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Construction and evaluation of luciferase reporter phages for the detection of active and non-replicating tubercle bacilli

Azger Dusthacker^a, Vanaja Kumar^{a,*}, Selvakumar Subbian^b, Gomathi Sivaramakrishnan^a, Guofang Zhu^c, Balaji Subramanyam^a, Sameer Hassan^a, Selvakumar Nagamaiah^a, John Chan^c, and Narayanan Paranj Rama^a

^a Department of Bacteriology, Tuberculosis Research Centre (TRC), Chennai-31, India

^b Department of Microbial and Molecular Pathogenesis, Texas A&M University Health Science Centre, College station, TX, USA

^c Department of Microbiology and Immunology, Albert Einstein College of Medicine (AECOM), New York, USA

Abstract

The luciferase reporter phages (LRP) show great promise for diagnostic mycobacteriology. Though conventional constructs developed from lytic phages such as D29 and TM4 are highly specific, they lack sensitivity. We have isolated and characterized Che12, the first true temperate phage infecting *M. tuberculosis*. Since the tuberculosis (TB) cases among HIV infected population result from the reactivation of latent bacilli, it would be useful to develop LRP that can detect dormant bacteria. During dormancy, pathogenic mycobacteria switch their metabolism involving divergent genes than during normal, active growth phase. Since the promoters of these genes can potentially function during dormancy, they were exploited for the construction of novel mycobacterial luciferase reporter phages. The promoters of *hsp60*, isocitrate lyase (*icl*), and alpha-crystallin (*acr*) genes from *M. tuberculosis* were used for expressing firefly luciferase gene (FFlux) in both Che12 and TM4 phages and their efficiency was evaluated in detecting dormant bacteria from clinical isolates of *M. tuberculosis*. These LRP constructs exhibited detectable luciferase activity in dormant as well as in actively growing *M. tuberculosis*. The TM4 *ts* mutant based constructs showed about one log increase in light output in three of the five tested clinical isolates and in *M. tuberculosis* H37Rv compared to conventional lytic reporter phage, phAE129. By refining the LRP assay format further, an ideal rapid assay can be designed not only to diagnose active and dormant TB but also to differentiate the species and to find their drug susceptibility pattern.

Keywords

Dormancy; Mycobacteriophage; Luciferase reporter phage assay

1. Introduction

Mycobacterium tuberculosis can persist within the human host for years without causing disease. During the initial stages of infection, transient bacteremia may result in the

Corresponding author: Dr. Vanaja Kumar, Department of Bacteriology, Tuberculosis Research centre, Chennai-31, India, Telephone No. +91-044-28369659, E.mail: vanajakumar@trcchennai.in, Fax.No: +91-(044)-28362528.

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dissemination of viable bacilli throughout the host (Parrish et al., 1998). The precise mechanism of host defense and the cause for the persistence of disease despite adequate chemotherapy remains unresolved. However, inside the immune-competent host, the bacteria undergoes gradual metabolic shutdown leading to non-replicating persistence marked by hypoxia. As an adaptation mechanism, these dormant bacteria actively express genes such as *hsp60*, isocitrate lyase (*icl*) (Li et al., 2005) and alpha crystallin protein (*acr*) (Park et al., 2003) in order to survive the hostile, nutrition-depleted intracellular niche. Non-acid fast granular forms have been reported in the presence of host defense mechanisms and anti tuberculosis drugs (Chandrasekar and Ratnam, 1992; Mattman, 1974).

It has been estimated that about 1.7 billion people harbor latent *M. tuberculosis* infection (Dye et al., 1999). More than half of the health care workers in high TB incidence and low/middle income countries are latently infected with TB (Joshi et al., 2007). The diagnosis of latent form of tuberculosis is either by a positive reaction to intradermal purified protein derivative or a chest radiograph (Parrish et al., 1998). Interferon gamma release assay is more specific than tuberculin skin testing in BCG vaccinated population but both are frequently discordant (Menzies et al., 2007). There is no simple, direct bacteriological tool available so far for the diagnosis of latent TB infection.

Over the past decade, luciferase reporter phages (LRP) have been developed that show great promise for diagnostic mycobacteriology (Jacobs et al., 1993). Researches aiming to develop LRP constructs with increased and sustained luciferase activity in *M. tuberculosis* have taken up different strategies. The first generation reporter phage phAE40 developed from TM4 mycobacteriophage were capable of detecting 10^4 bacteria/ml. (Jacobs et al., 1993). Subsequently phGS18, developed from the temperate phage L5, showed better sensitivity with *M. smegmatis* but was unable to infect *M. tuberculosis* (Sarkis et al., 1995). Alternatively, D29 a lytic mycobacteriophage showing close DNA homology with L5 was chosen to develop LRP constructs (Pearson et al., 1996). These constructs showed about a log increase in RLU output but still fell short in their sensitivity (unpublished observation) to be used for diagnosis. The poor sensitivity may be attributed to the lytic nature of the existing phage constructs, which lyse the bacteria leading to rapid disintegration of ATP. Che12, a temperate phage capable of infecting *M. tuberculosis* could delay the host cell lysis and LRP constructs developed from Che12 have shown promise in diagnosing TB.

In the present study, we report the construction of reporter phages from Che12 based phAETRC16 and TM4 based temperature-sensitive (*ts*) phage mutant, phAE159 (Bardarov et al., 2002) and evaluation of their potential as a diagnostic tool for tuberculosis. These phage constructs express the reporter fire fly luciferase (FFlux) gene through mycobacterial promoters of genes that are potentially functional during dormancy such as *hsp60*, *icl* and *acr*. This is the first report on developing reporter phages capable of detecting dormant bacilli in addition to actively growing cells of clinical isolates of *M. tuberculosis*.

2. Materials and Methods

2.1. Bacterial strains, Chemicals and Plasmids

The mycobacterial reference strains *M. tuberculosis* H37Rv and *M. smegmatis* mc² 155 and 5 clinical isolates of *M. tuberculosis* were grown at 37°C in Middlebrook 7H9 (Gifco) complete media (MADC). Luria-Bertani (LB) media was used to propagate *E. coli* cells. For solid media, agar at concentration of 2% was incorporated into the respective broth. Wherever necessary, kanamycin (25 µg/mL for *E. coli* and 20 µg/mL for mycobacteria) was added to the culture media. All chemicals were purchased from sigma (St. Louis, USA) unless otherwise mentioned. All enzymes were procured from New England Biolabs (MA, USA). Description of the plasmids and primers used in this study are listed in Table 1a & b.

2.2. Construction of LRPs

LRP construct phAETRC16 with hsp60 promoter driving FFlux gene was developed from Che12, the unique temperate phage capable of infecting *M. tuberculosis* isolated and characterized at Tuberculosis Research Centre, Chennai, India. A temperature-sensitive (*ts*) mutant phAE159 was earlier developed from TM4 at Albert Einstein College of Medicine, New York. Using phAETRC16 and phAE159, attempts were made to develop LRPs with FFlux driven by *icl*, *hsp60* and *acr* promoters. Standard molecular techniques were followed for the generation of recombinant plasmids and cosmids (Sambrook et al., 1989). One microgram of the integrating vector, pMVLux was digested sequentially with two units each of *KpnI* and *BbvCI* to remove the L5 integrase. The *cosB* region from cosmid vector pGJ2 was amplified by PCR with primers Cos-KpnI and Cos-AgeI that contain flanking restriction site for *KpnI* and *AgeI* respectively. The 772bp amplified product was purified and ligated with the linear pMVLux to generate a 5.2 kb cosmid, pMVcos, in which the *int* site was replaced with the *cos* region. The promoters of mycobacterial genes *icl* and *acr* were obtained as 270 and 205 base pairs of DNA fragment respectively. This was accomplished by PCR using *M. tuberculosis* H37RV genomic DNA and site-specific forward and reverse primers that contained flanking restriction sites for *AgeI* and *PvuII* respectively, for subsequent ligation reaction (Table 1a). The purified promoters were ligated to pMVcos to yield pACRLux and pICLLux that contain promoter of *acr* and *icl* respectively. The integrity of recombinant plasmids and cosmids were assessed through restriction enzyme mapping and sequencing. The recombinant cosmids thus generated were digested with *NotI* and ligated with either phAETRC16 or *ts* mutant phage phAE159 that were cut with *NotI* and lambda packaged *in vitro*. Standard procedures were followed for the generation of reporter phages from shuttle plasmids thus generated (Pearson et al., 1996). The plaques obtained on the lawn of *M. smegmatis* mc² 155 were purified, checked for the presence of the insert and propagated for standard LRP assays.

2.3. LRP assay

Suspensions of *M. smegmatis* mc² 155, *M. tuberculosis* H37Rv and clinical isolates of *M. tuberculosis* strains were prepared in Middlebrook 7H9 complete broth using growth on fresh Lowenstein Jensen (LJ) slopes. Suspensions of 1×10^7 cells/mL were added to equal volume of 1×10^8 pfu/mL of phage to get a multiplicity of infection (MOI) of 10 (phage: bacteria). The phage-bacteria mixture was incubated at 37°C for 3 to 4 hours. For experiments with phAETRC201 and phAETRC202 the phage-bacteria mixture was incubated both at optimum and restrictive temperatures of 37°C and 30°C respectively. For evaluating the constructs with both dormant and actively growing cells, 0.33 mM D-luciferin (R&D Systems, Minneapolis) in 0.05M sodium citrate buffer was added to phage-cell mixture and photons released were measured using the luminometer (Monolight 2010) with 10 seconds integration time at 4 hours and 24 hours after phage infection. The dark count was 80 RLU. RLU in the order of more than three times the dark count is taken as significant (Carrier et al, 1997). The reporter phages phAE129 and phAETRC16 were used as controls and the results were compared.

2.4. Dormancy model

Wayne's model was used to simulate *in vitro* dormancy in the mycobacterial strains tested (Wayne and Hayes, 1996). Briefly, the mycobacterial strains from LJ slopes were scrapped and suspended in 1 mL of MADC to make a stock inoculum. A bacterial suspension corresponding to # 1 MacFarland's standard was prepared from the stock and diluted to a final volume of 5 mL with MADC containing 1.5 µg/mL of methylene blue indicator. About 2.5 mL headspace volume was maintained to get 2:1 ratio of culture to airspace in the growth container. The bacteria were grown in sealed containers at 37°C with constant stirring. A week after the disappearance of blue color of methylene blue from the bacterial culture, 50 µL of the

five reporter phages were added to 250 μ L of culture and RLU was measured after 4 hours and 24 hours of incubation.

3. Results

3.1. Construction of reporter phages

Our attempt to develop reporter phages using Che12 based phAETRC16 and TM4 based *ts* mutant phAE159 expressing FFlux gene by *icl*, *acr* and *hsp60* promoters resulted in three reporter phages. The construct phAETRC21, developed from phAETRC16, had FFlux driven by *icl* promoter while phAE159 based constructs phAETRC201 and phAETRC202 had *hsp60* and *acr* promoters, respectively. Their integrity was confirmed through restriction mapping and DNA sequencing. The basic structure of the plasmid, cosmid and the recombinant LRPs are illustrated in Fig. 1 and Tables 1b & 1c.

3.2. Sensitivity of the constructs

When *M. tuberculosis* strains were titrated with the LRP constructs, phAETRC201 was more sensitive than other constructs. It was able to detect *M. tuberculosis* H₃₇Rv as low as 81 cells (Table 2) while 4 logs more cells of clinical isolate were required to be detected by the same. The sensitivity of all the constructs were lower by at least 1 log in the clinical isolate compared to *M. tuberculosis* H₃₇Rv irrespective of whether the construct is from a lytic, temperate or a *ts* mutant phage.

3.3. Luciferase production kinetics

The recombinant constructs phAETRC21, phAETRC201 and phAETRC202 were tested for light production after infection with *M. tuberculosis* H₃₇Rv and clinical isolates of *M. tuberculosis* strains for 3, 24, 48 and 72 hours. All of them showed measurable RLU with all the mycobacterial strains tested (Fig. 2). With phAE129, the RLU produced was the highest at 3 hours. TM4 *ts* mutant based phAETRC202 resulted in increased RLU at all the tested time points showing maximum light production. With the temperate LRP, phAETRC21 the maximum light output was seen at 72 hours while others showed maximum light production at 48 hours after infection of *M. tuberculosis* H₃₇Rv (Fig. 2). With clinical isolate-1 phAETRC201 did not produce significant RLU both at 30°C and 37°C. Interestingly, phAETRC202 developed from *ts* mutant produced optimum RLU at an infection temperature of 30°C while the other LRP phAETRC201 produced from the same *ts* mutant showed an optimum activity at 37°C (Table 3).

3.4. Evaluation of the LRP constructs in active and dormant cultures of *M. tuberculosis*

High level light production was observed in the laboratory grown standard strain H37Rv after 4 and 24 hours of infection with all the phage constructs in actively growing and dormant *M. tuberculosis* cells. However, both the Che12 based constructs failed to produce significant light at 4 hours in dormant cells. TM4 based constructs produced light with isolate1, an NTM strain, showing more pronounced activity in actively growing cells at 24 hours.

With isolate 2, low level light production was observed with phAETRC16 only with dormant cells at 24 hours. With both the isolates 2 & 3, phAETRC21 resulted in low level RLU production only with actively growing cells at 4 hours. The other constructs exhibited good activity both with actively growing and dormant cells at low levels in 4 hours showing notable increase when tested at 24 hours.

In dormant cells of isolates 3, 4 and 5 Che12 constructs, phAETRC16 and 21 did not produce significant light. With dormant cells of isolates 2, 3, 4 and 5 phAETRC201 showed maximum

light production. Similar high level RLU production was observed in the other *ts* mutant based construct phAETRC202 except in the isolate 5 at 24 hours.

4. Discussion

Since latent tuberculosis infection serves as a reservoir for reactivation-tuberculosis at a later stage, it is important to understand the pathophysiology of the bacilli during such conditions for effective control of tuberculosis. Clinically, these persisting bacilli from caseous necrosis disseminate to other parts of the lungs and to the outside world when the patient coughs vigorously (Jacques, 2003). One of the primary problems in studying dormant bacilli in latent infection is the lack of tools to identify such bacteria. There is an urgent need for the development of an improved diagnostic tool that can aid in the detection of latent bacilli in the host. In the present study, we hypothesized that a LRP construct, with promoters from mycobacterial persistence genes driving the FFlux gene, will infect heterogeneous population of both active and dormant bacilli leading to their detection. We took advantage of the true temperate phage Che12, capable of lysogenising and integrating into *M. tuberculosis* genome, and a TM4 based temperature sensitive mutant phAE159, behaving as a temperate phage at its restrictive temperature, for developing novel LRPs with improved efficiency compared to lytic phage constructs.

Isocitrate lyase, encoded by *icl*, plays an important role in the metabolism of *M. tuberculosis* during the latent state (Li et al., 2005). Activity of this enzyme has been shown to increase in long-term cultures of *M. tuberculosis* (Flynn et al., 2001). It has been demonstrated that *icl* promotes persistence of infection by enhancing bacterial survival within inflammatory macrophages (McKinney et al., 2000). The *acr* gene, which encodes an alpha crystalline homologue is specifically induced during hypoxia and potentially involved in mycobacterial latency (Park et al., 2003). Also, it is one of the most prominent stationary phase induced proteins (Parrish et al., 1998). Luciferase reporter gene under the control of *acr* promoter has been used previously to measure the hypoxic responsiveness in BCG (Park et al., 2003). We chose to use the promoters of *icl* and *acr* since their function reflects the *in vivo* situation where metabolically low-active tubercle bacilli are in a state of non-replicating persistence neither cultivable nor measurable by the conventional techniques.

The lytic phage construct phAE129 used as control produced maximum light at the end of 3 hours of incubation with *M. tuberculosis* H37Rv which decreased drastically at 24 hours and continued to decrease up to 72 hours. Temperate phage Che12 based constructs showed minimal light output at 3 hours gradually increasing up to 72 hours. Alternatively TM4 *ts* mutant based construct phAETRC201 with *hsp60* promoter to express FFlux gene exhibited moderately increased light output at 3 hours increasing up to 48 hours of incubation but decreasing thereafter. Another TM4 *ts* mutant based construct, phAETRC202 with *acr* promoter, produced RLU in 5 digits right from 3 hours up to 72 hours. It is inferred that the performance of these constructs was exemplary not only because they were *ts* mutant based but also due to the credibility of their promoters driving FFlux gene.

The construct phAETRC201 showed maximum light production at its restrictive temperature i.e. 37°C as expected. Whereas phAETRC202 showed increased light output at the nonrestrictive temperature 30°C. Both these phages were developed from TM4 *ts* mutant phAE159. At 30°C there is an increase in plaque forming units (PFU) in a *ts* mutant favoring increased luciferase production leading to increase in RLU. On the other hand, lysis of the host cells leads to rapid degradation of ATP resulting in decreased RLU. Since the host cell lysis is delayed at 37°C, there is an increased availability of ATP that results in more RLU. However, the phage replication is limited leading to less luciferase production compared to that at 30°C. Thus, RLU production observed at both temperatures is the net result of luciferase enzyme

production and prolonged availability of ATP. In this regard, the functional characteristics of the *acr* and *hsp60* promoters may also play a significant role.

Phages infecting and lysing dormant bacilli have been reported previously (Schrader et al., 1997). In this study, we report for the first time that constructs developed from TM4 based *ts* mutant viz, phAETRC201 and phAETRC202 could infect dormant tubercle bacilli that were maintained in anaerobic environment using Wayne's dormancy model. However, Che12 construct, phAETRC21 did not produce detectable RLU with most of the strains in the dormant stage. The inability of Che12 to successfully infect the dormant bacilli can be attributed to the lack of MT3 motif in the tape measure protein (TMP). Recently, Piuri and Hatfull, 2006 had reported that mycobacteriophages Che8, Omega, TM4 and Bxz1 have MT3 motif in their tape measure protein (TMP) conferring the ability to efficiently infect *M. smegmatis* in stationary phase having thicker or more highly cross-linked peptidoglycan layer. Similar thickening of the peptidoglycan layer can be expected in *M. tuberculosis* cells growing in hypoxic condition. In the present study, TM4 *ts* mutant phAE159 based constructs infected all *M. tuberculosis* strains in hypoxic condition resulting in light production whereas Che12 based, temperate phage constructs could only infect the standard strain. The BLAST search with repeated iterations, for the MT3 motif in the TMP revealed the absence of the MT3 motif in the Che12 genome. Also, entire length of TMP in all 10 mycobacteriophages is in between 1100–1900 residues whereas the Che12 TMP measured only about 800 residues, indicating possible loss of the motif during evolution (unpublished data).

Despite the absence of MT3, LRP constructs of Che12 and D29 origin are able to infect the stationary cells of *M. tuberculosis* H₃₇Rv but not of the clinical isolates. Moreover, at least 1 log more of actively growing cells of clinical isolate were needed to be detected by these constructs compared to *M. tuberculosis* H₃₇Rv. This difference can be attributed to the reported variability in gene sequence between the laboratory grown strains and the clinical isolates. Hobman reported that several genetic lesions accumulate during the life of a laboratory standard culture of *E. coli* K-12 (Hobman et al., 2007). The mean survival time of 2 of 3 clinical strains of *M. tuberculosis* were reported to vary significantly from that of the Erdman strain (Scanga et al., 2001). The presence of *moaA3* gene in a large number of clinical isolates of *M. tuberculosis* in Kerala, South India that is absent in H37Rv and H37Ra has been reported (Sarojini et al., 2005). Whole genome analysis of *M. tuberculosis* H₃₇Rv and the clinical strain CDC1551 revealed that a higher level of polymorphism is present among them (Fleishmann et al., 2002).

Phage constructs phAETRC201 and phAETRC202 resulted in high RLU production with one of the isolates, while other constructs did not. This isolate was later identified to be *M. kansasii* based on its mycolic acid pattern as observed in HPLC. The parental phage of these constructs, TM4, is capable of infecting slow growing mycobacteria (Ford et al., 1998). Use of NAP (Riska et al., 1997) or paranitrobenzoic acid containing medium helps in differentiating MAIS complex from *M. tuberculosis*. More than one kind of reporter phage could be exploited in differentiating the species of mycobacteria at the time of diagnosis.

Wayne had earlier documented that there was a decrease in ATP of more than 50% in NRP2 state compared to active cultures. Despite the decrease of ATP level in dormant bacteria, the *ts* mutant reporter phages constructed in the present work, were found to infect and produce photons comparable to actively growing cells. The observation also substantiates that the increased luciferase activity and infectivity of TM4 constructs can be attributed to the combined effect of the promoter activity and the presence of peptidoglycan hydrolase motif within the TMP. It may be postulated that after successful infection of the reporter phages through the MT3 motif, the mycobacterial promoters could manage to function effectively within the environment prevailing inside the bacteria, leading to sustained light production.

5. Conclusion

The TM4 based reporter phages expressing FFlux driven by promoters of mycobacterial persistence genes exhibited enhanced RLU production in both dormant and actively growing *M. tuberculosis* cultures. The success of TM4 based construct could be due to the presence of Mt3 motif in their TMP conferring the ability to infect mycobacterial cells in latent state, which is found to be absent in the temperate phage Che12. This explains the inability of Che12 based constructs to infect dormant bacilli probably with a thickened peptidoglycan outer layer. TM4 based constructs developed in the present attempt showed increased light output from three of five clinical isolates and *M. tuberculosis* H37Rv grown in dormant conditions.

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Fig. 1.

Schematic representation of the Plasmid, Cosmid and LRPs

A: pMVLux with integrase gene

B: pMVcos with cosB replacing int.gene in pMVLux

C: pICLLux with *icl* promoter replacing *hsp60* in pMVcos

D: pACRLux- with *acr* promoter replacing *hsp60* in pMVcos

Fig. 2. Light production kinetics of five reporter constructs in *M. tuberculosis* H37Rv. RLU was measured at 3, 24, 48 and 72 hours after phage infection. Reporter phages used: 1-phAE129, 2-phAETRC16, 3-phAETRC21, 4-phAETRC201, 5- phAETRC202.

Fig. 3.

Performance of LRP constructs in active and dormant cultures of *M. tuberculosis* H37Rv and five clinical isolates of *M. tb* (I to V).

RLU is measured after 4 and 24 hours of infection. Isolate number I is *M. kansasii* and TM4 constructs phAETRC201 and phAETRC202 produced significant RLU with this strain. Isolate number II and III are sensitive to isoniazid and rifampicin, whereas isolate number IV and V are multidrug resistant strains. Significant RLU was produced by all the constructs in both active and dormant cultures of *M. tuberculosis* H37Rv. TM4 constructs produced significant RLU in most of the dormant wild type strains.

Table 1

Table 1a. Plasmids used in this study		
Plasmid	Description	Source
pMVLux	A 4kb Integration plasmid containing <i>oriE</i> , <i>aph</i> and, <i>fflux</i> under BCG <i>hsp60</i> promoter	J.Drumm, AECOM
pGJ2	An Amp ^r cosmid with <i>oriE</i>	N.S.Gomathi, TRC
pMVcos	A 5.2kb cosmid derivative of pMVLux	This work
pACRLux	Derivative of pMVcos with <i>fflux</i> under MTB <i>acr</i> promoter	This work
pICLLux	Derivative of pMVcos with <i>fflux</i> under MTB <i>icl</i> promoter	This work
Table 1b. Primers used in this study		
Primer	Sequence (5'-3')	
Cos-KpnI	CCCGGTACCTTTAATTAATACGCGTTTTGGTGAGCAGCAATAT	
Cos-AgeI	CCCACCGGTGACGTGTCTAGATACGTCTGCTTT	
Acr-AgeI	TTTTTACCGGTAGGCCAACTGCACC	
Acr-PvuII	TTTTTCAGCTGTTGATGCCTCCTAATCG	
Icl-AgeI	TTTTTACCGGTGGGTGGACCAGCGTTGACAC	
Icl-PvuII	TTTTTCAGCTGGTCCTTCCAGCGCGGGTTCG	
Table 1c. LRP constructs and their promoters		
LRP construct	Promoter	Parental phage
phAETRC21	<i>icl</i>	Che12
phAETRC201	<i>hsp60</i>	TM4 (<i>ts</i> mutant)
phAETRC202	<i>acr</i>	TM4 (<i>ts</i> mutant)

Table 2

Sensitivity of the LRP constructs

LRP	Minimum cells detected (cells/ml)	
	H37Rv	Clinical isolate
phAE129	4×10^4	6×10^5
phAETRC16	5×10^5	1×10^7
phAETRC21	6×10^5	1×10^7
phAETRC201	8×10^1	1×10^5
phAETRC202	2×10^4	1×10^6

Table 3

Temperature preference of phAETRC201 and phAETRC202

LRP	Temperature (°C)	Relative light units (RLU)				
		H37Rv	Isolate 1	Isolate2	Isolate3	Isolate4
PhAETRC202	37	2242	8305	580	571	3986
	30	8076	13913	1295	1052	9738
PhAETRC201	37	18915	242	5819	6778	2898
	30	13069	237	1259	393	524

RLU – Average of two readings