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ORIGINAL ARTICLE

Using Pooled Recombinant Plasmids as Control Materials for Diagnostic Real-Time PCR

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SUMMARY

Background: **Despite the potential usefulness of plasmids as reference materials for PCR, there are very few reports documenting the experience with using these materials in quality control (QC) of laboratory developed tests (LDTs), in a clinical diagnostic setting. In this study, an approach to preparing and titering such controls is detailed. The practicality of using plasmids as QC materials was explored and presented.**
Methods: **Target DNA fragments (n = 11) were amplified from positive samples and cloned using TA-cloning. Identities of the fragments were ascertained using DNA sequencing. Real-time PCR was carried out using TaqMan probes on RGQ or CFX-96 thermal-cyclers.**
Results: **All the 11 targeted DNA fragments were successfully cloned into *E. coli* vectors. Plasmid pools could be repeatedly reconstituted to generate stable and usable positive controls for multiplex PCR assays in a simple workflow. Importantly, plasmid controls generated meaningful run data that could be used in QC processes for monitoring routine runs. Further, plasmid material could be spiked into clinical specimens for quality assurance (QA) purposes, avoiding the culture of live infectious organisms, showing another routine usefulness of plasmids.**
Conclusions: **Plasmid pools are useful and inexhaustible sources of reference materials for various routine QC uses in the diagnostic laboratory.**
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KEY WORDS

quality control, clinical diagnostics, plasmid pools, real-time PCR, TA cloning

INTRODUCTION

Accreditation guidelines for molecular diagnostic laboratories generally stipulate the use of positive and negative controls in every real-time PCR run, as well as a periodic review of quality control (QC) data generated from such controls. Exogenous QC controls are primarily used to monitor reagents (positive controls) and contamination (negative controls). The common approaches for exogenous positive control QC materials are synthetic nucleic acids or patient-derived positive samples. Although patient-derived samples simulate closely the samples being tested, they will eventually run out, and a continuous supply cannot be assured. Moreover, variations in threshold cycles (Ct values) between different

patient samples will necessitate dilutions to be determined for each new batch of positive controls to meet a pre-specified range of Ct values. In contrast, synthetic nucleic acid-based QC materials such as plasmids are easily quantifiable, infinitely renewable, and chemically stable. As such, they may permit more stable QC data to be generated, thereby enabling QC criteria to be defined more easily. Being synthetic, nucleic acid controls can be treated as a reagent and can be transported and stored relatively conveniently.

Importantly, while patient samples are potentially biohazardous, plasmid-derived materials can be regarded as safe and therefore are particularly suited as surrogates for the detection of highly contagious diseases [1, 2]. For example, a Control Set for *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis*, comprising defined copy numbers of linearized positive control plasmids in carrier nucleic acid, is available as exogenous positive control for BD's (Franklin Lakes, NJ, USA) ProbeTec Amplified DNA Assay for these organisms.

Exogenous controls may also undergo and verify the extraction steps. For example, one study [3] proposed the use of a plasmid-transfected cell line as a surrogate QC material for *Chlamydia trachomatis* detection, simulating clinical samples for nucleic acid extraction because *C. trachomatis* exists within epithelial cells [4-6]. Such controls are also useful as reference materials for quality assurance (QA) programs in accredited laboratories. Finally, internal control material may be added into a sample and is processed with the sample. Such controls usually carry detection targets unrelated to the disease being detected, such as those derived from phages or plants, so as not to interfere with the assay. Internal controls are commonly provided in many diagnostic amplification assays.

Despite the usefulness of plasmids as reference materials for PCR, there are very few reports describing the derivation and characterization of these materials for QC purposes [1,2,7,8]. The objective of this study is to construct eleven *Escherichia coli* plasmids, each of which carry a DNA fragment that is targeted by one of the six multiplex real time-PCR assays used in this laboratory. Data obtained from the use of these plasmids for routine QC were evaluated. A simplified workflow was established to QC these plasmids prior to their release for routine testing. Finally, we demonstrated the spike-in of plasmid material into clinical specimens, as a surrogate for an infectious and controlled organism *Burkholderia pseudomallei*, avoiding the culture of live organisms for routine QA purposes.

MATERIALS AND METHODS

PCR amplification, TA cloning, and DNA sequencing

Target amplicons were amplified using existing primers as per the Molecular Laboratory SOP (Supplementary Table S1) using HotStar Taq polymerase (Qiagen, Netherlands). Templates were either archival DNA of positive samples or DNA amplicons generated by assembly PCR (Supplementary Table S1). NTCs corresponding to each target were included in every run. Each PCR reaction comprise 10 µL 2x HotStar mastermix (Qiagen, Netherlands), 0.5 µM each forward and reverse primers, 5 µL template DNA, and 3 µL nuclease free water. TOPO TA Cloning (Life Technologies, Carlsbad, CA, USA) was carried out according to the manufacturer's instructions. Positive clones were identified by PCR and sequenced in a ABI3730 DNA sequencer (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Plasmid extraction was carried out using Qiagen Miniprep kit according to the manufacturer's instructions. Concentrations of resultant plasmids were quantitated using NanoDrop (Thermo Fisher Scientific).

Real-time PCR

Real-time PCR was carried out as per SOP using routine pre-aliquoted mastermixes on RGQs (Qiagen) or CFX-96 (Bio-Rad, Hercules, CA, USA). One set of runs (screening runs) was carried out using pooled plasmids in a range of dilutions to estimate the dilution at which a targeted Ct value could be obtained. A dilution was chosen such that the Ct value produced will be interpreted as positive based on established QC criteria, and a further 1:16 dilution should still be detected as positive. One set of pilot runs at a selected dilution was then conducted, with an appropriate DNA internal control (IC) incorporated, together with the corresponding 1:16 dilution, to confirm the performance of the plasmids at the selected dilution. The screening and pilot runs were run using the same reagent lot and on the same CFX-96 (for LEGN and CPMP) or on any of the four identical RGQs in the laboratory (for remaining tests).

The DNA internal control (IC) is 154 bp DNA construct consisting of a MS2 Bacteriophage sequence constructed by PCR amplification using overlapping primers (not shown). The IC can be amplified with the forward and reverse primers for IC (Supplementary Table S1). The amplified IC was quantitated using NanoDrop and added to each positive control at a pre-determined ratio. The IC is added to the testing samples during the automated extraction procedure (EZ1, Qiagen), and the QC criteria for the IC within a negative sample is Ct = 25 ± 5 or Ct = 30 ± 5, depending on the test. The IC does not play a critical role in the QC of the positive controls. Nevertheless, the IC was added to each positive control preparation and should be detectable in each positive control material.

Table 1. Summary of the performance of plasmid pools in pilot runs.

Assay	Plasmid	Conc. (ng/µL)	260/ 280	Vector bp	Target bp	Total bp	Copies/ µL	Channels	Thres-hold Ct	Lower limit	Upper limit	Predicted Ct	Copies/ µL	Dilution	Observed Ct[3]	1:16 Ct
HSV1/ HSV2/ VZV	H1-1	193.1	1.89	3956	143	4099	4.36E + 10	Green	32	22	27	25	110400	1.19E + 06	24	28
	H2-1	44.3	1.92	3956	143	4099	1.00E + 10	Yellow	32	22	27	26	11040	2.72E + 06	25	30
	V-1	135.2	3956	3956	113	4099	3.08E + 10	Orange	30	20	25	24	11200	8.25E + 06	24	28
	IC[1]														27	31
CET	Cb-1	97.9	1.92	3956	98	4054	2.24E + 10	Yellow	29	19	24	22	46000	1.46E + 06	19	25
	E-1	112.3	1.91	3956	115	4071	2.56E + 10	Orange	29	19	24	24	40800	1.88E + 06	24	28
	T-1	141.9	1.89	3956	152	4108	3.20E + 10	Green	30	20	25	22	36000	2.67E + 06	20	26
	IC[1]														30	34
LEGN	L-1	147.4	1.9	3956	266	4222	3.23E + 10	Green	36	25	30	25	160	1.00E + 08	26	30
								Orange	36	25	30	25	160	1.00E + 08	26	31
	IC[2]														31	35
BUPS	BU-1	43.4	1.9	3956	81	4037	9.96E + 09	Green	34	25	30	25	51	1.95E + 08	26	30
	IC[1]														27	Unde-tected
CPMP	CP-1	43.8	1.91	3956	74	4030	1.01E +10	Yellow	37	25	30	25	679	2.97E + 07	29	35
	MP-1	62.8	1.88	3956	117	4073	1.43E + 10	Green	37	25	30	25	676	4.23E + 07	29	34
	IC[2]														31	37
PCP	PCP-3	51.6	1.94	3956	72	4028	1.19E + 10	Green	32	25	30	25	50	2.37E + 08	25	30
	IC[1]														29	33

Abbreviations: HSV1/HSV2/VZV - Herpes simplex virus types 1 and 2/ Varicella-zoster virus, CET - Cytomegalovirus/Epstein-Barr virus/ - ***Toxoplasma gondii,*** **BUPS -** ***Burkholderia pseudomallei*****, LEGN -** ***Legionella*** **spp./*****L. pneumophila*****, CPMP -** ***Mycoplasma pneumoniae/ Chlamydophila pneumoniae,*** **PCP -** ***Pneumocystis jirovecii*****. [1] - 15 µL IC per 500 µl, [2] - 30 µL IC per 300 µl, [3] - Acceptable range for IC is 25 ± 5 for HSV1/HSV2/VZV, CET, and BUPS; or 30 ± 5 for LEGN, CPMP and PCP.**

Table 2. Inter- (a) and intra- (b) run performance of the HSV1/HSV2/VZV positive control.

a)

Target	Replicate	Final Ct	Ave	SD	1:16 Ct	Ave	SD
H1 Green	1	24	24	0.08	28	29	0.65
	2	24			29		
	3	24			29		
H2 Orange	1	25	24	0.52	30	29	1.18
	2	24			28		
	3	24			28		
VZV Yellow	1	24	25	1.14	28	29	1.25
	2	26			30		
	3	26			30		
IC Crimson	1	27	28	1.08	31	31	0.40
	2	29			32		
	3	27			31		

b)

Target	Replicate	Final Ct	Ave	SD	1:16 Ct	Ave	SD
H1 Green	2	24	25	0.68	29	30	0.66
	3	25			29		
	4	25			30		
H2 Orange	2	24	25	0.53	28	28	0.85
	3	25			28		
	4	25			29		
VZV Yellow	2	26	27	0.55	30	31	0.74
	3	27			30		
	4	27			32		
IC Crimson	2	29	28	0.85	32	31	1.10
	3	28			32		
	4	27			30		

The positive controls were added at 5 µL per 20 µL PCR reaction (for HSV1/HSV2/VZV, LEPN, CPMP, and PCP), 5 µL per 25 µL PCR reaction (for CET), or 10 µL per 20 µL PCR reaction (for BUPS).

Use of BUPS plasmid as a surrogate for *B. pseudomallei* in a clinical sample

An archival endotracheal tube aspirate (ETTA) sample (P150002743) stored at -80°C was em-ployed as the matrix for testing. 0.5 mL of the matrix was spiked with 10 µL of 5.1 x 10^4 copies/µL (1000x of positive control) of BUPS plasmid (BU-1) or 10 µL of nuclease-free water (NFW). The samples were then pro-cessed as per SOP for BUPS test.

RESULTS AND DISCUSSION

All 11 targets were successfully amplified and cloned into TOPO vectors (Figure 1A and B). For each target, one PCR-positive plasmid clone was selected and sequenced using M13 forward primer. The identities of the cloned inserts were confirmed by detection of expected DNA sequences of each target (not shown). The objective was to construct moderate positive controls, which were also used as weak positive controls after 1:16 dilution. A desired Ct-range was determined using pre-established Ct thresholds for each assay (Table 1). For example, for HSV1/HSV2/VZV multiplex assay, the Ct thresholds were 32 for HSV1/HSV2 and 30 for VZV (that is, Ct < 32 for HSV1 or HSV2, and < 30 for VZV will be reported as positive). The permitted range of Ct for the positive control would then be (Ct - 10) < Ct range < (Ct - 5). Hence, for HSV1/HSV2 and VZV, the desired Ct would be 22 - 27 and 20 - 25, res-

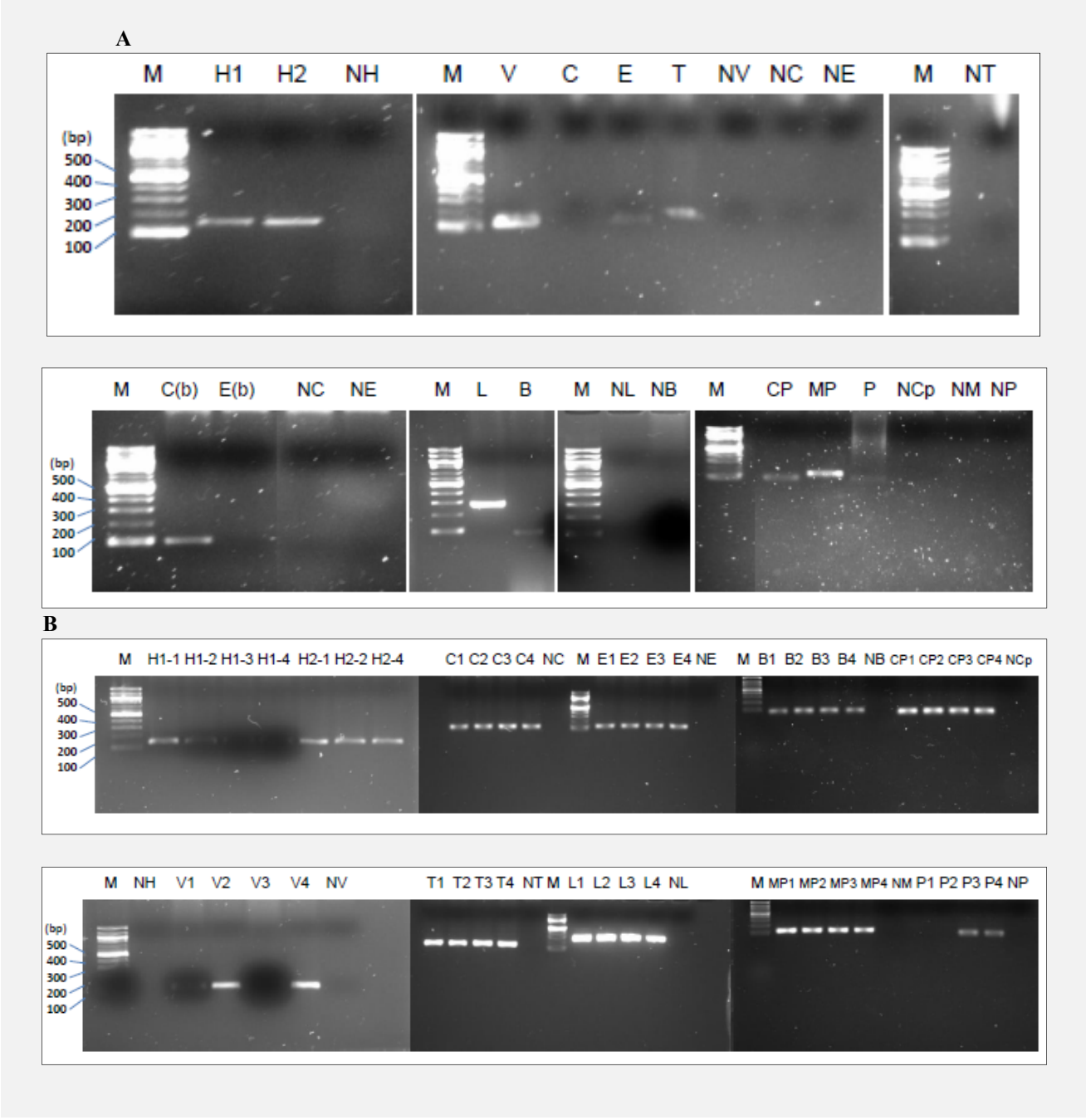
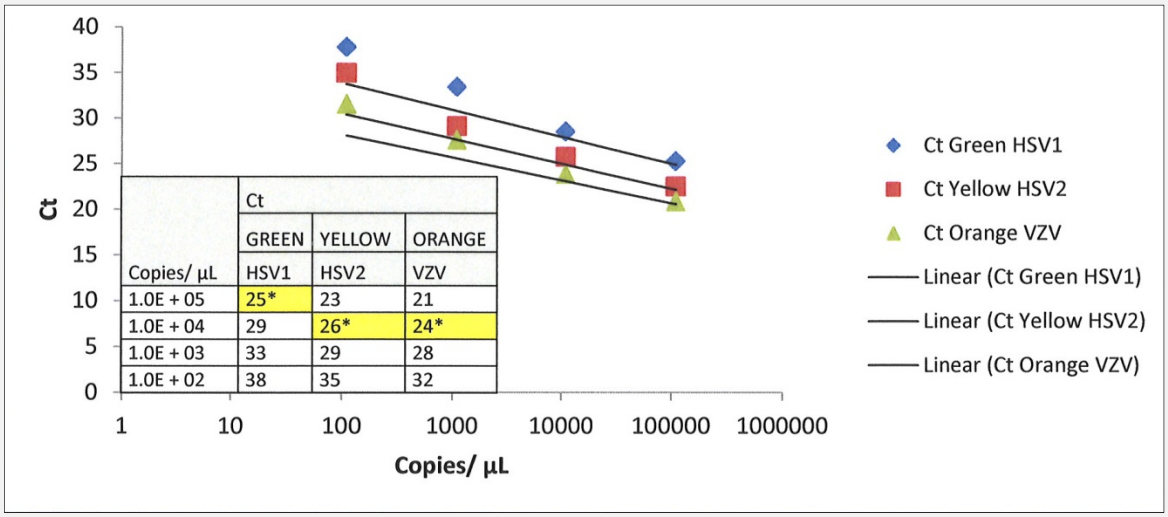


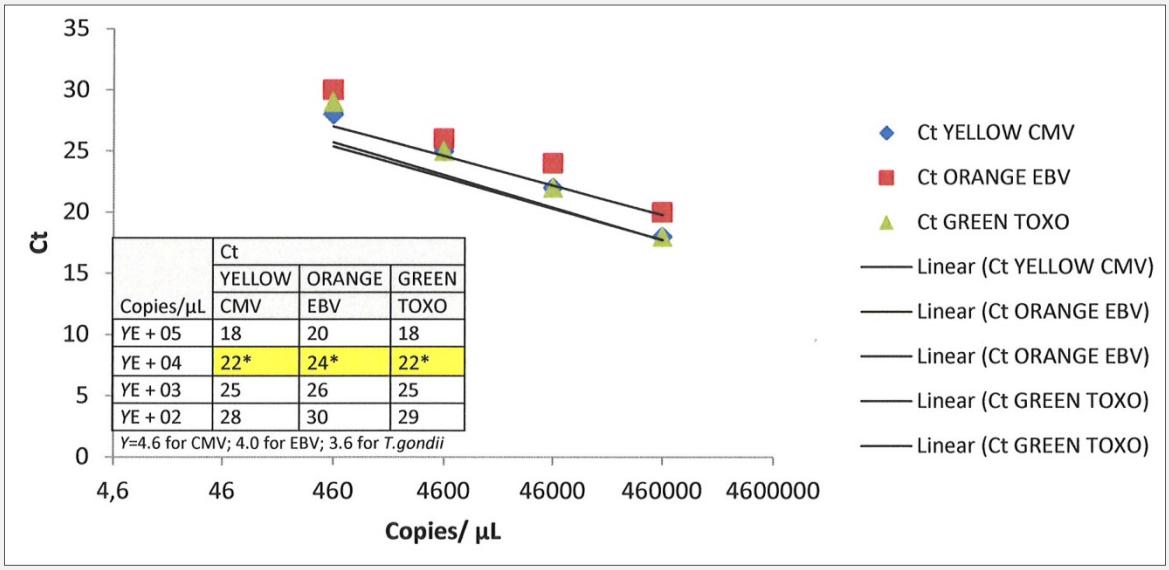
Figure 1. PCR amplification and TOPO cloning of targets.

(A) PCR amplification. M - 100 bp DNA ladder, H1 - HSV1, H2 - HSV2, NH - NTC for HSV PCR, V – VZV, C - CMV (failed), E – EBV, T - *T. gondii*, NV - NTC for VZV PCR, N(C) - NTC for CMV PCR, NE - NTC for EBV PCR, NT - NTC for *T. gondii* PCR, C(b) - CMV fragment, E(b) - EBV fragment (failed), NC - NTC for CMV PCR, NE - NTC for EBV PCR, L - LEGE fragment, B - BUPS fragment, NL - NTC for LEGE PCR, NB - NTC for BUPS PCR, CP - CP fragment, MP - MP fragment, P - PCP fragment, NCp - NTC for CP PCR, NM - NTC for MP PCR, NP - NTC for PCP PCR.

(B) PCR analysis of TOPO clones. M - 100bp DNA ladder, H1-1 to H1-4 - HSV1 clones 1 to 4, H2-1 to H2-4 - HSV2 clones 1, 2 and 4, C1 to C4 - CMV clones 1 to 4, from C(b), E1 to E4 - EBV clones 1 to 4, B1 to B4 - BUPS clones 1 to 4, CP1 to CP4 - CP clones 1 to 4, V1 to V4 - VZV clones 1 to 4 (no fragment in clone 3), T1 to T4 - *T. gondii* clones 1 to 4, L1 to L4 - LEGE clones 1 to 4, MP1 to MP4 - MP clones 1 to 4, P1 to P4 - PCP clones 1 to 4 (no fragments in clones 1 and 2).

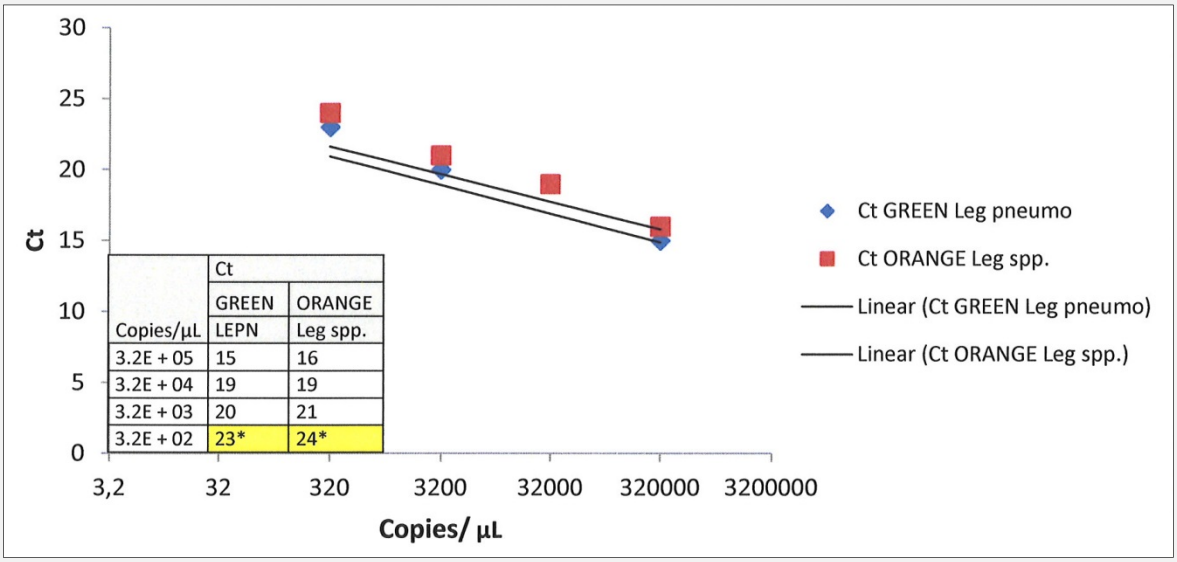


Copies/ µL	Ct GREEN HSV1	Ct YELLOW HSV2	Ct ORANGE VZV
1.0E + 05	25*	23	21
1.0E + 04	29	26*	24*
1.0E + 03	33	29	28
1.0E + 02	38	35	32

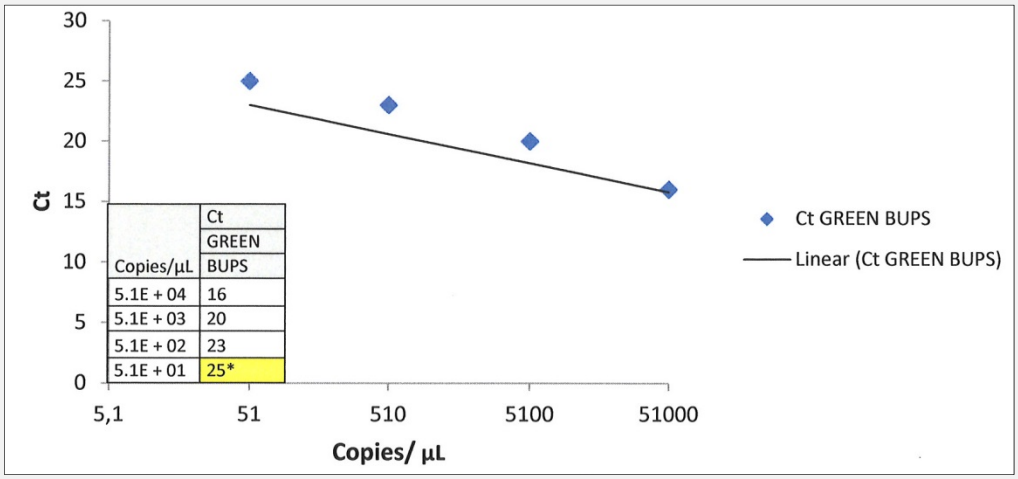


Copies/µL	Ct YELLOW CMV	Ct ORANGE EBV	Ct GREEN TOXO
YE + 05	18	20	18
YE + 04	22*	24*	22*
YE + 03	25	26	25
YE + 02	28	30	29

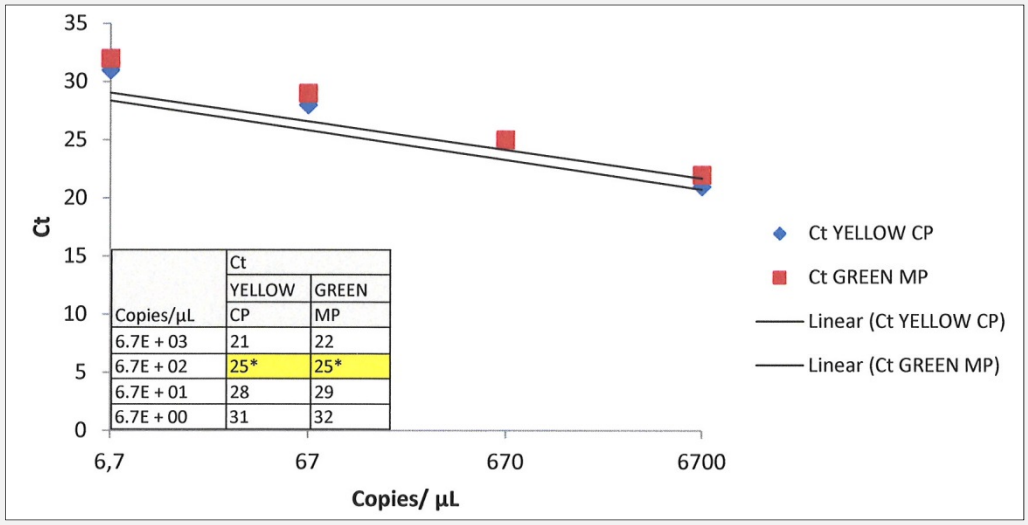
Y=4.6 for CMV; 4.0 for EBV; 3.6 for *T.gondii*



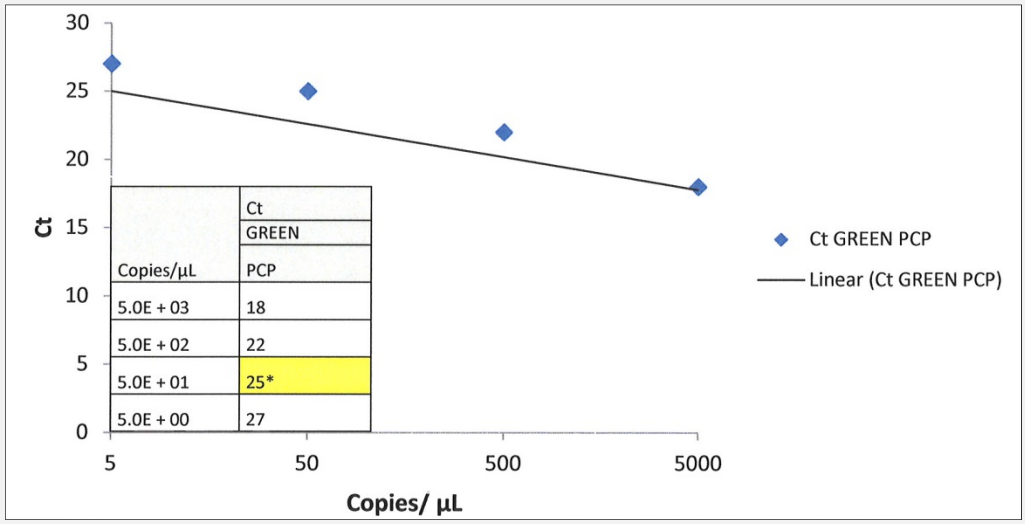
Copies/µL	Ct GREEN LEPN	Ct ORANGE Leg spp.
3.2E + 05	15	16
3.2E + 04	19	19
3.2E + 03	20	21
3.2E + 02	23*	24*



Copies/µL	Ct GREEN BUPS
5.1E + 04	16
5.1E + 03	20
5.1E + 02	23
5.1E + 01	25*



Copies/µL	Ct YELLOW CP	Ct GREEN MP
6.7E + 03	21	22
6.7E + 02	25*	25*
6.7E + 01	28	29
6.7E + 00	31	32



Copies/µL	Ct GREEN PCP
5.0E + 03	18
5.0E + 02	22
5.0E + 01	25*
5.0E + 00	27

Figure 2. Screening runs using a range of dilutions of pooled plasmids in various assays. The selected Ct values were indicated by asterisks (*). For LEGN (Leg. Pneumo/Leg. Spp), a further 2x dilution (160 copies/µL) was used in the testing run. NFW, nucelase-free water.

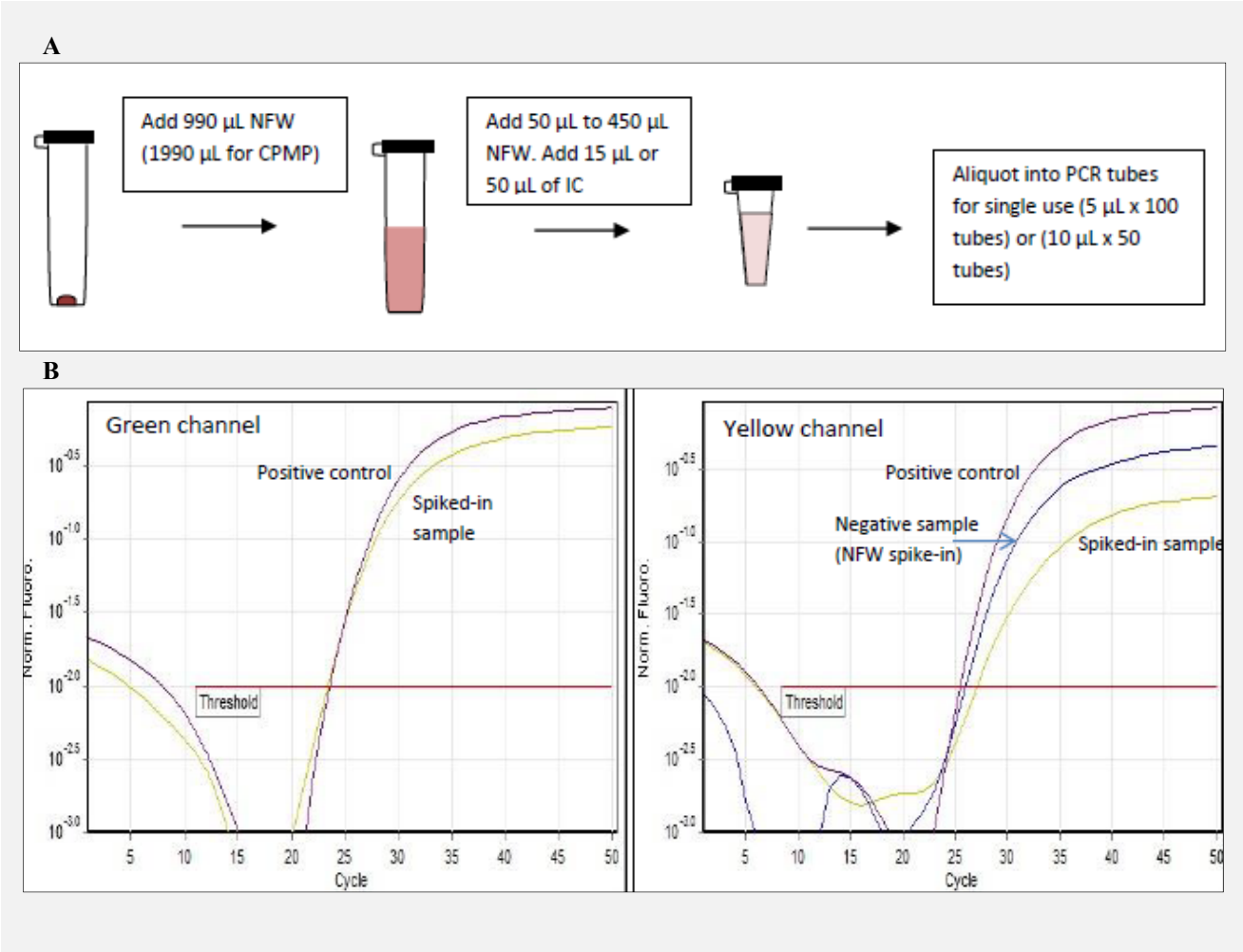


Figure 3. (A) Workflow for the routine production and QC of positive control; (B) use of BUPS plasmid as a surrogate for *B. pseudomallei.*

pectively. Similarly, for CET the pre-established Ct threshold for CMV/EBV and *T. gondii* were 29 and 30, respectively. Hence, the desired Ct would be 19 - 24 and 20 - 25, respectively. However, as the Ct cutoffs for LEGN, BUPS, CPMP and PCP were somewhat high (32 - 37), an arbitrary Ct range of 25 - 30 was used. The selection of Ct values was somewhat arbitrary, with the main purpose of obtaining positive controls that would stably pass QC, even after 1:16 dilution.

The plasmids were pooled and tested in a range of copy numbers in screening runs (Figure 2), so as to determine the dilution for each plasmid to give the desired Ct value. (Copy numbers were chosen using pre-established limit of detection (LoD) curves as guides; LoD curves not shown). These plasmids were then pooled again based on the selected dilutions, and the resultant pools were tested in pilot runs to demonstrate that the expected Ct values were obtained (Table 1). IC was incorporated and tested in the pilot runs. As a result, all the plasmid pools were successfully interpreted as positive and were within their respective desired Ct range. Following to this laboratory's current QC procedure, a 1:16 dilution of the positive control was also tested for each assay. This diluted positive sample represents a low positive sample to ascertain the sensitivity of each batch of PCR master-mix. As such, this low positive sample should also be positive for each QC run. All of the 1:16 diluted plasmid pools were successfully tested as positive. Although not critical, the IC was also detected within acceptable QC ranges for each assay. Although there is also no criteria for 1:16 diluted IC, the IC for most of the assays (except BUPS) was detectable after 1:16 dilution.

The inter-run (independent dilutions and runs) and intra-run (independent dilutions run in same run) reproducibility was investigated using HSV1/HSV2/VZV plasmid pool as a representative positive control (Table 2). Results suggested that comparable Ct values can be obtained over repeated dilutions and over different runs. A proposed workflow for routine production of plasmid

controls was derived (Figure 3A). All preparatory work for involving high concentrations of positive controls was done in a separate laboratory using a set of designated pipettes. The plasmids were combined to create a 1000X stock and aliquoted to make individual batches of controls according to the workflow described in Figure 3A. The workflow has been implemented in the laboratory and current data collected over a period of about 6 months (Supplementary Table S2) showed that several batches of plasmid-pool positive-controls passed QC and were usable over this period of time. This data would reflect sources of variability such as storage time (concentrated stock) and lot of diluent (nuclease-free water), but the batches were done by the same operator using the same set of pipettes. The current stock of plasmids could last many batches of positive control preparations. The respective *E. coli* clones are stored in Microbanks in a -20^{o}C freezer.

The BUPS plasmid could also be used as a surrogate for *B. pseudomallei* for quality assurance testing. The advantage of using plasmids is the avoidance of culturing, manipulating, and transporting live *B. pseudomallei*, an organism classified as a controlled infectious agent under the Biological Agents and Toxins Act (BATA) in Singapore. The positive sample contained BUPS plasmid spiked at 20x the concentration used as an external positive control. A negative sample was spiked with nuclease-free water. Indeed the plasmid was recovered by the routine extraction process (EZ1 from Qiagen) and was detected using the routine testing workflow (Figure 3B). In this laboratory, plasmids were also used as QC materials during the preparation of new batches of PCR mastermix. A tube from a new batch is run alongside that from an in-use batch. To assure the consistency between batches of mastermix preparations, the expectation is a difference of $\leq$ 1 Ct between the batches of mastermix and for the 1:16 dilution of the positive control to be detectable.

One challenge of this study was the use of Ct values as run QC parameters. As real-time PCR is generally regarded as a qualitative method, Ct values may not be consistent enough for use as a QC parameter. Data obtained in this study suggested that Ct values were sufficiently stable to be used for QC in routine LDTs. Challenges of using plasmids as positive controls also exist. The major risk for the use of plasmids is that they were notoriously difficult to contain. However, base mutations can be incorporated into the target sequences to permit tracing of possible plasmid contamination [1]. Other methods include monitoring and detecting plasmid DNA from the environment and the use of negative controls. Plasmid contamination can also be removed using nucleic acid removing agents (e.g. DNAZap or DNA-Away), 10% sodium hypochloride, or UV irradiation. The use of dUTP-modified primers together with a uracil-n-glycosylase (UNG) step will also contain amplified plasmid DNA, as amplicons with primer regions digested by UNG cannot be propagated by amplification. Further, it cannot be known that assays on pure plasmids will give the same signal as assays on DNA from other matrices which contain other DNA and contaminants. However, such bias can be easily detectable through comparative testing. In addition, the monitoring of the usability of the plasmids over a period of 6 months and across a few batches is insufficient to yield solid estimates of the characteristics of the plasmid controls.

CONCLUSION

Plasmid controls may serve as a renewable and stable source of QC materials. They can also be used as surrogate positive DNA for spiking into clinical samples for QA purposes, avoiding the use of contagious organisms for routine or daily QC purposes. Hence, plasmids may become increasingly widely used as reference materials in future.

Declaration of Interest:
All the authors declare no conflicts of interest.

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Supplementary Table S1. Primers and templates used for this study.

Assay		Target	Forward 5' - 3'	Reverse 5' – 3'	Probe 5' – 3'	Template
HSV1/HSV2/VZV (Herpes simplex virus types 1 and 2/Varicella-zoster virus)	HSV1	UL30	GGGGTGATCGGCGAGT AYTG	ATCTGCTGGCCGTCGTA RATG	FAM/TTTGCCCCATCTGGAGCTC TCG/BHQ1	Positive patient sample
	HSV2	UL30	GGGGTGATCGGCGAGT AYTG	ATCTGCTGGCCGTCGTA RATG	/HEX/TCTGCCGCACCTGGAGC TTTC/BHQ1	Positive patient sample
	VZV	ORF28	CGATTATGCCCGCAAAC TTGT	ACGGCATGGCCCGTCTA TT	Rox/GCGTCGTGTGTTTCCATTGC TGAAT/3IAbRQSp	Positive patient sample
CET (Cytomegalovirus/Epstein-Barr virus/*Toxoplasma gondii*)	CMV	UL55	ACGTAAGCCAGACAGCA GCC	ACTCACGACCCGTGGTC ATCTTTA	HEX/TTGTCCTCG/ZEN/CCCAGT TGACCGTACTG/3IABkFQ	Positive patient sample
	EBV	BamH1-W	ACGTAAGCCAGACAGCA GCC	TTTGTGTGGACTCCTGG CG	ROXN/TCCTGCAGCTATTTCTGG TCGCATCA/3IAbRQSp	Positive patient sample
	TG	529b prepeat	AGGAGAGATATCAGGAC TGTAG	GCGTCGTCTCGTCTAGA TCG	FAM/CCGGCTTGG/ZEN/CTGCTT TTCCTG/3IABkFQ	PCR assembled target fragment
BUPS (*Burkholderia pseudomallei*)		A0179	ATCGAATCAGGGCGTTC AAG	CATTCGGTGACGACACG ACC	FAM-CGCCGCAAGACGCCA TCGTTCAT-BHQ1	Positive patient sample
LEGN (*Legionella*spp./*L.pneumophila*)	LEGN	23S-5S	GTACTAATTGGCTGATT GTCTTGACC	CCTGGCGATGACCTACT TTCG	FAM-ATCGTGTAA/ZEN/ACTCTGACTC TTTACCAAACCTGTGG-3IABkFQ	PCR assembled target fragment
	L.spp				ROX-ATCTC|+G|AA|+C|T|+C|A|+G|AA|+G|T|+G|AAAC-BHQ-2	
CPMP (*Mycoplasma pneumoniae*/*Chlamydophila pneumoniae*)	MP	PTS	GTCCACGGCTATAACAG GATGA	AACACTGAAGCGGGCAT GA	FAM-TGCTCGGTG/ZEN/ ATCGTGGGATCGTT-3IABkFQ	PCR assembled target fragment
	CP	Arg	CGTGGTGCTCGTTATTC TTTACC	TGGCGAATAGAGAGCAC CAA	HEX-CTTCAACAG/ZEN/AGAAGACCA CGACCCGTCA-3IABkFQ	PCR assembled target fragment
PCP (*Pneumocystis jirovecii*)	PLASMID CONTROLSP	β-tubulin	GATCCGAGACATGGTCG CTATT	TTCAACCTCCTTCATGG AAACAG	FAM-TGTTGCAGCGA TTTTCCGCGGTA-BHQ1	Positive patient sample
IC	IC	MS2	TTAAATCGGCTACGGGG TTG	GAGGAGAGCCGTACCCA CAC	TYE705/CGGAGGTATGTCAGATC CACGC/IAbRQSp	PCR assembled target fragment

Supplementary Table S2. Summary of the usability of plasmid pools across batches. The date of preparation is shown in brackets.

Assay	Plasmid	Cutoff Ct	Lower limit	Upper limit	Targeted Ct	Pilot Run		Batch 1		Batch 2		Batch 3	
						Final Ct	1:16 Ct	Final Ct	1:16 Ct	Final Ct	1:16 Ct	Final Ct	1:16 Ct
						(Aug 2015)		(Dec 2015)		(Jan 2016)		(Feb 2016)	
HSV1/ HSV2/ VZV	H1-1	32	27	22	25	24	28	26	30	27	31	27	31
	H2-1	32	27	22	26	25	30	28	32	28	33	28	33
	V-1	30	25	20	24	24	28	26	30	26	31	26	28
	IC					27	31	27	undetected	26	32	26	undetected
						(Aug 2015)		(Jan 2016)		(Jan 2016)		(Feb 2016)	
CET	Cb-1	29	24	19	22	19	25	23	27	20	27	22	26
	E-1	29	24	19	24	24	28	22	26	20	27	22	26
	T-1	30	25	20	22	20	26	24	28	23	30	24	29
	IC					30	34	31	undetected	28 *	undetected	28 *	32
						(Aug 2015)		(Feb 2016)					
LEGN	L-1	36	30	25	25	26	30	28	32	unavailable			
		36	30	25	25	26	31	28	31				
	IC					31	35	31	35				
						(Aug 2015)		(Jan 2016)		(Mar 2016)		(Jun 2016)	
BUPS	BU-1	34	30	25	25	26	30	26	29	26	30	26	31
	IC					27	undetected	28	undetected	28	34	28	undetected
						(Aug 2015)		(Nov 2015)					
CPMP	CP-1	37	30	25	25	29	35	30	36	unavailable			
	MP-1	37	30	25	25	29	34	29	34				
	IC					31	37	29	33				
						(Aug 2015)		(Nov 2015)		(Apr 2016)			
PCP	PCP-3	32	30	25	25	25	30	26	29	26	30		
	IC					29	33	27	undetected	29	29		

*** - Changed to 50 µL IC per 500 µL mixture.**