Technical evaluation of a Real-time polymerase chain reaction (PCR) assay for the detection of *Bartonella* spp for diagnostic purposes

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PART A: DECLARATION

I, Ghowa Booley, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been/is being, or is to be submitted for another degree in this or any other university. This work has not been reported or published prior to registration for the above-mentioned degree.

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PART B: ABSTRACT

Infective endocarditis (IE) is a rare disease affecting heart tissues. The laboratory diagnosis of culture-negative endocarditis is complicated, and largely based on the combination of nucleic acid detection methods and serological investigation. There is a paucity of published data on microbes causing culture-negative endocarditis, but a recent report indicated that the bacterium *Bartonella* was the commonest cause of culture-negative endocarditis at a tertiary care facility in Cape Town, South Africa. This laboratory-based, non-clinical pilot study, evaluated the utility of a previously published real-time PCR assay for detecting *Bartonella* spp. on cats. This will be the first time this target will be evaluated in a real-time PCR assay to detect *Bartonella* spp. in human samples.

For this study, we constructed a plasmid vector containing an insert of 83bp, derived from the *Bartonella nuo*G gene. In this non-clinical, laboratory evaluation, we used one laboratory sample to amplify the *nuo*G bacterial DNA fragment and cloned it into a plasmid vector. Using this plasmid in a technical validation, we demonstrated that the previously described assay could detect *nuo*G when using the LightCycler 480 Probes Master Mix. The results indicated that the assay reliably detected as little as 1000 copies of the target DNA, and infrequently also detected 10-100 copies of the target. The study showed no amplification using some commonly encountered organisms found in our clinical setting, thus indicating 100% specificity for *Bartonella*. We demonstrated that a plasmid construct containing an internal fragment from the *nuo*G gene successfully detected the target using a real-time PCR assay.

Future testing should include further optimisation to improve reaction efficiency of the assay with spiked diagnostic samples, including peripheral blood, and DNA extracted from heart valve samples. The utility of the RT-PCR for diagnostic purposes should be evaluated by comparing assay turnaround time, sensitivity, and specificity of this assay versus the conventional PCR and Sanger sequencing currently in use to detect *Bartonella* spp. in heart valves.

We concluded that the assay exhibited strong potential for use as a diagnostic PCR using the constructed plasmid, but that further optimization to improve PCR efficiency, and work to determine the clinical sensitivity and specificity are needed before the assay can be applied to blood samples.

Diagnostic PCR for the detection of *Bartonella* spp. from peripheral blood specimens

Validation Protocol

National Health Laboratory Service

Groote Schuur Hospital

Conducted by: Dr G. Booley

Co-investigator: Dr L. Paul

Co-investigators: Dr C. Moodley and Dr P. Naicker

April 2020

Contents

1. Background and Rationale

Bartonellae are Gram-negative, intracellular organisms, capable of causing infections in humans and animals. It is a zoonotic bacterium transmitted via vectors such as ticks, fleas and lice. Infection by *Bartonella* is associated with an array of clinical presentations, from asymptomatic individuals, mild skin manifestations, cat-scratch disease, Carrion's disease, bacillary angiomatosis and infective endocarditis (IE) (1). The most common species infecting humans are *B. henselae* and *B. quintana* (2). Risk factors for infection by *Bartonella* include deprivations and poor living conditions related to war, immunosuppression, homelessness and alcoholism, as well as the presence of body or head lice harbouring *Bartonella* (3, 4). *Bartonella* evades the host immune system by persisting in the bloodstream as an intra-erythrocytic parasite, and may be carried asymptomatically (5). It possesses unique virulence factors allowing for angiogenesis, angiomatosis, suppression of apoptosis and promotion of vegetation formation, such as those found in valvular heart disease (1).

In South Africa the majority of IE is attributed to underlying valvular heart disease (6). The diagnosis of IE is based on the modified Duke's criteria, a set of clinical features and diagnostic criteria (7-9). Laboratory diagnosis is complicated by the fact that South Africa has a 55% rate of culture-negative endocarditis (10, 11), attributed to inappropriate blood culture collection, antibiotic therapy prior to cultures, and infection with fastidious organisms such as *Bartonella* spp. Epidemiological data on culture-negative endocarditis data is lacking. The most recent detail only pertains to provincial data from the Western Cape from the public health system from 2000 and 2004 (10, 11).

In a retrospective folder review, of culture-negative endocarditis conducted at Groote Schuur Hospital between 2009 – 2013, a total of 118 heart-valve tissue samples submitted for routine culture and bacterial screening using the broad range 16S rDNA diagnostic PCR (hereafter 16S NAAT, described on page 3) for bacterial identification, was utilised. Of these, 26/118 (22%) were culture negative, and based on 16S NAAT results, 18/26 (69%) were confirmed to be due to infection with *Bartonella quintana* (Ntuli and Bamford, unpublished data). 16S NAAT was limited to those individuals referred for valve surgery and replacement only. Furthermore, at the time of surgery many may have been on treatment for endocarditis and in some instances 16S rRNA NAAT was negative because other bacterial species were responsible (e.g. *Streptococcus* spp.) (6).

Empiric antibiotic therapy is routinely administered for suspected IE, but the spectra covered by these antibiotics does not include *Bartonella* spp. Only when patients do not respond to empiric therapy, or when repeat blood cultures are negative and symptoms do not resolve, are additional *Bartonella*-specific agents administered (1, 12). The associated health care costs, morbidity, and mortality are high (6). Patients considered for this would be those who have had three blood cultures taken prior

to antibiotic administration and found to be culture negative, and meeting Duke's diagnostic criteria (6).

Laboratory diagnosis of *Bartonella* spp. infection is challenging – routine culture methods for this organism from heart valve specimens are labour intensive and growth may only appear after 12 – 14 days. Serologic testing with enzyme-linked immunosorbent assays (ELISA) have limited specificity, with cross-reactivity to other organisms (13). Indirect fluorescent antibody assay (IFA) tests for changes in levels of IgM and IgG due to bacterial infection are also not specific, and changes may be due to infection with any bacterial pathogen. Furthermore, organism-specific IFAs are not stand-alone tests, requiring correlation with histopathology or culture results (1).

A broad-spectrum, conventional polymerase chain reaction (cPCR), targeting the bacterial 16S rRNA gene, combined with Sanger sequencing, can be performed on DNA extracted from culture, tissue biopsy, or whole blood. This assay has previously been tested using DNA obtained from pure culture, with a reported sensitivity and specificity of 72% and 98%, respectively. Assays using DNA extracted from blood are routinely less sensitive (58%) potentially due to PCR inhibition by co-purified blood components or low level of infection (14, 15).

Improved sensitivity of detection can be achieved using real-time PCR based assays. Various in-house real-time assays have been described for identifying all bartonellae to species level (15,16). The *nuo*G gene target (encoding the gamma sub-unit of nicotinamide adenine dinucleotide dehydrogenase) produced no false positive results, nor did it amplify any closely related bacteria (viz. *Ehrlichia* and *Rickettsia*). Furthermore, when *nuo*G was used in a real time PCR assay, it was able to detect as little as 10 copies of the target gene in samples (16). Improved molecular testing would provide a reliable solution for identifying a fastidious organism such as *Bartonella* spp., allowing for earlier identification and subsequent targeted therapy, reducing morbidity and potentially mortality.

The *nuo*G gene thus could be a good target for a diagnostic real-time PCR assay to detect *Bartonella* spp. in peripheral blood samples in a subset of patients in whom the diagnosis is likely. Therefore, in this study we aim to develop and optimise a real-time PCR using a target previously validated for use in cats.

2. **Strategy**

This will be a laboratory-based, diagnostic accuracy study, aimed at validating a real-time polymerase chain reaction, previously described on cats (16). This genus-specific assay, targeting the *nuo*G gene of the genus *Bartonella*, will be evaluated for possible future use in our laboratory. An internal fragment of the gene, present on a plasmid, will be used to evaluate and optimise the RT-PCR.

Technical validation

3. Experimental objectives for technical validation

3.1. DNA extraction methods

DNA will be extracted from peripheral blood using a suitable automated extraction platform, as per the manufacturer's instructions, by trained laboratory personnel as per SOP VIRO243v7, Q-Pulse.

3.2. Preparation of control vector

Previously described primers (16), will be used to amplify an 83 bp fragment from the *Bartonella* spp. *nuo*G gene, and the resulting amplicon cloned using a commercial plasmid cloning kit. A recombinant plasmid will be isolated from transformed *Escherichia coli* cells, and Sanger sequencing used to confirm the insert. This plasmid construct (pTnuo) will be used for real-time assay optimisations, and as a positive control in the optimised assay.

3.3. Primers and probes

The *nuo*G primers, probes, and real-time assay conditions to identify *Bartonella* spp. have previously been published and optimised extensively, although not with human clinical samples (16). For this study, the *nuo*G- specific probe (Table 1) will be labelled with FAM (instead of Texas Red, as in original paper) for detection on the Bio-Rad CFX-96 real time PCR thermocycler currently in use in our setting. A previously validated internal amplification control (GFP) will be included to evaluate the impact of inhibitors in the assay (17). Primer and probe ratios for optimal PCR results will be experimentally determined by testing different ratios using the same PCR conditions.

Table 1. Primer and probe sequences used in this assay.

Target	Primer	Sequence (5' – 3')	Fluorescent tags	Species	Reference
*nuo*G	BarF	CAATCTTCTTTTGCTTCACC		*Bartonella* spp.	(16)
	BarR	TCAGGGCTTTATGTGAATAC			
	BarP	TTYGTCATTTGAACACG	(FAM-ZEN - IBFQ)		
GFP	GFPF	CCTGTCCTTTTACCAGACAACCA		Internal Amplification Control (IAC)	(17)
	GFPR	GGTCTCTCTTTTCGTTGGGATC			
	GFPP	TACCTGTCCACACAATCTGCCCTTTCG	(HEX-ZEN - IBFQ)		

The conditions set out for this set of primers and probes as outlined by Andre *et al.* (16), are as follows:

- A final concentration of 1.2 µM of each primer and probe

- Amplification conditions were
    - 95°C for 3 mins, followed by
    - 40 cycles at 95°C for 10 mins, and
    - 52.8°C for 30 s

3.4. Interpretation of results

The guide in Table 2 will be used to interpret the results obtained from the PCR assay.

Table 2. PCR Interpretation guide.

nuoG	IAC	Identification and Result
+	+	*Bartonella* spp. Detected
-	+	*Bartonella* spp. Not Detected
+	-	*Bartonella* spp. Detected, internal amplification control not detected. (Repeat test optional.)
-	-	Assay failed. Repeat PCR (and DNA extraction if needed).

3.5. Analytical Sensitivity (LOD)

The analytical limit of detection (LOD) of the assay will be determined to evaluate the assay's analytic sensitivity. This will be accomplished by testing the recombinant vector (pTnuo) containing the PCR target, at least five concentrations – including the approximated LOD, a concentration above the LOD, and one below the LOD. The LOD will be determined with a 95 % confidence interval, by testing at least 20 replicates of each of the selected concentrations, over a period of 3 days (18).

3.6. Precision

This parameter will be determined from the analytic LOD experiments. The repeatability and inter-run reproducibility will be determined by evaluating the variations in Ct values of all 10 replicates spanning the LOD, on different days.

3.7. Analytical Specificity

The potential for cross-reactivity will be evaluated by testing DNA from bacteria prevalent in our clinical setting. The organisms to be tested are those which are likely to be encountered in cultures and associated with infective endocarditis (Table 3). Purified DNA of the organisms in Table 3 (if

available) will be tested using the conditions and limits determined from the preceding experiments. All tests will be conducted in duplicate. *Coxiella burnetii*, routinely included in the differential diagnosis for IE, will not be evaluated here, since this is a BSL 3 organism and requires cell culture, which are not available in our setting.

Table 3. Organisms to be tested for cross-reactivity with the new assay.

Bacteria		**Fungi**	**Viruses**
Klebsiella pneumoniae	*Staphylococcus aureus*	*Candida albicans*	*Cocksackie virus*
Haemophilus influenzae	*Streptococcus pneumoniae*		
Haemophilus parainfluenzae	*Streptococcus pyogenes*		
Pseudomonas aeruginosa	*Streptococci mitis*		
Streptococcus agalactiae	*Staphylococcus epidermidis*		
Aggregatibacter spp.	*Eikenella corrodens*		
Legionella pneumophila	*Brucella melitensis*		
Legionella longbeachae			

The specificity of the primer set has previously been determined (See Table 4) (16). This validation included species of *Bartonellae* and will not be evaluated again.

Clinical validation

4. Experimental objectives for clinical validation

4.1. Sample types

This assay is being validated for the detection of *Bartonella* spp. DNA in peripheral blood samples. These samples are routinely collected in patients with suspected IE and will be readily available for further molecular testing when routine testing is unsuccessful. For clinical LOD determination, residual EDTA-peripheral blood (EDTA-PB) samples will be collected from patient samples submitted to GSH haematology for routine testing. These samples will have been collected by trained professional medical staff, as part of routine collection for diagnostic analysis. No patients will be recruited for this study.

4.2. Clinical Sensitivity

To determine impact of potential inhibitors and assay performance in a laboratory diagnostic workflow, EDTA-PB samples will be used as mock-positive samples. EDTA-PB samples will be collected, and DNA extracted to confirm they are *Bartonella* spp.-negative, using the conventional laboratory 16S rDNA PCR and previously described assay designed. PCR-negative EDTA-PB samples will be pooled and spiked with pTnuo, to prepare a dilution series of at least 3 concentrations spanning the analytic LOD.

The clinical LOD will be determined with a 95 % confidence interval, by testing at least 20 replicates each of the selected concentrations, over a period of 3 days (19-22).

4.3. Precision

This parameter will be determined from the clinical LOD experiments. The repeatability and inter-run reproducibility will be determined by evaluating the variations in Ct values of all 20 replicates spanning the LOD, on different days.

4.4. Inhibition

The presence and effect of inhibiting substances will be evaluated in the clinical LOD experiments using spiked clinical samples and amplification of the internal amplification control.

4.5. Accuracy

To determine the accuracy of the newly optimise nuoG assay to detect *Bartonella spp.* in patients with infective endocarditis, DNA from previously 16S PCR-confirmed *Bartonella*-positive heart valves, stored in the departmental repository (HREC REF: R020/2015), will run on the newly optimise nuoG assay, and the results compared.

If feasible, a prospective, parallel comparison will be used to determine the accuracy of the assay being validated. All EDTA-PB and DNA extracted from heart valves will be tested on the new assay, and the results compared to those obtained on routine 16S PCR on DNA extracted from heart valves. A commercial assay may also be included as a comparator, if available.

4.6. Specimen types:

- Correct (acceptable samples):
  - Peripheral blood collected in (EDTA-containing tubes) (EDTA-PB)

- Incorrect (reject and request correct sample type):
  - Clotted blood (containing acid citrate, in yellow top tubes)
  - Blood in sodium citrate containing tubes (blue top tubes)
  - Heparinised blood (green tops)
  - Blood culture bottles

4.7. Specimen Processing and Storage:

- Stored at 4°C fridge in C18 Microbiology Laboratory (NHLS) until processing is to be performed weekly.

4.8. Quality control:

- The prepared and sequence-confirmed plasmid vector will be included in every run as a positive control. A separate negative extraction control, processed with each batch of extractions will be included as a negative control in each run. Once the test has been validated and will be done routinely, control plasmids, primers and probes will be purchased from IDT or similar commercial provider producing high quality, synthesised nucleic acids.

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6. Approvals

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PART D: ACKNOWLEDGEMENTS

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1. INTRODUCTION

Infective endocarditis (IE) is a condition affecting either prosthetic or native heart valves, abnormal structures, as well as the lining of the heart or any other prosthetic material within the heart (6).

Considered to be a rare disease globally, it is reported to affect approximately 3-10/100 000 persons annually (23). In South Africa, the majority of infective endocarditis cases is attributed to underlying valvular heart disease, often as a result of underlying rheumatic heart disease and therefore causes infective endocarditis in younger individuals (24). In developed countries, an older population with valvular degeneration or prosthetic valves is usually affected with endocarditis (6).

The organisms commonly implicated in infective endocarditis include viridans streptococci, *Streptococcus gallolyticus* including nutritionally variant streptococci, *Staphylococcus aureus, Enterococci,* and the HACEK group of organisms (*Haemophilus* spp., *Aggregatibacter, Cardiobacterium hominis, Eikenella* spp., and *Kingella kingae),* or those commonly associated with culture-negative endocarditis such as *Coxiella, Brucella* spp. and *Bartonella* spp. The last three organisms are all zoonotic pathogens (8, 25, 26). *Brucella*-associated disease has an incidence of 0.1-0.3% per 100 000 population per annum for the years 1979-1984 in South Africa (28) without an update since. The seroprevalence data of *Coxiella* for humans is not well known, however, in cats and cattle, seroprevalences are 2% and 8% respectively (27, 28).

The diagnosis of infective endocarditis is based on clinical findings as well as laboratory testing. The modified Duke's criteria (Table 1) are often used to aid in the diagnosis of this condition (7-9). This list encompasses a group of major and minor diagnostic criteria. Major criteria include one or more positive blood cultures with an organism commonly associated with endocarditis, evidence of endocardial involvement and new valvular regurgitation (8). Minor criteria include a pre-existing heart condition, fever, vascular and immunological phenomena (8). In order to make a clinical diagnosis one requires the presence of two major criteria, or five minor criteria, or one major and three minor criteria (6).

Confirmation of a clinical diagnosis is often complicated when no organisms are cultured successfully. This may be due to a false-negative test when the clinical manifestation is highly

suggestive of IE. Other reasons for the overwhelming high rate of negative blood cultures are attributed to inappropriate blood culture collection, too little blood collected or non-viable or non-culturable microbes due to initiation of antibiotic administration prior to sampling. Fastidious organisms such as *Bartonella* spp., *Coxiella burnetii* or *Brucella* spp. may result in negative blood cultures where they fail to grow (11).

TABLE 4 | Modified Duke's Criteria[b]

[b]*Li, Sexton (8)*

MAJOR CRITERIA

Positive blood cultures for IE (1 of the following):

Typical microorganisms consistent with IE from 2 separate blood cultures with:

- *S aureus*
- *Streptococci: Viridans, S. gallolyticus, Granulicatella* spp and *Abiotrophia defectiva,*
- HACEK group – *Haemophilus aphrophilus* (subsequently called *Aggregatibacter aphrophilus* and *Aggregatibacter paraphrophilus), Actinobacillus actinomycetemcomitans (*subsequently called *Aggregatibacter actinomycetemcomitans), Cardiobacterium hominis, Eikenella corrodens, Kingella kingae*
- Community-acquired enterococci in the absence of a primary focus

Persistently positive blood culture:

- For organisms that are typical causes of IE – At least 2 positive blood cultures from blood samples drawn >12 hours apart
- For organisms that are more commonly skin contaminants – 3 or a majority of ≥4 separate blood cultures (with first and last drawn at least 1 hour apart)
- For organisms that are more commonly skin contaminants – 3 or a majority of ≥4 separate blood cultures (with first and last drawn at least 1 hour apart)

Evidence of endocardial involvement (1 of the following):

Echocardiogram positive for IE:

- Vegetation (oscillating intracardiac mass on a valve or on supporting structures, in the path of regurgitant jets, or on implanted material, in the absence of an alternative anatomic explanation)
- Abscess, or
- New partial dehiscence of the prosthetic valve

New valvular regurgitation

MINOR CRITERIA

- Predisposition – Intravenous drug use or presence of a predisposing heart condition (prosthetic heart valve or a valve lesion associated with significant regurgitation or turbulence of blood flow)
- Fever – Temperature ≥38.0°C (100.4°F)
- Vascular phenomena – Major arterial emboli, septic pulmonary infarcts, mycotic aneurysm, intracranial hemorrhage, conjunctival hemorrhages, or Janeway lesions
- Immunologic phenomena – Glomerulonephritis, Osler nodes, Roth spots, or rheumatoid factor

DIAGNOSTIC CRITERIA

2 major criteria

5 minor criteria or

1 major and three minor criteria **(6)**

2. CULTURE-NEGATIVE INFECTIVE ENDOCARDITIS IN SOUTH AFRICA

In South Africa, a 55% rate of culture-negative endocarditis (CNE) has previously been reported (6, 11). The genus *Bartonella* was identified in most of the culture-negative IE cases, in an unpublished, retrospective folder review, conducted at Groote Schuur Hospital between 2009 – 2013. In this review of diagnostic data, 26/118 (22%) heart-valve tissue samples submitted for routine culture were culture negative. Furthermore, 16S rDNA diagnostic PCR and Sanger sequencing for bacterial identification further showed that 18/26 (69%) culture-negative samples were due to infection with *Bartonella quintana* (Ntuli and Bamford, unpublished data).

There is a scarcity of published literature on the causes of CNE in South Africa, where only two reports, published in the last 5 years, highlighted *Bartonella* as a cause of CNE locally. One report is a case study describing the bacterium in an infected heart valve using 16S rRNA gene amplification and Sanger sequencing, in a patient attending a private health care facility in Cape Town, South Africa (29).

A more recent study from Tygerberg hospital indicated that *Bartonella* was the most prevalent cause of IE where no organisms were isolated from blood cultures. This study made use of serological and 16S PCR methods to identify *Bartonella* spp. (30). The implication is that endocarditis attributed to infection with *Bartonella* spp. might be underdiagnosed due to the organism's fastidious nature, and that improved diagnostic tests are needed to identify this organism reliably.

3. THE GENUS *BARTONELLA* AND HUMAN DISEASE

The genus *Bartonella* (family Bartonellaceae) are grouped in the α-proteobacteria. More than 13 species of *Bartonella* are implicated in human disease. The species most often identified in humans are *Bartonella quintana and Bartonella henselae* and to a lesser degree *Bartonella bacilliformis;* the latter is endemic to the Andes mountain range (1, 31).

The reservoirs of these Gram-negative, intracellular, fastidious, and slow-growing organisms are arthropod vectors such as ticks, fleas, or lice (32). The microbe is transmitted to humans, via direct inoculation upon taking a blood meal (33). The organism is historically famous for causing trench fever in soldiers during World War I and II. In modern times, trench fever is associated with the homeless and the carriage of body lice (1).

Other *Bartonella* species infrequently associated with human disease are *B. koehlerae*, *B. vinsonii*, *B. bovis*, *B. tamiae*, *B. doshiae*, *B. grahamii*, *B. mayotimonensis*, *B. elizabethae*, *B. washoensis*, *B. rochalimae* and *B. vinsonii* (34). Infection with *Bartonella* spp. is regarded as an emerging zoonosis and underestimated public health issue.

The risk factors for infection with *Bartonella* include wars, homelessness, immune-incompetence and alcoholism, as well as the presence of body or head lice harbouring *Bartonella* (3, 4). In a study of *Pediculus humanus* morphotype *humanus* (body lice) and *P. humanus* morphotype *capitis* (head lice), collected from nine African countries, the presence of *Bartonella* spp. was detected in both species of lice. Poverty, as evidence by gross domestic product, was identified as a main factor associated with the occurrence of body and head lice carrying *Bartonella* (35) This implies that poverty, combined with the presence of body lice, would be risk factors for human infection. Indeed, studies report on *Bartonella* spp. infection in the homeless (35-37). In a study involving homeless persons in San Francisco, one third of the participants had body lice, most of which were infected with a specific strain of *B. quintana* (38).

Locally, a study on Gauteng participants revealed the presence of *Bartonella* in 22.5% in HIV-positive patients tested and 9.5% of healthy volunteers (58). Although rare, infection in immune-competent individuals has been reported, where a recent case of atypical cat scratch disease (CSD) was recently identified in an immune-competent child presenting with typical CSD symptoms (39).

Bartonellae spp. can cause a wide range of disease among humans, factors contributing to its development is not well established (57). Disease manifestation is dependent on the infecting species as well as the immune status of the individual (33). This may range from asymptomatic individuals to trench fever, bacillary angiomatosis, or IE (1). Culture-negative endocarditis is strongly associated with more severe disease, therefore requiring improved diagnostic detection methods, especially for likely causal organisms. As mentioned before, a study from Tygerberg Hospital, found *Bartonella* to be the commonest cause of culture-negative endocarditis

INFOGRAPHIC: Associated diseases, diagnostic confirmation, antimicrobial therapy

ASSOCIATED DISEASES

Cat scratch disease
Carrion's disease
Trench fever
Other, more rare complications

- granulomatous hepatitis
- splenitis
- osteomyelitis
- encephalopathy
- neuro-retinitis
- Parinaud's syndrome preauricular (adenopathy and conjunctivitis)
- Vasculitis
- Endocarditis
- Peliosis
- Arthritis
- Bacillary angiomatosis
- Peruvian warts

DIAGNOSTIC CONFIRMATION

- Culture, organism fastidious, slow growth
- Serology
- DNA Detection, presence in infected tissue or blood
- Histology

Some disease manifestations confused with

- Kaposi's sarcoma (BA)

EFFECTIVE ANTIMICROBIALS

Azithromycin
Doxycycline

FIGURE 1 | Infographic on *Bartonella* spp

in Cape Town, South Africa (30). *Bartonella henselae* is distributed worldwide (1, 33). Infection is often associated with cats infested with fleas and domestic cats being responsible for disease transmission to humans causing bacillary angiomatosis (BA) and fever of unknown origin in immunosuppressed individuals. In persons with advanced human immunodeficiency virus (HIV) disease, which has a high prevalence in South Africa due to its large HIV-positive population, BA may be confused with deep seated fungal infections such as histoplasmosis or Kaposi's sarcoma, due to the similarities in the clinical appearance of the skin lesions (40).

Bartonella quintana is globally distributed and, as mentioned previously, often associated with poor hygiene, homelessness and the presence of human body lice (33). This species is also commonly associated with trench fever (an acute self-limiting condition associated with fevers), infective endocarditis and in the immunocompromised individual, a similar spectrum of disease as with *B. henselae* (1).

Infections with *Bartonella bacilliformis* causes a biphasic illness, characterised by haemolytic anaemia and fever, in its acute phase. This is followed by skin lesions and verruga peruana in the late phase (31). The

acute phase carries with it a high mortality, with its late disease manifestations due to its vascular proliferative pattern.

Bartonella occurs as an intracellular pathogen in humans, evading the host immune system and persisting in the bloodstream as an intra-erythrocytic parasite, and carriage may be asymptomatic (14). A number of virulence factors (Table 2) enable invasion, angiogenesis, angiomatosis, suppression of apoptosis, and promotion of vegetation formation, such as those found in valvular heart disease (1). These sequelae are evidence of an established chronic infection with transient episodes of bacteraemia (30).

TABLE 5 | *Bartonella* virulence factors

Important virulence factors[a]	**Functions**
Trimeric autotransporter adhesins (TAA) proteins: *Bartonella* adhesion A (*Bad*A) protein and variable outer membrane protein (VOMP)	Important in attachment to host cells via β1-integrins, activation of HIF-1 and NF-B, inhibition of macrophage phagocytosis. It also enables angiogenesis by upregulation of vascular endothelial growth factor (VEGF)
***Bartonella* effector proteins (Beps)(41-43)** - **VirB/D4,** - **Trw**	Inhibition of apoptosis, proinflammatory activation, angiogenesis, invasome formation allows organism to establish host uptake and endothelial proliferation
Hemin binding proteins	Binding to heme, host cell adhesion
BafA autotransporter	Mimics vascular endothelial growth factor VEGF and thus interacts with VEGF receptor 2, to induce pathways(s) responsible for cell proliferation, tube formation and sprouting of micro-vessels in mice (44).

Lipopolysaccharide A	Inhibits TLR4 proinflammatory response and inhibits complement activation and DC maturation
Type IV Secretion systems (TSS)	Essential for establishing intraerythrocytic infection of the host

[a]*Harms and Dehio (33), Schulein and Dehio (41)*

4. LABORATORY DIAGNOSTICS OF IE CAUSED BY *BARTONELLA* SPECIES

Laboratory diagnosis of *Bartonella* spp. infection can be challenging due the fastidious nature of the organism (36). The mainstay of diagnosis is based on serological and molecular techniques. Culture-based methods remain onerous and laborious. The organism can be isolated on routine culture media such as chocolate agar (incubated for a minimum of 28 days). However, the sensitivity of culture is reported to only be 20% when compared to molecular-based techniques (45).

The most commonly utilised serological diagnostic tests include enzyme-immunoassays (EIA) or immunofluorescent assay (IFA)-based methods, with either in-house or commercial microscopic IFA being the most common application. Despite the increased utility of IFA, the method is time-consuming and cumbersome when compared to EIA. EIA and IFA both determine titres of *Bartonella*-associated IgG. Good correlation with higher IgG titres of greater than 1:800 has been associated with infective endocarditis, but this should be interpreted in conjunction with other tests including molecular tests and/or histopathology, which rely greatly on testing the heart valve tissue itself (1, 46). These tests, do however, have reduced specificity, with cross-reactivity noted between *B. quintana* and *B. henselae*, *Coxiella burnetii and Mycoplasma* spp., which may also be implicated in culture-negative endocarditis (13, 46).

Detection of nucleic acid provides a reliable solution for identifying fastidious organisms such as *Bartonella* spp. and organisms which cannot be cultured in biosafety level two facilities, which are not approved to culture zoonotic organisms. A broad-spectrum, conventional polymerase chain reaction (cPCR) assay targeting the bacterial 16S rRNA gene, combined with Sanger sequencing can be performed on DNA extracted from culture, tissue biopsy, or whole blood. More frequently, this test is performed on valve tissues obtained from invasive procedures such as surgery with removal of infected or damaged valvular tissue. This assay

has previously been evaluated using DNA obtained from tissue valves, with a high sensitivity of 92% (47). Assays using DNA extracted from blood or serum are less sensitive and specific, 36% and 33% respectively (48) (14, 15). Another gene target commonly used to identify bartonellae is based on a conventional PCR assay with Sanger sequencing of the *rib*C gene target, but this assay only provides identification to genus level (12, 49). The genus identification may still assist with the clinical management of the patient.

Improved sensitivity of detection can be achieved using real-time PCR-based assays. A commercial real-time PCR assay targeting *Bartonella henselae,* but excluding *B. quintana,* is available (http://*Bartonella*.genesig.com/assets/files/b_henselae_std.pdf). Various in-house real-time PCR assays have been described for identifying all bartonellae to species level (15, 16, 50). These target the 16S-23S intergenic region, the *ssr*A gene, *glt*A gene, *fts*Z or *nuo*G gene amongst others, with *glt*A being the most commonly used gene target (Table 3) (15, 16, 50). To improve sensitivity, commercial kits may include two targets, and this can also be applied to in-house-developed molecular tests.

The *nuo*G gene target, encoding the gamma subunit of NADH (nicotinamide adenine dinucleotide dehydrogenase), was found to not yield false positive results, nor did it amplify any closely related bacteria (such as *Ehrlichia* and *Rickettsia*). Furthermore, when *nuo*G was used as the target in a real time PCR assay, it was able to detect as few as 10 copies of the target gene in positive animal samples (16). The *nuo*G gene could thus be an excellent target to detect *Bartonella* spp. in human samples as well; but has not been evaluated for this purpose as yet, with RT assays on human blood samples.

TABLE 6 | Examples of some in-house targets and commercially available kits

IN-HOUSE PCR GENE TARGETS	COMMERCIAL KITS TO DETECT *BARTONELLA*
***glt*A** ***pap*31** ***rpo*B** ***nuo*G** ***rib*C**	BactoReal® qRT-PCR detection of the gltA gene of *Bartonella* spp. specific to the detection of *B. clarridgeiae, B. elizabethae, B. grahamii, B. henselae, B. koehlerae, B. quintana, B. volans* and *B. washoensis. Bartonella rochalimae, B. vinsonii* and *B. bovis* are detected with very low sensitivity. *Bartonella bacilliformis* is not detected (51). *Bartonella henselae* Real-time PCR Kit, RUO-NZYTech Real-time PCR Kit for *B. quintana* is designed for the in vitro quantification of *B. quintana* genomes (52). Targeting *rib*D

Bartonella spp. detection using PCR methods on direct blood samples has been performed for cat-scratch disease looking for *B. henselae*, but this was unfortunately limited due to its small sample size, and produced a sensitivity of only 18% when performed on serum (53). In Carrion's disease, DNA-based methods for the detection of *B. bacilliformis* using both specific molecular methods and universal 16S rRNA, has proven to assist with diagnosis when correlated with clinical findings, in a high prevalence setting (54). With the alarmingly high number of culture-negative endocarditis infections in our setting, a molecular test coupled with clinical findings may greatly improve disease diagnosis rates, and subsequently patient outcomes (6, 10, 11).

The introduction of a rapid, in-house, molecular assay for the detection of *Bartonella* DNA directly from peripheral blood samples would greatly impact on patient management. Empiric antibiotic therapy is routinely administered for suspected infective endocarditis and only de-escalated to specific regimens once a specific microbe is identified using routine diagnostic testing methods. Empiric therapy for IE usually consists of dual therapy with penicillin and gentamicin, and in few cases may include cloxacillin if *S. aureus* is suspected (based on history or clinical findings). This regimen would not routinely be effective for IE with organisms such as *Bartonella* and other organisms commonly associated with culture-negative endocarditis.

This therefore results in unnecessary exposure to inappropriate antibiotics, and a delay of appropriate targeted antibiotic therapy with doxycycline (1, 55).

Prolonged hospital stays and subsequent surgical intervention are associated with increased health care costs, morbidity, and mortality (3, 29, 49). Furthermore, *Bartonella* spp. associated endocarditis is often associated with destructive disease with resultant surgical intervention (49, 56). Therefore, in the absence of a positive blood culture, or failure to respond to therapy, or when repeated cultures remain negative, a diagnosis of culture-negative endocarditis should be considered.

5. IN SUMMARY

The introduction of a reliable diagnostic test for the rapid identification of *Bartonella* spp. infection may greatly assist in early diagnosis and promote effective antibiotic stewardship. Its prompt diagnosis may also assist in shorter hospital stay and possibly even fewer complications. In addition to identifying *Bartonella* spp. in infective endocarditis, a molecular test could also be useful to diagnose other conditions associated with bartonella. It may be used in the diagnosis of bacillary angiomatosis (BA), ocular infections, cat scratch disease, and for differentiation between Kaposi's sarcoma and BA (49). A molecular test could potentially be performed on peripheral blood samples and may potentially serve as an adjunct to the diagnosis of BA. With a rapid turnaround time and its less invasive nature - the diagnosis for infective endocarditis due to *Bartonella* spp. may well eliminate the uncertainties of serological tests performed early on in the diagnosis of infective endocarditis. In fact, such a test performed at the time of the first set of blood cultures may assist with earlier initiation of treatment for bartonella endocarditis, if the pathogen is considered.

Whilst there is an emphasis on early diagnostics, eradication of the disease would be the ideal goal. As a zoonotic disease a One Health approach may prove useful in an attempt to reduce the transmission to mammals and humans by means of ectoparasite control (31). Another strategy would be to ensure good sanitary practices and in an ideal world eradication of homelessness and poverty.

A multisectoral collaboration, in-line with One Health strategies, may prove to be quite challenging with the vast number of arthropod and mammalian reservoirs involved, but targeted screening could aid identification of hotspot areas where public health improvement is required, similar to that used for malaria.

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PART H: JOURNAL MANUSCRIPT

Technical evaluation of a Real-time polymerase chain reaction (PCR) assay for the detection of *Bartonella* spp for diagnostic purposes

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Abstract

The laboratory diagnosis of culture-negative endocarditis is complex, and largely based on the combination of PCR-based methods and serological investigations performed on heart valve tissues. The turnover time for molecular testing could be improved with the use of real-time polymerase amplification assays (RT-PCR), which is more sensitive and therefore able to detect low levels of target bacteria in a sample. This non-clinical, laboratory-based study evaluated the utility of a published RT-PCR assay to detect *Bartonella* spp. for future use in human samples such as blood and tissues.

For this study, we constructed a plasmid vector containing an insert of 83bp, derived from the *Bartonella nuo*G gene. In this laboratory evaluation, we demonstrated that the previously described assay could detect *nuo*G when using the LightCycler 480 Probes Master Mix. The results indicated that the assay reliably detected as little as 1000 copies of the target DNA, and infrequently also detected 10-100 copies of the target. The study showed no amplification using some commonly encountered organisms found in our clinical setting, thus 100% specificity for *Bartonella*. We demonstrated that a plasmid construct containing an internal fragment from the *nuo*G gene successfully detected the target using a RT-PCR assay.

Future testing should include further optimisation to improve reaction efficiency of the assay with spiked diagnostic samples, including peripheral blood, and DNA extracted from heart valve samples. The utility of the RT-PCR for diagnostic purposes should be evaluated by comparing assay turnaround time, sensitivity, and specificity of this assay versus the conventional PCR and Sanger sequencing currently in use to detect *Bartonella* spp in heart valves.

1 Introduction

Infective endocarditis (IE) is a rare disease globally, affecting approximately 3-10/100 000 persons annually (Khan et al., 2016). In South Africa, the majority of infective endocarditis cases is attributed to underlying valvular heart disease, when compared to elderly patients in more developed countries (Hitzeroth et al., 2015). The diagnosis of infective endocarditis is often based on the modified Duke's criteria (Durack et al., 1994; Hitzeroth et al., 2015; Nishimura et al., 2008). The organisms commonly implicated in infective endocarditis include Viridans streptococci, *Streptococcus gallolyticus* including nutritionally variant streptococci, *Staphylococcus aureus, Enterococci,* and the HACEK group of organisms (*Haemophilus* spp., *Actinobacillus actinomycetemcomitans, Cardiobacterium hominis, Eikenella spp*, and *Kingella kingae)* (Habib et al., 2015; Li et al., 2000; Prendergast, 2006).

When organisms are not isolated using culture-based diagnostic methods of suspected IE heart valve tissues or blood samples, culture-negative endocarditis (CNE) is considered. CNE is often associated with more severe disease warranting surgical intervention (Koegelenberg, 2003; Pecoraro et al., 2021). Organisms often implicated in this condition include *Bartonella* spp., *Coxiella burnettii*, or *Brucella* spp. In South Africa, a 55% rate of culture-negative endocarditis (CNE) has previously been reported (Koegelenberg et al., 2004; Koegelenberg, 2003; Pecoraro et al., 2021). *Bartonella* was identified in the majority of the CNE cases in two tertiary-care hospitals in the Western Cape, with the diagnosis confirmed using serology and/or 16S PCR methods to identify *Bartonella* (Pecoraro et al., 2021).

Bartonella spp. are Gram-negative, intracellular, slow-growing organisms associated with arthropod vectors and are transmitted to humans during a blood meal (Stacey R. Rose, 2020). Two important species associated with human CNE include *Bartonella henselae* and *Bartonella quintana*. *B. henselae* is distributed worldwide (Okaro et al., 2017; Stacey R. Rose, 2020) and often associated with cats infested with fleas. It is responsible for disease transmission to humans causing bacillary angiomatosis (BA) and fever of unknown origin in immunosuppressed individuals. In persons with advanced Human Immunodeficiency Virus (HIV) disease, BA may be confused with deep seated fungal infection such as histoplasmosis or Kaposi's sarcoma due to the similarities in its clinical appearance of the skin lesions (Forrestel et al., 2015). *B. quintana* is globally distributed and often associated with poor hygiene, homelessness and the presence of human body lice (Harms & Dehio, 2012). *B. quintana* is commonly associated with trench fever, an acute self-limiting condition associated with fevers, infective endocarditis, and in the immunocompromised individual a similar spectrum of disease as with *B. henselae* (Okaro et al., 2017).

Laboratory diagnosis of *Bartonella* infection can be challenging due its fastidious nature, with the sensitivity of culture only about 20% when compared to molecular based techniques (Fournier et al., 2002; Lam et al., 2019). The mainstay of diagnosis is serological and molecular based methods.

Detection of nucleic acids provides a reliable solution for identifying fastidious organisms such as *Bartonella.* A broad-spectrum, conventional polymerase chain reaction (cPCR) targeting the bacterial 16S rRNA gene is useful to detect the organism in infected heart valve tissues and this method is used in local laboratories. This assay has previously been tested using DNA obtained from endocardial tissue with a reported sensitivity 97,8% and this despite the use of antibiotics a specificity of 92% in a recent study (Edouard et al., 2015). Assays using DNA extracted from blood or serum are less sensitive, 36% and 33% respectively and thought to be due to the prior use of antibiotics (Diaz et al., 2012; Edouard et al., 2015).

A number of in-house, real-time assays have been described for identifying all *Bartonellae* to the genus level, these include one or more of the following gene targets: *ssr*A, *glt*A, *fts*Z, or *nuoG*

(Kosoy et al., 2018). The *nuo*G target is a well described gene target with high specificity and sensitivity, with a described the limit of detection (LOD) as 100 copies (95% confidence interval), although as low as 10 copies of the target gene in an animal (Andre et al., 2016; Colborn et al., 2010). This real-time PCR assay has since been used to detect the organism in the peripheral blood of animals, as well as a heart valve of a dog with infective endocarditis (Andre et al., 2016). Therefore, the *nuoG* gene may be a strong target candidate for detecting *Bartonella* in human samples.

In this study we evaluated a previously described assay (Andre et al., 2016) using *nuoG* as the PCR target, to discern assay performance with an in-house constructed plasmid, a commercial PCR kit and real-time PCR, as a potential assay for human diagnosis of *Bartonella* infection.

2 Materials and methods

2.1 Construction of control plasmid vectors

A plasmid vector containing an internal fragment of the proposed target gene was constructed to serve as the PCR target and subsequent positive control in the validation experiments (*Supplementary Fig. 1*). For this, previously described primers (Table 1) (Andre et al., 2016), were used to amplify an internal fragment of the *nuoG* gene (83bp) from a residual clinical sample previously confirmed to contain *Bartonella* DNA, using an in-house 16S diagnostic PCR. The target fragment was amplified using conventional PCR, and amplicons were ligated into a commercial plasmid cloning vector (pTZ57R/T) (Thermo Scientific) as per kit insert instructions (Hoffmann et al., 2008; Zilinskiene, 2016). Recombinant plasmids were transformed into competent *Escherichia coli* cells and selected for using ampicillin (100µg/ml) and blue-white screening, with IPTG and X-Gal (Zilinskiene, 2016). White colonies were sub-cultured in the presence of ampicillin, followed by plasmid isolation using a commercial extraction kit (Monarch Plasmid Miniprep Kit, NEB). The cloned inserts were confirmed using Sanger sequencing (Inqaba Biotec, Pretoria, South Africa). Sequences were analysed using Chromas (Technelysium Pty Ltd) and identified using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The resulting plasmid construct containing the internal target sequence for the *nuo*G gene (pT*Nuo*8) was used for further work.

A previously validated internal amplification control (IAC) plasmid (pGFP), containing a DNA fragment from the green-fluorescent protein encoding gene in Aequorea victoria was included to monitor the impact of inhibitors in the assay (Murphy et al., 2007).

TABLE 1 | *Bartonella* specific real-time PCR parameters with primer and probe details. Details of forward and reverse probes and primers with their sequences with specific PCR conditions used for the optimising processes.

Target	Primer	Sequence (5'-3')	Fluorescent tags	PCR Conditions	Reference
***nuo*G**	BarF	CAATCTTCTTTTGCTTCACC	(FAM-ZEN - IBFQ) *	95ºC for 3 minutes, 40 cycles at 95ºC for 10 minutes 52.8ºC for 30 second/per Roche package insert at 60ºC	(Andre et al., 2016)
	BarR	TCAGGGCTTTATGTGAATAC			
	BarP	TTYGTCATTTGAACACG			
GFP	GFPF	CCTGTCCTTTTACCAGACAACCA	(HEX** -ZEN - IBFQ)		(Murphy et al., 2007)
	GFPR	GGTCTCTCTTTTCGTTGGGATC			
	GFPP	TACCTGTCCACACAATCTGCCCTTTCG			

*FAM - Fluorescein amidites, ZEN – propriety quencher developed by Integrated DNA Technologies, IBFQ - Iowa Black® Fluorescent Quencher
**HEX: Hexachloro-fluorescein

2.2 Bartonella specific real time PCR

The RT-PCR using the published *nuoG* primers, probe, and assay conditions to identify *Bartonella* spp. has previously been described and optimised extensively, although not using human clinical samples (Andre et al., 2016). For this study, the *nuo*G specific probe *(Table 1*) was labelled with FAM for detection on the Bio-Rad CFX-96 Touch (Bio-Rad). PCR reactions of 25 µl contained LightCycler® 480 Probes Master Mix (Roche) (Hoffmann et al., 2008), both sets of primers and probes to simultaneously detect *nuoG* and GFP, and 5µl of target DNA. Primer and probe ratios for optimal PCR results were experimentally determined as below.

2.3 PCR optimisation

The assay as defined by Andre *et al.,* was optimised for use with the IQ SYBR Green kit, with an annealing temperature of 52.8ºC and 1.2µM primer concentration. To adapt this for use in our setting, the assay was performed using the LC480 Probes Master kit (Roche) which requires an annealing and elongation temperature of 60ºC. To determine the effect of this change in temperature as well as the impact of changes in primer concentrations, the assay was run separately at both temperatures (52.8ºC and 60.0ºC), as well as using different primer concentrations (1.0, 1.2, 1.4µM), with the *pTnuo*8 and pGFP plasmids, and LC480 Probes Master Mix kit. Primer concentration was tested in 2 separate runs, in triplicate at 60ºC, but only once at 52.8ºC, in triplicate. The PCR conditions were 95ºC for 3 minutes followed by 40 cycles at 95ºC for 10 minutes and 52.8ºC for 30 seconds (*Table 2*) (Andre et al., 2016).

2.4 Limit of detection studies

To determine the assay's lower LOD, ten-fold serial dilutions (10^6 ng/µl – 10^1 ng/ul) of the control vector (pT*nuo*8) were prepared around the reported LOD (Andre et al., 2016). The LOD was determined by repeated testing of the prepared DNA series, with minimum of 24 replicates of each concentration, over a period of 3 days, to produce an estimate with a 95% confidence interval.

2.5 Repeatability and Reproducibility

Repeatability and reproducibility were determined using the limit of detection experiments to assess intra- and inter-assay variability.

2.6 Amplification efficiency

Amplification efficiency (E) was calculated by the Bio-Rad CFX-96 instrument, using the following formula ($E = 10^{-1/slope}$) which is performed by the software available on the instrument.

2.7 Specificity

The primers were previously tested using a subset of bacterial pathogens (Andre et al., 2016). Therefore, we only tested whether the primer set would amplify DNA from pathogens frequently encountered in our clinical setting. The specificity of the assay was assessed using the DNA from several organisms commonly encountered.

Descriptive statistics was used to analyse the generated data.

3 Results

3.1 Temperature and primer optimisation

To determine the optimal temperature and primer concentrations, the assay was tested using 2 temperatures and 3 primer concentrations. The results of these experiments showed that at a temperature of 52.8ºC, as outlined by Andre *et al*., all primer concentrations of 1.0µM, 1.2µM and 1.4µM were reliably amplified and within 1 Ct-value of each other, and for each primer concentration. However, internal amplification controls were not included in the first run at 52.8ºC.

The results of the temperature and primer concentration optimization assays are summarised in Table 2.

TABLE 2 | Primer and temperature optimisation of *nuo*G. Primer concentrations of 1.0µM, 1.2µM, 1.4µM are used for all runs. One run is performed at 52.8 ºC and two runs were performed at 60ºC as per the Roche kit. These runs are compared to assess for any differences in runs.

Temperature	**Primer concentration (µM)**	***nuo*G (Ct-value)**			**IAC (Ct-value)**		
Run 1		1	2	3	1	2	3
52.8ºC	**1.0**	20,14	19,94	20,04	N/A	N/A	N/A
	1.2	20,27	20,18	20,08	N/A	N/A	N/A
	1.4	20,21	20,15	20,07	N/A	N/A	N/A
Run 2							
60.0ºC	**1.0**	21,61	21,78	21,29	39,96	31,81	20,99
	1.2	21,44	21,44	21,28	22,93	42,14	35,03
	1.4	22,26	22,19	22,11	1,8	37,24	40,07
Run 3							
60.0ºC	**1.0**	23,43	23	23,08	N/A	43,66	40,69
	1.2	22,88	22,96	23,13	41,95	N/A	42,65
	1.4	21,08	22,78	23,08	43,6	37,99	N/A

*N/A, (not amplified: CFX software presents this as N/A)

At 60°C the concentrations of 1.0µM, 1.2µM and 1.4µM demonstrated reliable amplification of the target with good repeatability - within 2 Ct-values when performed at 52.8°C for *nuo*G, as well as for the internal amplification control. This is visible in all runs as seen in *Table 2*. In view of the negligible Ct-value change, we chose to run our experiments at a primer concentration of 1.2µM and temperature of 60°C.

3.2 Limit of detection experiments

Data from experiments, 8 replications for each of 6 different concentrations tested per run (repeated over 3 days) were used to calculate the limit of detection. Except for the runs using 100 and 10 copies, all other calculations were done using 24 replicates in total.

Ct-values vary according to the concentration or copy numbers of target DNA present in a sample. It is expected that for a change of copy number by a tenfold dilution – the corresponding change in Ct-value is approximated to be 3 (Bustin et al., 2009; Schrader et al., 2012). As demonstrated in Fig. 1, the median value increases by a Ct of 3-5 with an increase in copy number of tenfold, above what is expected.

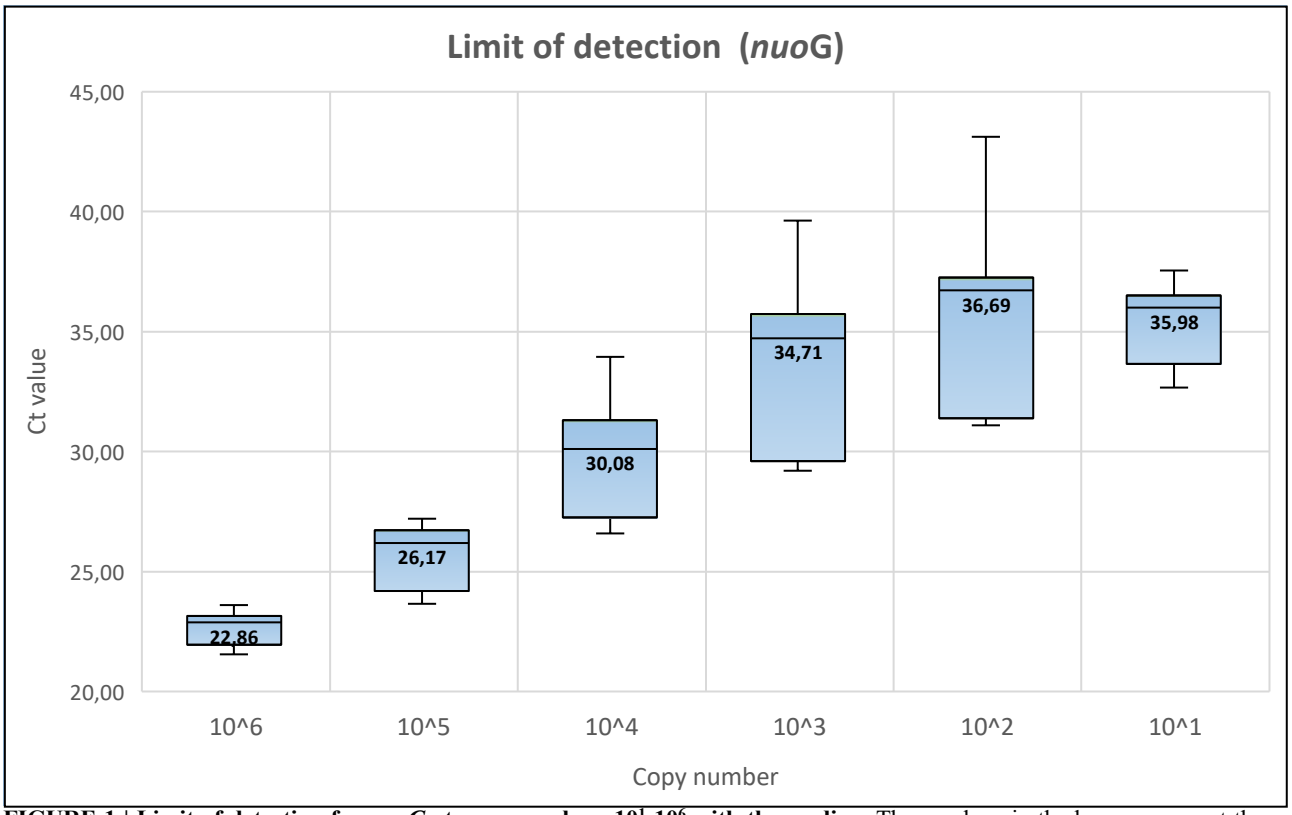

FIGURE 1 | Limit of detection for *nuoG* at copy numbers 10^1-10^6 with the median. The numbers in the boxes represent the median Ct value calculated from 20-24 repeat assays; Error bars represent the SE. Seven technical repeats was done per run, and repeated thrice, lower concentrations resulted in non-reproducible amplification, thus were less than those at higher amplification concentrations.

In addition, the range of Ct-values for each concentration appear to increase, however in lower values of DNA such as 10-100 copies, variability or spread of data appears to be reduced. This observation is likely skewed due to the inconsistency of amplification in the lower concentrations of DNA. Therefore, for this assay the lowest copy number to reliably detect 100%, was at a copy number of 1000. The corresponding Ct-value for 1000 copies would be between 34-37 (Fig. S2). Run 1 and run 2 were very similar (Fig. S3), with the third run being very different to these and may therefore result in a few false negatives should this cut-off be used. The internal amplification control (IAC) distribution of Ct-values at higher concentrations demonstrated a larger range of data points across all copy numbers which highlights potential technical error, likely from pipetting technique.

The Ct-values for the median values are around 31-32 (Fig. 2) throughout all copy numbers. The limit of detection for the IAC was not determined in these experiments as this was previously

determined and recorded to be between 30-37 cycle thresholds.

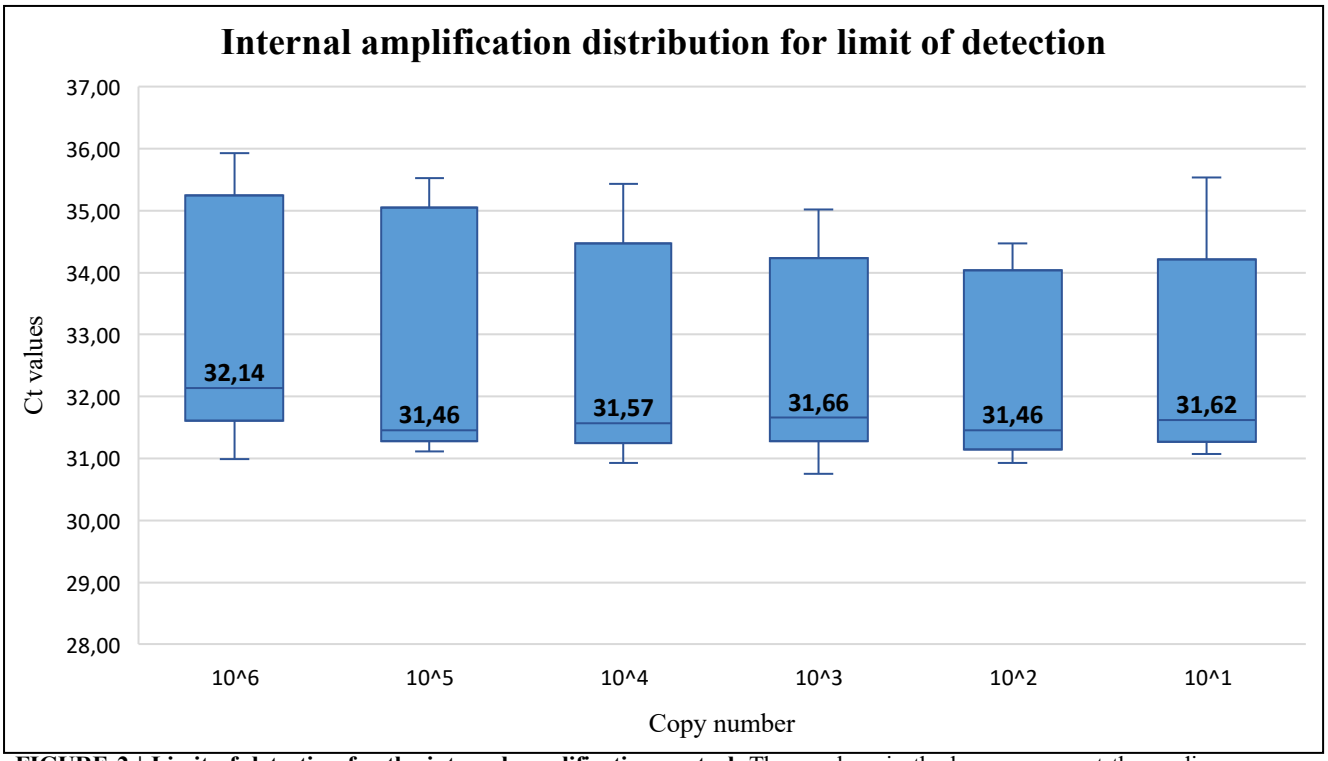

FIGURE 2 | Limit of detection for the internal amplification control. The numbers in the boxes represent the median copy number calculated from 20-24 data points.

3.3 Repeatability and Reproducibility

To determine inter-assay and intra-assay variability, samples were assayed in octuplicate, and inter-run variation assessed for the three consecutive runs performed. Runs 1 and 2 are very similar when compared to run 3 with a difference of 2 Ct-values compared to the former (Fig. 5).

Runs 1 and 2 appear to have little variability within the higher copy numbers. At a copy number of 10^3-10^4, for runs 1 and 2, the range appears larger which may be indicative of technical error from pipetting. Variability in 10-100 copies is also noted, bearing in mind that less than 24 data points are available for these. Whilst run 3 is very different, variability is evidently much smaller.

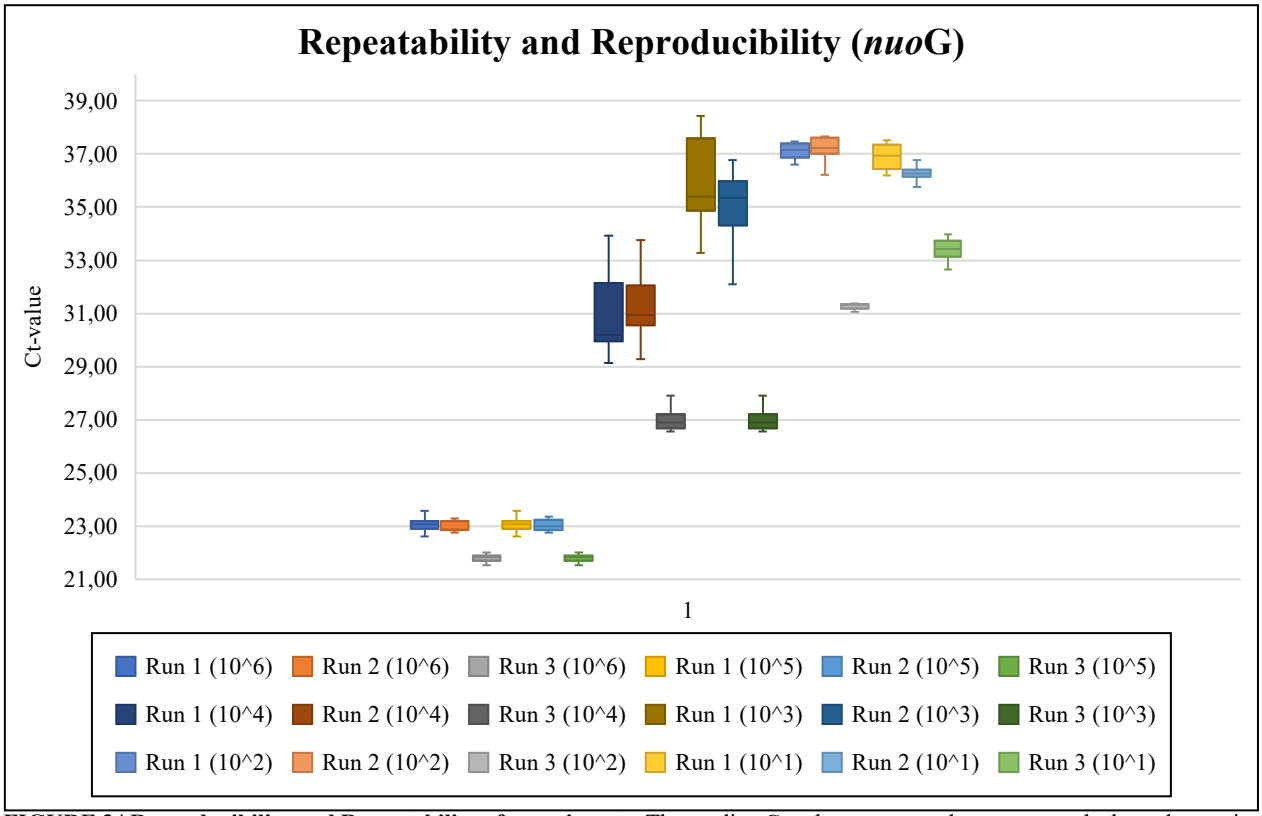

FIGURE 3 | Reproducibility and Repeatability of experiments. The median Ct values were used to construct the box plots, using 8 replicates. Error bars represents the calculated SE. These runs show the similarities of runs 1 and 2, with increased variability for runs 10^3-10^4. Run 3 is vastly different to Run 1 and Run 2 with good repeatability.

3.4 Efficiency

Amplification efficiency (Fig. 4 and 5) should ideally range between 90-110%. Run 1 and 2 was less than 110% (Fig. 4), and acceptable. Run 3 (Fig. 7C) was grossly unacceptable with an amplification efficiency of 165%. This may once again highlight the technical variation in the setup of this run (Andre et al., 2016).

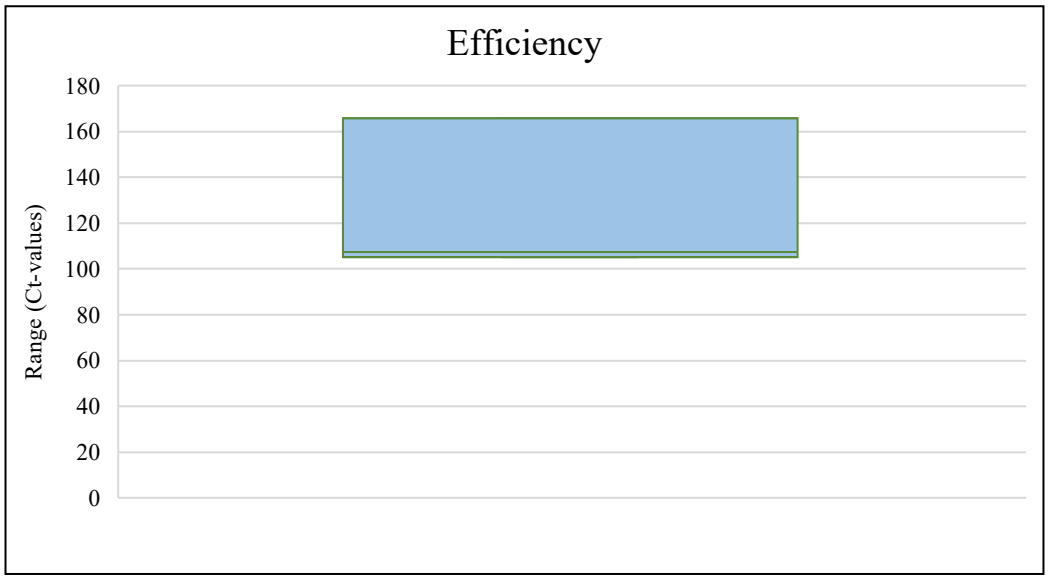

FIGURE 4 | Amplification efficiency. This represents the efficiency of three runs only, between100-160.

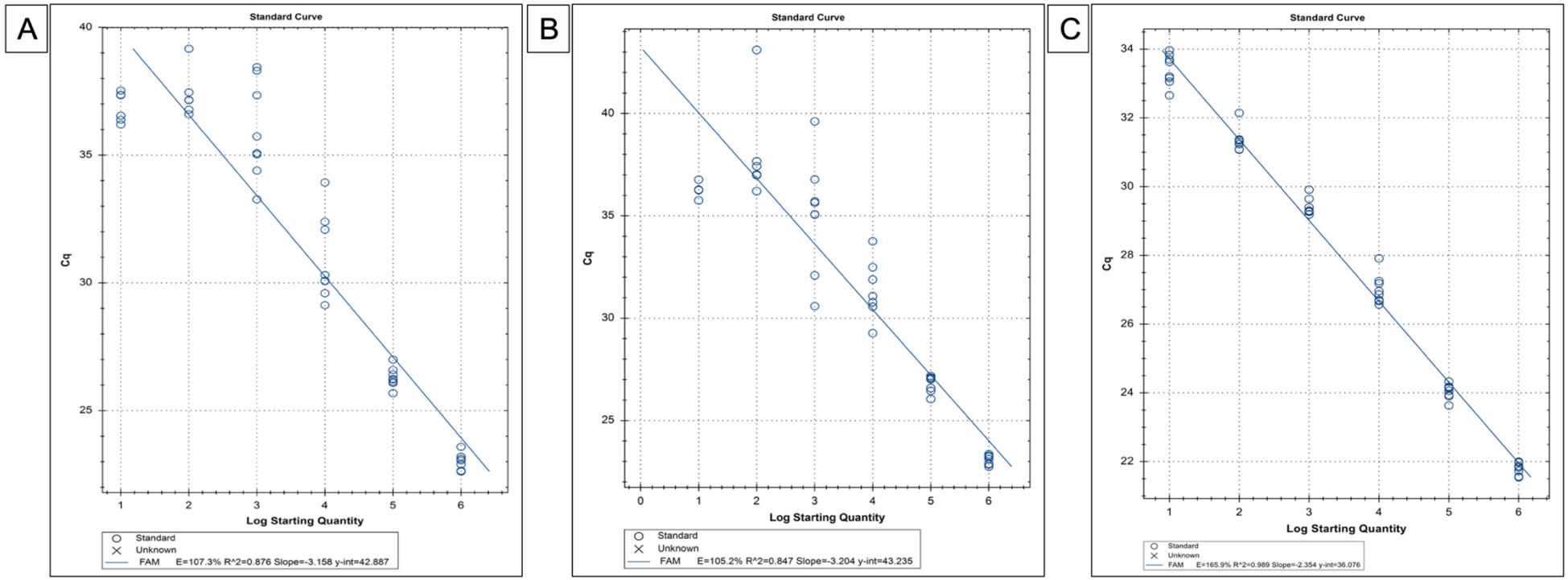

FIGURE 5 | Amplification efficiency for three RT-PCR runs targeting *nuoG*. A: Run 1. demonstrating acceptable efficiency but poor repeatability, **B**: Run 2, showing acceptable efficiency and poor repeatability **C**: Run 3, unacceptable efficiency, yet reasonable repeatability. Each copy number was represented by 8 (including a negative control) replicates per run. (Graphs were generated by the CFX instrument software)

3.5 Specificity

The specificity of the primers used in this assay had previously been tested and we therefore only assessed specificity using DNA extracted from a panel of microbes prevalent in our clinical setting. Genomic DNA (20ng per reaction) from the selected bacterial isolates *(*Table 2) were used in PCR reactions.

E. faecalis amplified at a Ct-value of 38 (Fig. 8*),* and a repeat run was repeated to determine whether this would consistently amplify, however this was absent on repeat experiments.

TABLE 3 | Pathogen panel tested to evaluate the specificity of *nuoG* primers and probe

Bacteria	Fungi	Viruses
Escherichia coli	*Candida albicans*	*None*
Staphylococcus aureus		
Streptococcus agalactiae		
Haemophilus influenzae		
Klebsiella Pneumoniae		
Pseudomonas aeruginosa		
Enterococcus spp		
Enterobacter cloacae complex		

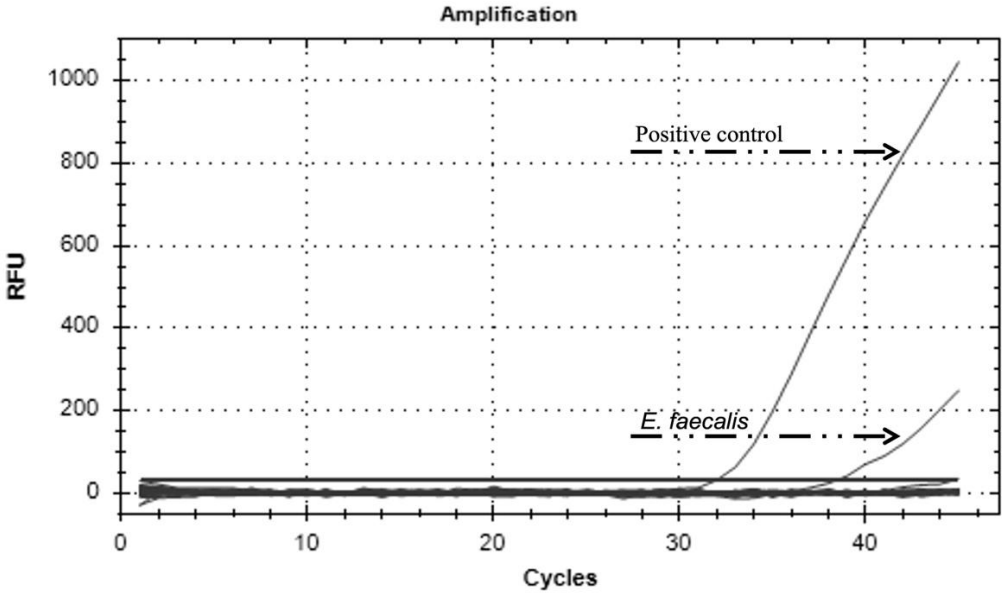

FIGURE 6 | Amplification of pathogen panel with *nuoG*. Demonstrating the absence of amplification of organisms from Table 3, and despite *E. faecalis* this is late amplification and would be interpreted as negative. (Graphs were generated by the CFX instrument software)

4 Discussion

The study reported here evaluated a bacterial target previously only used to detect *Bartonellae* spp, in animals. For this assay, we successfully amplified bacterial DNA present in a residual human sample. We developed a RT-PCR assay, using amplified DNA which was cloned into a plasmid, for the detection and quantification of *Bartonella* spp. This is a broad range assay for detection of most *Bartonella* species, using a previously described primer set that reportedly detects most *Bartonella* species, including those of human clinical significance.

A plasmid was constructed successfully, and the insert identity confirmed as identical to an internal fragment of the *Bartonella nuo*G gene. At the time, this was one of the best performing targets utilising RT-PCR for the detection of *Bartonella* spp on blood specimens, however only performed in veterinary samples. *Glt*A, a well-used gene target for conventional and RT-PCR is well known to identify *B. bacilliformis* which is not endemic to South Africa. *Nuo*G has been compared to *glt*A (also the commonest gene used in reported literature) in veterinary samples and able to detect *Bartonella* spp., including those associated with human disease. There is a lack of published literature on gene targets to detect *Bartonella* spp. in human subjects. However, *glt*A, *ssr*A, *rib*C, *fts*Z have all been used previously for the detection of *Bartonella* spp from animals whereby *glt*A has been the more commonly used gene target (Colborn et al., 2010; Kosoy et al., 2018). Identification of the *Bartonella* to the species level has been challenging due the large number of species (Diaz et al., 2012). Thus far, *nuo*G has been identified as the most sensitive target in reported studies to detect the microbe in insect and animal studies (Andre et al., 2016; Colborn et al., 2010). No data regarding use of this gene target to identify *Bartonella* spp in humans has been identified (Kosoy et al., 2018), however current data suggests that *nuo*G may better detect *Bartonella* spp. such as *B. quintana* and *B. henselae* (Andre et al., 2016), which are important culprits in human disease.

Preliminary RT-PCR assays showed that the target was detected using the previously described primers and probes, when using a commercial kit, and real-time instrument available in our routine setting. For diagnostic purposes, an *E. coli* containing plasmid should be stored and extracted as required. Chemically synthesised pure plasmid could also be procured from companies which can construct this. This pure plasmid would be supplied in a lyophilized form in nanomolar concentration, more than adequate for a respectable number of runs. When ordering from a company, you are also supplied with data sheets, to store for quality assurance purposes. The lack of available commercial kits to detect *Bartonella* spp. requires the development of in-house assays, which in a clinical setting such as ours, may be infrequently requested. A further drawback of commercial kits is availability. Once a specific assay is validated, they may be removed from the market and another validation will need to be done should a new commercial kit be used. For a rare diseases such as CNE, an in-house assay using a multi-purpose PCR ready-mix, could be adapted for use with multiple in-house designed assays which could be more beneficial by avoiding wastage if commercially procured kits are utilised. This avoids unnecessary expenditure and wastage from expiration due to the lack of its utilisation. As more than one pre-mixed RT-PCR mixes are on the market and available locally, one could validate an in-house assay with more than one commercially available kit on the market. This allows for an alternative, verified assay to be available if a specific commercial PCR mix is not available any longer.

Temperature for the kit appears to be reasonable and we should aim to work with this kit (i.e., Roche), regularly available kit when performing further optimisation. In-house developed RT-PCR assays could also be customised to be performed on more than one real-time instrument, as opposed to some commercial kits which limit you to the use of a specified instrument.

As expected, it was observed that the IAC (of which a standard amount to each well), amplified within the same Ct-value range was between 35-40 cycles. A lower concentration of the IAC is used in to ensure reagents are not consumed and inhibit detection of the actual PCR-target, at lower concentrations (Schrader et al., 2012).

The limit of detection should ideally be attempted to further find the true LOD by means of assessing values between 100-1000 to achieve a 95% positivity rate and furthermore, perform clinical LOD studies. These experiments may include spiking of whole blood with varying concentrations of the pure plasmid or added to digested human tissue prior to DNA extraction. This may reduce the LOD further from inhibitors present in these samples.

Repeatability across runs showed a large variation in each copy number, once again highlighting the technical variation due to pipetting error as confirmed with the internal amplification control data.

Amplification in this initial study was not satisfactory in run 3, and more optimizations should be done. Amplification efficiency could be due to potential inhibition of the polymerase in the assay, a consequence of chemical impurities in DNA samples. In the case of the plasmid, it could be residual chemicals such as salts, phenol, alcohol and chaotropic agents such as sodium dodecyl sulphate and guanidinium isothiocyanate. For clinical samples inhibitors present in tissues and blood may also interfere with PCR. Therefore, a clinical evaluation should include determination of the quantity and quality of DNA obtained from different extraction methods.

An excess of DNA in a PCR reaction also could result in inhibition; although not reported here, we observed this at the higher concentration of target tested initially. Ideally, Ct values for tenfold dilutions should be approximately 3 Ct values apart. In our assay, this was not consistent, ranging from slightly above to just below 3 Ct values. This is suggestive of either impurity associated with the extracted plasmid, or technical error. In future work, the assays should be repeated, and new plasmid extracts used. Purchased plasmids obtained from a company should also be tested; these are supplied in lyophilized form potentially and potentially could minimize technical variation if plasmids are extracted in the laboratory. In the original paper, herring sperm DNA was included in PCR, and it was claimed to improve efficiency and sensitivity. This is not ideal, as samples from the clinical setting will already contain human DNA; excess DNA is inhibitory to PCR assays. Lastly, efficiency should be evaluated using lower concentrations of primers and probe, as the 480 Probes master advise that a maximum of 1µM of primers and probes are used in assays. Therefore, for our purposes, efficiency should be evaluated in assays containing lower amounts of reagents, and not at the concentration of 1.2µM as indicated in the previous publication (Andre et al., 2016).

As per real-time PCR guidelines, efficiency amplification is ideally between 90-110% with influence usually due to inhibitors, reagent concentration, non-optimal PCR parameters such as temperature. The wide variation in this study implies potential technical variation, but also highlight the need for further analysis and optimization of this PCR. Future optimisation should be done with mock clinical samples, newly extracted plasmid (potentially testing different commercial kits to discern which kits provides best quality for diagnostic use), as well as evaluation of a readymade, synthesized plasmid obtained from a biotechnology company such as IDT (Integrated DNA technologies); the latter was used in the in the original paper.

Assay precision was not satisfactory in this study. Particularly, one out of 3 runs differed from the other 2 runs, potentially the result of technical variation in the preparation of the plasmid or setup of the real-time PCR runs. Due to time constraints, the assay could not be repeated a 4th time, to discern if observed differences were due to technical error.

The assay demonstrated specificity to detect only *Bartonella*, as it did not detect DNA from any of our panel of pathogens commonly encountered in our clinical setting. However, this assay only included a limited number of species relevant in our clinical environment although *Andre et al.* tested a range of closely related organisms.

5 Conclusion

In conclusion, we demonstrate that a previously designed real-time PCR targeting *Bartonella nuo*G, could be used with a locally available commercial real-time kit. However, further optimisation as well as a clinical validation is necessary to discern a diagnostic cut-off for a real-time assay for use in clinical samples. Finally, the manuscript presented here does not contain sufficient data for publication. Further evaluation and optimisation are required to generate relevant data, in addition to a clinical validation will generate further data to ascertain whether this test is fit for purpose.

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7. Tables and Figures

Journal Manuscript Tables

Supplementary material Tables

Journal Manuscript Figures

FIGURE 1 | Limit of detection for *nuo*G

FIGURE 2 | Limit of detection for the internal amplification control

FIGURE 3 | Reproducibility and Repeatability of experiments

FIGURE 4 | Amplification efficiency

FIGURE 5 | A: represents Run 1 E=107.3% R^2=0.876 slope=-3.158,

B: represents Run 2 E=105.2% R^2=0.847 slope=-3.204,

C: represents Run 3 E=165.9% R^2=0.989 slope=-2.3

Supplementary Material Figures

FIGURE S1 | Plasmid Construction

FIGURE S2 | Approximation of Positivity

TABLE 1 | ALL DATA COMPILED FOR EACH RUN INCLUDING DESCRIPTIVE DATA

Please note that original JPEG images with better resolution are available as per journal pre-requisite

								Mean	Standard Error	Median	Mode	Standard Deviation	Sample Variance	Kurtosis	Skewness	Range	Minimum	Maximum	Sum	Count	Confidence Level(95,0%)
IAC	Run 1	A01	Cy5	IAC	Std	10^6	32,09														
		A02	Cy5	IAC	Std	10^6	31,65														
		A03	Cy5	IAC	Std	10^6	31,39														
		A04	Cy5	IAC	Std	10^6	31,49														
		A05	Cy5	IAC	Std	10^6	31,75														
		A06	Cy5	IAC	Std	10^6	31,45														
		A07	Cy5	IAC	Std	10^6	31,38														
		A08	Cy5	IAC	Std	10^6	30,99														
	Run 2	A01	Cy5	IAC	Unkn	10^6	32,50														
		A02	Cy5	IAC	Unkn	10^6	32,18														
		A03	Cy5	IAC	Unkn	10^6	32,47														
		A04	Cy5	IAC	Unkn	10^6	32,04	33,07	0,38	32,14	#N/A	1,86	3,45	-1,46	0,67	4,94	30,99	35,93	793,68	24,00	0,78
		A05	Cy5	IAC	Unkn	10^6	31,51														
		A06	Cy5	IAC	Unkn	10^6	32,00														
		A07	Cy5	IAC	Unkn	10^6	32,25														
		A08	Cy5	IAC	Unkn	10^6	31,89														
	Run 3	A01	Cy5	IAC	Std	10^6	35,92														
		A02	Cy5	IAC	Std	10^6	35,19														
		A03	Cy5	IAC	Std	10^6	35,93														
		A04	Cy5	IAC	Std	10^6	35,91														
		A05	Cy5	IAC	Std	10^6	35,68														
		A06	Cy5	IAC	Std	10^6	35,11														
		A07	Cy5	IAC	Std	10^6	35,42														
		A08	Cy5	IAC	Std	10^6	35,49														
	Run 1	B01	Cy5	IAC	Std	10^5	31,37														
		B02	Cy5	IAC	Std	10^5	31,32														
		B03	Cy5	IAC	Std	10^5	31,17														
		B04	Cy5	IAC	Std	10^5	31,16														
		B05	Cy5	IAC	Std	10^5	31,15														
		B06	Cy5	IAC	Std	10^5	31,15														
		B07	Cy5	IAC	Std	10^5	31,11														
		B08	Cy5	IAC	Std	10^5	31,17														
	Run 2	B01	Cy5	IAC	Unkn	10^5	31,33														
		B02	Cy5	IAC	Unkn	10^5	31,65														
		B03	Cy5	IAC	Unkn	10^5	31,74														
		B04	Cy5	IAC	Unkn	10^5	31,61	32,61	0,37	31,46	31,17	1,83	3,34	-1,54	0,74	4,41	31,11	35,52	782,70	24,00	0,77
		B05	Cy5	IAC	Unkn	10^5	31,45														
		B06	Cy5	IAC	Unkn	10^5	31,41														
		B07	Cy5	IAC	Unkn	10^5	31,40														
		B08	Cy5	IAC	Unkn	10^5	31,47														
	Run 3	B01	Cy5	IAC	Std	10^5	34,97														
		B02	Cy5	IAC	Std	10^5	35,05														
		B03	Cy5	IAC	Std	10^5	35,10														
		B04	Cy5	IAC	Std	10^5	35,52														
		B05	Cy5	IAC	Std	10^5	35,08														
		B06	Cy5	IAC	Std	10^5	35,08														
		B07	Cy5	IAC	Std	10^5	35,05														
		B08	Cy5	IAC	Std	10^5	35,19														
	Run 1	C01	Cy5	IAC	Std	10^4	31,31														
		C02	Cy5	IAC	Std	10^4	31,15														
		C03	Cy5	IAC	Std	10^4	31,06														
		C04	Cy5	IAC	Std	10^4	31,21														
		C05	Cy5	IAC	Std	10^4	31,24														
		C06	Cy5	IAC	Std	10^4	31,25														
		C07	Cy5	IAC	Std	10^4	30,93														
		C08	Cy5	IAC	Std	10^4	31,11														
	Run 2	C01	Cy5	IAC	Unkn	10^4	31,80														
		C02	Cy5	IAC	Unkn	10^4	31,46														
		C03	Cy5	IAC	Unkn	10^4	31,34														
		C04	Cy5	IAC	Unkn	10^4	31,40	32,50	0,34	31,57	31,31	1,67	2,79	-1,40	0,75	4,50	30,93	35,43	780,04	24,00	0,71
		C05	Cy5	IAC	Unkn	10^4	31,31														
		C06	Cy5	IAC	Unkn	10^4	31,69														
		C07	Cy5	IAC	Unkn	10^4	31,68														
		C08	Cy5	IAC	Unkn	10^4	31,90														
	Run 3	C01	Cy5	IAC	Std	10^4	34,23														
		C02	Cy5	IAC	Std	10^4	35,43														
		C03	Cy5	IAC	Std	10^4	35,02														
		C04	Cy5	IAC	Std	10^4	34,94														
		C05	Cy5	IAC	Std	10^4	34,68														
		C06	Cy5	IAC	Std	10^4	34,83														
		C07	Cy5	IAC	Std	10^4	34,67														
		C08	Cy5	IAC	Std	10^4	34,40														

	Run 1	D01	FAM	nuoG	Std	10^3	35,07																				
		D02	FAM	nuoG	Std	10^3	33,27																				
		D03	FAM	nuoG	Std	10^3	38,31																				
		D04	FAM	nuoG	Std	10^3	37,34																				
		D05	FAM	nuoG	Std	10^3	35,73																				
		D06	FAM	nuoG	Std	10^3	34,39																				
		D07	FAM	nuoG	Std	10^3	38,44																				
		D08	FAM	nuoG	Std	10^3	35,03																				
	Run 2	D01	FAM	nuoG	Std	10^3	35,06																				
		D02	FAM	nuoG	Std	10^3	35,7																				
		D03	FAM	nuoG	Std	10^3	35,64																				
		D04	FAM	nuoG	Std	10^3	30,59	33,47	0,71	34,71	35,07	3,49	12,18	-1,41	0,04	10,42	29,19	39,61	803,38	24,00	1,47
		D05	FAM	nuoG	Std	10^3	36,78																				
		D06	FAM	nuoG	Std	10^3	35,07																				
		D07	FAM	nuoG	Std	10^3	39,61																				
		D08	FAM	nuoG	Std	10^3	32,09																				
	Run 3	D01	FAM	nuoG	Std	10^3	29,64																				
		D02	FAM	nuoG	Std	10^3	29,27																				
		D03	FAM	nuoG	Std	10^3	29,91																				
		D04	FAM	nuoG	Std	10^3	29,28																				
		D05	FAM	nuoG	Std	10^3	29,4																				
		D06	FAM	nuoG	Std	10^3	29,27																				
		D07	FAM	nuoG	Std	10^3	29,3																				
		D08	FAM	nuoG	Std	10^3	29,19																				
	Run 1	E01	FAM	nuoG	Std	10^2																					
		E02	FAM	nuoG	Std	10^2	37,15																				
		E03	FAM	nuoG	Std	10^2	36,78																				
		E04	FAM	nuoG	Std	10^2	37,46																				
		E05	FAM	nuoG	Std	10^2																					
		E06	FAM	nuoG	Std	10^2	37,16																				
		E07	FAM	nuoG	Std	10^2	36,6																				
		E08	FAM	nuoG	Std	10^2	39,16																				
	Run 2	E01	FAM	nuoG	Std	10^2	43,11																				
		E02	FAM	nuoG	Std	10^2	37,42																				
		E03	FAM	nuoG	Std	10^2	37,02																				
		E04	FAM	nuoG	Std	10^2																					
		E05	FAM	nuoG	Std	10^2		35,18	0,78	36,69	#N/A	3,50	12,24	-0,59	0,25	12,04	31,07	43,11	703,64	20,00	1,64
		E06	FAM	nuoG	Std	10^2	36,21																				
		E07	FAM	nuoG	Std	10^2	36,99																				
		E08	FAM	nuoG	Std	10^2	37,67																				
	Run 3	E01	FAM	nuoG	Std	10^2	31,07																				
		E02	FAM	nuoG	Std	10^2	31,37																				
		E03	FAM	nuoG	Std	10^2	31,09																				
		E04	FAM	nuoG	Std	10^2	31,36																				
		E05	FAM	nuoG	Std	10^2	31,3																				
		E06	FAM	nuoG	Std	10^2	31,23																				
		E07	FAM	nuoG	Std	10^2	31,35																				
		E08	FAM	nuoG	Std	10^2	32,14																				
	Run 1	F01	FAM	nuoG	Std	10^1																					
		F02	FAM	nuoG	Std	10^1	37,34																				
		F03	FAM	nuoG	Std	10^1	37,36																				
		F04	FAM	nuoG	Std	10^1	37,52																				
		F05	FAM	nuoG	Std	10^1	36,2																				
		F06	FAM	nuoG	Std	10^1	36,38																				
		F07	FAM	nuoG	Std	10^1	36,54																				
		F08	FAM	nuoG	Std	10^1																					
	Run 2	F01	FAM	nuoG	Std	10^1	35,76																				
		F02	FAM	nuoG	Std	10^1																					
		F03	FAM	nuoG	Std	10^1																					
		F04	FAM	nuoG	Std	10^1																					
		F05	FAM	nuoG	Std	10^1	36,77	35,20	0,41	35,98	#N/A	1,74	3,01	-1,76	-0,14	4,87	32,65	37,52	633,61	18,00	0,86
		F06	FAM	nuoG	Std	10^1	36,28																				
		F07	FAM	nuoG	Std	10^1	36,26																				
		F08	FAM	nuoG	Std	10^1																					
	Run 3	F01	FAM	nuoG	Std	10^1	33,05																				
		F02	FAM	nuoG	Std	10^1	33,63																				
		F03	FAM	nuoG	Std	10^1	33,16																				
		F04	FAM	nuoG	Std	10^1	32,65																				
		F05	FAM	nuoG	Std	10^1	33,2																				
		F06	FAM	nuoG	Std	10^1	33,84																				
		F07	FAM	nuoG	Std	10^1	33,7																				
		F08	FAM	nuoG	Std	10^1	33,97																				

Run 1	D01	Cy5	IAC	Std	10^3	31,24														
	D02	Cy5	IAC	Std	10^3	31,24														
	D03	Cy5	IAC	Std	10^3	31,68														
	D04	Cy5	IAC	Std	10^3	31,29														
	D05	Cy5	IAC	Std	10^3	31,10														
	D06	Cy5	IAC	Std	10^3	31,31														
	D07	Cy5	IAC	Std	10^3	30,75														
	D08	Cy5	IAC	Std	10^3	31,02														
Run 2	D01	Cy5	IAC	Unkn	10^3	31,76														
	D02	Cy5	IAC	Unkn	10^3	31,62														
	D03	Cy5	IAC	Unkn	10^3	31,78														
	D04	Cy5	IAC	Unkn	10^3	31,63	32,41958333	0,305189107	31,66	31,24	1,495115174	2,235369384	-1,41024212	0,707199916	4,27	30,75	35,02	778,07	24	0,631331769
	D05	Cy5	IAC	Unkn	10^3	31,25														
	D06	Cy5	IAC	Unkn	10^3	31,71														
	D07	Cy5	IAC	Unkn	10^3	31,46														
	D08	Cy5	IAC	Unkn	10^3	31,64														
Run 3	D01	Cy5	IAC	Std	10^3	35,02														
	D02	Cy5	IAC	Std	10^3	34,23														
	D03	Cy5	IAC	Std	10^3	34,70														
	D04	Cy5	IAC	Std	10^3	34,54														
	D05	Cy5	IAC	Std	10^3	34,23														
	D06	Cy5	IAC	Std	10^3	34,11														
	D07	Cy5	IAC	Std	10^3	34,27														
	D08	Cy5	IAC	Std	10^3	34,49														
Run 1	E01	Cy5	IAC	Std	10^2	31,02														
	E02	Cy5	IAC	Std	10^2	31,12														
	E03	Cy5	IAC	Std	10^2	31,28														
	E04	Cy5	IAC	Std	10^2	30,97														
	E05	Cy5	IAC	Std	10^2	31,20														
	E06	Cy5	IAC	Std	10^2	31,12														
	E07	Cy5	IAC	Std	10^2	31,04														
	E08	Cy5	IAC	Std	10^2	31,48														
Run 2	E01	Cy5	IAC	Unkn	10^2	31,66														
	E02	Cy5	IAC	Unkn	10^2	31,43														
	E03	Cy5	IAC	Unkn	10^2	31,16														
	E04	Cy5	IAC	Unkn	10^2	31,80	32,24	0,29	31,46	31,12	1,42	2,01	-1,44	0,73	3,54	30,93	34,47	773,81	24,00	0,60
	E05	Cy5	IAC	Unkn	10^2	31,68														
	E06	Cy5	IAC	Unkn	10^2	30,93														
	E07	Cy5	IAC	Unkn	10^2	31,15														
	E08	Cy5	IAC	Unkn	10^2	31,44														
Run 3	E01	Cy5	IAC	Std	10^2	34,29														
	E02	Cy5	IAC	Std	10^2	34,41														
	E03	Cy5	IAC	Std	10^2	34,01														
	E04	Cy5	IAC	Std	10^2	34,11														
	E05	Cy5	IAC	Std	10^2	34,47														
	E06	Cy5	IAC	Std	10^2	34,38														
	E07	Cy5	IAC	Std	10^2	34,18														
	E08	Cy5	IAC	Std	10^2	33,48														
Run 1	F01	Cy5	IAC	Std	10^1	31,52														
	F02	Cy5	IAC	Std	10^1	31,28														
	F03	Cy5	IAC	Std	10^1	31,07														
	F04	Cy5	IAC	Std	10^1	31,07														
	F05	Cy5	IAC	Std	10^1	31,09														
	F06	Cy5	IAC	Std	10^1	31,16														
	F07	Cy5	IAC	Std	10^1	31,25														
	F08	Cy5	IAC	Std	10^1															
Run 2	F01	Cy5	IAC	Unkn	10^1	31,62														
	F02	Cy5	IAC	Unkn	10^1	32,05														
	F03	Cy5	IAC	Unkn	10^1	31,90														
	F04	Cy5	IAC	Unkn	10^1	31,52	32,58	0,32	31,62	31,52	1,54	2,36	-1,46	0,56	4,47	31,07	35,54	749,28	23,00	0,66
	F05	Cy5	IAC	Unkn	10^1	31,40														
	F06	Cy5	IAC	Unkn	10^1	31,26														
	F07	Cy5	IAC	Unkn	10^1	31,61														
	F08	Cy5	IAC	Unkn	10^1	33,55														
Run 3	F01	Cy5	IAC	Std	10^1	34,72														
	F02	Cy5	IAC	Std	10^1	34,15														
	F03	Cy5	IAC	Std	10^1	34,55														
	F04	Cy5	IAC	Std	10^1	34,28														
	F05	Cy5	IAC	Std	10^1	34,41														
	F06	Cy5	IAC	Std	10^1	34,01														
	F07	Cy5	IAC	Std	10^1	34,27														
	F08	Cy5	IAC	Std	10^1	35,54														

nuoG	Run 1	A01	FAM	nuoG	Std	10^6	22,62	22,62	0,13	22,86	22,86	0,64	0,41	-1,28	-0,46	2,04	21,54	23,58	542,87	24,00	0,27
		A02	FAM	nuoG	Std	10^6	23,06														
		A03	FAM	nuoG	Std	10^6	23,11														
		A04	FAM	nuoG	Std	10^6	22,65														
		A05	FAM	nuoG	Std	10^6	22,90														
		A06	FAM	nuoG	Std	10^6	23,20														
		A07	FAM	nuoG	Std	10^6	23,05														
		A08	FAM	nuoG	Std	10^6	23,58														
	Run 2	A01	FAM	nuoG	Std	10^6	23,36														
		A02	FAM	nuoG	Std	10^6	23,23														
		A03	FAM	nuoG	Std	10^6	23,28														
		A04	FAM	nuoG	Std	10^6	22,75														
		A05	FAM	nuoG	Std	10^6	23,13														
		A06	FAM	nuoG	Std	10^6	22,86														
		A07	FAM	nuoG	Std	10^6	22,86														
		A08	FAM	nuoG	Std	10^6	22,89														
	Run 3	A01	FAM	nuoG	Std	10^6	21,97														
		A02	FAM	nuoG	Std	10^6	21,83														
		A03	FAM	nuoG	Std	10^6	21,87														
		A04	FAM	nuoG	Std	10^6	21,54														
		A05	FAM	nuoG	Std	10^6	21,56														
		A06	FAM	nuoG	Std	10^6	21,84														
		A07	FAM	nuoG	Std	10^6	22,00														
		A08	FAM	nuoG	Std	10^6	21,73														
	Run 1	B01	FAM	nuoG	Std	10^5	26,59	25,72	0,26	26,17	26,59	1,27	1,62	-1,48	-0,50	3,54	23,63	27,17	617,18	24,00	0,54
		B02	FAM	nuoG	Std	10^5	26,08														
		B03	FAM	nuoG	Std	10^5	26,13														
		B04	FAM	nuoG	Std	10^5	26,41														
		B05	FAM	nuoG	Std	10^5	26,21														
		B06	FAM	nuoG	Std	10^5	27,00														
		B07	FAM	nuoG	Std	10^5	26,24														
	Run 2	B08	FAM	nuoG	Std	10^5	25,69														
		B01	FAM	nuoG	Std	10^5	27,02														
		B02	FAM	nuoG	Std	10^5	27,11														
		B03	FAM	nuoG	Std	10^5	27,05														
		B04	FAM	nuoG	Std	10^5	26,05														
		B05	FAM	nuoG	Std	10^5	26,45														
		B06	FAM	nuoG	Std	10^5	27,05														
		B07	FAM	nuoG	Std	10^5	27,17														
		B08	FAM	nuoG	Std	10^5	26,59														
	Run 3	B01	FAM	nuoG	Std	10^5	24,06														
		B02	FAM	nuoG	Std	10^5	23,94														
		B03	FAM	nuoG	Std	10^5	24,33														
		B04	FAM	nuoG	Std	10^5	24,16														
		B05	FAM	nuoG	Std	10^5	23,90														
		B06	FAM	nuoG	Std	10^5	23,63														
		B07	FAM	nuoG	Std	10^5	24,14														
		B08	FAM	nuoG	Std	10^5	24,18														
	Run 1	C01	FAM	nuoG	Std	10^4	32,39	29,75	0,48	30,08	30,56	2,33	5,42	-1,07	0,10	7,36	26,57	33,93	714,09	24,00	0,98
		C02	FAM	nuoG	Std	10^4	29,13														
		C03	FAM	nuoG	Std	10^4	32,08														
		C04	FAM	nuoG	Std	10^4	33,93														
		C05	FAM	nuoG	Std	10^4	30,08														
		C06	FAM	nuoG	Std	10^4	30,07														
		C07	FAM	nuoG	Std	10^4	29,59														
		C08	FAM	nuoG	Std	10^4	30,31														
	Run 2	C01	FAM	nuoG	Std	10^4	31,08														
		C02	FAM	nuoG	Std	10^4	29,27														
		C03	FAM	nuoG	Std	10^4	30,56														
		C04	FAM	nuoG	Std	10^4	33,76														
		C05	FAM	nuoG	Std	10^4	30,56														
		C06	FAM	nuoG	Std	10^4	31,89														
		C07	FAM	nuoG	Std	10^4	30,79														
		C08	FAM	nuoG	Std	10^4	32,49														
	Run 3	C01	FAM	nuoG	Std	10^4	27,25														
		C02	FAM	nuoG	Std	10^4	27,91														
		C03	FAM	nuoG	Std	10^4	27,19														
		C04	FAM	nuoG	Std	10^4	26,96														
		C05	FAM	nuoG	Std	10^4	26,67														
		C06	FAM	nuoG	Std	10^4	26,7														
		C07	FAM	nuoG	Std	10^4	26,86														
		C08	FAM	nuoG	Std	10^4	26,57														

TABLE 2 | INTERNAL AMPLIFICATION DESCRIPTIVE DATA

IAC											
10^6		10^5		10^4		10^3		10^2		10^1	
Column1		Column1		Column1		Column1		Column1		Column1	
Mean	33,07	Mean	32,6125	Mean	32,5016667	Mean	32,4195833	Mean	32,2420833	Mean	32,5773913
Standard Error	0,3788828	Standard Error	0,37308412	Standard Error	0,34086722	Standard Error	0,30518911	Standard Error	0,28941469	Standard Error	0,32060665
Median	32,135	Median	31,46	Median	31,57	Median	31,66	Median	31,46	Median	31,62
Mode	#N/A	Mode	31,17	Mode	31,31	Mode	31,24	Mode	31,12	Mode	31,52
Standard Deviation	1,85613905	Standard Deviation	1,82773143	Standard Deviation	1,6699015	Standard Deviation	1,49511517	Standard Deviation	1,41783662	Standard Deviation	1,53757547
Sample Variance	3,44525217	Sample Variance	3,34060217	Sample Variance	2,78857101	Sample Variance	2,23536938	Sample Variance	2,01026069	Sample Variance	2,36413834
Kurtosis	-1,4644478	Kurtosis	-1,5377313	Kurtosis	-1,4013561	Kurtosis	-1,4102421	Kurtosis	-1,4378025	Kurtosis	-1,4583831
Skewness	0,66838016	Skewness	0,73921474	Skewness	0,74860876	Skewness	0,70719992	Skewness	0,725119	Skewness	0,56383703
Range	4,94	Range	4,41	Range	4,5	Range	4,27	Range	3,54	Range	4,47
Minimum	30,99	Minimum	31,11	Minimum	30,93	Minimum	30,75	Minimum	30,93	Minimum	31,07
Maximum	35,93	Maximum	35,52	Maximum	35,43	Maximum	35,02	Maximum	34,47	Maximum	35,54
Sum	793,68	Sum	782,7	Sum	780,04	Sum	778,07	Sum	773,81	Sum	749,28
Count	24	Count	24	Count	24	Count	24	Count	24	Count	23
Confidence Level(95,0%)	0,78377878	Confidence Level(95,0%)	0,77178329	Confidence Level(95,0%)	0,70513756	Confidence Level(95,0%)	0,63133177	Confidence Level(95,0%)	0,5986999	Confidence Level(95,0%)	0,6648975

TABLE 3 | *nuo*G DESCRIPTIVE DATA

nuoG																			
10^6		10^5			10^4			10^3			10^2			10^1					
	Column1			Column1			Column1			Column1			Column1			Column1		
Mean	22,6195833	Mean		25,7158333	Mean		29,75375	Mean		33,4741667	Mean		35,182	Mean		35,2005556		
Standard Error	0,13091301	Standard Error		0,25952641	Standard Error		0,47509069	Standard Error		0,71242312	Standard Error		0,78226681	Standard Error		0,40924007		
Median	22,86	Median		26,17	Median		30,075	Median		34,71	Median		36,69	Median		35,98		
Mode	22,86	Mode		26,59	Mode		30,56	Mode		35,07	Mode		#N/A	Mode		#N/A		
Standard Deviation	0,64134017	Standard Deviation		1,27141454	Standard Deviation		2,32745955	Standard Deviation		3,49014627	Standard Deviation		3,49840355	Standard Deviation		1,73625856		
Sample Variance	0,41131721	Sample Variance		1,61649493	Sample Variance		5,41706793	Sample Variance		12,181121	Sample Variance		12,2388274	Sample Variance		3,01459379		
Kurtosis	-1,280886	Kurtosis		-1,4767564	Kurtosis		-1,0742338	Kurtosis		-1,4128296	Kurtosis		-0,5884315	Kurtosis		-1,7630262		
Skewness	-0,4603379	Skewness		-0,5032831	Skewness		0,10432296	Skewness		0,04472266	Skewness		0,2475755	Skewness		-0,1366167		
Range	2,04	Range		3,54	Range		7,36	Range		10,42	Range		12,04	Range		4,87		
Minimum	21,54	Minimum		23,63	Minimum		26,57	Minimum		29,19	Minimum		31,07	Minimum		32,65		
Maximum	23,58	Maximum		27,17	Maximum		33,93	Maximum		39,61	Maximum		43,11	Maximum		37,52		
Sum	542,87	Sum		617,18	Sum		714,09	Sum		803,38	Sum		703,64	Sum		633,61		
Count	24	Count		24	Count		24	Count		24	Count		20	Count		18		
Confidence Level(95,0%)	0,2708142	Confidence Level(95,0%)		0,53687127	Confidence Level(95,0%)		0,98279997	Confidence Level(95,0%)		1,47375952	Confidence Level(95,0%)		1,63730326	Confidence Level(95,0%)		0,86342107		

TABLE 4 | DATA FOR SEPARATE RUNS INCLUDING DESCRIPTIVE DATA

								Mean	Standard Error	Median	Mode	Standard Deviation	Sample Variance	Kurtosis	Skewness	Range	Minimum	Maximum	Sum	Count	Confidence Level(95,0%)
IAC	Run 1	A01	Cy5	IAC	Std	10^6	32,09	31,52	0,11	31,47	#N/A	0,32	0,10	1,22	0,22	1,10	30,99	32,09	252,19	8,00	0,27
		A02	Cy5	IAC	Std	10^6	31,65														
		A03	Cy5	IAC	Std	10^6	31,39														
		A04	Cy5	IAC	Std	10^6	31,49														
		A05	Cy5	IAC	Std	10^6	31,75														
		A06	Cy5	IAC	Std	10^6	31,45														
		A07	Cy5	IAC	Std	10^6	31,38														
		A08	Cy5	IAC	Std	10^6	30,99														
	Run 2	A01	Cy5	IAC	Unkn	10^6	32,50	32,11	0,11	32,11	#N/A	0,32	0,10	0,44	-0,61	0,99	31,51	32,50	256,84	8,00	0,27
		A02	Cy5	IAC	Unkn	10^6	32,18														
		A03	Cy5	IAC	Unkn	10^6	32,47														
		A04	Cy5	IAC	Unkn	10^6	32,04														
		A05	Cy5	IAC	Unkn	10^6	31,51														
		A06	Cy5	IAC	Unkn	10^6	32,00														
		A07	Cy5	IAC	Unkn	10^6	32,25														
		A08	Cy5	IAC	Unkn	10^6	31,89														
	Run 3	A01	Cy5	IAC	Std	10^6	35,92	35,58	0,12	35,59	#N/A	0,33	0,11	-1,64	-0,27	0,82	35,11	35,93	284,65	8,00	0,28
		A02	Cy5	IAC	Std	10^6	35,19														
		A03	Cy5	IAC	Std	10^6	35,93														
		A04	Cy5	IAC	Std	10^6	35,91														
		A05	Cy5	IAC	Std	10^6	35,68														
		A06	Cy5	IAC	Std	10^6	35,11														
		A07	Cy5	IAC	Std	10^6	35,42														
		A08	Cy5	IAC	Std	10^6	35,49														
	Run 1	B01	Cy5	IAC	Std	10^5	31,37	31,20	0,03	31,17	31,17	0,09		0,38	1,34	0,26	31,11	31,37	249,60	8,00	0,08
		B02	Cy5	IAC	Std	10^5	31,32														
		B03	Cy5	IAC	Std	10^5	31,17														
		B04	Cy5	IAC	Std	10^5	31,16														
		B05	Cy5	IAC	Std	10^5	31,15														
		B06	Cy5	IAC	Std	10^5	31,15														
		B07	Cy5	IAC	Std	10^5	31,11														
		B08	Cy5	IAC	Std	10^5	31,17														
	Run 2	B01	Cy5	IAC	Unkn	10^5	31,33	31,51	0,05	31,46	#N/A	0,14	0,02	-1,01	0,56	0,41	31,33	31,74	252,06	8,00	0,12
		B02	Cy5	IAC	Unkn	10^5	31,65														
		B03	Cy5	IAC	Unkn	10^5	31,74														
		B04	Cy5	IAC	Unkn	10^5	31,61														
		B05	Cy5	IAC	Unkn	10^5	31,45														
		B06	Cy5	IAC	Unkn	10^5	31,41														
		B07	Cy5	IAC	Unkn	10^5	31,40														
		B08	Cy5	IAC	Unkn	10^5	31,47														
	Run 3	B01	Cy5	IAC	Std	10^5	34,97	35,13	0,06	35,08	35,05	0,17	0,03	5,14	2,14	0,55	34,97	35,52	281,04	8,00	0,14
		B02	Cy5	IAC	Std	10^5	35,05														
		B03	Cy5	IAC	Std	10^5	35,10														
		B04	Cy5	IAC	Std	10^5	35,52														
		B05	Cy5	IAC	Std	10^5	35,08														
		B06	Cy5	IAC	Std	10^5	35,08														
		B07	Cy5	IAC	Std	10^5	35,05														
		B08	Cy5	IAC	Std	10^5	35,19														
	Run 1	C01	Cy5	IAC	Std	10^4	31,31	31,16	0,04	31,18	#N/A	0,12	0,01	0,35	-0,81	0,38	30,93	31,31	249,26	8,00	0,10
		C02	Cy5	IAC	Std	10^4	31,15														
		C03	Cy5	IAC	Std	10^4	31,06														
		C04	Cy5	IAC	Std	10^4	31,21														
		C05	Cy5	IAC	Std	10^4	31,24														
		C06	Cy5	IAC	Std	10^4	31,25														
		C07	Cy5	IAC	Std	10^4	30,93														
		C08	Cy5	IAC	Std	10^4	31,11														
	Run 2	C01	Cy5	IAC	Unkn	10^4	31,80	31,57	0,08	31,57	#N/A	0,22	0,05	-1,71	0,21	0,59	31,31	31,90	252,58	8,00	0,19
		C02	Cy5	IAC	Unkn	10^4	31,46														
		C03	Cy5	IAC	Unkn	10^4	31,34														
		C04	Cy5	IAC	Unkn	10^4	31,40														
		C05	Cy5	IAC	Unkn	10^4	31,31														
		C06	Cy5	IAC	Unkn	10^4	31,69														
		C07	Cy5	IAC	Unkn	10^4	31,68														
		C08	Cy5	IAC	Unkn	10^4	31,90														
	Run 3	C01	Cy5	IAC	Std	10^4	34,23	34,78	0,13	34,76	#N/A	0,37	0,14	0,32	0,31	1,20	34,23	35,43	278,20	8,00	0,31
		C02	Cy5	IAC	Std	10^4	35,43														
		C03	Cy5	IAC	Std	10^4	35,02														
		C04	Cy5	IAC	Std	10^4	34,94														
		C05	Cy5	IAC	Std	10^4	34,68														
		C06	Cy5	IAC	Std	10^4	34,83														
		C07	Cy5	IAC	Std	10^4	34,67														
		C08	Cy5	IAC	Std	10^4	34,40														

	Run 1	D01	Cy5	IAC	Std	10^3	31,24													
		D02	Cy5	IAC	Std	10^3	31,24													
nuoG	Run 1	A01	FAM	nuoG	Std	10^6	22,62													
		A02	FAM	nuoG	Std	10^6	23,06													
		A03	FAM	nuoG	Std	10^6	23,11													
		A04	FAM	nuoG	Std	10^6	22,65													
		A05	FAM	nuoG	Std	10^6	22,90	23,02	0,11	23,06	#N/A	0,31	0,10	0,51	0,41	0,96	22,62	23,58	184,17	8,00	0,26
		A06	FAM	nuoG	Std	10^6	23,20													
		A07	FAM	nuoG	Std	10^6	23,05													
		A08	FAM	nuoG	Std	10^6	23,58													
	Run 2	A01	FAM	nuoG	Std	10^6	23,36													
		A02	FAM	nuoG	Std	10^6	23,23													
		A03	FAM	nuoG	Std	10^6	23,28													
		A04	FAM	nuoG	Std	10^6	22,75													
		A05	FAM	nuoG	Std	10^6	23,13	23,05	0,08	23,01	22,86	0,23	0,05	-1,97	0,14	0,61	22,75	23,36	184,36	8,00	0,19
		A06	FAM	nuoG	Std	10^6	22,86													
		A07	FAM	nuoG	Std	10^6	22,86													
		A08	FAM	nuoG	Std	10^6	22,89													
	Run 3	A01	FAM	nuoG	Std	10^6	21,97													
		A02	FAM	nuoG	Std	10^6	21,83													
		A03	FAM	nuoG	Std	10^6	21,87													
		A04	FAM	nuoG	Std	10^6	21,54													
		A05	FAM	nuoG	Std	10^6	21,56	21,79	0,06	21,84	#N/A	0,17	0,03	-0,98	-0,52	0,46	21,54	22,00	174,34	8,00	0,14
		A06	FAM	nuoG	Std	10^6	21,84													
		A07	FAM	nuoG	Std	10^6	22,00													
		A08	FAM	nuoG	Std	10^6	21,73													
	Run 1	B01	FAM	nuoG	Std	10^5	26,59													
		B02	FAM	nuoG	Std	10^5	26,08													
		B03	FAM	nuoG	Std	10^5	26,13													
		B04	FAM	nuoG	Std	10^5	26,41													
		B05	FAM	nuoG	Std	10^5	26,21	26,29	0,14	26,23	#N/A	0,39	0,15	1,11	0,48	1,31	25,69	27,00	210,35	8,00	0,32
		B06	FAM	nuoG	Std	10^5	27,00													
		B07	FAM	nuoG	Std	10^5	26,24													
		B08	FAM	nuoG	Std	10^5	25,69													
	Run 2	B01	FAM	nuoG	Std	10^5	27,02													
		B02	FAM	nuoG	Std	10^5	27,11													
		B03	FAM	nuoG	Std	10^5	27,05													
		B04	FAM	nuoG	Std	10^5	26,05													
		B05	FAM	nuoG	Std	10^5	26,45	26,81	0,14	27,04	27,05	0,40	0,16	0,17	-1,14	1,12	26,05	27,17	214,49	8,00	0,34
		B06	FAM	nuoG	Std	10^5	27,05													
		B07	FAM	nuoG	Std	10^5	27,17													
		B08	FAM	nuoG	Std	10^5	26,59													
	Run 3	B01	FAM	nuoG	Std	10^5	24,06													
		B02	FAM	nuoG	Std	10^5	23,94													
		B03	FAM	nuoG	Std	10^5	24,33													
		B04	FAM	nuoG	Std	10^5	24,16													
		B05	FAM	nuoG	Std	10^5	23,90	24,04	0,08	24,10	#N/A	0,22	0,05	0,94	-0,86	0,70	23,63	24,33	192,34	8,00	0,18
		B06	FAM	nuoG	Std	10^5	23,63													
		B07	FAM	nuoG	Std	10^5	24,14													
		B08	FAM	nuoG	Std	10^5	24,18													
	Run 1	C01	FAM	nuoG	Std	10^4	32,39													
		C02	FAM	nuoG	Std	10^4	29,13													
		C03	FAM	nuoG	Std	10^4	32,08													
		C04	FAM	nuoG	Std	10^4	33,93													
		C05	FAM	nuoG	Std	10^4	30,08	30,95	0,59	30,20	#N/A	1,66	2,76	-0,37	0,87	4,80	29,13	33,93	247,58	8,00	1,39
		C06	FAM	nuoG	Std	10^4	30,07													
		C07	FAM	nuoG	Std	10^4	29,59													
		C08	FAM	nuoG	Std	10^4	30,31													
	Run 2	C01	FAM	nuoG	Std	10^4	31,08													
		C02	FAM	nuoG	Std	10^4	29,27													
		C03	FAM	nuoG	Std	10^4	30,56													
		C04	FAM	nuoG	Std	10^4	33,76													
		C05	FAM	nuoG	Std	10^4	30,56	31,30	0,49	30,94	30,56	1,38	1,91	0,42	0,54	4,49	29,27	33,76	250,40	8,00	1,15
		C06	FAM	nuoG	Std	10^4	31,89													
		C07	FAM	nuoG	Std	10^4	30,79													
		C08	FAM	nuoG	Std	10^4	32,49													
	Run 3	C01	FAM	nuoG	Std	10^4	27,25													
		C02	FAM	nuoG	Std	10^4	27,91													
		C03	FAM	nuoG	Std	10^4	27,19													
		C04	FAM	nuoG	Std	10^4	26,96													
		C05	FAM	nuoG	Std	10^4	26,67	27,01	0,15	26,91	#N/A	0,44	0,19	1,86	1,32	1,34	26,57	27,91	216,11	8,00	0,36
		C06	FAM	nuoG	Std	10^4	26,7													
		C07	FAM	nuoG	Std	10^4	26,86													
		C08	FAM	nuoG	Std	10^4	26,57													

	Run 1	D01	FAM	nuoG	Std	10^3	35,07														
		D02	FAM	nuoG	Std	10^3	33,27														
		D03	FAM	nuoG	Std	10^3	38,31														
		D04	FAM	nuoG	Std	10^3	37,34	35,95	0,67	35,40	#N/A	1,89	3,57	-1,35	0,20	5,17	33,27	38,44	287,58	8,00	1,58
		D05	FAM	nuoG	Std	10^3	35,73														
		D06	FAM	nuoG	Std	10^3	34,39														
		D07	FAM	nuoG	Std	10^3	38,44														
		D08	FAM	nuoG	Std	10^3	35,03														
	Run 2	D01	FAM	nuoG	Std	10^3	35,06														
		D02	FAM	nuoG	Std	10^3	35,7														
		D03	FAM	nuoG	Std	10^3	35,64														
		D04	FAM	nuoG	Std	10^3	30,59	35,07	0,97	35,36	#N/A	2,76	7,60	0,53	-0,15	9,02	30,59	39,61	280,54	8,00	2,30
		D05	FAM	nuoG	Std	10^3	36,78														
		D06	FAM	nuoG	Std	10^3	35,07														
		D07	FAM	nuoG	Std	10^3	39,61														
		D08	FAM	nuoG	Std	10^3	32,09														
	Run 3	D01	FAM	nuoG	Std	10^3	29,64														
		D02	FAM	nuoG	Std	10^3	29,27														
		D03	FAM	nuoG	Std	10^3	29,91														
		D04	FAM	nuoG	Std	10^3	29,28	29,41	0,09	29,29	29,27	0,24	0,06	1,78	1,57	0,72	29,19	29,91	235,26	8,00	0,20
		D05	FAM	nuoG	Std	10^3	29,4														
		D06	FAM	nuoG	Std	10^3	29,27														
		D07	FAM	nuoG	Std	10^3	29,3														
		D08	FAM	nuoG	Std	10^3	29,19														
	Run 1	E01	FAM	nuoG	Std	10^2															
		E02	FAM	nuoG	Std	10^2	37,15														
		E03	FAM	nuoG	Std	10^2	36,78														
		E04	FAM	nuoG	Std	10^2	37,46	37,39	0,38	37,16	#N/A	0,92	0,85	3,90	1,87	2,56	36,60	39,16	224,31	6,00	0,97
		E05	FAM	nuoG	Std	10^2															
		E06	FAM	nuoG	Std	10^2	37,16														
		E07	FAM	nuoG	Std	10^2	36,6														
		E08	FAM	nuoG	Std	10^2	39,16														
	Run 2	E01	FAM	nuoG	Std	10^2	43,11														
		E02	FAM	nuoG	Std	10^2	37,42														
		E03	FAM	nuoG	Std	10^2	37,02														
		E04	FAM	nuoG	Std	10^2		38,07	1,03	37,22	#N/A	2,52	6,34	5,23	2,23	6,90	36,21	43,11	228,42	6,00	2,64
		E05	FAM	nuoG	Std	10^2															
		E06	FAM	nuoG	Std	10^2	36,21														
		E07	FAM	nuoG	Std	10^2	36,99														
		E08	FAM	nuoG	Std	10^2	37,67														
	Run 3	E01	FAM	nuoG	Std	10^2	31,07														
		E02	FAM	nuoG	Std	10^2	31,37														
		E03	FAM	nuoG	Std	10^2	31,09														
		E04	FAM	nuoG	Std	10^2	31,36	31,36	0,12	31,33	#N/A	0,34	0,11	5,34	2,12	1,07	31,07	32,14	250,91	8,00	0,28
		E05	FAM	nuoG	Std	10^2	31,3														
		E06	FAM	nuoG	Std	10^2	31,23														
		E07	FAM	nuoG	Std	10^2	31,35														
		E08	FAM	nuoG	Std	10^2	32,14														
	Run 1	F01	FAM	nuoG	Std	10^1															
		F02	FAM	nuoG	Std	10^1	37,34														
		F03	FAM	nuoG	Std	10^1	37,36														
		F04	FAM	nuoG	Std	10^1	37,52	36,89	0,24	36,94	#N/A	0,58	0,34	-2,79	-0,09	1,32	36,20	37,52	221,34	6,00	0,61
		F05	FAM	nuoG	Std	10^1	36,2														
		F06	FAM	nuoG	Std	10^1	36,38														
		F07	FAM	nuoG	Std	10^1	36,54														
		F08	FAM	nuoG	Std	10^1															
	Run 2	F01	FAM	nuoG	Std	10^1	35,76														
		F02	FAM	nuoG	Std	10^1															
		F03	FAM	nuoG	Std	10^1															
		F04	FAM	nuoG	Std	10^1		36,27	0,21	36,27	#N/A	0,41	0,17	1,49	-0,04	1,01	35,76	36,77	145,07	4,00	0,66
		F05	FAM	nuoG	Std	10^1	36,77														
		F06	FAM	nuoG	Std	10^1	36,28														
		F07	FAM	nuoG	Std	10^1	36,26														
		F08	FAM	nuoG	Std	10^1															
	Run 3	F01	FAM	nuoG	Std	10^1	33,05														
		F02	FAM	nuoG	Std	10^1	33,63														
		F03	FAM	nuoG	Std	10^1	33,16														
		F04	FAM	nuoG	Std	10^1	32,65	33,40	0,16	33,42	#N/A	0,45	0,21	-0,95	-0,36	1,32	32,65	33,97	267,20	8,00	0,38
		F05	FAM	nuoG	Std	10^1	33,2														
		F06	FAM	nuoG	Std	10^1	33,84														
		F07	FAM	nuoG	Std	10^1	33,7														
		F08	FAM	nuoG	Std	10^1	33,97														

TABLE 5 | INTERNAL AMPLIFICATION DESCRIPTIVE DATA: 10^6

10^2					
Column1		Column1		Column1	
Mean	37,385	Mean	38,07	Mean	31,36375
Standard Error	0,37613606	Standard Error	1,0281472	Standard Error	0,11847148
Median	37,155	Median	37,22	Median	31,325
Mode	#N/A	Mode	#N/A	Mode	#N/A
Standard Deviation	0,92134141	Standard Deviation	2,51843602	Standard Deviation	0,33508794
Sample Variance	0,84887	Sample Variance	6,34252	Sample Variance	0,11228393
Kurtosis	3,89745071	Kurtosis	5,23449351	Kurtosis	5,33815876
Skewness	1,86545327	Skewness	2,23185742	Skewness	2,122319
Range	2,56	Range	6,9	Range	1,07
Minimum	36,6	Minimum	36,21	Minimum	31,07
Maximum	39,16	Maximum	43,11	Maximum	32,14
Sum	224,31	Sum	228,42	Sum	250,91
Count	6	Count	6	Count	8
Confidence Level(95,0%)	0,96688852	Confidence Level(95,0%)	2,64293652	Confidence Level(95,0%)	0,28014053

TABLE 6 | INTERNAL AMPLIFICATION DESCRIPTIVE DATA: 10^5

10^1					
Column1		Column1		Column1	
Mean	36,89	Mean	36,2675	Mean	33,4
Standard Error	0,23657275	Standard Error	0,20621085	Standard Error	0,16057931
Median	36,94	Median	36,27	Median	33,415
Mode	#N/A	Mode	#N/A	Mode	#N/A
Standard Deviation	0,57948253	Standard Deviation	0,41242171	Standard Deviation	0,45418687
Sample Variance	0,3358	Sample Variance	0,17009167	Sample Variance	0,20628571
Kurtosis	-2,7943612	Kurtosis	1,48898166	Kurtosis	-0,9498836
Skewness	-0,091022	Skewness	-0,0363402	Skewness	-0,3599016
Range	1,32	Range	1,01	Range	1,32
Minimum	36,2	Minimum	35,76	Minimum	32,65
Maximum	37,52	Maximum	36,77	Maximum	33,97
Sum	221,34	Sum	145,07	Sum	267,2
Count	6	Count	4	Count	8
Confidence Level(95,0%)	0,60812962	Confidence Level(95,0%)	0,65625497	Confidence Level(95,0%)	0,37970973

TABLE 7 | INTERNAL AMPLIFICATION DESCRIPTIVE DATA: 10^4

10^4					
Column1		Column1		Column1	
Mean	30,9475	Mean	31,3	Mean	27,01375
Standard Error	0,58757598	Standard Error	0,48807933	Standard Error	0,15412121
Median	30,195	Median	30,935	Median	26,91
Mode	#N/A	Mode	30,56	Mode	#N/A
Standard Deviation	1,66191585	Standard Deviation	1,3804968	Standard Deviation	0,43592062
Sample Variance	2,76196429	Sample Variance	1,90577143	Sample Variance	0,19002679
Kurtosis	-0,3725839	Kurtosis	0,42414719	Kurtosis	1,86319276
Skewness	0,86692148	Skewness	0,53997584	Skewness	1,32426398
Range	4,8	Range	4,49	Range	1,34
Minimum	29,13	Minimum	29,27	Minimum	26,57
Maximum	33,93	Maximum	33,76	Maximum	27,91
Sum	247,58	Sum	250,4	Sum	216,11
Count	8	Count	8	Count	8
Confidence Level(95,0%)	1,38939642	Confidence Level(95,0%)	1,15412421	Confidence Level(95,0%)	0,36443876

TABLE 8 | INTERNAL AMPLIFICATION DESCRIPTIVE DATA: 10^3

10^3					
Column1		Column1		Column1	
Mean	35,9475	Mean	35,0675	Mean	29,4075
Standard Error	0,66797869	Standard Error	0,97475592	Standard Error	0,08655613
Median	35,4	Median	35,355	Median	29,29
Mode	#N/A	Mode	#N/A	Mode	29,27
Standard Deviation	1,88932906	Standard Deviation	2,75702609	Standard Deviation	0,24481772
Sample Variance	3,56956429	Sample Variance	7,60119286	Sample Variance	0,05993571
Kurtosis	-1,3456879	Kurtosis	0,53060001	Kurtosis	1,7840366
Skewness	0,19599464	Skewness	-0,1542211	Skewness	1,56615592
Range	5,17	Range	9,02	Range	0,72
Minimum	33,27	Minimum	30,59	Minimum	29,19
Maximum	38,44	Maximum	39,61	Maximum	29,91
Sum	287,58	Sum	280,54	Sum	235,26
Count	8	Count	8	Count	8
Confidence Level(95,0%)	1,57951862	Confidence Level(95,0%)	2,30493149	Confidence Level(95,0%)	0,20467273

TABLE 9 | INTERNAL AMPLIFICATION DESCRIPTIVE DATA: 10^2

10^2R1		10^2R2		10^2R3	
Column1		Column1		Column1	
Mean	31,15375	Mean	31,40625	Mean	34,16625
Standard Error	0,05846053	Standard Error	0,10766909	Standard Error	0,11270913
Median	31,12	Median	31,435	Median	34,235
Mode	31,12	Mode	#N/A	Mode	#N/A
Standard Deviation	0,16535136	Standard Deviation	0,30453419	Standard Deviation	0,31878956
Sample Variance	0,02734107	Sample Variance	0,09274107	Sample Variance	0,10162679
Kurtosis	1,16242228	Kurtosis	-1,1834206	Kurtosis	2,9594991
Skewness	1,12457875	Skewness	-0,2820925	Skewness	-1,6050796
Range	0,51	Range	0,87	Range	0,99
Minimum	30,97	Minimum	30,93	Minimum	33,48
Maximum	31,48	Maximum	31,8	Maximum	34,47
Sum	249,23	Sum	251,25	Sum	273,33
Count	8	Count	8	Count	8
Confidence Level(95,0%)	0,13823719	Confidence Level(95,0%)	0,25459695	Confidence Level(95,0%)	0,26651474

TABLE 10 | INTERNAL AMPLIFICATION DESCRIPTIVE DATA: 10^1

10^1R1		10^1R2		10^1R3	
Column1		Column1		Column1	
Mean	31,2057143	Mean	31,86375	Mean	34,49125
Standard Error	0,06144518	Standard Error	0,25707793	Standard Error	0,16923607
Median	31,16	Median	31,615	Median	34,345
Mode	31,07	Mode	#N/A	Mode	#N/A
Standard Deviation	0,16256867	Standard Deviation	0,72712619	Standard Deviation	0,4786719
Sample Variance	0,02642857	Sample Variance	0,5287125	Sample Variance	0,22912679
Kurtosis	1,71758095	Kurtosis	5,26429548	Kurtosis	3,48617116
Skewness	1,35207731	Skewness	2,18499554	Skewness	1,72572615
Range	0,45	Range	2,29	Range	1,53
Minimum	31,07	Minimum	31,26	Minimum	34,01
Maximum	31,52	Maximum	33,55	Maximum	35,54
Sum	218,44	Sum	254,91	Sum	275,93
Count	7	Count	8	Count	8
Confidence Level(95,0%)	0,15035094	Confidence Level(95,0%)	0,60789271	Confidence Level(95,0%)	0,40017972

TABLE 11 | *NUO*G DESCRIPTIVE DATA: 10^6

TABLE 12 | *NUO*G DESCRIPTIVE DATA: 10^5

10^6					
nuoG					
Column1		Column1		Column1	
Mean	23,02125	Mean	23,045	Mean	21,7925
Standard Error	0,1092088	Standard Error	0,08187534	Standard Error	0,06064387
Median	23,055	Median	23,01	Median	21,835
Mode	#N/A	Mode	22,86	Mode	#N/A
Standard Deviation	0,30888914	Standard Deviation	0,23157843	Standard Deviation	0,17152676
Sample Variance	0,0954125	Sample Variance	0,05362857	Sample Variance	0,02942143
Kurtosis	0,51162474	Kurtosis	-1,9659246	Kurtosis	-0,9820213
Skewness	0,4096392	Skewness	0,13987535	Skewness	-0,5193068
Range	0,96	Range	0,61	Range	0,46
Minimum	22,62	Minimum	22,75	Minimum	21,54
Maximum	23,58	Maximum	23,36	Maximum	22
Sum	184,17	Sum	184,36	Sum	174,34
Count	8	Count	8	Count	8
Confidence Level(95,0%)	0,25823778	Confidence Level(95,0%)	0,19360442	Confidence Level(95,0%)	0,14339996

TABLE 12 | *NUO*G DESCRIPTIVE DATA: 10^5

10^5					
Column1		Column1		Column1	
Mean	26,29375	Mean	26,81125	Mean	24,0425
Standard Error	0,13673407	Standard Error	0,14233308	Standard Error	0,07631584
Median	26,225	Median	27,035	Median	24,1
Mode	#N/A	Mode	27,05	Mode	#N/A
Standard Deviation	0,38674235	Standard Deviation	0,40257874	Standard Deviation	0,21585379
Sample Variance	0,14956964	Sample Variance	0,16206964	Sample Variance	0,04659286
Kurtosis	1,11404395	Kurtosis	0,16685801	Kurtosis	0,93508585
Skewness	0,47702582	Skewness	-1,1384623	Skewness	-0,8568521
Range	1,31	Range	1,12	Range	0,7
Minimum	25,69	Minimum	26,05	Minimum	23,63
Maximum	27	Maximum	27,17	Maximum	24,33
Sum	210,35	Sum	214,49	Sum	192,34
Count	8	Count	8	Count	8
Confidence Level(95,0%)	0,32332469	Confidence Level(95,0%)	0,33656425	Confidence Level(95,0%)	0,18045828

TABLE 13 | *NUO*G DESCRIPTIVE DATA: 10^4

10^4					
Column1		Column1		Column1	
Mean	30,9475	Mean	31,3	Mean	27,01375
Standard Error	0,58757598	Standard Error	0,48807933	Standard Error	0,15412121
Median	30,195	Median	30,935	Median	26,91
Mode	#N/A	Mode	30,56	Mode	#N/A
Standard Deviation	1,66191585	Standard Deviation	1,3804968	Standard Deviation	0,43592062
Sample Variance	2,76196429	Sample Variance	1,90577143	Sample Variance	0,19002679
Kurtosis	-0,3725839	Kurtosis	0,42414719	Kurtosis	1,86319276
Skewness	0,86692148	Skewness	0,53997584	Skewness	1,32426398
Range	4,8	Range	4,49	Range	1,34
Minimum	29,13	Minimum	29,27	Minimum	26,57
Maximum	33,93	Maximum	33,76	Maximum	27,91
Sum	247,58	Sum	250,4	Sum	216,11
Count	8	Count	8	Count	8
Confidence Level(95,0%)	1,38939642	Confidence Level(95,0%)	1,15412421	Confidence Level(95,0%)	0,36443876

TABLE 13 | *NUO*G DESCRIPTIVE DATA: 10^4

10^3					
Column1		Column1		Column1	
Mean	35,9475	Mean	35,0675	Mean	29,4075
Standard Error	0,66797869	Standard Error	0,97475592	Standard Error	0,08655613
Median	35,4	Median	35,355	Median	29,29
Mode	#N/A	Mode	#N/A	Mode	29,27
Standard Deviation	1,88932906	Standard Deviation	2,75702609	Standard Deviation	0,24481772
Sample Variance	3,56956429	Sample Variance	7,60119286	Sample Variance	0,05993571
Kurtosis	-1,3456879	Kurtosis	0,53060001	Kurtosis	1,7840366
Skewness	0,19599464	Skewness	-0,1542211	Skewness	1,56615592
Range	5,17	Range	9,02	Range	0,72
Minimum	33,27	Minimum	30,59	Minimum	29,19
Maximum	38,44	Maximum	39,61	Maximum	29,91
Sum	287,58	Sum	280,54	Sum	235,26
Count	8	Count	8	Count	8
Confidence Level(95,0%)	1,57951862	Confidence Level(95,0%)	2,30493149	Confidence Level(95,0%)	0,20467273

TABLE 14 | *NUO*G DESCRIPTIVE DATA: 10^3

10^2					
Column1		Column1		Column1	
Mean	37,385	Mean	38,07	Mean	31,36375
Standard Error	0,37613606	Standard Error	1,0281472	Standard Error	0,11847148
Median	37,155	Median	37,22	Median	31,325
Mode	#N/A	Mode	#N/A	Mode	#N/A
Standard Deviation	0,92134141	Standard Deviation	2,51843602	Standard Deviation	0,33508794
Sample Variance	0,84887	Sample Variance	6,34252	Sample Variance	0,11228393
Kurtosis	3,89745071	Kurtosis	5,23449351	Kurtosis	5,33815876
Skewness	1,86545327	Skewness	2,23185742	Skewness	2,122319
Range	2,56	Range	6,9	Range	1,07
Minimum	36,6	Minimum	36,21	Minimum	31,07
Maximum	39,16	Maximum	43,11	Maximum	32,14
Sum	224,31	Sum	228,42	Sum	250,91
Count	6	Count	6	Count	8
Confidence Level(95,0%)	0,96688852	Confidence Level(95,0%)	2,64293652	Confidence Level(95,0%)	0,28014053

10^1					
Column1		Column1		Column1	
Mean	36,89	Mean	36,2675	Mean	33,4
Standard Error	0,23657275	Standard Error	0,20621085	Standard Error	0,16057931
Median	36,94	Median	36,27	Median	33,415
Mode	#N/A	Mode	#N/A	Mode	#N/A
Standard Deviation	0,57948253	Standard Deviation	0,41242171	Standard Deviation	0,45418687
Sample Variance	0,3358	Sample Variance	0,17009167	Sample Variance	0,20628571
Kurtosis	-2,7943612	Kurtosis	1,48898166	Kurtosis	-0,9498836
Skewness	-0,091022	Skewness	-0,0363402	Skewness	-0,3599016
Range	1,32	Range	1,01	Range	1,32
Minimum	36,2	Minimum	35,76	Minimum	32,65
Maximum	37,52	Maximum	36,77	Maximum	33,97
Sum	221,34	Sum	145,07	Sum	267,2
Count	6	Count	4	Count	8
Confidence Level(95,0%)	0,60812962	Confidence Level(95,0%)	0,65625497	Confidence Level(95,0%)	0,37970973

FIGURE 1 | PLASMID CONSTRUCTION

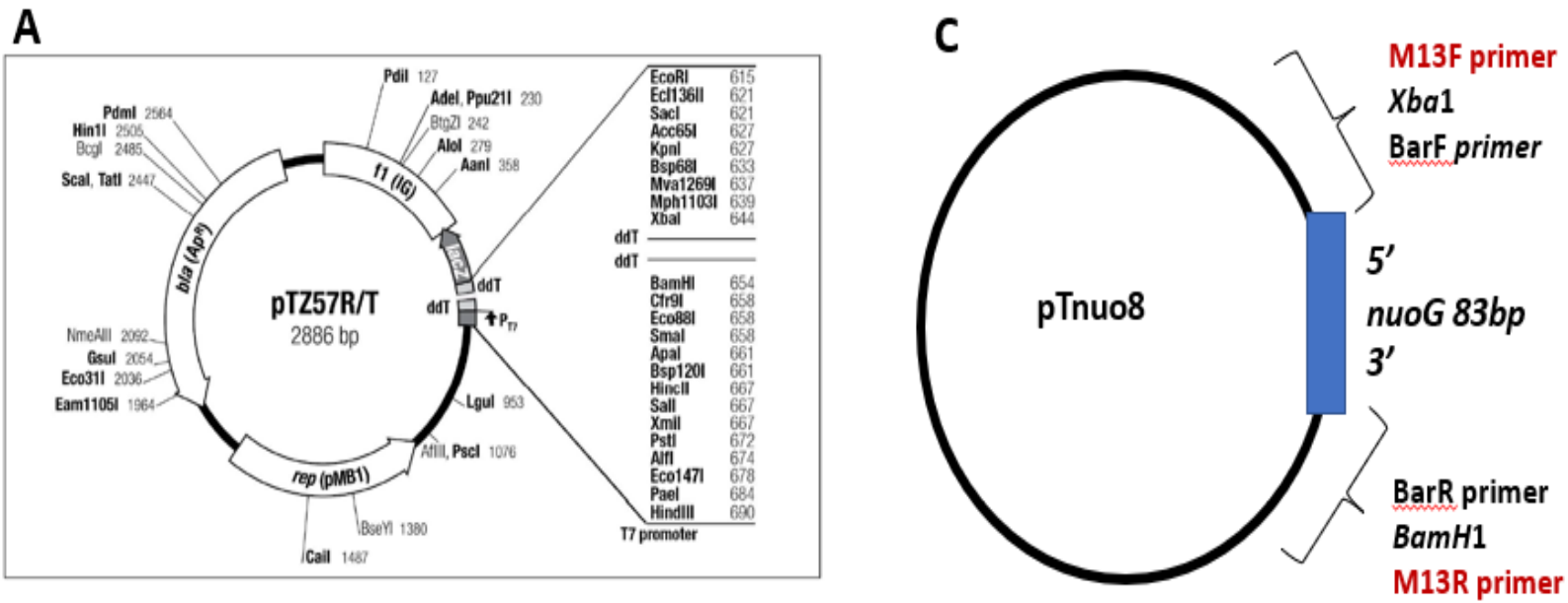


FIGURE 2 | APPROXIMATION OF POSITIVITY

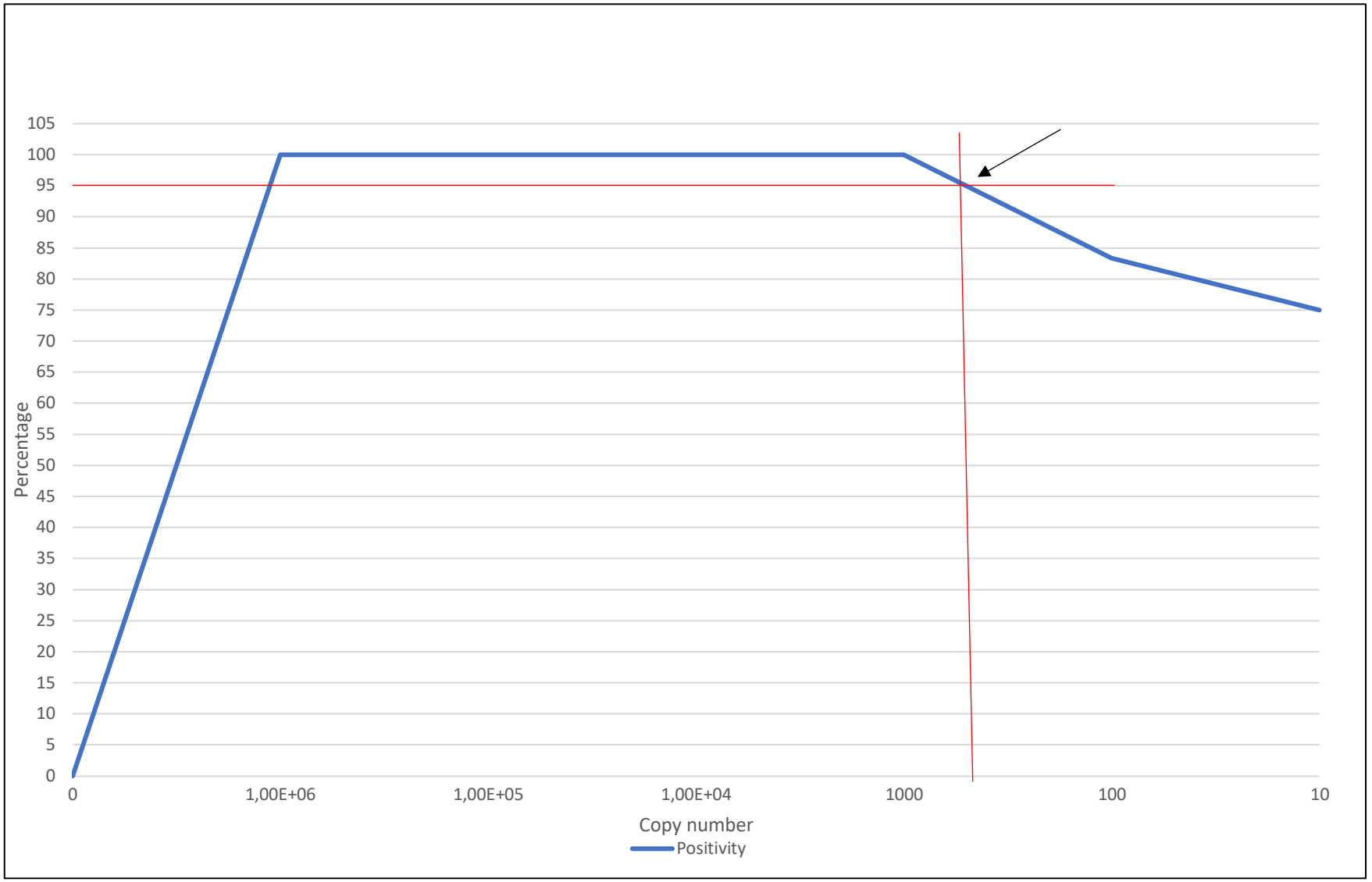
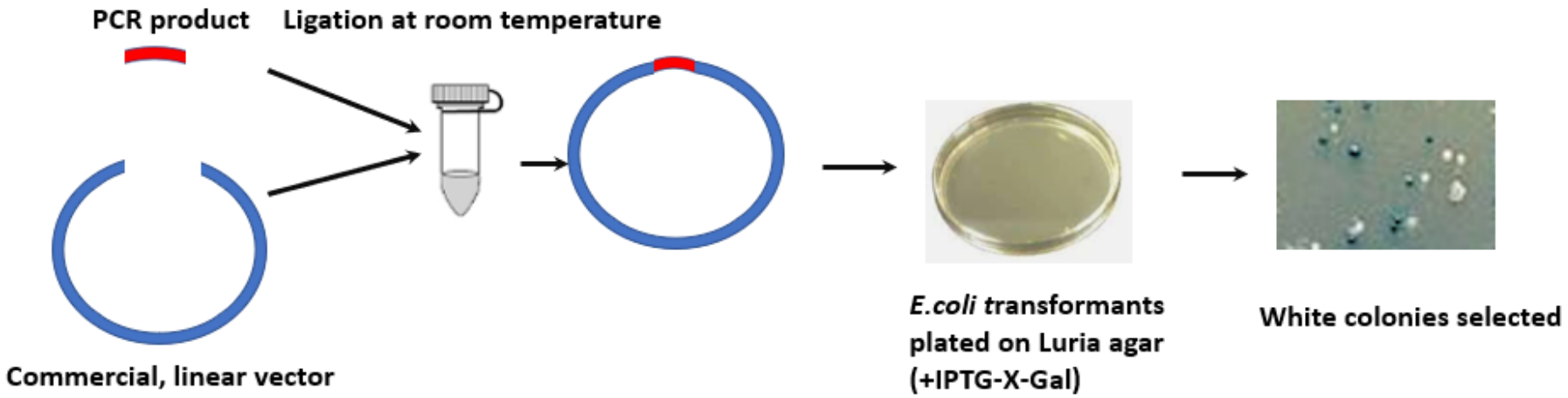


FIGURE 3 | ISOLATION OF POSITIVE CONTROL pTnuo



PART J: GUIDELINES TO AUTHORS FOR FRONTIER'S JOURNAL PUBLICATION

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Title, Abstract, and Key Words: All submitted manuscripts must include a title, an abstract of no greater than 150 words, and a list of up to five key terms than can be used by readers/researchers to find the published material. This information should be included in the online submission form, as well as appear on the first page of the submitted document. Upon acceptance, authors will be invited to submitted a translation of the abstract into a second language of their choice for publication.

Author information: All submitted manuscripts must include the first and last names, institutional/organizational affiliations, and short (75-word) biographies for all authors. Contact information, especially an email address, must be provided for at least the first or corresponding author as identified. This information should be included in the online submission form.

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- Font: Times New Roman, 12 pt.; **Title should appear in bold**, *Headings in italics.* Use numbering system (2, 2.1, 2.2.3) to indicate heading levels.*

- Spacing: Double-spaced
- Margins: 1 inch on all sides
- Page numbers: Centred in the bottom margin

*Please DO type your entire manuscript in “Normal” style. DO NOT use style sheets or other advanced formatting options.

DO NOT:

- include running titles
- include headers or footers.

Style: ***All citations must be in APA style. The work cited list must include DOIs (Digital Object Identifiers) for all digital content for which they are available.*** Please check your manuscript, including references, for correct APA style and completeness.

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Last updated: June 23, 2021

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3. **How does *Frontiers* use and share your information?**

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- Have your data deleted or otherwise removed. You have the right, under certain circumstances, to obtain the erasure of your data by The Forum.
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- Lodge a complaint. You have the right to bring a claim before the competent data protection authority.

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Effective Date
This policy is effective beginning September 12, 2018. Last updated: June 23, 2021.

Supplementary Material

Supplementary Data

Supplementary Material should be uploaded separately on submission. Please include any supplementary data, figures and/or tables. All supplementary files are deposited to FigShare for permanent storage and receive a DOI.

Supplementary material is not typeset so please ensure that all information is clearly presented, the appropriate caption is included in the file and not in the manuscript, and that the style conforms to the rest of the article. To avoid discrepancies between the published article and the supplementary material, please do not add the title, author list, affiliations, or correspondence in the supplementary files.

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Supplementary Figures

Supplementary Figure 1. The figure legends are required to have the same font as the main text, 12 point normal Times New Roman, single spaced. Please use a single paragraph for each legend and prepare the figures keeping in mind the PDF layout.

PART K: INSTITUTIONAL APPROVALS

UNIVERSITY OF CAPE TOWN — FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

FHS016: Annual Progress Report / Renewal

HREC office use only (FWA00001637; IRB00001938) This serves as notification of annual approval, including any documentation described below.			
☑ Approved	Annual progress report	Approved until/next renewal date	28·02·22
☐ Not approved	See attached comments		
Signature Chairperson of the HREC/ Designee		Date Signed	24/5/21

Note: Please note that incomplete submissions will not be reviewed
Please email this form and supporting documents (if applicable) in a combined pdf-file to hrec-enquiries@uct.ac.za.
Please clarify your plan for research-related activities during COVID-19 lockdown

Comments to PI from the HREC

Principal Investigator to complete the following:

1. Protocol information

Date (when submitting this form)	04/03/2021		
HREC REF Number	073/2018	Current Ethics Approval was granted until	28Feb2021
Protocol title	DEVELOPMENT AND VALIDATION OF AN IN-HOUSE, REAL-TIME POLYMERASE CHAIN REACTION ASSAY FOR THE DETECTION OF *BARTONELLA* IN PERIPHERAL BLOOD OF PATIENTS WITH CULTURE-NEGATIVE INFECTIVE ENDOCARDITIS (MMED CANDIDATE - DR G BOOLEY) sub-study linked to R020/2015		
Protocol number (if applicable)	V1		
Are there any sub-studies linked to this study?	☐ Yes X No		
If yes, could you please provide the HREC Ref's for all sub-studies? **Note:** A separate FHS016 must be submitted for each sub-study.			
Principal Investigator	Dr Lynthia Paul		

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)

Department / Office Internal Mail Address	Division of Medical Microbiology, 5th Floor, Falmouth Building, Faculty of Health Sciences, UCT

1.1 Does this protocol receive US Federal funding?	☐ Yes	x No
1.2 If the study receives US Federal Funding, does the annual report require full committee approval? **Note:** Any annual approvals for **Full Committee** review MUST be submitted on the monthly HREC submission dates. (Please send electronic copy for full committee review to hrec-enquiries@uct.ac.za)	☐ Yes	☐ No
If yes in 1.2 please complete section 1.3 below for invoicing purposes		
1.3 Annual Approval for **full committee** review	- R 3450 (inclusive of vat)	

For invoicing purposes, please provide:	
Sponsor's name	
Contact person	
Address	
Telephone number	
Email Address	

2. List of documentation for approval

Synopsis for renewal and cover letter

FHS016 -renewal

3. Protocol status (tick ✓)

	Open to enrolment
☐	Closed to enrolment (tick ✓)
x	Research-related activities are ongoing (student study 99% complete)
☐	Research-related activities are complete, long-term follow-up only
☐	Research-related activities are complete, data analysis only
☐	Main study is complete but sub-study research-related activities are ongoing
☐	Study is closed → Please submit a Study Closure Form (FHS010)

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)

4. Enrolment

Number of participants enrolled to date	0
Number of participants enrolled, since last HREC Progress report (continuing review)	0
Additional number of participants still required	0

5. Refusals

Total number of refusals (participants invited to join the study, but refused to take part)	0

6. Cumulative summary of participants

Total number of participants who provided consent	NA
Number of participants determined to be ineligible (i.e. after screening)	NA
Number of participants currently active on the study	NA
Number of participants completed study (without events leading to withdrawal)	NA
Number of participants withdrawn at participants' request (i.e. changed their mind)	NA
Number of participants withdrawn by PI due to toxicity or adverse events	NA
Number of participants withdrawn by PI for other reasons (e.g. pregnancy, poor compliance)	NA
Number of participants lost to follow-up. Please comment below on reasons for loss of follow-up.	NA
Number of participants no longer taking part for reasons not listed above. Please provide reasons below:	NA

7. Progress of study

Please provide a brief summary of the research to date including the overall progress and the progress since the last annual report as well as any relevant comments/issues you would like to report to the HREC:
The MMed student (Dr Bhooley) done a technical, laboratory validation and shown that, using a pure plasmid containing a fragment of DNA from the target bacterium, that this real time PCR will detect between 100-1000 copies of the target gene if present. She is now using this data, preparing a thesis for her MMed degree. We still need to show specificity (it do not detect pathogens other than the intended target)- although we use primerset that had been published and validated by others that it is specific. Follow up work to do in future: a clinical validation, using real patient specimens (consented and with all relevant permission to do prospective collection). A protocol amendment will be done in near future and submitted to HREC to enable a clinical validation with real life specimens.

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)

8. Protocol violations and exceptions (tick ✓ all that apply)

x	No prior violations or exceptions have occurred since the original approval
☐	Prior violations or exceptions have been reported since the last review and have already been acknowledged or approved
☐	Unreported minor violations that have occurred since the last review, as well as significant deviations not yet reported, are attached for review

9. Amendments (tick ✓ all that apply)

x	No prior amendments have been made since the original approval
☐	Prior amendments have been reported since the last review and have already been approved
☐	New protocol changes/ amendments are requested as part of this continuing review (See note below)

Note: If new protocol changes are being requested in this review, please complete an amendment form (FHS006).
Specific changes in the amended protocol and consent/assent forms must be **bolded**, *italicised* or tracked and all changes must include a rationale.

10. Adverse events

10.1 Please provide below or attach a narrative summary of serious adverse events and/ or unanticipated problems since the last progress report. Please indicate changes made to the protocol and informed consent document(s) as a result (if not already reported to the HREC). Please comment on whether causality to any study procedure or intervention could be established.
No adverse events as no recruitment occurred in this project.

10.2 Have participants received appropriate treatment/ follow-up/ referral when indicated (e.g. in the case of abnormal or incidental clinical findings, distress or anxiety)?		
☐ Yes	☐ No	x Not applicable
If yes, please describe:		
Not applicable.		

11. Summary of Monitoring and Audit Activities (tick ✓)

11.1 Was this study monitored or audited by an external agency (e.g. SAHPRA, FDA)?		
☐ Yes	☐ No	x Not applicable

11.2 Did a Data and Safety Monitoring Board publish a report?		
☐ Yes	☐ No	x Not applicable

11.3 If yes, please identify the agency and attach a summary of the findings.

Agency Name		Report attached	☐ Yes	☐ No	☐ Not applicable
		DSMB report attached	☐ Yes	☐ No	☐ Not applicable

11.4 Has there been any agency, institutional or other inquiry into non-compliance in this study, or any finding of non-compliance concerning a member of the research team?	
x Yes	☐ No
If yes, please explain:	
Previously reported deviation whereby a student was not added as "study staff member" (due to inexperience of new PI) – been and dealt with >2yrs ago. None other since.	

12. Level of risk (tick ✓)

12.1 In light of your experience of this research, please indicate whether the level of risk to participants has:	
☐	Increased
☐	Decreased
X	Shown no change
If there has been a change, please explain:	

12.2 Please provide a narrative summary of recent relevant literature that may have a bearing on the level of risk.
Not applicable to current protocol

13. Statement of conflict of interest

Has there been any change in the conflict of interest status of this protocol since the original approval? (tick ✓)	
☐ Yes	x No
If yes, please explain and if necessary, attach a revised conflict of interest statement (Section #7 in the New Protocol Application Form FHS013):	

14. Signature

My signature certifies that the above is complete and correct.			
Signature of PI		Date	4 March 2021

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)