA-3′	F = ATTO 700 Q = BHQ2
	655	5′-TCGTGCCTCATCGAAACTGACGAGGFFQFFGGTTTGTGGT/3C6/-3′	F = ATTO 655 Q = BHQ2
	594	5′-CTCCAACGGACCCTAACGCACTGGAFFQCTTATGTATTGT-3′	F = ATTO 594 Q = BHQ2
	532	5′-TCACTCATGGAACATCACTCCTCCGFFQTTCCTTTCCAGA/3C6/-3′	F = ATTO 532 Q = BHQ1
LIG	**700**	5′-/5Phos/GGACCGAGTCAGAACTAGATGTAGG/3InvdT/-3	
	655	5′-/5Phos/GTTTCGATGAGGCACGTCTTAGATGTACG/3InvdT/-3′	
	594	5′-/5Phos/GTTAGGGTCCGTTGGAGCTTAGATGTACG/3InvdT/-3′	
	532	5′-/5Phos/TGATGTTCCATGAGTGTCTTAGATGTACG/3InvdT/-3′	
CAP	**700**	5′-TCCGTGAGTAATGTGGTGAAATAAACTGGGCC*C-3′	
	655	5′-TCGCTGAGCCTCCACAAACCAATAACCTCGT*C-3′	
	594	5′-TCGCTGAGCCTCAATACATAAGAAATCCAGT*G-3′	

	532	5′-TCGCTGAGCCACTGGAAAGGAAAAACGGAGG*A-3′	
Captured	**700**	5′-TCCGTGAGTAATGTGGTGAAATAAACTGGGCC*CT-3′	
	655	5′-TCGCTGAGCCTCCACAAACCAATAACCTCGT*CA-3′	
	594	5′-TCGCTGAGCCTCAATACATAAGAAATCCAGT*GC-3′	
	532	5′-TCGCTGAGCCACTGGAAAGGAAAAACGGAGG*AG-3′	
Target	**700**	5′-TCCGTGAGTAATGTGGTGAAATAAACTGGGCC*CTGGACCGAGTCTTAACTAGATGTAGG/3InvdT/-3′	
	655	5′-TCGCTGAGCCTCCACAAACCAATAACCTCT*CAGTTTCGATGAGGCACGTCTTAGATGTACG/3InvdT/-3′	
	594	5′-TCGCTGAGCCTCAATACATAAGAAATCCAGT*GCGTTAGGGTCCGTTGGAGCTTAGATGTACG/3InvdT/-3′	
	532	5′-TCGCTGAGCCACTGGAAAGGAAAAACGGAGG*AGTGATGTTCCATGAGTGTCTTAGATGTACG/3InvdT/-3′	

Choice of Dyes

Dyes were chosen for their photostability, solubility in aqueous media, and, most importantly, compatibility with our enzymes. Multiplexing the fluorophores is achieved by matching the dye excitation wavelengths to laser lines at 532 nm (ATTO 532), 594 nm (ATTO 594), 640 nm (ATTO 655), and 701 nm (ATTO 700). We found ATTO 700 to be superior, with respect to enzymatic compatibility, to the ATTO 488 and other dyes of similar spectral properties that are traditionally used in multiplexing applications.(1, 2) Use of the shorter wavelength dyes resulted in very high false signal due to unusually rapid single-strand digestion by the exonuclease. The dyes were attached to the Probe Oligo using standard postsynthetic protocols (-NH2 – NHS-ester amide chemistry(3)). As quenchers, we found that the quenchers BHQ-1 (for ATTO 532) and BHQ-2 (for ATTO 594, 655 and 700) provided >99% quenching efficiency, thereby guaranteeing a large difference between quenched and unquenched states(4). The quenchers were attached during the solid-state DNA oligo synthesis by using the corresponding quencher-labelled phosphoramidites.

Preparation of the 100x Oligo Mix

For ease of use, all the Probe, Ligation and Capture oligos were pre-mixed, in nuclease-free water, at a 100 times the required final concentration; before addition (of the oligo mix) to the detection reaction (Supplementary Table 7).

Supplementary Table 7: Description of the Probe, Ligation and Capture oligos used for the 100x Oligo Mix.

Oligo	100x Oligo Mix (nM)	Final Oligo Concentration (nM)
Probe, 700 nm	600	6
Probe, 655 nm	600	6
Probe, 594 nm	600	6
Probe, 532 nm	1000	10
LIG, 700 nm	4000	40
LIG, 655 nm	100	10
LIG, 594 nm	1000	10
LIG, 532 nm	2000	20
CAP, 700 nm	50	0.5
CAP, 655 nm	50	0.5
CAP, 594 nm	50	0.5
CAP, 532 nm	50	0.5

Optimisation of the Buffer used in the Detection Reaction

The reaction buffer had to be chosen in a way that would allow all enzymes to work in the same environment. We found that the limiting factor in buffer optimization was *Pfu*Ultra II Fusion HS DNA polymerase, with respect to the specificity of its dsDNA exonuclease-activity. Both *Bst* L.F. DNA polymerase and *E. coli* DNA ligase tolerated a much wider range of buffer conditions. While these two enzymes did not work in the proprietary buffer supplied with *Pfu*Ultra II Fusion HS DNA polymerase, it was found that using a modified version of this buffer made it possible to have all enzymes work in the same buffer (Supplementary Table 8). Optimisation of the Tris-HCl, KCl and $MgCl_2$ components of the buffer are described below (Supplementary Figures 1 and 2).

Supplementary Table 8: Final Concentration of the components that make up the 1x buffer used in a typical, four-color detection reaction.

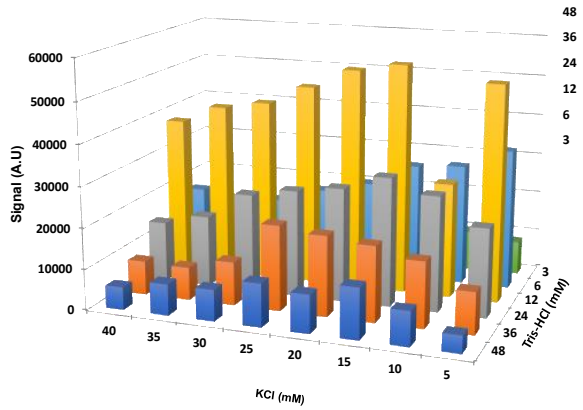
Component	Concentration in 1x buffer	Concentration in 5x buffer stock
Tris-HCl, pH 7.6	12 mM	60 mM
$MgCl_2$	5 mM	25 mM
KCl	20 mM	100 mM
Triton X-100	0.1%	0.5%
DTT	0.5 mM	2.5 mM
NAD	400 nM	2 µM

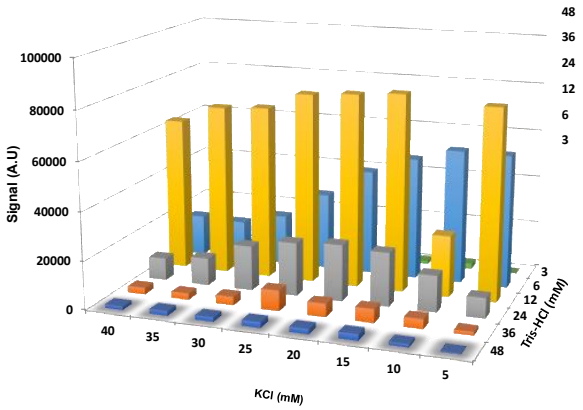
a. Titration of Tris-HCl, pH 7.6 vs. KCl at 5 mM $MgCl_2$

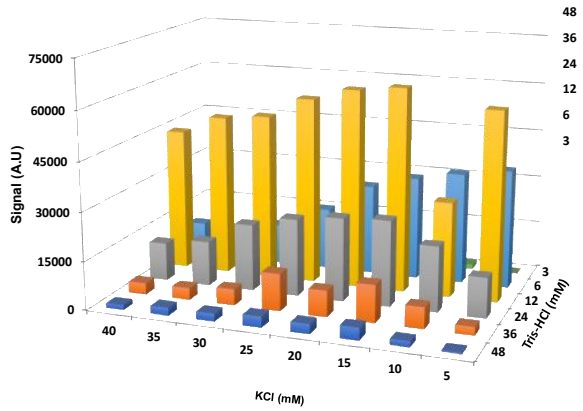
To optimise the Tris-HCl, pH 7.6 and KCl concentrations a cross-titration experiment was preformed, leaving the $MgCl_2$ concentration at 5 mM. The cross-titration showed that concentrations of 12 mM Tris-HCl and 20 mM KCl provided a good operating window for the reaction, which could accommodate slight increases and decreases in salt concentration without adversely affecting the performance of the detection reaction (Supplementary Figure 1). Accommodation of additional salts is desirable as it allows for some tolerance to water loss from microdroplets.

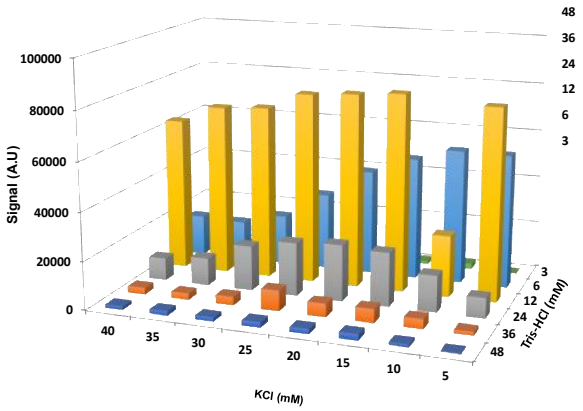
Signal (700 Channel)		Tris-HCl, pH 7.6 (mM)					
		48	36	24	12	6	3
KCl (mM)	40	5596	8380	14838	38219	17597	446
	35	7673	8087	17488	42525	14658	2341
	30	7721	10733	24115	44442	16832	6813
	25	10606	20919	26178	49164	20431	4806
	20	9384	19681	27819	54010	23243	4984
	15	12456	18545	31519	56013	28764	7310
	10	8447	16186	28209	28075	29688	9857
	5	4333	10343	21737	52902	34484	8319

Signal (655 Channel)		Tris-HCl, pH 7.6 (mM)					
		48	36	24	12	6	3
KCl (mM)	40	1450	2829	9387	63752	17023	71
	35	1893	2756	11461	70824	16279	68
	30	1793	3658	18750	71977	20816	2558
	25	2326	8591	22197	78985	32061	709
	20	2122	5592	23435	80342	43824	603
	15	2626	5815	22352	81812	50957	1208
	10	1292	4200	15183	25551	55942	2138
	5	416	1602	8435	79244	55666	-4097









Signal (594 Channel)		Tris-HCl, pH 7.6 (mM)					
		48	36	24	12	6	3
KCl (mM)	40	1493	3593	11917	44321	10327	121
	35	2458	3727	13883	49871	9620	80
	30	2479	5092	20762	51305	12493	1532
	25	3463	11537	23945	57773	19233	329
	20	3065	8123	25770	61556	28174	506
	15	3873	11600	26509	63164	32059	745
	10	1839	6696	20284	29457	34644	1348
	5	522	2670	12442	58521	36896	-25136

Signal (532 Channel)		Tris-HCl, pH 7.6 (mM)					
		48	36	24	12	6	3
KCl (mM)	40	888	1347	7373	70962	25197	384
	35	623	735	8378	80093	23383	104
	30	553	731	15090	85830	30135	5366
	25	656	3213	18570	102300	43890	1291
	20	15	1494	19990	110125	57950	1780
	15	223	2837	18814	113412	65217	3263
	10	436	1008	11987	35427	68051	6330
	5	-95	38632	6129	87224	65313	-10012

Supplementary Figure 1: Cross-Titration of Tris-HCl, pH 7.6 vs. KCl, using 5 mM $MgCl_2$. Tris-HCl, pH 7.6 was titrated from 3 to 48 mM, while KCl was titrated from 5 to 40 mM using the four color standard reaction with 0 and 25 pM dNTPs. The fluorescent signal (25 pM dNTPs– 0 pM dNTPs) for the four colors is shown in the tables and plotted; 700 nm (top left), 655nm (top right), 594 nm (bottom left) and 532 nm (bottom right).

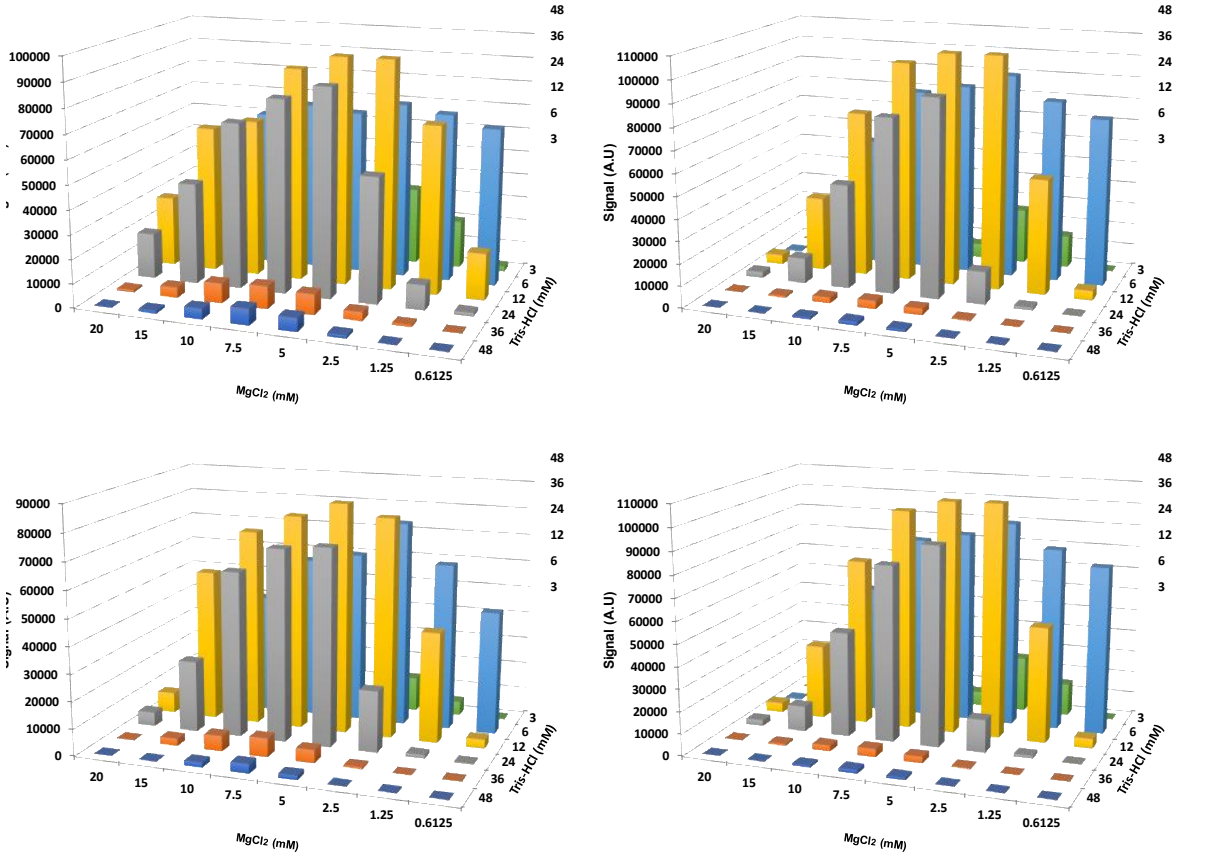
b. Titration of Tris-HCl, pH 7.6 vs. $MgCl_2$ at 20 mM KCl

Following on from the KCl optimisation (Supplementary Figure 1), a cross-titration of Tris-HCl, pH 7.6 and $MgCl_2$ was performed with a fixed KCl concentration of 20 mM. 12 mM Tris-HCl, pH 7.6 and 5 mM $MgCl_2$ provided a good operating window for the reaction. Therefore,

12 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$ and 20 mM KCl were used as the final concentrations for the 1x buffer.

Signal (700 Channel)		Tris-HCl, pH 7.6 (mM)					
		48	36	24	12	6	3
MgCl ² (mM)	20	295	1039	19007	29379	9046	237
	15	1249	4371	42097	61091	40467	1655
	10	4529	8355	68902	65543	64546	19140
	8	6867	9487	80055	88639	69910	19167
	5	5704	8578	86198	94672	67958	33123
	3	1347	3670	52153	94780	72931	32297
	1.25	138	1053	10484	69753	70101	20090
	0.6125	-414	204	1555	19493	65668	2335

Signal (655 Channel)		Tris-HCl, pH 7.6 (mM)					
		48	36	24	12	6	3
MgCl ² (mM)	20	137	345	2802	4541	547	-14
	15	91	910	11634	34125	6010	122
	10	1001	2797	47846	75778	58151	1374
	8	1723	3820	79934	100088	83004	1264
	5	1118	2957	90169	105536	86962	6592
	3	176	581	15000	105898	93345	25445
	1.25	-57	133	1400	52610	82987	14848
	0.6125	-187	33	254	4477	76482	320



Signal (594 Channel)		Tris-HCl, pH 7.6 (mM)					
		48	36	24	12	6	3
MgCl ² (mM)	20	197	331	5251	7784	234	137
	15	175	2745	26640	56488	6053	217
	10	1882	5847	61663	73105	44271	612
	8	3596	7041	71330	79922	60190	633
	5	1687	4857	72998	85563	63440	4576
	3	-51	788	22822	81431	76417	12832
	1.25	-54	24	1409	41167	62137	5217
	0.6125	-522	213	441	3400	45844	469

Signal (532 Channel)		Tris-HCl, pH 7.6 (mM)					
		48	36	24	12	6	3
MgCl ² (mM)	20	160	302	-67155	13192	525	156
	15	-119	1122	24037	108424	13203	418
	10	660	3047	84597	117514	95877	1211
	8	1156	4017	109785	166429	130208	1684
	5	359	1920	107652	175157	138494	17174
	3	-145	-34	7248	160655	155851	47503
	1.25	-43	-141	433	47890	115409	29229
	0.6125	-537	226	217	1656	71684	3587

Supplementary Figure 2: Cross-Titration of Tris-HCl, pH 7.6 vs. $MgCl_2$, using 20 mM KCl. Tris-HCl, pH 7.6 was titrated from 3 to 48 mM, while $MgCl_2$ was titrated from 0.6125 to 20 mM using the four color standard reaction with 0 and 25 pM dNTPs. The fluorescent signal (25 pM dNTPs– 0 pM dNTPs) for the four colors is shown in the tables and plotted; 700 nm (top left), 655nm (top right), 594 nm (bottom left) and 532 nm (bottom right).

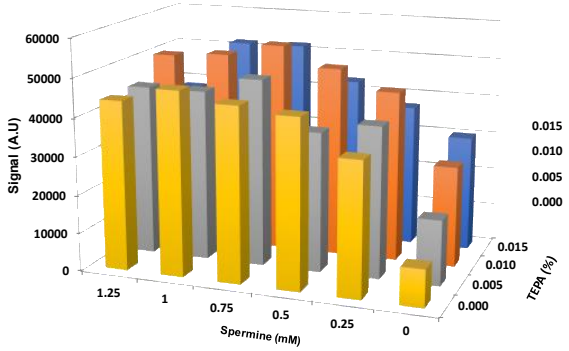
Optimisation of Spermine, Spermidine and TEPA Additives

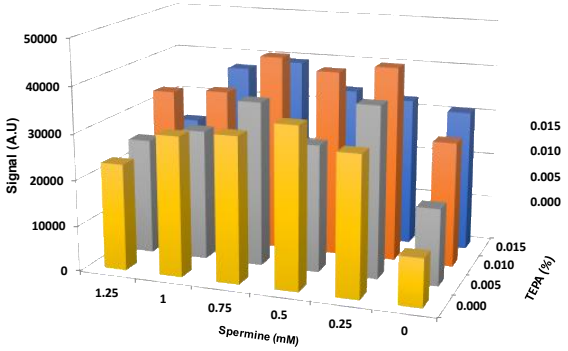
Reaction additives spermine, spermidine and TEPA were found to enhance the detection reaction and in an identical way as to how the buffer was optimised, cross-titrations were used to optimise the concentrations of spermine, spermidine and TEPA in the detection reaction

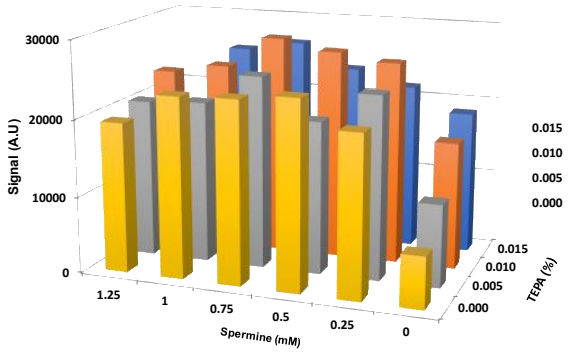
a. Titration of Spermine vs. TEPA at 1.0 mM Spermidine

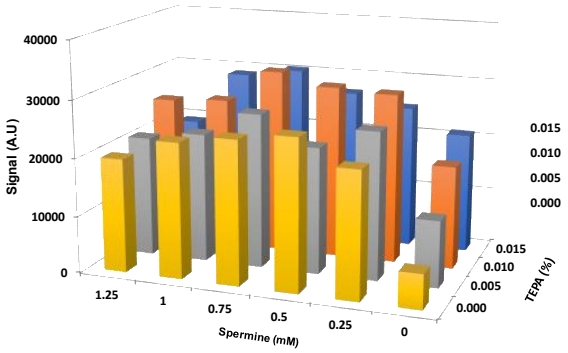
Signal (700 Channel)		Spermine (mM)					
		1.25	1.00	0.75	0.50	0.25	0.00
TEPA (%)	0.015	38419	51735	51883	43142	37226	30218
	0.010	50139	51231	54521	49478	44434	26312
	0.005	44445	44441	48544	36313	39221	17022
	0.000	44086	47806	45280	43781	34726	9740

Signal (655 Channel)		Spermine (mM)					
		1.25	1.00	0.75	0.50	0.25	0.00
TEPA (%)	0.015	24079	37247	39447	33834	32593	30870
	0.010	33444	34335	42879	40517	42322	27203
	0.005	25240	28386	35677	27606	37014	16597
	0.000	23218	30346	31513	34790	30175	10387









Signal (594 Channel)		Spermine (mM)					
		1.25	1.00	0.75	0.50	0.25	0.00
TEPA (%)	0.015	16864	25363	26616	23533	21631	18605
	0.010	23143	24351	28443	27156	26211	16514
	0.005	20564	21002	24923	19784	23724	10733
	0.000	19473	23391	23631	24383	20861	6693

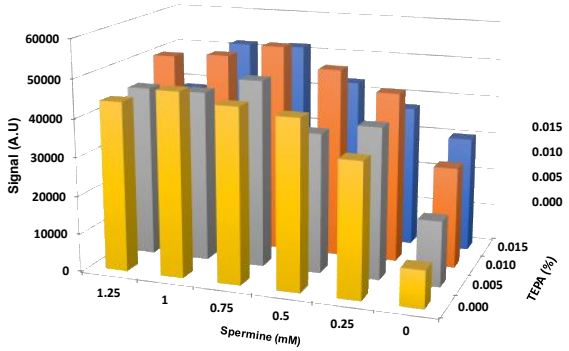
Signal (532 Channel)		Spermine (mM)					
		1.25	1.00	0.75	0.50	0.25	0.00
TEPA (%)	0.015	19399	29009	30415	26993	25070	21013
	0.010	25626	26294	32092	30089	29574	17954
	0.005	20956	22462	26822	21990	25619	11597
	0.000	19801	23543	24952	26245	21941	6058

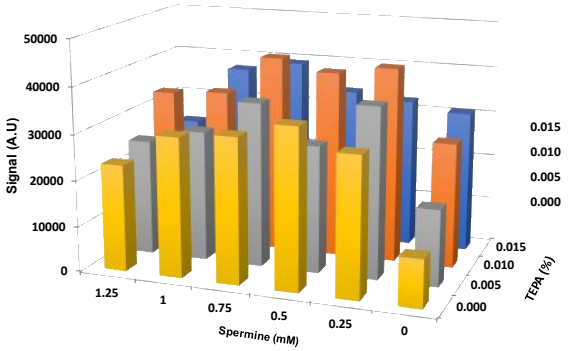
Supplementary Figure 3: Cross-Titration of Spermine vs. TEPA, using 1 mM Spermidine. Spermine was titrated from 0 to 1.25 mM, while TEPA was titrated from 0 – 0.015 % using the four color standard reaction with 0 and 25 pM dNTPs. The fluorescent signal (25 pM dNTPs– 0 pM dNTPs) for the four colors is shown in the tables and plotted; 700 nm (top left), 655nm (top right), 594 nm (bottom left) and 532 nm (bottom right).

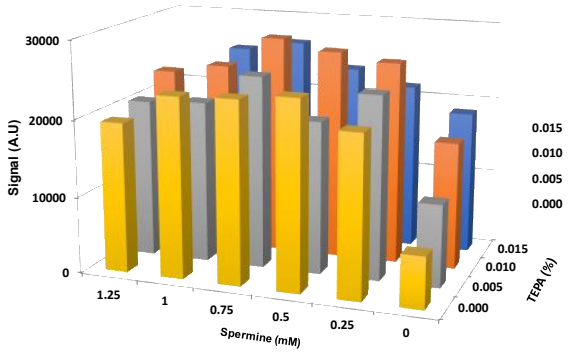
b. Titration of Spermine vs. TEPA at 1.5 mM Spermidine

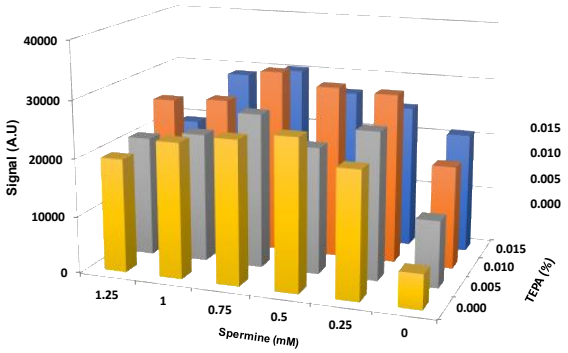
Signal (700 Channel)		Spermine (mM)					
		1.25	1.00	0.75	0.50	0.25	0.00
TEPA (%)	0.015	38419	51735	51883	43142	37226	30218
	0.010	50139	51231	54521	49478	44434	26312
	0.005	44445	44441	48544	36313	39221	17022
	0.000	44086	47806	45280	43781	34726	9740

Signal (655 Channel)		Spermine (mM)					
		1.25	1.00	0.75	0.50	0.25	0.00
TEPA (%)	0.015	24079	37247	39447	33834	32593	30870
	0.010	33444	34335	42879	40517	42322	27203
	0.005	25240	28386	35677	27606	37014	16597
	0.000	23218	30346	31513	34790	30175	10387









Signal (594 Channel)		Spermine (mM)					
		1.25	1.00	0.75	0.50	0.25	0.00
TEPA (%)	0.015	16864	25363	26616	23533	21631	18605
	0.010	23143	24351	28443	27156	26211	16514
	0.005	20564	21002	24923	19784	23724	10733
	0.000	19473	23391	23631	24383	20861	6693

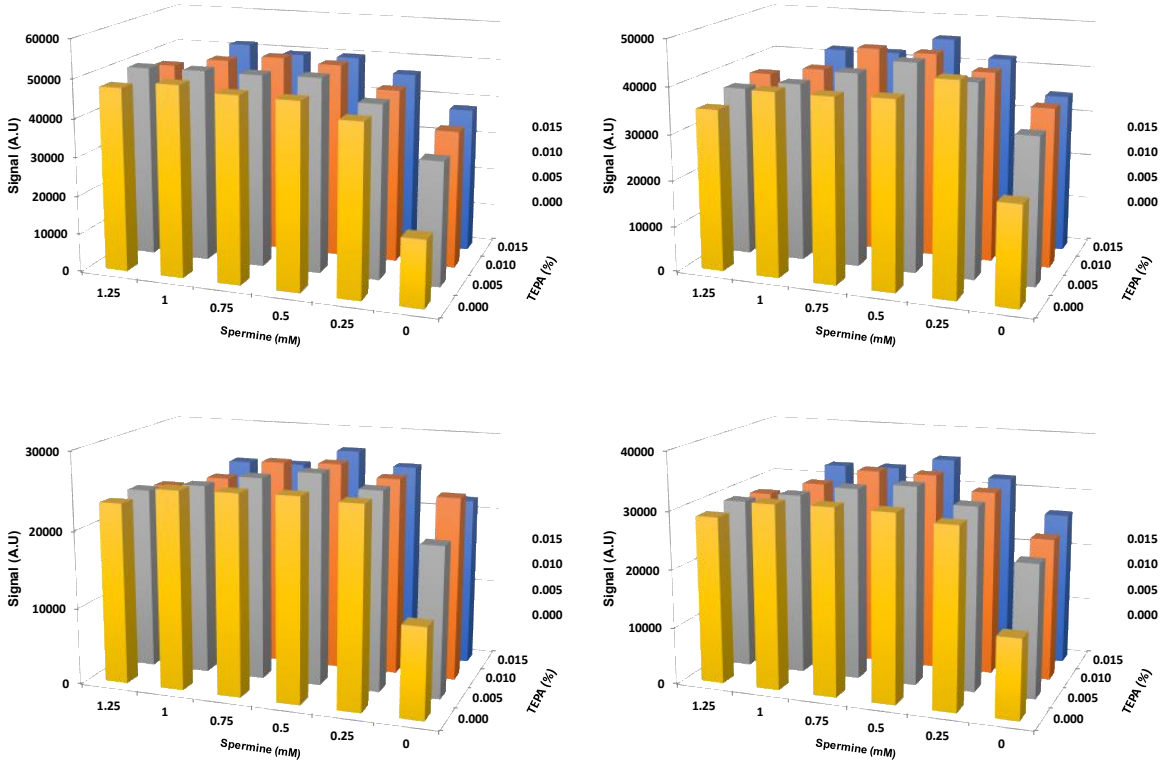
Signal (532 Channel)		Spermine (mM)					
		1.25	1.00	0.75	0.50	0.25	0.00
TEPA (%)	0.015	19399	29009	30415	26993	25070	21013
	0.010	25626	26294	32092	30089	29574	17954
	0.005	20956	22462	26822	21990	25619	11597
	0.000	19801	23543	24952	26245	21941	6058

Supplementary Figure 4: Cross-Titration of Spermine vs. TEPA, using 1.5 mM Spermidine. Spermine was titrated from 0 to 1.25 mM, while TEPA was titrated from 0 – 0.015 % using the four color standard reaction with 0 and 25 pM dNTPs. The fluorescent signal (25 pM dNTPs– 0 pM dNTPs) for the four colors is shown in the tables and plotted; 700 nm (top left), 655nm (top right), 594 nm (bottom left) and 532 nm (bottom right).

c. Titration of Spermine vs TEPA at 2.0 mM Spermidine

Signal (700 Channel)		Spermine (mM)					
		1.25	1.00	0.75	0.50	0.25	0.00
TEPA (%)	0.015	35935	51597	49758	49933	46394	38004
	0.010	47613	49941	51650	50738	45223	35856
	0.005	49703	50027	49996	50360	44989	32116
	0.000	47622	49419	48057	47849	44127	17263

Signal (655 Channel)		Spermine (mM)					
		1.25	1.00	0.75	0.50	0.25	0.00
TEPA (%)	0.015	25332	41804	41913	45707	42077	34689
	0.010	37802	39647	45011	44698	41548	34848
	0.005	36998	38854	42058	45139	41949	31980
	0.000	35076	39767	39753	40239	44904	21554



Signal (594 Channel)		Spermine (mM)					
		1.25	1.00	0.75	0.50	0.25	0.00
TEPA (%)	0.015	15544	25082	25184	27438	25763	21849
	0.010	22666	24181	26772	27041	25632	23752
	0.005	23518	24628	26142	27229	25623	19436
	0.000	23349	25507	25703	25898	25571	11620

Signal (532 Channel)		Spermine (mM)					
		1.25	1.00	0.75	0.50	0.25	0.00
TEPA (%)	0.015	20818	32735	32971	35032	32277	26530
	0.010	28802	31229	34172	34139	31838	24588
	0.005	29433	31177	33034	34133	31567	22991
	0.000	28806	31744	32017	31942	30738	13648

Supplementary Figure 5: Cross-Titration of Spermine vs. TEPA, using 2 mM Spermidine. Spermine was titrated from 0 to 1.25 mM, while TEPA was titrated from 0 – 0.015 % using the four color standard reaction with 0 and 25 pM dNTPs. The fluorescent signal (25 pM dNTPs– 0 pM dNTPs) for the four colors is shown within the tables and plotted; 700 nm (top left), 655 nm (top right), 594 nm (bottom left) and 532 nm (bottom right).

Cross-titrations of Spermine vs. TEPA were performed at fixed concentrations of spermidine (Supplementary Figures 3, 4 & 5). From this data 1 mM spermine, 1 mM spermidine and 0.015% TEPA were used as the final additive concentration in the four color detection reaction.

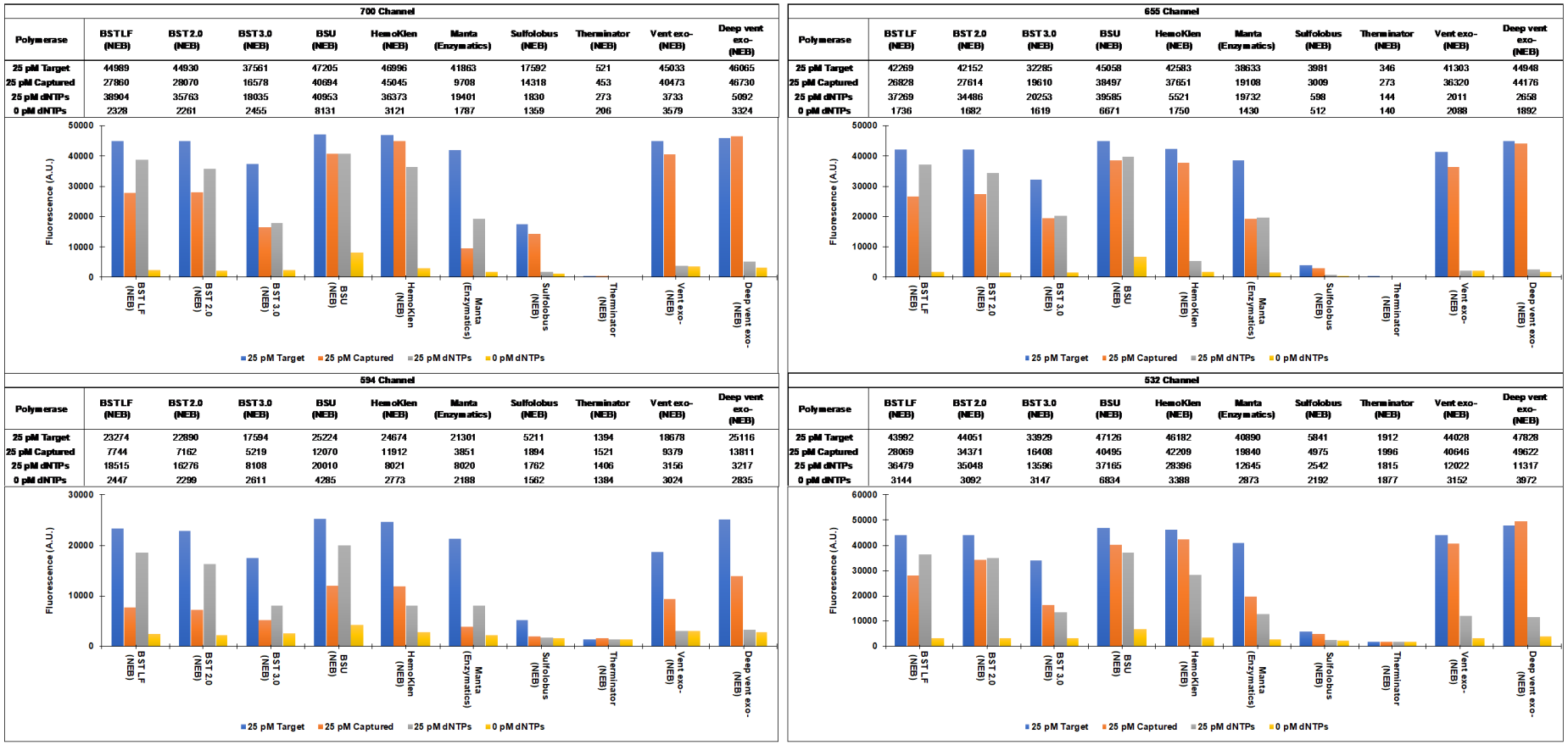
Screening of Capture Polymerase and Ligation Enzyme

The choice of capture polymerase was based on initial screenings using a variety of commercially available DNA polymerases. In these reactions, the other enzymes were the same as described earlier, i.e., *E. coli* DNA ligase and *Pfu*Ultra II Fusion HS DNA polymerase. The tables and graphs below show the fluorescence intensity after 100 minutes of incubation. Note that conditions were not yet fully optimized for capture and ligation, giving results that differ from those in reactions performed under finalized conditions.

The choice of ligase was based on initial screenings using a variety of commercially available DNA ligases. In these reactions, the other enzymes were the same as described earlier, i.e., *Bst* L.F. DNA polymerase and *Pfu*Ultra II Fusion HS DNA polymerase. The tables and graphs below show the fluorescence intensity after 100 minutes of incubation. Note that conditions were not yet fully optimized for capture and ligation, giving results that differ from those in reactions performed under finalized conditions.

As before, “captured” refers to a construct of CAP+N (simulating that capture has taken place), and “target” refers to a construct of CAP+N+LIG (simulating that both capture and ligation have taken place).

a. Screening of Capture Polymerase – Part 1



700 Channel

Polymerase	BSTLF (NEB)	BST2.0 (NEB)	BST3.0 (NEB)	BSU (NEB)	HemoKlen (NEB)	Manta (Enzymatics)	Sulfolobus (NEB)	Therminator (NEB)	Vent exo- (NEB)	Deep vent exo- (NEB)
25 pM Target	44989	44930	37561	47205	46996	41863	17592	521	45033	46065
25 pM Captured	27860	28070	16578	40694	45045	9708	14318	453	40473	46730
25 pM dNTPs	38904	35763	18035	40953	36373	19401	1830	273	3733	5092
0 pM dNTPs	2328	2261	2455	8131	3121	1787	1359	206	3579	3324

655 Channel

Polymerase	BSTLF (NEB)	BST2.0 (NEB)	BST3.0 (NEB)	BSU (NEB)	HemoKlen (NEB)	Manta (Enzymatics)	Sulfolobus (NEB)	Therminator (NEB)	Vent exo- (NEB)	Deep vent exo- (NEB)
25 pM Target	42269	42152	32285	45058	42583	38633	3981	346	41303	44948
25 pM Captured	26828	27614	19610	38497	37651	19108	3009	273	36320	44176
25 pM dNTPs	37269	34486	20253	39585	5521	19732	598	144	2011	2658
0 pM dNTPs	1736	1682	1619	6671	1750	1430	512	140	2088	1892

594 Channel

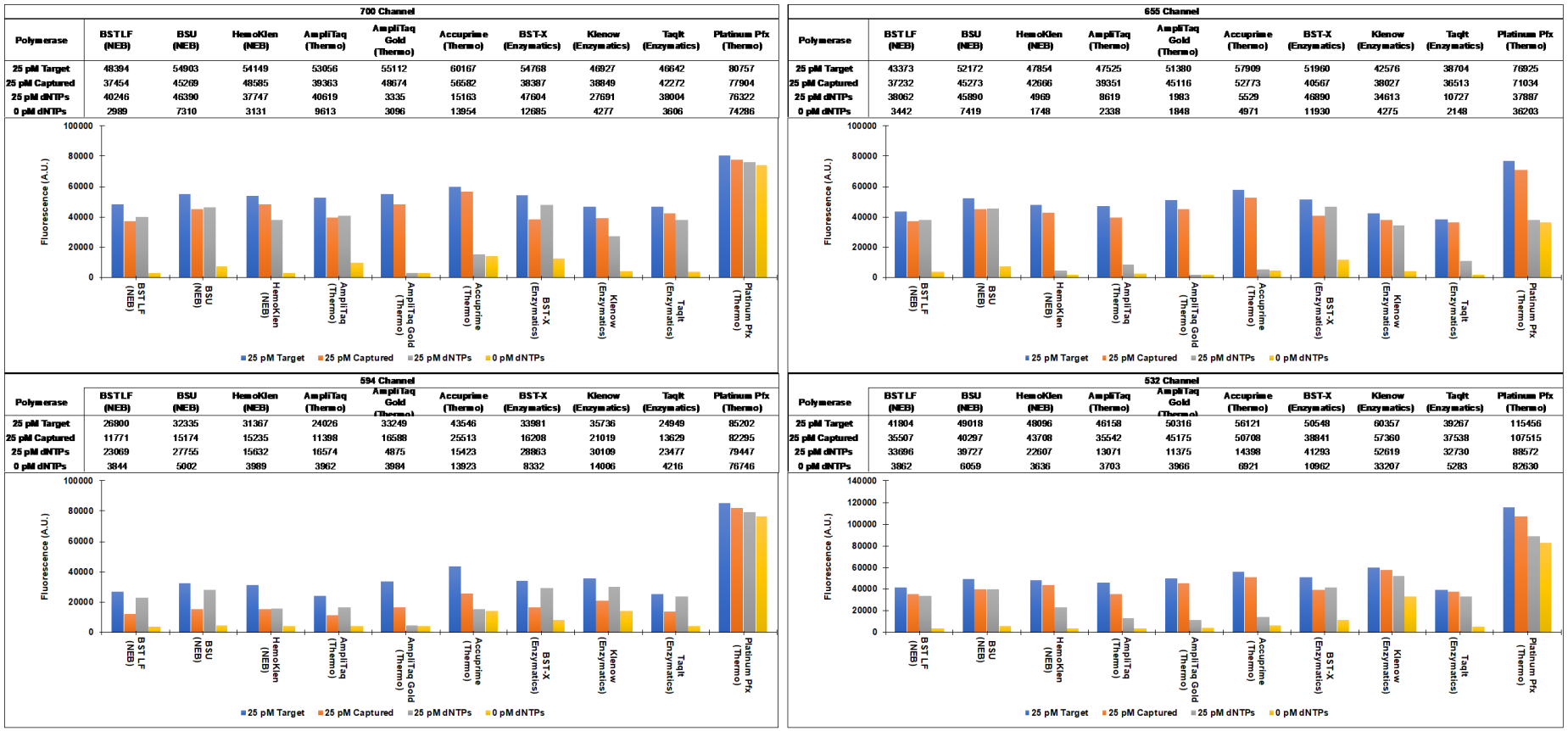
Polymerase	BSTLF (NEB)	BST2.0 (NEB)	BST3.0 (NEB)	BSU (NEB)	HemoKlen (NEB)	Manta (Enzymatics)	Sulfolobus (NEB)	Therminator (NEB)	Vent exo- (NEB)	Deep vent exo- (NEB)
25 pM Target	23274	22890	17594	25224	24674	21301	5211	1394	18678	25116
25 pM Captured	7744	7162	5219	12070	11912	3851	1894	1521	9379	13811
25 pM dNTPs	18515	16276	8108	20010	8021	8020	1762	1406	3156	3217
0 pM dNTPs	2447	2299	2611	4285	2773	2188	1562	1384	3024	2835

532 Channel

Polymerase	BSTLF (NEB)	BST2.0 (NEB)	BST3.0 (NEB)	BSU (NEB)	HemoKlen (NEB)	Manta (Enzymatics)	Sulfolobus (NEB)	Therminator (NEB)	Vent exo- (NEB)	Deep vent exo- (NEB)
25 pM Target	43992	44051	33929	47126	46182	40890	5841	1912	44028	47828
25 pM Captured	28069	34371	16408	40495	42209	19840	4975	1996	40646	49622
25 pM dNTPs	36479	35048	13596	37165	28396	12645	2542	1815	12022	11317
0 pM dNTPs	3144	3092	3147	6834	3388	2873	2192	1877	3152	3972

Supplementary Figure 6: Screening of capture polymerase – Part 1. Different polymerases (from different suppliers) were used to test their ability to capture dNTPs at 25 pM, using the four color standard reaction, compared to 25 pM Captured and 25 pM Target oligos. The four colors are shown in the tables and plotted; 700 nm (top left), 655 nm (top right), 594 nm (bottom left) and 532 nm (bottom right).

b. Screening of Capture Polymerase – Part 2



700 Channel

Polymerase	BST LF (NEB)	BSU (NEB)	HemoKlen (NEB)	AmpliTaq (Thermo)	AmpliTaq Gold (Thermo)	Accuprime (Thermo)	BST-X (Enzymatics)	Klenow (Enzymatics)	TaqIt (Enzymatics)	Platinum Pfx (Thermo)
25 pM Target	48394	54903	54149	53056	55112	60167	54768	46927	46642	80757
25 pM Captured	37454	45269	48585	39363	48674	56582	38387	38849	42272	77904
25 pM dNTPs	40246	46390	37747	40619	3335	15163	47604	27691	38004	76322
0 pM dNTPs	2989	7310	3131	9613	3096	13954	12685	4277	3606	74286

655 Channel

Polymerase	BST LF (NEB)	BSU (NEB)	HemoKlen (NEB)	AmpliTaq (Thermo)	AmpliTaq Gold (Thermo)	Accuprime (Thermo)	BST-X (Enzymatics)	Klenow (Enzymatics)	TaqIt (Enzymatics)	Platinum Pfx (Thermo)
25 pM Target	43373	52172	47854	47525	51380	57909	51960	42576	38704	76925
25 pM Captured	37232	45273	42666	39351	45116	52773	40567	38027	36513	71034
25 pM dNTPs	38062	45890	4969	8619	1983	5529	46890	34613	10727	37887
0 pM dNTPs	3442	7419	1748	2338	1848	4971	11930	4275	2148	36203

594 Channel

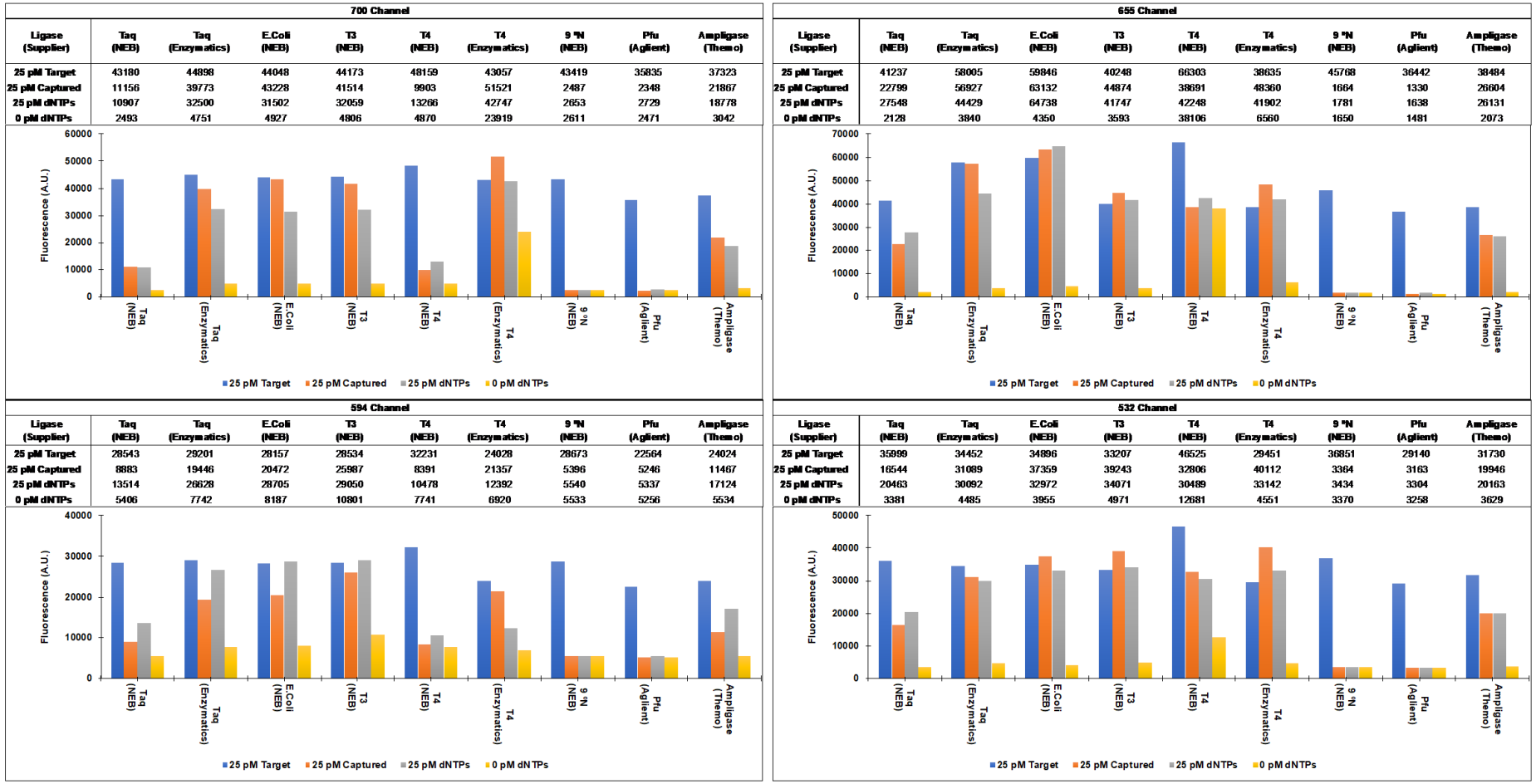
Polymerase	BST LF (NEB)	BSU (NEB)	HemoKlen (NEB)	AmpliTaq (Thermo)	AmpliTaq Gold (Thermo)	Accuprime (Thermo)	BST-X (Enzymatics)	Klenow (Enzymatics)	TaqIt (Enzymatics)	Platinum Pfx (Thermo)
25 pM Target	26800	32335	31367	24026	33249	43546	33981	35736	24949	85202
25 pM Captured	11771	15174	15235	11398	16588	25513	16208	21019	13629	82295
25 pM dNTPs	23069	27755	15632	16574	4875	15423	28863	30109	23477	79447
0 pM dNTPs	3844	5002	3989	3962	3984	13923	8332	14006	4216	76746

532 Channel

Polymerase	BST LF (NEB)	BSU (NEB)	HemoKlen (NEB)	AmpliTaq (Thermo)	AmpliTaq Gold (Thermo)	Accuprime (Thermo)	BST-X (Enzymatics)	Klenow (Enzymatics)	TaqIt (Enzymatics)	Platinum Pfx (Thermo)
25 pM Target	41804	49018	48096	46158	50316	56121	50548	60357	39267	115456
25 pM Captured	35507	40297	43708	35542	45175	50708	38841	57360	37538	107515
25 pM dNTPs	33696	39727	22607	13071	11375	14398	41293	52619	32730	88572
0 pM dNTPs	3862	6059	3636	3703	3966	6921	10962	33207	5283	82630

Supplementary Figure 7: Screening of capture polymerase – Part 2. Different polymerases (from different suppliers) were used to test their ability to capture dNTPs at 25 pM, using the four color standard reaction, compared to 25 pM Captured and 25 pM Target oligos. The four colors are shown in the tables and plotted; 700 nm (top left), 655 nm (top right), 594 nm (bottom left) and 532 nm (bottom right).

c. Screening of Ligation Enzyme



Supplementary Figure 8: Screening of Ligation Enzyme. Different Ligases (from different suppliers) were used to test their ability to capture dNTPs at 25 pM, using the four color standard reaction, compared to 25 pM Captured and 25 pM Target oligos. For the four colors the data from 0 pM dNTPs, 25 pM dNTPs, 25 pM Captured and 25 pM Targets are shown in the tables and plotted; 700 nm (top left), 655 nm (top right), 594 nm (bottom left) and 532 nm (bottom right).

Digestion of oligo mix using the exonuclease activity of different polymerases

Finding a suitable exonuclease proved the most difficult among the enzymes needed in our reaction cascade. “Classical” exonucleases (Exonuclease T, Exonuclease I, Lambda Exonuclease) all lacked sufficient dsDNA selectivity for our intended purpose, resulting in unwanted digestion of Probe Oligo even in absence of dNTPs. Most polymerases with 3’ -> 5’ exonuclease activity were either insufficiently selective as well, or their exonuclease activity was too slow to result in sufficient signal being generated. The three polymerases that exhibited suitable levels of selectivity and reactivity were Phusion II DNA polymerase, Phire Hot Start II DNA Polymerase and *Pfu*Ultra II Fusion HS DNA polymerase. All three polymerases required different buffer systems and reaction temperatures for optimal reactivity (Supplementary Table 9):

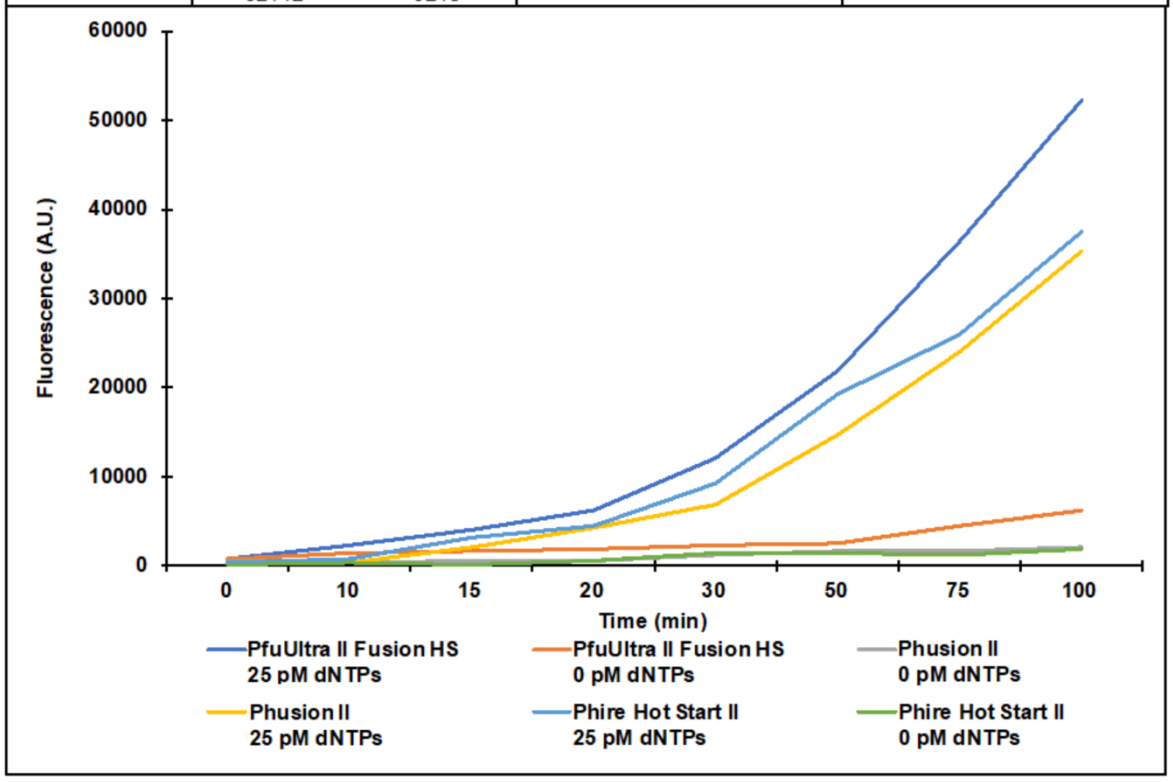
a. Polymerase - Required Buffers and Temperatures

Supplementary Table 9: Description of the three Polymerase buffer conditions used.

1x Buffer Component			
Polymerase	***Pfu*Ultra II Fusion HS**	**Phusion II**	**Phire Hot Start II**
pH	7.6	8.0	8.0
Tris-HCl	12 mM	40mM	5 mM
$MgCl_2$	5 mM	15mM	15 mM
KCl	20 mM	100mM	60 mM
Triton X-100	0.10 %	0.10%	0.10%
DTT	0.5 mM	0.5 mM	0.5 mM
NAD	400 nM	400 nM	400 nM
Temperature	69.5 ºC	68.5 ºC	69.0 ºC

b. Screening of different Polymerases

Time (min)	PfuUltra II Fusion HS		Phusion II		Phire Hot Start II	
	25 pM dNTPs	0 pM dNTPs	25 pM dNTPs	0 pM dNTPs	25 pM dNTPs	0 pM dNTPs
0	847	836	250	522	250	177
10	2213	1519	267	444	758	355
15	3995	1739	2065	658	3158	184
20	6267	1884	4226	656	4502	619
30	12095	2204	6955	1195	9276	1494
50	21737	2533	14631	1619	19281	1408
75	36395	4428	23899	1737	26042	1285
100	52112	6218	35210	1975	37354	1920

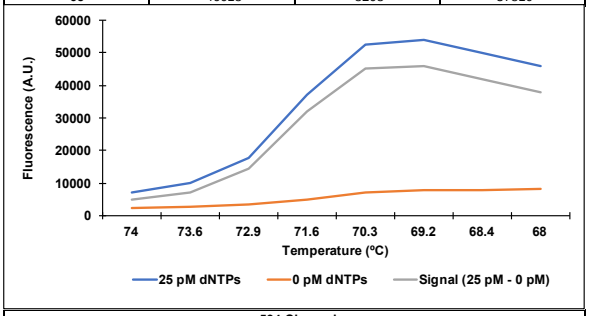


Supplementary Figure 9: Screening of different Polymerases. Different Polymerases were tested for their ability to digest the probe DNA, using the four color standard reaction, at 0 pM and 25 pM dNTPs concentration (data from the 655 nm channel is shown).

c. Digestion temperature for *Pfu*Ultra II Fusion HS DNA polymerase

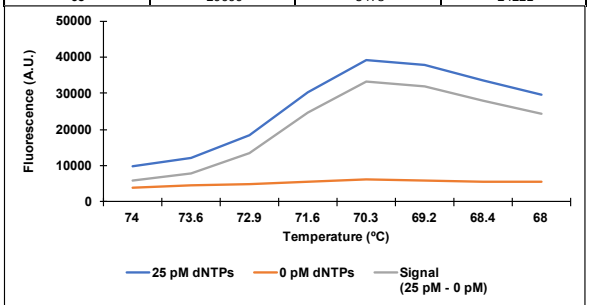
700 Channel

Temperature (ºC)	25 pM dNTPs	0 pM dNTPs	Signal (25 pM - 0 pM)
74	7227	2377	4850
73.6	9933	2840	7093
72.9	17883	3440	14443
71.6	37012	4933	32079
70.3	52437	7272	45165
69.2	53978	8023	45955
68.4	50020	8003	42017
68	46018	8208	37810



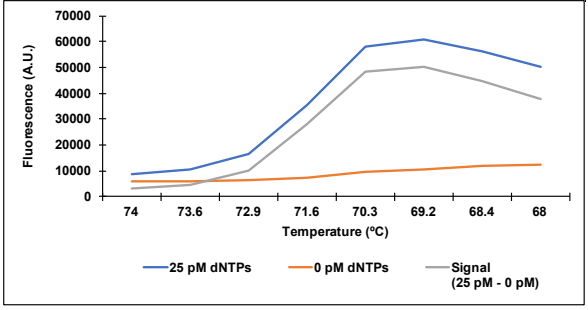
655 Channel

Temperature (ºC)	25 pM dNTPs	0 pM dNTPs	Signal (25 pM - 0 pM)
74	9888	3966	5922
73.6	12115	4363	7752
72.9	18279	4834	13445
71.6	30105	5452	24653
70.3	39141	6087	33054
69.2	37752	5864	31888
68.4	33406	5627	27779
68	29699	5478	24221



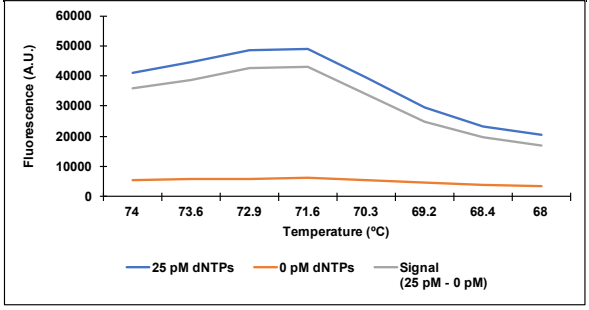
594 Channel

Temperature (ºC)	25 pM dNTPs	0 pM dNTPs	Signal (25 pM - 0 pM)
74	8821	5654	3167
73.6	10357	5912	4445
72.9	16595	6492	10103
71.6	35600	7376	28224
70.3	57805	9371	48434
69.2	60711	10543	50168
68.4	56234	11752	44482
68	50175	12429	37746



532 Channel

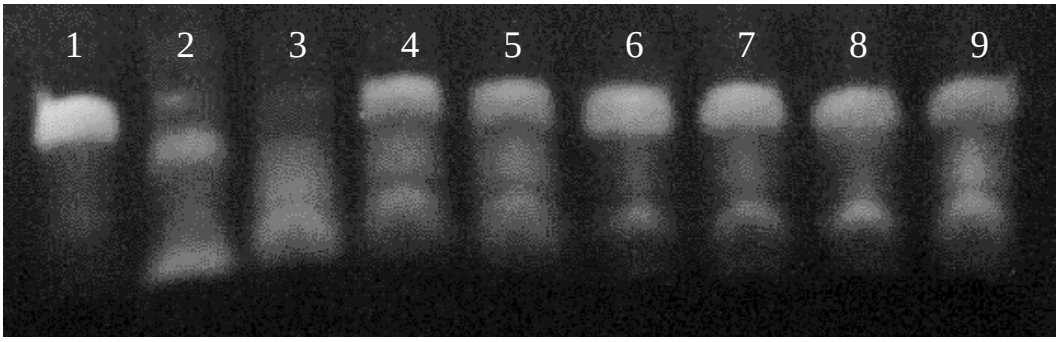
Temperature (ºC)	25 pM dNTPs	0 pM dNTPs	Signal (25 pM - 0 pM)
74	41086	5310	35776
73.6	44651	5971	38680
72.9	48365	5912	42453
71.6	48871	6058	42813
70.3	39287	5412	33875
69.2	29664	4663	25001
68.4	23385	3870	19515
68	20347	3411	16936



Supplementary Figure 10: Screening of digestion temperature for *Pfu*Ultra II Fusion HS DNA polymerase. *Pfu*Ultra II Fusion HS DNA polymerase was screened from 60 to 74 ºC for its ability to digest the probe DNA, using the four color standard reaction, at 0 and 25 pM dNTPs concentration. For the four colors the data from 0 pM and 25 pM dNTPs as well as the signal (0 pM – 25 pM) is shown within the tables and plotted; 700 nm (top left), 655nm (top right), 594 nm (bottom left) and 532 nm (bottom right).

Choice of Protecting Group on LIG

In order to protect the oligos from unwanted exonuclease digestion, a protecting group was needed at the 3'-end of LIG. Several commercially available end modifications were tested for their suitability by subjecting the corresponding 3'-modified LIG oligos to an excess of *Pfu*Ultra II Fusion HS DNA polymerase during a 2-hour incubation at 70°C. The figure below shows a picture of the resulting 8% denaturing PAGE analysis of the reactions.

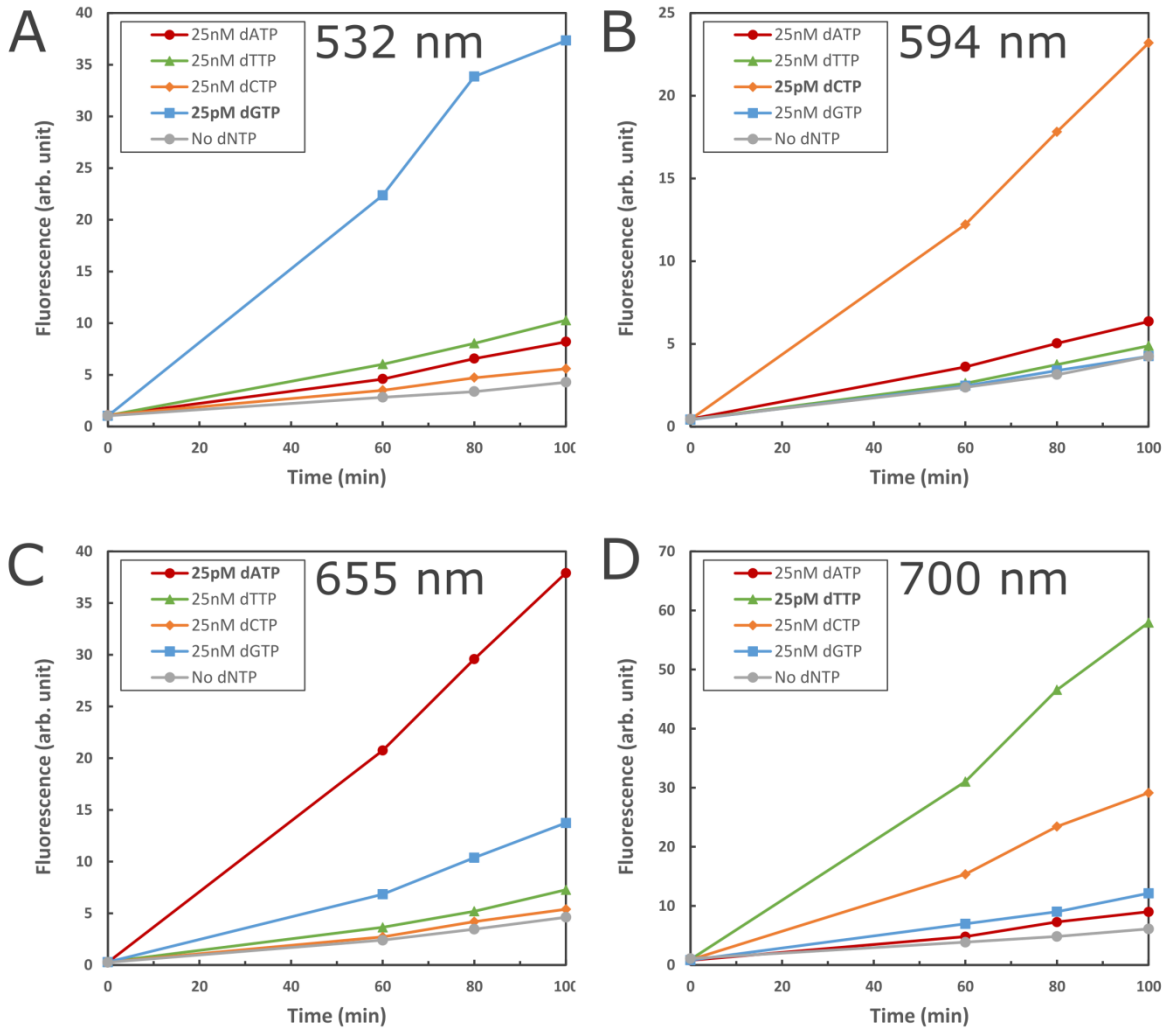


Lane number	End modification
1	(none), unreacted reference sample
2	(none)
3	RNA base (Uracil)
4	Phosphorothioate
5	Hexanediol
6	5'-3' inverted dT
7	Spacer 9
8	C3 spacer
9	dspacer (abasic furan)

Supplementary Figure 11: Image (detail) of 8% denaturing (formamide) polyacrylamide gel showing the digestion of LIG by PfuUltra II Fusion HS bearing a variety of 3' end modifications.

It is apparent that unprotected oligos are digested almost completely under these conditions, and that the RNA base offers no protection. The most efficient protecting group is the 5'-3' inverted dT, which was consequently used for our experiments.

Specificity of Detection



Supplementary Figure 12: Testing for chemical cross-talk by adding either intended or "incorrect" dNTP to the reaction. The individual capture channels were tested separately (A – Probe, LIG and CAP for dGTP detection only, B – Probe, LIG and CAP for dCTP detection only, C – Probe, LIG and CAP for dATP detection only, D – Probe, LIG and CAP for dTTP detection only). Note that in each case the intended dNTP is present at 1000-fold lower concentration than the three others.

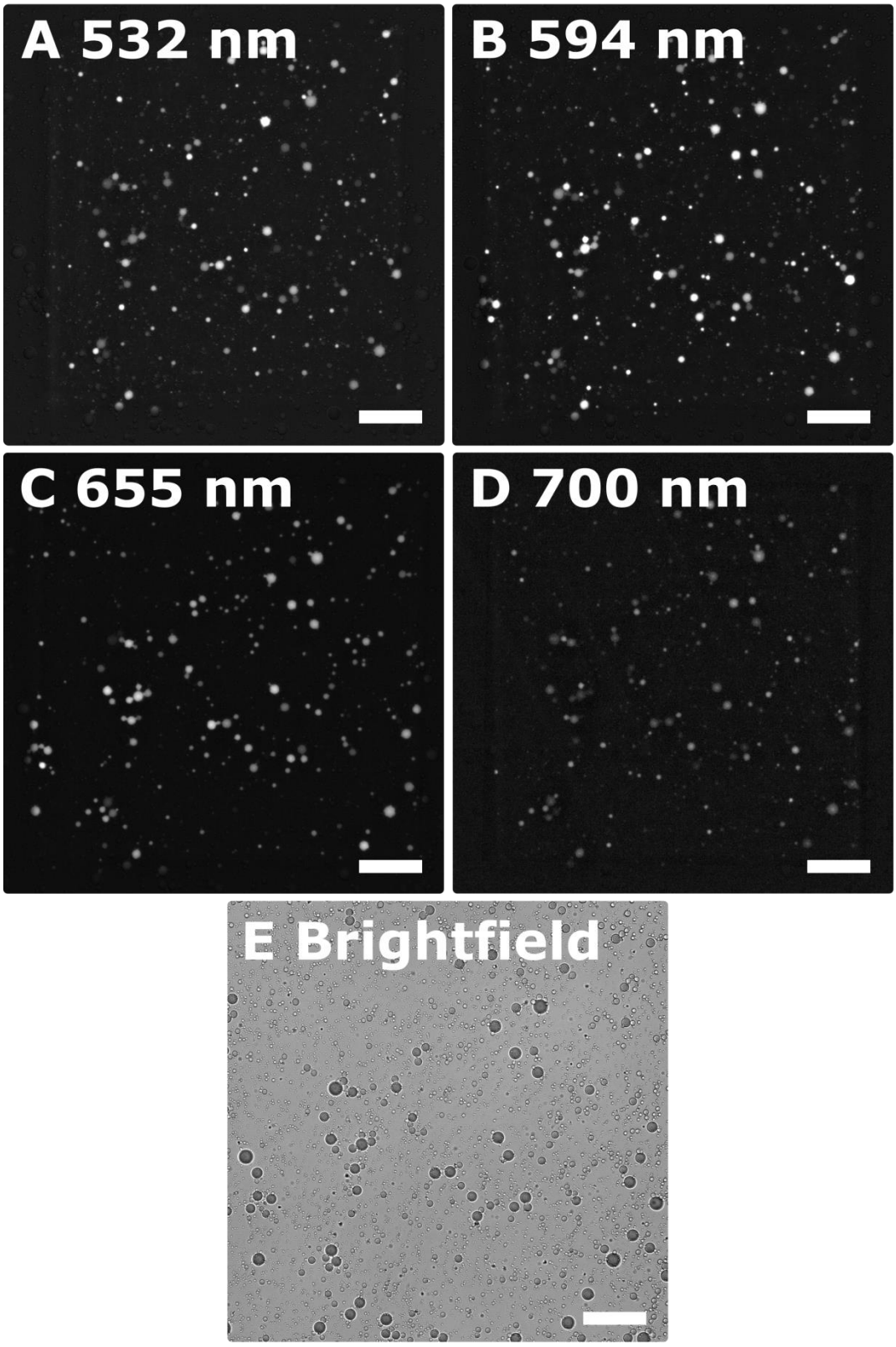
Fluorescence Measurements of Droplets

Fluorescent images of microdroplets were acquired using a custom fluorescent microscope consisting of an Andor Zyla 5.5 sCMOS camera, four CW laser excitation lines (Vortran Stradus 532 nm, Cobalt Mambo 594 nm, Vortran Stradus 640 nm, Vortran Stradus 701 nm), a filter cube changer to select the appropriate excitation, dichroic and emission filters (see Supplementary Table 10), and a galvanometer scanning mirror system to raster the laser over the area of interest. The exposure time per laser for each 300 µm x 300 µm field of view was 30 s.

Supplementary Table 10: Filter sets used for fluorescence measurements of droplets

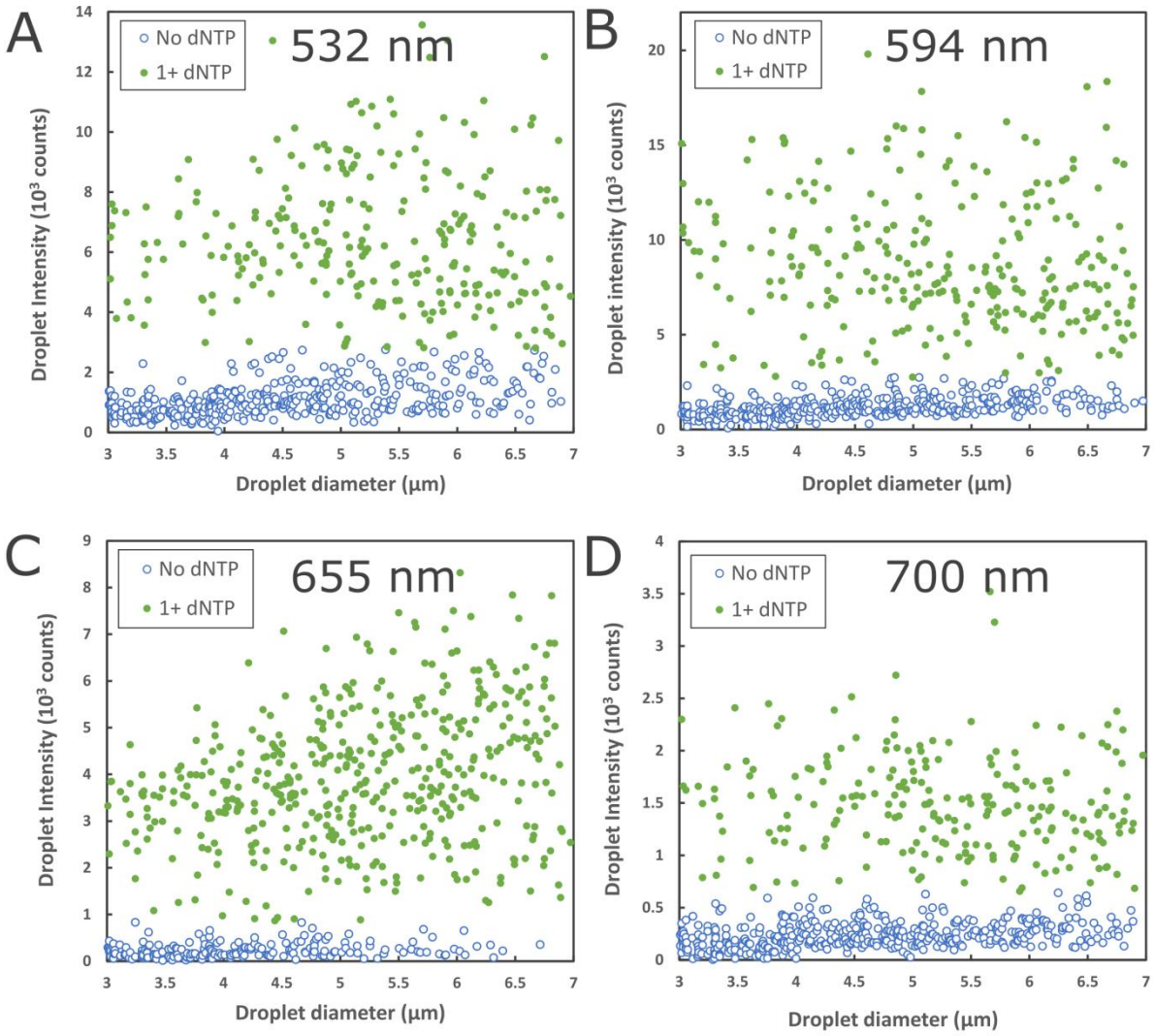
Laser Wavelength	Excitation filter	Dichroic	Emission filter
701 nm	Semrock FF01-692/40	Chroma 725dcxxr	Semrock FF01-795/150
640 nm	Semrock FF01-640/14	Semrock Di02-R635	Semrock FF01-679/41
594 nm	Semrock FF01-591/6	Semrock Di02-R594	Semrock FF01-647/57
532 nm	Semrock FF01-532/3	Semrock Di02-R532	Semrock FF01-582/75 + FF01-562/40

A. Example Images – All Color Channels



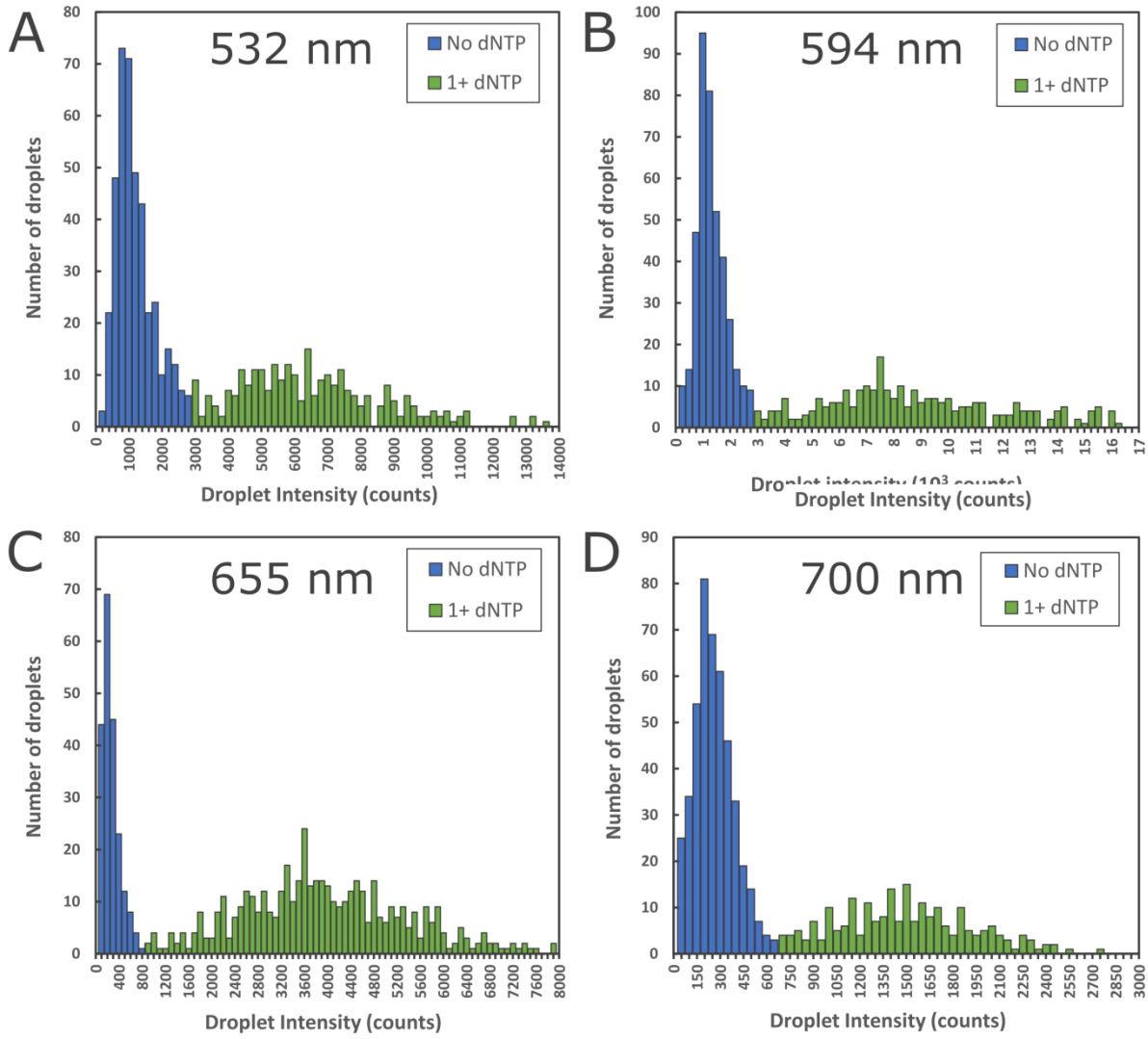
Supplementary Figure 13: Example images of a field of view of droplets in: A-D) the four color channels used for dNTP detection, and E) brightfield used for droplet position and size detection. Scale bars are 50 µm.

B. Fluorescence Intensity vs. Droplet Diameter – All Color Channels



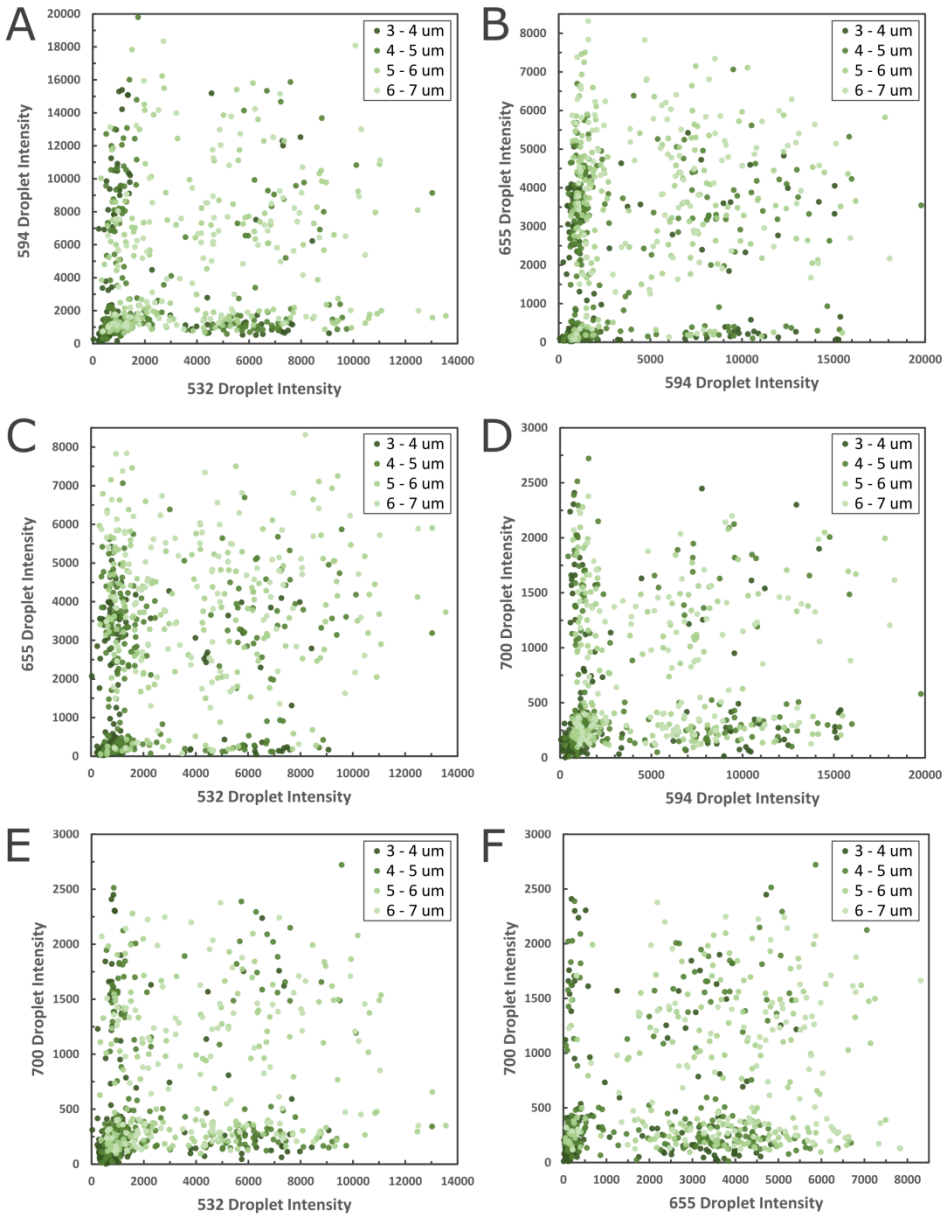
Supplementary Figure 14: Droplet intensity vs. droplet diameter extracted from each of the four fluorescence channels: A) 532 nm, B) 594 nm, C) 655 nm, D) 700 nm.

C. Fluorescence Intensity histograms – All Color Channels



Supplementary Figure 15: Histograms showing frequency of occurrence vs droplet intensity for all four color channels (A 532 nm, B 594 nm, C 655 nm, D 700 nm) in the 3-7 μm diameter range. In each case, two peaks are visible, one for droplets which contain no nucleotide (blue) and one for droplets which contain one (or more) nucleotides (green).

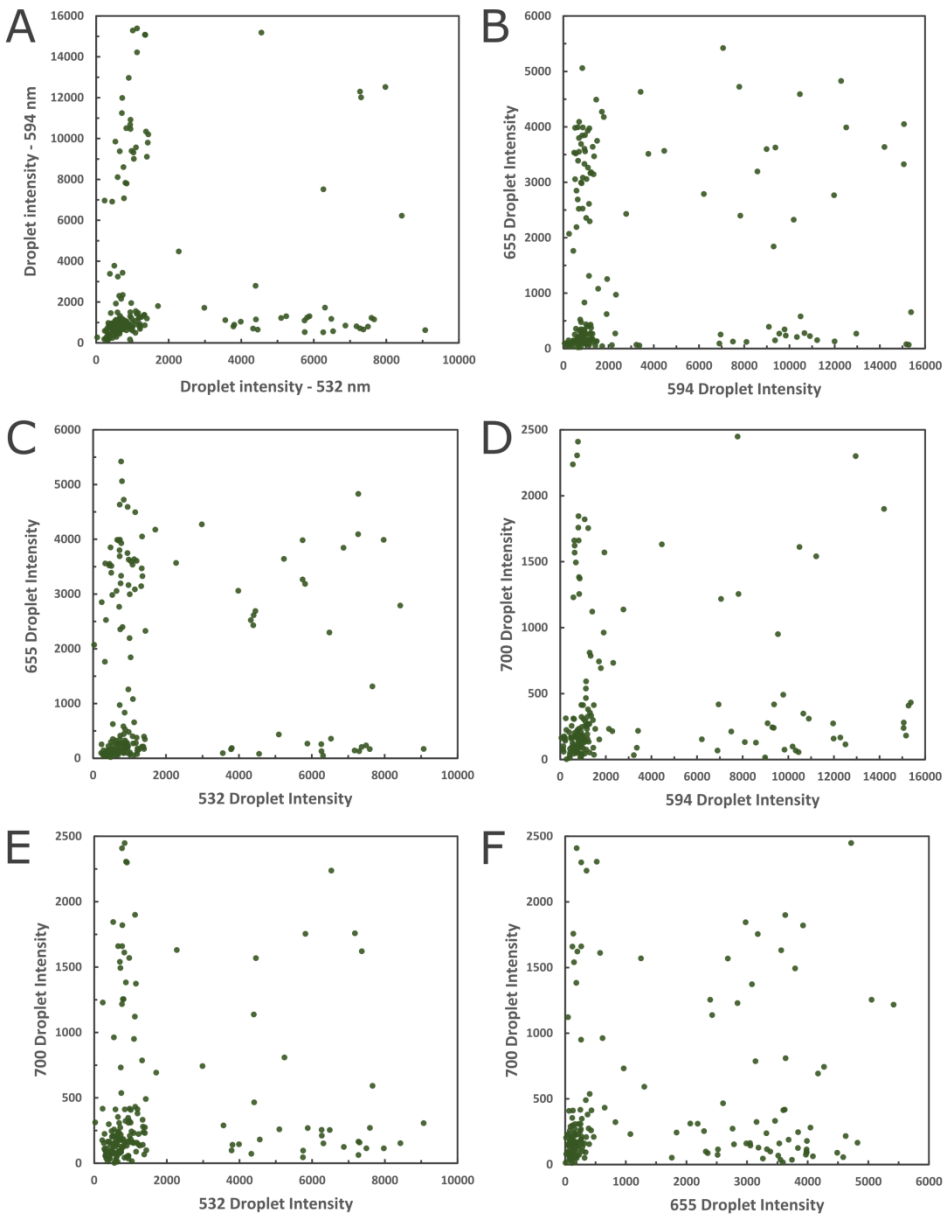
D. Fluorescence Intensity – Channel Correlations (3 - 7 micrometer diameters)



Supplementary Figure 16: Droplet intensity in a given color channel plotted against droplet intensity in another channel. All six of the possible pairs are shown: A) 532 nm vs. 594 nm, B) 594 nm vs. 655 nm, C) 532 nm vs. 655 nm, D) 594 nm vs. 700 nm, E) 532 nm vs. 700 nm, F) 655 nm vs. 700 nm. These plots allow for a visual examination of the correlation between color channels. The different diameter subsets are colored from dark green at small droplets to light green at larger droplets.

For higher dNTP concentration or larger droplets a fraction of the droplets will begin to appear on the diagonal as the probability of a droplet containing more than one type of dNTP becomes larger. For instance, 12.5 pM of each dNTP in 5 µm droplets gives $P(\geq 1)_{532}P(\geq 1)_{594} \approx 15\%$.

E. Fluorescence Intensity – Channel Correlations (3 - 4 micrometer diameters)



Supplementary Figure 17: Droplet intensity in a given color channel plotted against droplet intensity in another channel. All six of the possible pairs are shown: A) 532 nm vs. 594 nm, B) 594 nm vs. 655 nm, C) 532 nm vs. 655 nm, D) 594 nm vs. 700 nm, E) 532 nm vs. 700 nm, F) 655 nm vs. 700 nm. These plots allow for a visual examination of the correlation between color channels. Only the 3-4 μm subset of the droplets is shown in each case. The Pearson correlation coefficient (and r^2 value) for sub-figures A - F are 0.072 (0.005), 0.149 (0.022), 0.135 (0.018), 0.124 (0.015), 0.090 (0.008), and 0.157 (0.025) respectively.

Supplementary Figure 17 shows the 3 - 4 μm diameter droplet range. As an example, consider the following. From Table 1 in the manuscript, the expected fraction of droplets with

signal for 12.5 pM at 3 μm and 4 μm diameter is 10% and 22% respectively. For two colours to be positive, this implies between 1% (if all droplets are 3 μm) and 4.8% (if all droplets are 4 μm). We see between 3.4% and 5.0% for the combinations of colours (532 nm, 594 nm and 700 nm) which have 12.5 pM dNTP concentration, which is close to the expected range. The 655 nm channel has 37.5 pM dNTP concentration so a higher fraction would be expected. At 3 μm diameter we expect 28% positive, at 4 μm 53% positive, which then gives a 2.8% to 11.7% range for double positive between the 12.5pM and 37.5pM channels. We observe 9.5% to 10.6%, again within expected bounds.

Droplet Finding Algorithm

First the gradient of the brightfield droplet image is taken to get the directional change in the intensity of the image. The circle finding algorithm is based on performing a matched filter(5). This involves correlating a known template image with the gradient image of interest. In this case the template is a mathematically-defined ring, $exp^{\frac{-(r-r_0)^2}{w^2}}$, with direction pointing radially outwards, where *r* is distance from the droplet's centre, r_0 is the template's size parameter, and *w* is the template's width parameter. This is performed multiple times, with a range of template image sizes, r_0, to allow a range of droplet sizes to be found. Suitable thresholds are chosen such that droplets are identified and spurious matches are minimised.

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