Novel Bioluminescent Quantitative Detection of Nucleic Acid Amplification in Real-Time

Olga A. Gandelman1,2*, Vicki L. Church1,2, Cathy A. Moore1,2, Guy Kiddle1, Christopher A. Carne3, Surendra Parmar4, Hamid Jalal4, Laurence C. Tisi1, James A. H. Murray2,5

1 Lumora Ltd., Ely, United Kingdom, 2 Institute of Biotechnology, University of Cambridge, Cambridge, United Kingdom, 3 Clinic 1A, Addenbrooke's Hospital, Cambridge, United Kingdom, 4 Clinical Microbiology and Public Health Laboratory, Addenbrooke's Hospital, Cambridge, United Kingdom, 5 Cardiff School of Biosciences, Cardiff University, Cardiff, United Kingdom

Introduction

In recent years the molecular amplification of polynucleotides has become increasingly important in life sciences. Many variants of these technologies exist, and they increasingly underpin commercial diagnostic tests as well as a large number of research applications. Most diagnostic applications rely on detection of a target nucleic acid through the process of amplification whose specificity is determined by the use of oligonucleotide primers complementary to the target sequence. The full potential of these analytical tools is only realised if the analysis can detect, report and quantify the amplification occurring in a closed-tube format that minimises the risk of contaminating other samples with amplified DNA.

The most common real-time detection solutions utilize fluorescence technologies to report the in-vitro synthesis of polynucleotides during the polymerase chain reaction (PCR) [4]. Intercalating dyes and fluorescently-labelled oligonucleotides are the most widely used methods of detection of the ongoing synthesis of the target amplicon, despite their requirement for relatively sophisticated optical equipment to excite the fluorophore of choice and detect the emitted light [5,6]. Unfortunately, the elaborate nature of such machinery has constrained attempts to produce robust, low-cost instruments.

Alternative approaches of amplification detection have been adopted that determine the production of inorganic pyrophosphate (PPi), a low-molecular weight by-product of all polynucleotide amplification [7–9]. One molecule of PPi is synthesised each time a nucleotide base is added during the polymerization reaction.
In any given polynucleotide amplification process, the amount of PPi liberated is therefore proportional to the amount of polynucleotide synthesized and hence the starting template concentration; detected PPi can thus be used to quantify the amount of the original target molecule present in a sample. To date turbidimetry is the only method available for detecting PPi continuously in an ongoing amplification reaction. This method utilises the relative insolubility of the Mg²⁺ salt of PPi, which precipitates at high concentrations and can be quantified by monitoring the increasing turbidity of the solution. However, relatively high concentrations of PPi are required and so this approach is limited to isothermal nucleic acid amplification technologies (iNAATs) such as loop-mediated amplification (LAMP) [10,11] that tend to produce large amounts of PPi. There are several distinct iNAATs available as alternatives to PCR, which use strand-displacing polymerases instead of heat denaturable enzymes; additional advantages is that they run at a constant temperature with strand-displacing polymerases instead of heat denaturable enzymes; and, iv) potential additional contamination with PPi through non-specific priming and non-specific amplification; iii) the possible release of alternative substrate for firefly luciferase; ii) the inevitable presence of abundant ATP-producing enzymes allows the quantitative determination of PPi, and hence of the progress of DNA amplification despite the above-mentioned limitations. Such BART (Biomiluorescent Assay in Real Time) assays are characterised by a unique kinetic signature, common to several coupled iNAATs tested, that allows not only the real-time detection, but also the quantitation of the nucleic acid target, as well as facile determination of negative samples. The BART signal can be detected using simple instruments capable of controlling a heating block and of detecting the significant levels of light produced using photodiodes or a charge-coupled device (CCD) camera. We confirm the robustness of the coupled iNAAT- BART assays to potentially inhibitory components of clinical samples by presenting the results of a pilot trial evaluating the use of LAMP-BART in Chlamydia trachomatis (CT) diagnosis from human urine samples.

Results

BART kinetic curves

Among currently available iNAATs, LAMP [19] typically generates high amplicon yields in reactions normally run at around 65 °C and has been shown to produce sufficient PPi, to be detected either by precipitation as its Mg²⁺ salt or through colorimetry using hydroxy naphthol blue [20]. LAMP has also been shown to produce quantitative results in a real-time fluorogenic assay [21], and was therefore selected for initial investigation of the potential of a coupled bioluminometric assay. LAMP primers were designed as described in Materials and Methods complementary to sequences present on the plasmid of Chlamydia and assayed using a plasmid template synthesized to contain this sequence, referred to as Chlamydia Artificial plasmid Template (ChAT). Reactions were conducted in a closed one-tube format that contained all enzymes and reagents necessary for both DNA amplification and ELIDA and incubated at 55 °C, a temperature selected as suitable for primer annealing, DNA synthesis, conversion of PPi to ATP and light emission, as well as ATP sulfurylase and luciferase stability. Such assays are referred to as LAMP-BART assays.

To carry out LAMP-BART reactions, hardware was assembled as described in Materials and Methods, comprising a programmable heating block simply housed within a commercially available chemiluminescence system (essentially a dark box containing a CCD camera viewing the top of the heating block). Light measurements from the camera were recorded every minute for the field of view and analysed by the attached computer. A profile of light emitted during a positive and a negative ChAT LAMP-BART reactions was recorded over 60 min (Figure 2). A light signal from a negative sample that did not contain any specific template started with a high background and then showed a continual near-exponential decay throughout the reaction. A positive sample had a distinct light output profile characterised by the initially high background decaying for some time in parallel with the negative sample. Unlike the negative sample, however, this initial decay was followed by a rapid increase in light intensity followed by an abrupt decline that diminished below initial
baseline levels to an almost undetectable level. This was visualised in a graph of light output against time as a sharp peak of light output (Figure 2A). By the end of the assay, the negative sample maintained a higher light output compared to the positive sample (Figure 2B). Positive ChAT LAMP-BART profiles therefore resulted in highly unusual kinetic curves, very different from the curves reported when LAMP is monitored using either fluorescence or turbidimetry, both of which usually result in sigmoid curves for positive samples, resembling those associated with real-time quantitative PCR [11,22].

Effects of ATP, dNTPs, APS and PP_i on the light output in BART

There are two major differences between the sigmoid curves described above and the BART curve: the first is the high starting background and the second is the rapid reduction in bioluminescence following the increase that occurs during DNA amplification. To understand further the origin of these differences, we considered the effect on light output in BART of the nucleotides and PP_i present in the full reaction.

With respect to nucleotides, the LAMP-BART reaction mixture initially contains high concentrations of all four dNTPs required for nucleic acid amplification as well as the ATP sulfurylase substrate, APS. When a positive sample is amplified in LAMP-BART, it is anticipated that dNTPs will be depleted as PP_i is released, APS is converted to ATP through reaction with PP_i, and ATP is then hydrolysed by luciferase to yield AMP and PP_i. Therefore, in a positive LAMP-BART assay, a continuous change of concentration of all four dNTPs, APS, PP_i and ATP will occur. All these substances with the exception of APS are known to affect firefly luciferase.
activity [23–25] and changes in their levels should therefore have a significant impact on BART light output. ATP and dATP are luciferase substrates, dCTP, dGTP and dTTP are competitive inhibitors of luciferase and PPi may have either a stimulating or inhibitory effect depending on its concentration [24,25]. To understand better the biochemistry underlying the observed BART curves, so-called ‘deficient’ formulations of ChAT LAMP-BART mixture, containing all ingredients except the primers and Bst DNA polymerase (omitted to prevent any possible specific or non-specific amplification and primer-dimer formation), were investigated with different concentrations of dNTPs, ATP, PPi, and APS.

The effect of the equimolar mixture of dNTPs (0–1 mM each) on light output revealed no background light in the absence of dNTPs and substantial amount of light in the presence of dNTPs (125 µM and above), with a plateau reached at concentrations higher than 250 µM (Figure 3A). This is most likely to be due to the saturation of luciferase with all four dNTPs. Among the four dNTPs present in the mixture dATP is the most likely luciferase substrate causing light emission [23]. The level of light signal was similar to the initial background observed in ChAT LAMP-BART assays, with a gradual decay of light closely resembling that seen in the negative ChAT LAMP-BART (Figure 2A) (data not shown). This decay is typical for all bioluminescent assays utilising firefly luciferase in the presence of high concentrations of substrates and is due to the loss of luciferase enzymatic activity through inhibition by the reaction products, as well as to the gradual thermal inactivation of the enzyme [26,27]. We therefore consider that the initial high light output and gradual decay is explained by the interaction of luciferase with the high concentrations of dNTPs present in a LAMP-BART reaction.

The effect of ATP was evaluated using ‘deficient’ ChAT LAMP-BART mixture containing 250 µM each dNTP and varying

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**Figure 3. Effect of dNTPs, ATP, PPI and APS on light output in BART.** Simulation of effects of different ingredients on the light output in LAMP-BART in a “deficient mix” lacking primers and Bst polymerase but containing all other components as described in each case below. (A) Light output detected using varying concentrations of an equimolar mixture of four dNTPs. Light output peaks at 500 µM total dNTP concentration. (B) Light output detected using varying concentrations of ATP in the presence of 250 µM equimolar dNTPs. Light output is higher than in panel (A) and reaches saturation at 100 µM ATP, showing greater sensitivity to ATP. (C) Inhibitory effect of different concentrations of PPI on the light emission in the presence of 250 µM dNTPs and 100 µM ATP. (D) Stimulatory effect of increasing concentrations of APS on the light emission in the presence of 250 µM dNTPs and 100 µM PPI. (E) Effect of different concentrations of APS on BART curves in complete LAMP-BART formulation with 10^7 ChAT target DNA (red – 100 µM, navy – 200 µM, brown – 500 µM, green – 750 µM, blue - 1000 µM). As APS concentration is increased, there is little effect on peaking time but more PPi, is converted to ATP resulting in a lower rate of inhibition of luciferase and a slower “switch off” of light output.

doi:10.1371/journal.pone.0014155.g003
concentrations of ATP (0–1 mM; Figure 3B). Increasing levels of ATP caused a substantial further increase in the background followed by steady decay, similar to that described above (Figure 3A). Addition of 10 μM ATP raised light brightness nearly 4-fold, and 100 μM ~6-fold. Further increase in ATP concentration was not accompanied by any significant changes in the light output, suggesting that in these conditions luciferase was saturated above 100 μM ATP (Figure 3B). The overall higher light output in this experiment suggests that, even though there was no depletion of dNTPs, ATP outcompeted dATP as a luciferase substrate and because of its much higher light-producing efficiency caused an increase in the total light output [28].

The analogous situation is likely to explain the “flash” of light in a positive LAMP-BART amplification, where the increase in light is also facilitated by the depletion of dNTPs.

To address the rapid decline in light output after the “flash”, the effect of PPi was evaluated using a ‘deficient’ ChAT LAMP-BART mixture comprising 250 μM each dNTP, 100 μM ATP and varying concentrations of PPi (0–0.5 mM; Figure 3C). Inclusion of 10 μM PPi had almost no impact on the resulting light output from dNTPs and ATP, while 50 μM PPi reduced light by 30%, 100 μM PPi by 50%, and 250 μM PPi brought it down to the level of typical background coming from dNTPs in the absence of ATP; 500 μM PPi reduced it even further (Figure 3C). Hence, in the presence of high PPi, light output decreased to the level below that observed with dNTPs alone - a result similar to that observed at the end of a positive LAMP-BART reaction.

These data are consistent with the explanation that in a positive LAMP-BART, when amplification occurs, PPi is produced and converted into ATP, consuming APS. As long as there is sufficient APS to convert PPi into ATP, the latter is made and provides the substrate for light production by luciferase. The rapid accumulation of PPi, and its conversion to ATP during the exponential phase of the amplification then leads to a peak in light output (flash). As APS is exhausted, and if amplification continues, free PPi accumulates and inhibits luciferase, as shown above [24,25]. This implies that APS concentration should thus have a significant effect on the shape of BART curves.

The effect of APS on BART light output was investigated using both ‘deficient’ and full ChAT LAMP-BART formulations. The ‘deficient’ formulation contained 250 μM each dNTP, 100 μM PPi, and different concentrations of APS (0–250 μM) but neither Bst polymerase nor primers. The increase in APS concentration in the presence of a fixed concentration of PPi caused an increase in light production due to the formation of ATP (Figure 3D). The overall result was similar to that shown in Figure 3A, when varying amounts of ATP were introduced directly into the system. The highest light level achieved was close to that observed from the direct addition of 100 μM ATP (compare Figure 3B). In the absence of APS, 100 μM PPi strongly inhibited the background light produced by 250 μM dNTPs (Figure 3D).

Further investigations were carried out with a full ChAT LAMP-BART formulation containing Bst polymerase, primers, 250 μM dNTPs, 10⁵ copies of ChAT template per reaction and varying amounts of APS (0–1 mM). In the presence of 100 and 200 μM APS, a rapid and sharp switch-off of the BART flash was observed. At higher APS concentrations, the light output peaks became broader and did not decline below background even after 60 minutes (Figure 3E), suggesting continuing conversion of PPi to ATP. In line with the explanation suggested above, the final concentration of PPi released through the amplification utilising 250 μM each dNTP could potentially reach 1 mM (assuming full utilization of all dNTPs). With APS limited to 100–200 μM, only part of the PPi released would therefore be able to be converted to ATP, with the further PPi accumulation inhibiting luciferase activity and further light output [24,25]. The rapid “switch-off” observed as the characteristic feature of a positive LAMP-BART curve is therefore likely to result from the build-up of PPi, which cannot be converted into ATP once APS is exhausted.

We conclude that the high initial background in BART is due to the high content of dATP with a possible slight contribution from contaminating PPi, and ATP. The characteristic “flash” from positive assays results from rapid ATP production, and the subsequent switch-off is a consequence of inhibition with PPi dependent on ATP concentration and ATP sulfurylase enzyme activity.

**Quantitative BART**

We next sought to investigate whether the timing of the flash peak, defined by the signal switch-off unique to BART, provides potential for quantitative real-time iNAATs. In a defined LAMP-BART formulation, one may expect that the time required for the same amount of PPi to be released by virtue of amplification process to cause a luminescent flash and its switch-off would be dependent on the amount of nucleic acid target present in the assay. Therefore, a direct relationship between the time-to-peak and the starting target concentration might be anticipated.

Quantitative assessment of LAMP-BART was carried out using the ChAT target (5.5–5.5×10⁶ target molecules per reaction) in the presence or absence of 100 ng non-specific salmon sperm carrier DNA (Figure 2). A positive correlation was observed between both the time to peak (tmax) and time to first inflexion point (tinfl) with the template abundance. The apparent tmax values varied between 12 and 50 minutes and tinfl between 5 and 40 minutes, the timings correlating well with template abundance (Figure 2C). A logarithmic analysis of tmax and tinfl plotted against ChAT copy number reveals a linear correlation over seven orders of magnitude, and down to 55 copies per reaction (Figure 2D). Below 55 copies, template DNA amplification was still reported, but a linear relationship with respect to template concentration was not observed in these conditions. The tmax and tinfl data achieved for a given copy number were shown to be highly reproducible, although increased variability was observed when lower concentrations of template were amplified. Time to first inflexion point and time to peak are thus directly correlated with target DNA copy number; this is similar to the correlation observed in qPCR between cycle time (Ct value) and DNA template load. Both correlations showed identical gradients with tinfl having a 6-minute smaller intercept. Time to peak is easy to define from the raw data output, while calculating tinfl requires some additional data processing, although tinfl can be used for faster detection or quantitation of the target present in a sample. We also note that the presence of 100 ng/assay (5 ng/μl) of background salmon sperm DNA had no effect on the quantitation of the target DNA, demonstrating that there is no interference between measured BART signal and this amount of exogenous non-specific nucleic acid present in the assay; a key consideration in measuring unknown samples. It also indicates that reduced quantitation at low copy number is not due to absolute DNA concentration.

**Correlation of DNA synthesis, PPi release and light output in LAMP-BART**

To determine how much DNA and PPi is produced to generate a BART light peak, BART output was monitored in parallel with the independent assessment of DNA synthesis. The real-time bioluminescent output reported during a LAMP-BART reaction with two different starting amounts of the ChAT DNA target
(1 pg/ml and 100 pg/ml), was compared to end-point assays for DNA amplification (monitored using fluorescence and gel-analysis), at various time intervals during the assay (Figure 4). Attempted direct end-point measurements of PPi by ELISA turned out to be unreliable due to the strong interference from the varying concentrations of ATP, APS, dATP and other deoxynucleotides.

In end-point fluorescent DNA analysis, amplicon became detectable at 30 min for the higher target concentration and after 35 min for the lower target concentration and reached 200 μg/ml at approximately 40 and 50 min, respectively (Figure 4B). The corresponding calculated concentration of PPi released by that time in the reaction is approximately 600 μM, a very high level at which APS would already have been depleted and excess PPi would therefore be present in the assay. By gel electrophoresis, LAMP amplicon became visible after 30 min for the higher target concentration and 40 min for the lower, while saturation of the fluorescence from ethidium bromide was reached at 45 and 50 min, respectively (Figure 4C and 4D). Direct comparison of measured amplicon accumulation with the BART signal (Figure 4A) showed that the detectable appearance of amplicon coincided with the time to the first inflexion point in the light output curve. We therefore propose that the increase in light signal started after a prolonged lag-period required for sufficient amplicon to be synthesised and PPi released, at which time nucleic acid amplification became exponential and resulted in the light flash. With the continuing amplification and further release of PPi into the system the light levels of the BART assay then diminished to the lowest point recorded throughout the assay. This corroborates the mechanism proposed above for the strong inhibition of firefly luciferase by free PPi, which cannot be converted into ATP because of APS exhaustion [,24,25]. BART therefore produces a peak of light in real-time when DNA amplification goes into exponential phase.

Intensity of light output in BART

Unlike conventional bioluminescent assays, BART measurements are intensity-independent. Conventional luciferase bioluminescent assays measure absolute light intensity and correlate its brightness with the levels of the analyte of interest [29]. An attempt to assess and compare the intensity of background light emitted from a ChAT LAMP-BART assay mix using a plate luminometer (BMG) failed, because the photomultiplier was overloaded even when small volumes (down to 5 μl) were measured with the lowest possible voltage setting and shortest integration time (20 ms). Though it was impossible accurately to quantify brightness of BART signals using the plate luminometer, it became clear that BART signals integrated over 60 s could be several orders of magnitude higher than those measured in traditional bioluminescent assays.

BART quantitation is based on temporal parameters, so it is not expected that absolute light output levels affect quantitation. ChAT LAMP-BART amplifications were carried out containing the same concentration of ChAT DNA (10⁶ copies per μl) in different reaction volumes (0.2-50 μl). The intensity of light decreased proportionally with the reduction in volume, but the times to peak remained unchanged, except for a slight increase with the smallest reaction volume (0.2 μl). Reaction volume did not affect either observed tmax or peak profile (Figure 5). We therefore conclude that BART quantitation depends on kinetic parameters of the coupled reactions affecting the time to light peak, and not on absolute light output intensity. Taken together with the high level of light signal, this suggests that assays are likely to be tolerant of turbid assay samples, and points to the potential to use low-cost lower sensitivity detection methods for measuring BART light output such as charge-coupled devices or photodiodes.

Figure 4. Correlation between bioluminescent output and DNA production in LAMP-BART. Light output in BART (A) and DNA yield assayed by end-point fluorescence method (B) and visualised by gel-analysis (C, D) for two different amounts of ChAT (1 pg/ml – blue, 100 pg/ml - red). Each curve represents one of three replicates. 2% agarose gel shows LAMP amplicon as a ladder of bands representing multiple concatamer repeats of the ChAT template using 1 pg/ml (C) and 100 pg/ml (D) of starting template. The strong band in all lanes corresponds to luciferin, which is strongly fluorescent under UV illumination. doi:10.1371/journal.pone.0014155.g004
Instruments for measuring INAAAT-BART

Unlike the majority of traditional bioluminescent assays, where highly sensitive detection systems are absolutely essential for measuring low-level light, BART produces such bright light outputs that much simpler light detection systems can be employed. Further, since light is emitted from within the reaction mixture itself, no external illumination is required as for fluorescence, and since thermal cycling is not required, substantially simpler hardware can be used to follow BART reactions.

Two instruments were therefore designed: a CCD-based detector currently suitable for 96- or 384-well formats, where light reflected by a mirror is detected by a camera from the top of the assay tube (Figure 6B and C), and very small stand-alone photodiode-based 8 or 16-well device, suitable for point-of-use applications or low-resource settings, that reads the emitted light from the bottom of each tube (Figure 6D). Neither machine has moving parts and optical design is simple since the samples do not need to be irradiated. Since quantitation is based upon the measurements of rates of change of light intensity, the need to measure accurately absolute light intensity is much less significant than in conventional bioluminescent assays. BART output can be monitored directly by imaging emitted light (Figure 6F and Movie S1) or represented graphically (Figure 6E). Both instruments utilize algorithms integrated within firmware for data processing to calculate the time to peak and generate a positive-negative call for individual samples by evaluating changes in the rate of light emission. These instruments allow BART to be applied in a wide range of applications from high-throughput screening to point-of-care (POC) and other low-throughput applications.

Application of LAMP-BART for detection of *Chlamydia trachomatis* in clinical specimens

To assess the application of BART for *in vitro* diagnostics, an evaluation of *Chlamydia trachomatis* (CT) diagnosis in human urine samples was performed, since there is a demand for high sensitivity molecular assays capable of diagnosis at POC [30]. To assess microbial range and selectivity of LAMP-BART for CT infection in clinical urine samples, DNA purified from 14 different strains of CT was assayed and found to be reliably detected by the ChAT LAMP-BART assay (Table S1). Analytical specificity was assessed using DNA purified from 28 pathogenic bacteria and commensal organisms of the oropharynx and genital tract (Table S2). No false-positives were detected, demonstrating the 100%-specificity of the assay.

Bacterial DNA was isolated as described in Materials and Methods from 105 clinical urine specimens of unknown CT status, analysed for CT DNA by ChAT LAMP-BART and the results compared to those from qPCR analysis (Table 1). Samples were defined as positive if a light peak was observed within 1 hour in BART and/or had Ct≤40 qPCR cycles. 45 urine samples were diagnosed positive for CT by qPCR, of which LAMP-BART reported 43 as CT-positive. Importantly, no LAMP-BART false positives occurred. The two samples identified as CT-positive only by qPCR had marginal Ct values of 40 cycles. In this comparison, LAMP-BART showed the same specificity as qPCR and 95.6% sensitivity (relative to qPCR). Moreover, it took BART less than 60 min to detect CT-positive samples, compared to 120 minutes with the qPCR used. LAMP-BART thus showed robust behaviour with these clinical samples and did not appear to be susceptible to inhibition by potential contaminants present in urine-derived samples subjected to rapid DNA preparation.

A side-by-side comparison of LAMP-BART with a TaqMan PCR currently used for clinical diagnosis [31] by the Health Protection Agency (Cambridge, UK) was carried out using samples from a Quality Control for Molecular Diagnostics (QCMD; http://www.qcmd.org) CT panel containing a range of clinically relevant CT loads. Accurate CT quantification is considered less significant for the clinical management of an infection than reliable detection [31] and in both methods the cryptic plasmid was used as the target for amplification to enhance sensitivity of detection, there being a multiple but variable number of copies of cryptic plasmid in CT. Samples were prepared as described in Materials and Methods, and volumes used in LAMP-BART were adjusted to those used in TaqMan PCR to achieve an identical target load in both assays [31]. The samples used and results are presented in Table 2, and correlation between $t_{\text{max}}$ values in LAMP-BART and Ct values in TaqMan PCR is shown in Figure 7A. A linear relationship was observed across a wide range of target copy number, two different clinical sampling methods (swabs and urines) and two CT variants (Swedish isolate and Dutch isolate) (Table 2). The higher level of
potential inhibitors in urine compared to swabs [32] was reflected in the results from urine sample 5, which in spite of a five-time higher CT load demonstrated later t\text{max} and Ct value than the swab sample 4. The linear relationship between t\text{max} in LAMP-BART and Ct values in TaqMan PCR supports their similar quantitative ability and the potential use of LAMP-BART for applications requiring quantification of a target. These results also mirror the quantitative nature of real-time LAMP using fluorogenic detection [21].

Detection of CT cryptic plasmid in clinical samples can be challenged by high levels of additional non-target DNA. Although a total carrier DNA load of 100 ng (equivalent to 5 ng/\mu l) did not affect LAMP-BART quantification (Figure 2C), the effect of higher levels of DNA on ChAT LAMP-BART was modelled using salmon sperm carrier DNA. A dilution series of ChAT plasmid (4–2.7×10^6 copies per reaction) was made in 100 ng/\mu l salmon

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**Table 1. Results of Chlamydia trachomatis testing by LAMP-BART and qPCR.**

|                      | LAMP-BART | qPCR |
|----------------------|-----------|------|
| Total number of samples | 105       | 105  |
| CT-positive samples  | 43 (t\text{max}＜60 min) | 45 (Ct≥40 cycles) |
| CT-negative samples  | 62        | 60   |
| Sensitivity, %       | 95.6      | 100  |
| Specificity, %       | 100       | 100  |
| Assay time           | 60 min    | 2.5 hours |
| Mean t\text{max} or Ct/equivalent time | 33.6 min  | 35.2 cycles ~1 h 46 min |

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**Figure 6. Devices for BART assays and different formats of BART data output.** (A) Original laboratory set-up for BART used in the research presented in this paper. (B–D) Later designs of custom equipment for BART assays. High-throughput CCD-camera based system for laboratory use available in 96/384-well format. (B) Exploded diagram and picture (C) of the CCD-camera-based device: 1 – light box, 2 – CCD-camera, 3- samples in standard 0.2 ml PCR tubes or 8-well strips or 96-well plate, 4 – heating block. (D) Portable diode-based device for one or two 8-well strips. (E) Graphical representation of the data for a dilution series: red – 1 ng, orange – 100 pg, green – 10 pg, blue – 1 pg, black - NTC. (F) Corresponding images of LAMP-BART reactions taken at 10 (a), 21 (b), 24 (c), 25 (d), 26 (e) and 30 min (f): top row – ChAT DNA dilution series with the decreasing amount of template 1 ng, 100 pg, 10 pg, 1 pg (left to right); bottom row – no-template control.

doi:10.1371/journal.pone.0014155.g006
sperm DNA and analysed under exactly the same conditions as in the side-by-side comparison with TaqMan PCR (Figure 7B). In the presence of 1.2 μg of overall total amount of carrier DNA in the assay ChAT plasmid was detected down to single copies within 50 minutes. Below 70 copies of the target the linearity between the \( t_{\text{max}} \) and target copy number was lost and reproducibility of the assay was significantly reduced but neither specificity nor sensitivity of LAMP-BART were affected by the presence of large amounts of foreign DNA.

**Application of RT-LAMP-BART for the detection of classical swine fever virus**

To demonstrate the applicability of BART to the detection of RNA templates, a model system based on classic swine fever virus (CSFV) was investigated. Purified RNA from an in vitro transcribed artificial template was amplified in a closed-tube one-step format, which included reverse transcription, LAMP amplification and BART detection reagents. For a wide dilution series of RNA \( (10^3-10^{10}) \) RT-LAMP-BART resulted in a sequence of light peaks with \( t_{\text{max}} \) showing inverse linear proportionality to RNA target copy number (Figure 7C and 7D). In the absence of AMV reverse transcriptase, neither amplification nor light peaks were detected, indicating the absence of background DNA and non-specific amplification. BART successfully reported on the exponential release of PPi through amplification of the cDNA copies generated from the RNA target in the coupled assay in the same tube. BART kinetic curves in this coupled RNA-cDNA amplification had exactly the same profile as in DNA amplification and the linear correlation between the starting copy number and \( t_{\text{max}} \) was retained. This points to the potential for coupled RT-LAMP-BART detection and quantification of RNA viral genome loads for diagnostics in low-resource settings.

**Table 2. Summary of the samples used in the comparison of ChAT LAMP-BART with TaqMan PCR**

| Sample | Matrix | CT variant | CT load, cells/ml | Ct | \( t_{\text{max}} \), min |
|--------|--------|------------|-------------------|----|---------------------|
| 1      | Urine  | n/a        | 0                 | n/d| n/d                 |
| 2      | Swab   | n/a        | 0                 | n/d| n/d                 |
| 3      | Swab   | Dutch clinical isolate LGV L2 | 285 | 29.8 | 24.5             |
| 4      | Swab   |            | 5700              | 24.6| 19.2               |
| 5      | Urine  |            | 28500             | 28.2| 22.4               |
| 6      | Urine  | Swedish variant | unknown | 22.4| 16.0               |

doi:10.1371/journal.pone.0014155.t002

**Figure 7. DNA and RNA analysis using LAMP-BART.** (A) Correlation between \( t_{\text{max}} \) values in LAMP-BART (vertical axis) and Ct values in TaqMan PCR (horizontal axis) obtained in the side-by-side analysis of the samples from the CT QCMD panel. (B) Effect of increased levels of foreign DNA (1.2 μg salmon sperm DNA/assay) on the sensitivity and speed of ChAT LAMP-BART assay carried out in 26-μl at 60°C. (C) Real-time bioluminescent assay of CSFV RNA fragment dilution series amplified by RT-LAMP at 55°C for 100 min (raw experimental data): \( 10^3 \) – red, \( 10^4 \) – orange, \( 10^5 \) – yellow, \( 10^6 \) – green, \( 10^7 \) – light-blue, \( 10^8 \) – dark-blue, \( 10^9 \) – violet, \( 10^{10} \) – pink, NTC – black. Each curve represents one of three replicates. (D) Semi-logarithmic plot of the time-to-peak versus CSFV RNA copy number in the same RT-LAMP-BART reactions.

doi:10.1371/journal.pone.0014155.g007
Discussion

Molecular diagnostic tests provide the “gold standard” in terms of sensitivity and specificity, and are in principle capable of detecting single copies of a specific nucleic acid sequence in a sample through the process of repeated copying by nucleic acid amplification. There is a rapidly increasing demand for such molecular diagnostic tests driven by the requirement for sensitive and accurate determination of contaminating or disease organisms, the presence of adventitious genetic material, or the diagnosis of genetically determined disease states. In particular, there is a need for tests providing speed, simplicity and robustness in both molecular assay and the necessary equipment. Such attributes are also vital in the low resource settings of the developing world, where molecular diagnostics have yet to have a widespread impact.

Currently available molecular diagnostic systems are predominantly based on qPCR, with the amplification reported by the increasing fluorescent signal from an intercalating dye, dye-labelled primer or labelled probe [1–4]. However, qPCR imposes strict requirements on the assay equipment, because of the combined need for temperature cycling, and wavelength-specific fluorescent excitation and emission measurement. These in turn pose limitations through the power consumption and optical arrangements required and have therefore restricted the production of low-cost, simple and robust instruments.

A solution to both the temperature cycling and fluorescence excitation and detection problems is provided by combining alternative amplification methods based on isothermal amplification using strand-displacing polymerases with the bioluminescent reporting of amplification. We show here that a real-time bioluminescent assay, BART can be produced by the simultaneous amplification of a nucleic acid target, conversion of the pyrophosphate produced to ATP, and its determination with a thermostable firefly luciferase. Importantly, this assay can be carried out as a simultaneous combined assay in a single closed tube without further additions, greatly reducing the risk of amplicon contamination of further samples. We further show that such assays can be effectively used on patient-derived samples, that straightforward and cost-effective instruments can be devised for the performance of BART assays, and that they are applicable to RNA targets through coupled reverse transcription.

The BART reporter is unique and clearly distinguishable from any other system used for real-time monitoring of nucleic acid amplification. The characteristic BART bioluminescent signature does not have the sigmoidal shape typical of fluorescent and turbidimetry measurements. It initiates with a high but rapidly declining background signal, followed in the case of a positive sample by a brighter flash and a rapid decline in light intensity. BART curves of this shape were observed not only when amplifying the ChAT template using LAMP, but also using other iNAATs and a range of DNA or viral RNA targets, the latter involving a simultaneous reverse-transcription step with LAMP. The BART light output was found to have the same characteristic shape independent of template, reaction conditions or iNAAT involved, reflecting the exponential production of the amplicon and release of PPi. BART assays therefore depend on the coupled amplification technology used, as BART simply reports on any resultant exponential release of PPi. We conclude that the dynamics of light output are characteristic of the coupled reactions involved in BART and not any specific amplification.

The high bioluminescent background observed in BART is an inevitable consequence of the reagents required for amplification, but is not problematic for the assay because the BART bioluminescent output reflects the rapid dynamic changes in the relative levels of PPi and ATP. The range of these changes is over two orders of magnitude (0.01–1 mM) and is unique among existing bioluminescent methods. The detectable light output at the beginning of the assay serves as an indicator of BART-reagent viability, and the residual background signal clearly signals non-amplified samples where target nucleic acid is not present. The ability of BART to cope with the presence of dATP, ATP and PPi, emphasises its distinction from previous manifestations of ELIDA [7–9,13], which are intolerant to their presence as contaminants and strongly depend on minimising the non-specific background. This tolerance of BART to high background light levels eliminates the need for alternative but less satisfactory solutions such as the use of apyrase to remove ATP, or of d-α-S-ATP, an analogue of dATP which is not a substrate for firefly luciferase and hence does not generate a bioluminescent signal, but which can be incorporated into a nucleic acid, albeit at a much slower rate [33].

Because of the continuous monitoring of light and the measurement of the rate of change of light intensity rather than absolute light levels, BART is quintessentially different from conventional bioluminescent methods based on firefly luciferase in its tolerance to high bioluminescent background, brightness of its light output and quantitation relying on peak timing rather than absolute light intensities. This also provides tolerance to contaminating ATP or PPi, from the sample, since rate of change not absolute levels are determined.

Dynamic changes in light intensity are therefore a key feature of BART, allowing analysis to be based on the rate of change of light production rather than absolute light intensity values. A theoretical drawback of BART might be the possible difficulty in distinguishing different sources of PPi production, for example from non-specific processes. However, specific amplification can be differentiated from non-specific by analysing kinetic rates of light output. In non-specific amplification, PPi release is usually slow, non-exponential and not followed by a rapid switch off due to the slower rate. Hence when a wide peak is observed, either with or without a subsequent reduction of the light signal below the background level, it most likely originates from non-specific amplification. We note that the occurrence and frequency of any non-specific amplification is an inherent property of the amplification technology used rather than the BART reporter system, as BART has been found to have no effect on the specificity of amplification. The LAMP-BART combination is particularly favourable as LAMP relies on six primers and eight recognition sites as opposed to the two amplification primers and third detection primer if used in PCR and thereby facilitates a higher specificity of amplification. Nevertheless, assays must be designed and validated to ensure that off-target exponential amplification does not occur, since BART will report on all exponential amplification occurring within the assay, and does not offer the potential for melt-curve analysis or primer binding detection that can be used with qPCR.

We further show that the BART reporter system allows quantitation of the target nucleic acid initially present. It is the only known quantifiable real-time reporter of amplification characterized by time-to-peak rather than by absolute signal output, suggesting a potential greater tolerance of inhibitors or turbid samples resulting from rapid sample preparation methods. The reported profile yields more information than either fluorescence or turbidimetry, both of which generate sigmoid curves. In BART it is possible to derive values for quantitation from either time to the first inflexion point (tinfl) of the curve or time to its maximal light output (tmax). Accurate analysis of time to peak can be performed with minimal data processing, and it is easy
to compare samples by visual assessment of the raw data. While both parameters can be used for quantification of the target in a way similar to using Ct values in conventional qPCR, time to the first inflexion may be of particular value in applications where time to result is of the essence.

Detection and measurement of BART signals does not require sophisticated optics or light detection methods. The high tolerance to absolute light intensity means that hardware specifications can tolerate wide variances, enabling low-cost manufacture. The high tolerance to absolute light intensity also widens the range of possible assay volumes. The potential for reducing BART reaction volume without alterations to hardware is practically attractive. BART assays on both instruments described here can be performed in total volumes as low as 2.5 or even 1 μl. The volume reduction results in a lower light output but has no effect on rates of change. Reduction in reaction volume offers savings in reagent costs without sacrificing test parameters and confers potential for miniaturisation. BART cannot be multiplexed conventionally, since if multiple targets of unknown initial concentration are simultaneously amplified, BART will report conventional, since if multiple targets of unknown initial concentration are simultaneously amplified, BART will report

A further advantage that follows from BART’s measurements of the kinetics of light output is its robustness to sample contaminants. This includes compounds that could affect luciferase activity, but also the ability of BART to tolerate addition of turbid samples or solid particulates. If the latter causes light absorbance or scattering and reduces absolute light intensity without effecting changes of reaction rates, both qualitative and quantitative analysis of the data are still feasible. This feature is important for molecular diagnostic applications where sample preparation contributes substantially to the cost and time of the whole assay and tolerance to magnetic beads or any other solid particles or pigments represents a significant advantage. Indeed the small trial performed on a panel of human urine specimens demonstrated that LAMP-BART showed robust behaviour, reliably detected CT DNA and was not susceptible to inhibition by potential contaminants present in urine-derived samples subjected to rapid DNA preparation.

Conclusions
BART - the bioluminescent monitoring using coupled conversion of inorganic pyrophosphate to ATP and the simultaneous monitoring of ATP levels using thermostable firefly luciferase - provides an effective system for reporting isothermal nucleic acid amplification in real time. It measures light generated in the process of amplification in a closed tube format and offers the potential for both quantitative and qualitative assays that are simple, fast, robust and low-cost in terms of equipment requirements. BART addresses requirements of molecular diagnostics and is well suited for use in a range of settings and in a wide variety of formats.

Methods
Materials and reagents
Unless otherwise noted, chemicals were purchased from Sigma with the exception of luciferin potassium salt (LH2; Europa Biotech, Ely, UK), UltraGlow firefly luciferase (UGrLuc; Promega, WI, USA), adenosine-5'-O-phosphosulphate (APS; Biolog Life Science Institute, Bremen, Germany), Bst DNA polymerase large fragment (Bst) and Thermopol buffer (New England Biolabs, MA, USA), QuantiTech SYBR Green PCR kit (Qiagen, Hilden, Germany), cloned AMV reverse transcriptase and PicoGreen dsDNA Quantitation kit (Invitrogen, CA, USA). Oligonucleotides were synthesized at the Department of Biochemistry, University of Cambridge (UK).

Template selection and primer design
A 224-base pair (bp) Chlamydia trachomatis (CT) artificial template (ChAT) was constructed using the Expand High Fidelity PCR System (Roche Applied Science, Indianapolis, IN, USA) from two overlapping oligonucleotides with a 25 bp overlap that reproduces a unique sequence from CT cryptic plasmid ORF8 (Genbank accession NC_001372; positions 1088–1311) which is identical in all GT strains harbouring this gene. A 224-base pair fragment was cloned into pCR2.1 Topo vector (Invitrogen). Sequences of six oligonucleotides including two Lamp, two loop and two displacing primers designed against the same sequence as described in [19] are shown in Table 3.

| Name  | Sequence                             |
|-------|--------------------------------------|
| LampB | gaccgcaagactaaacatgttttttgttgaatctttgtaagg |
| LampF | cgcatcagggatgattagtttattttggtcattgtcctggg |
| LoopB | cgcagacagactatatt                  |
| LoopF | aaacctgtcagacatcc                |
| DisplB | tattctcggatctcc             |
| DisplF | gatcatatatccgagttct            |

doi:10.1371/journal.pone.0014155.t003

LAMP-BART assay
Optimised LAMP-BART reagent contained 0.2 μM of each displacing primer, 0.4 μM each loop primer, 0.8 μM each Lamp primer, 200 μM each dNTP, 0.16 U/μl Bst DNA polymerase large fragment, 100 μg/ml LH2, 100 μM APS, 0.5 U/ml ATP sulphurylase, 5.6–6.2 μg/ml UGrLuc, 60 mM KCl, 0.4 mg/ml polyvinylpyrrolidone and 10 mM DTT in 1x ThermoPol buffer (final concentrations in assay tube are given). BART reactions were run at 55°C in 20 μl total volume containing 15 μl reagent mix with 5 μl added template solution unless otherwise stated. Template was pre-denatured (5 min, 95°C). Reaction mixtures were covered with mineral oil to prevent evaporation. Each sample was run in triplicate.

Hardware
The LAMP-BART assay was carried out on an assembled instrument comprising a PG-controlled TRobot thermocycler (Biometrax, Göttingen, Germany) placed beneath a CCD camera within a ‘Chemi Genius Bio Imaging System’ (Syngene, Cambridge, UK; Figure 6A). This allowed the light emissions to be quantified at any position on the 96-well heating block and measure simultaneously from either a 0.2 ml PCR tubes, 8-well strips or a 96-well PCR plate. Light was integrated over a 60 second intervals using custom software ‘ReactIVD’ (Synoptics, Cambridge, UK) and data saved as images, graphs and Excel spreadsheets.

LAMP-BART kinetics
1 pg and 100 pg of the ChAT plasmid were run in 50 μl LAMP-BART reactions at 55°C. Full BART kinetic curves from each ChAT concentration were recorded over 60 min. One
sample of each ChAT concentration was taken out and placed on ice at 0, 10, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min. DNA concentration in collected samples was determined using PicoGreen® dsDNA Quantiitation Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol using a Cary Eclipse fluorimeter (Varian, CA, USA), or by gel electrophoresis run on 2% agarose gels with ethidium bromide visualisation.

**LAMP-BART detection of *Chlamydia trachomatis* in clinical specimens**

Analytical specificity and microbial range of LAMP-BART were tested on DNA from 14 different strains of *CT* (Table S1) and from a panel of 28 other pathogenic bacteria and commensals from the oropharynx and genital tract (Table S2). LAMP-BART reactions were run with 2–20 pg DNA. Bacterial DNA was isolated from 0.5 ml urine specimens obtained upon approval of the NHS Research Ethics Committee from 105 patients presenting to the Genitourinary clinic at Addenbrooke’s Hospital (Cambridge, UK) using ChargeSwitch gDNA Mini Bacteria Kit (Invitrogen, CA, USA) following the manufacturer’s protocol. The data were analyzed anonymously and did not require patient consent. All urine specimens were either stored at +4°C for ≤5 days or frozen within 5 days of collection and stored at −20°C. Each sample was analysed in duplicate by BART and in-house qPCR in parallel with a ChAT dilution series used for calibration. qPCR was run in 10 µl reactions containing 1 × QuantiTech SYBR Green PCR reagent, 0.4 µM forward and reverse primers (TTCCTTGAGTCATCCTGTTAGG and TTGTCCTTGGATATGAATCTGC, respectively) and 2.5 µl of the sample. qPCR was run on Rotor Gene 3000 (Corbett Research, Australia) using the following profile: 10 min at 94°C, then 50 cycles of 30 s at 94°C, 30 s at 56°C, 45 s at 72°C, then 15 s at 72°C and a melt step.

**LAMP-BART comparison with TaqMan PCR**

*CT QCMD 2010 panel* (Qnostics, UK) was used in the side-by-side comparison of ChAT LAMP-BART and TaqMan PCR. Samples were resuspended in 200 µl of molecular grade water (except for sample 5 resuspended in 1 ml). 200 µl of each sample were extracted using Qiagen DX Reagent Pack on Corbett Robotics Extractor connected to a vacuum pump. Extracted DNA was eluted in 100 µl of molecular grade water. Fully evaluated TaqMan PCR used for *CT* routine screening of urine and swab clinical specimens at Health Protection Agency laboratories in Cambridge, UK, was carried out by introducing 12 µl of extracted samples to 14 µl of the reagent [31]. qPCR was run on Rotor Gene 6000 (Qiagen, Germany). To make target loads identical between the amplification assays the volumes of sample and reagent used in LAMP-BART were adjusted to 12 and 14 µl, respectively, with the final concentrations of all ingredients remaining as described above. LAMP-BART was run at 60°C for 90 min.

**RT-LAMP-BART of purified classic swine fever RNA**

A pGEM construct containing 163-base pair (bp) DNA fragment complementary to the classic swine fever viral RNA (CSFV) sequence was a kind gift from Friedrich-Loeffler-Institut (Germany). Sequences of the CSFV target and five oligonucleotides including two Lamp, two loop and one displacing primer designed against the sequence as described in [19] are shown in Table 4.

RNA was in-vitro transcribed from the pGEM construct using AmpliScribe™ T7 High Yield Transcription Kit (EPICEN-
TRE Biotechnologies, Madison, USA) according to the manufacturer’s recommendations. To achieve full removal of DNA the mixture was treated twice with RNase free DNase. Quality and concentration of RNA preparations was assessed spectrophotometrically using a Nanodrop spectrophotometer (Thermo Scientific).

RT-LAMP-BART reagent contained 0.4 μM of displacing primer F, 0.8 μM each loop primer, 1.6 μM each LAMP primer, 300 μM each dNTP, 0.16 U/μl Bst DNA polymerase large fragment, 1.5 U/μl cloned AMV reverse transcriptase, 100 μg/ml LH2, 100 mM APS, 0.5 U/μl ATP sulfurylase, 5.6–6.2 μg/ml UGrLuc, 50 mM KCl, 0.4 mg/ml polyvinylpyrrolidone and 10 mM DTT in 1× ThermoPol buffer (final concentrations in assay tube are given). BART reactions were run at 55°C in 20 μl total volume containing 15 μl reagent mix with 5 μl added template solution. Reaction mixtures were covered with mineral oil to prevent evaporation. Each sample was run in triplicate.

**Supporting Information**

**Table S1** Chlamydia strains tested for inclusivity.

Found at: doi:10.1371/journal.pone.0014155.s001 (0.03 MB DOC)

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**Table S2** Pathogenic bacteria and commensal organisms of the oropharynx and genital tract tested for cross-reactivity.

Found at: doi:10.1371/journal.pone.0014155.s002 (0.04 MB DOC)

**Movie S1** LAMP-BART of a ChAT dilution series: red - 1 ng, orange - 100 pg, green - 10 pg, blue - 1 pg, black - no-template control. Top row - ChAT DNA dilution series with decreasing amount of template (left to right); bottom row - no-template control. Found at: doi:10.1371/journal.pone.0014155.s003 (0.85 MB MOV)

**Acknowledgments**

We would like to thank Nigel Appleton from Lumara Ltd for proof-reading the manuscript and the Friedrich-Loeffler-Institut for the cloned classic swine fever fragment.

**Author Contributions**

Conceived and designed the experiments: OG VC CM GK HJ LT JAHM. Performed the experiments: OG VC CM GK SP. Analyzed the data: the GO VC CM GK SP HJ LT JAHM. Contributed reagents/materials/analysis tools: OG VC CM GK CC SP HJ LT JAHM. Wrote the paper: OG LT JAHM.