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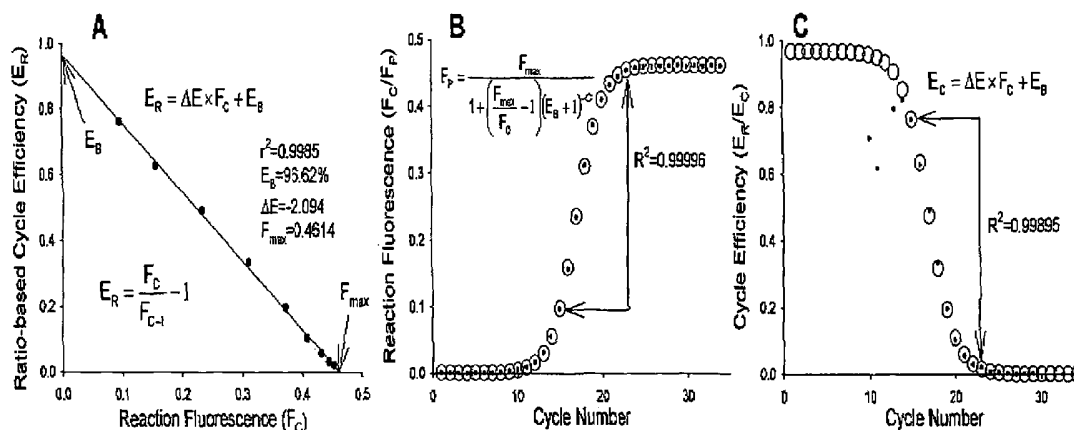
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(54) Title: APPARATUS, PROCESS AND METHODS FOR USE WITH QANTITATIVE PCR



Arrows indicate the cycles that were included in the linear regression analysis conducted in (A)

(57) Abstract: There is provided herein apparatus, machine-readable media, methods and processes for performing quantitative real-time polymerase chain reaction (PCR) and to quantify the amount of a unique target polynucleotide sequences contained within a sample. This is accomplished by a linear regression analysis of data generated during PCR amplification from which values from two parameters are derived: maximal amplification efficiency and rate-of-decay of amplification efficiency. These values are then inputted into a mathematical function from which the maximal fluorescence, target quantity or amount of amplified nucleic acid at a specific cycle are calculated. The final step is the conversion of quantitative units to the number of targets molecules, based upon the correlation of reaction fluorescence to DNA mass. A patient having a condition involving the abundance of a selected nucleic acid sequence is identified by comparing the calculated number with a predetermined number of polynucleic acid molecules.



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

**Title: Apparatus, Process and Methods for Use With
 Quantitative PCR**

Field of the Invention

The invention relates to the field of nucleic acid quantification, particularly as it relates to the polymerase chain reaction ("PCR").

Background to the Invention

Quantitative kinetic PCR

The polymerase chain reaction (PCR) is a reiterative process in which as little as a single target DNA molecule can be amplified to quantifiable amounts. This is conventionally accomplished using an enzyme-based replication mechanism, in which reaction temperature is repetitively cycled (called thermocycling) for a sufficient number of times to amplify the target to saturating amounts (generally 50 thermocycles). The introduction of DNA fluorescence for monitoring PCR amplification in real time, led to a major advancement for quantitative PCR. Although several variants in fluorescence chemistry have been developed, they all share the same key property; that is fluorescence intensity is related to the mass of amplified DNA. Fluorescent detection of amplified DNA not only allowed the kinetics of PCR amplification to be determined with high resolution, it also allowed automation via software data analysis.

In addition to indicating whether a specific DNA target is present within a sample (e.g. the presence of a specific pathogen such as HIV), real time PCR also potentially allows the number of target DNA molecules to be quantified with accuracy and sensitivity (down to a single target molecule), and with an enormous dynamic range ($1-10^7$ target molecules). PCR is the basis for a number of new biomedical diagnostic applications, in addition to quantitative applications for molecular genetics and genomics research (e.g. gene expression analysis, pathogen detection, genetic analysis, among many others).

Despite the nearly eight years since the commercial introduction of fluorescence-based quantitative PCR, several caveats still remain. In simple

terms not only is the accuracy and reliability unsatisfactory, but also the extent and source(s) of these deficiencies are largely unrecognized.

To date, all known real time PCR quantification methodologies are based upon the principle of defining a common point within the amplification curves, that allow amplification reactions to be compared to each other. Generally referred to as the "threshold method", the inventors of this approach (Higuchi et al. 1993; EP0640828) defined this point by selecting a threshold fluorescence, from which a fractional threshold cycle (C_t ; also called the crossing point or CP) is derived for each amplification reaction. This approach is illustrated by Rutledge and Côté (2003), which describes the underlying mathematics and the application of standard curves for converting C_t values into the number of target molecules.

The threshold approach has historically dominated the field of quantitative PCR. Although several variants have appeared which attempt to automate the selection of the fluorescence threshold, e.g. based upon maximum or minimum values of the first and/or second derivatives of the reaction fluorescence curve (Wittwer, US 6,303,305 B1; McMillan, US 6,713,297 B2), none of these modify how target quantification is accomplished.

The threshold approach as currently practiced has several weaknesses, directly relating to two aspects encompassing amplification efficiency. First is that amplification efficiency must be known in order to achieve target quantification. Amplification efficiencies in turn can only be determined accurately via construction of standard curves (US 2002/0058262 A1, US 6,691,041 B2), which are difficult to construction, prone to errors and are impractical for high-throughput applications. Second, it must be assumed that amplification efficiencies of samples are identical to that of DNA standards used in the construction of the standard curves (US 6,691,041 B2), an assumption that has been found to be invalid in many cases.

Liu and Saint (2002) provide the only known exception to the threshold method for quantitative application of real time PCR. They demonstrated that curve fitting of fluorescence readings to the classic Boltzmann sigmoid function (a process commonly used for nonlinear regression analysis of

sigmoidal datasets (Motulsky and Christopoulos 2003), allowed target quantity to be determined from a single amplification reaction, and equally important, without the need to know amplification efficiency. The application of sigmoidal mathematics furthermore illustrates that, contrary to that demanded by the exponential mathematics upon which the threshold methods are based, amplification efficiency is never constant during PCR amplification, but rather each thermocycle produces progressively lower amplification efficiency, a process that eventually leads to cessation of amplification.

Notwithstanding these revelations, several deficiencies of the Liu and Saint approach have prevented its implementation and have led to a general disregard of this approach. In addition to the complexities of curve fitting that compromises its practicality, a significant weakness of the Liu and Saint approach is its inability to convert fluorescence units into the number of target molecules.

Current research and diagnostic tools involving quantitative real time PCR are not fully satisfactory. The "threshold-based method" that has dominated quantitative application of real time PCR to date can give rise to difficulties in implementation, insufficient reliability, and difficulties with quality control. In particular, the necessary assumption that amplification efficiency of samples is equal to that of the DNA standards used for absolute quantification has been shown to be invalid in many applications, resulting in large and unpredictable errors. Occurrences of such errors are of paramount importance for many diagnostic applications that require a high level of quantitative accuracy and reliability, such as for determination of viral load or residual disease. The innate limitations of the threshold-based methods have thus precluded broad implementation of quantitative kinetic PCR in many types of biomedical and diagnostic applications.

Summary of the Invention

There is disclosed herein an apparatus process, and method that permit PCR quantification of unique target polynucleic acid sequences, including DNA and RNA sequences (called the "target") contained within a sample. This is accomplished by linear regression analysis of data generated

during PCR amplification (using standard, commercially available instrumentation and enzyme kit), from which values for two parameters are derived. These are then inputted into a novel mathematical function from which target quantity is calculated. The final step is conversion of quantitative
5 units to the number of target molecules, based upon correlation of reaction fluorescence to DNA mass, a process called herein "optical calibration".

The present invention greatly simplifies target quantification by circumventing the need for curve fitting, using instead an easy to implement approach based upon linear regression analysis. Combined with methods for
10 establishing and monitoring the accuracy of quantitative scale via optical calibration, the present invention not only permits conversion of fluorescence units into the number of target molecules, but as well provides effective quality control and assurance over quantitative scale. This enables unprecedented capabilities for verification of quantitative accuracy, and for development of
15 generally applicable quantitative standards for calibrating real time PCR thermocyclers.

Brief Description of the Figures

20

FIGURE 1A is a graphical depiction of an embodiment of sigmoidal functions describing amplicon accumulation and cycle efficiency.

FIGURE 1B is a graphical depiction of an example of cycle efficiency plotted against reaction fluorescence.

25 FIGURE 2A is a graphical depiction of an example of loss in amplicon efficiency in relation to DNA accumulation.

FIGURE 2B is a graphical depiction of an example of fluorescence readings (actual and predicted) in relation to cycle number.

30 FIGURE 2C is a graphical depiction of an example of ratio-derived amplification efficiency plotted against cycle number.

FIGURE 3A is a graphical depiction of an example of amplification profiles of reaction fluorescence vs. cycle number with varying reaction volume.

FIGURE 3B is a graphical depiction of an example of ratio-derived cycle efficiency vs. reaction fluorescence with varying reaction volume.

FIGURE 4A is a graphical depiction of an example of amplification profiles of reaction fluorescence vs. cycle number with varying annealing and elongation

5 times.

FIGURE 4B is a graphical depiction of an example of ratio-derived cycle efficiency vs. reaction fluorescence with varying annealing and elongation times.

FIGURE 5 is a graphical depiction of an example of drifting plateau produced
10 by low base amplification efficiencies.

FIGURE 6 is a graphical depiction of an example of the relationship between fluorescence and DNA mass.

FIGURE 7 is a graphical depiction of mathematical modeling of PCR amplification.

15 FIGURE 8 is a depiction of a screen shot of an MS Excel TM worksheet used in an example of LRE quantification.

Detailed Description of the Preferred Embodiments

Linear Regression of Efficiency (LRE)

It is disclosed herein that a linear relationship exists between reaction
5 fluorescence and amplification efficiency (Figures 1 and 2). This relationship permits determination of two fundamental kinetic parameters of PCR amplification via linear regression, that in combination with a novel sigmoid function, enables target quantification.

Also disclosed herein is the extension of this quantitative capability to
10 calibration-of-scale by correlating reaction fluorescence to DNA mass that in turn permits the number of target molecules to be calculated. Based upon optical calibration, this aspect of the invention also permits effective monitoring accuracy-of-scale, in addition to greatly facilitating high-throughput applications, in part by circumventing the need to prepare quantitative
15 standards for each individual target, as is required using current threshold-based methodologies.

PCR quantification via LRE is an eight-step process, although certain steps may be useful alone or in sub-combinations. For LRE, the process can be conducted in eight steps, for example:

20

1. A typical real time PCR amplification is conducted and fluorescence is assayed at a selected temperature such that reaction fluorescence is directly proportional to the amount of DNA present in the reaction; reaction fluorescence is measured
25 following each thermocycle, producing an profile of the DNA amplification process (e.g. Figure 2B)
2. Raw reaction fluorescence readings (F_C) are exported for analysis (e.g. into a spreadsheet)
3. Background fluorescence is subtracted from all reaction
30 fluorescence readings
4. Amplification efficiency is calculated for each thermocycle from the ratio of reaction fluorescence (F_C) to the reaction

fluorescence from the previous thermocycle (F_{C-1}), generating what is called the ratio-based estimate of amplification efficiency (E_R):

$$E_R = \frac{F_C}{F_{C-1}} - 1 \quad \text{Equation 1}$$

5. E_R is plotted against reaction fluorescence (F_C), producing a line from which the Y intercept and slope are determined using a standard linear regression analysis (Figure 2). These represent two key kinetic parameters that define the shape of the amplification curve, called maximal amplification efficiency ($E_{\max}$) and rate-of-decay of amplification efficiency (ΔE) as described by:

$$E_R = \Delta E \times F_C + E_{\max} \quad \text{Equation 2}$$

The maximal fluorescence ($F_{\max}$) produced by the amplification is in turn determined by the ratio of $E_{\max}$ to its rate-of-decay (ΔE):

$$F_{\max} = \frac{E_{\max}}{-\Delta E} \quad \text{Equation 3}$$

Equation 2 can also be used to predict the efficiency of each cycle (E_C) based upon its corresponding fluorescence (F_C), which enables quality assessment of the fluorescence dataset based upon the level of correlation between E_R with E_C :

$$E_C = \Delta E \times F_C + E_{\max} \quad \text{Equation 4}$$

6. $E_{\max}$ and $F_{\max}$ are placed into a novel mathematical function (equation 5) from which target quantity is calculated using cycle number (C) and the corresponding reaction fluorescence reading (F_C). This produces a target quantity in the fluorescence units (F_0), calculated for each thermocycle (within the amplification curve), from which an average and standard deviation of target quantity is calculated:

$$F_0 = \frac{F_{\max}}{1 + \left(\frac{F_{\max}}{F_C} - 1 \right) (E_{\max} + 1)^C} \quad \text{Equation 5}$$

A related sigmoid function can be used to predict reaction fluorescence (F_P) once F_0 is calculated. This enables assessment of the quality of the fluorescence dataset based upon the level of correlation between F_P and F_C :

$$F_P = \frac{F_{\max}}{1 + \left(\frac{F_{\max}}{F_0} - 1 \right) (E_{\max} + 1)^{-C}} \quad \text{Equation 6}$$

7. Target quantity is converted into DNA mass (M_0) by multiplication with a calibration factor (CF) that relates reaction fluorescence units (FU) to DNA mass (nanogram) and is expressed in units of nanograms/FU.

$$M_0 = CF \times F_0 \quad \text{Equation 7}$$

8. DNA mass of the target quantity is converted into number of DNA molecules based upon the size of the amplified region (amplicon size; A_S) where N_0 is the number of target molecules:

$$N_0 = (M_0 \times 9.1 \times 10^{11}) / A_s \quad \text{Equation 8}$$

5

Attributes of LRE quantification

Many capabilities provided by LRE in certain embodiments are not provided by threshold-based methods, such as:

10

1. Target quantification that does not require construction of standard curves; threshold-based quantification cannot be accurately accomplished without standard curves
2. Variation in maximal amplification efficiency (E_{max}) within
15 samples does not greatly impact target quantification accuracy; using threshold-based quantification even small differences in maximal amplification efficiency from that of the standard curve can produce large errors
3. Amplification efficacy can be assessed for individual
20 amplification reactions via maximal amplification efficiency; this enables presence of PCR inhibitors or ineffective reaction preparation to be assessed for each individual amplification reaction
4. Target quantification can be accomplished from data obtained
25 from a single amplification reaction; threshold-based quantification can only be accomplished by comparison to other samples, with absolute quantification (i.e. the number of target molecules) requiring PCR amplification of DNA standards in which the quantity of target is known
- 30 5. Facilitates development of high-throughput applications by abrogating the need for preparing quantified standards for each

target combined with effective assessment of accuracy-of-scale through optical calibration

6. Rapid optimization of reaction conditions is greatly facilitated via accurate but simple to conduct assessment of amplification efficacy within individual amplification reactions
7. Easy to implement, requiring little or no setup; even the most basic application of a threshold method requires extensive setup and testing

Establishing Quantitative Scale via Optical Calibration

The accuracy of quantitative determinations using real time PCR is directly dependent upon the accuracy of quantitative scale. Historically, quantitative scale has been acquired from PCR-generated standard curves constructed for each target, a process prone to errors including ineffective quantification of the DNA standard (Rasmussen 2002, Bustin 2002, Rutledge and Côté 2003). This situation that is further exacerbated by the requirement that a DNA standard be prepared for each target. Accuracy-of-scale is paramount to the success of most quantitative applications of real time PCR, particularly for biomedical applications (e.g. measuring viral load and residual disease).

An alternative approach encompasses a process called "optical calibration" that exploits the discovery that quantitative scale is directly linked to amplicon fluorescence through its relationship to DNA mass, such that target quantification can be achieved by correlating reaction fluorescence to the DNA mass present in the reaction mixture. Optical calibration is important in some applications due to the uncalibrated nature of fluorescence units produced by real time PCR, such that fluorescence units generated by each individual thermocycler is unique to both the optical characteristics of its instrumentation and the optical characteristics of the reaction mixture. Thus it is generally important to derive a conversion factor for each thermocycler and reaction configuration. DNA mass can be accurately determined based upon the reaction fluorescence as disclosed herein. This in turn enables the

number of target molecules to be calculated, without having to resort to a PCR-generated standard curve.

Founded upon this principle, absolute quantification of multiple targets can be effectively achieved using a single, preestablished quantitative scale, circumventing the need to prepare quantified DNA standards for each target, as well as enabling an unprecedented level of quantitative accuracy to be established.

In addition to facilitating development of large-scale applications such as high throughput analyses for clinical diagnostics and genomics research, optical calibration as described herein enables a higher level of quality control and assurance of quantitative scale, than attainable using current methodologies. Furthermore, it enables development of universal standards that could facilitate development of worldwide standards for establishment and verification of quantitative scale, a deficiency that currently plagues quantitative PCR (Bustin 2002).

LRE quantification generates target quantity in fluorescence units (Equation 5), which although useful for comparing the relative quantities of target in different samples, is in some applications less useful than quantification in units of target molecules. This is similar to the deficiency of quantification via curve-fitting as described by Liu and Saint (2002) that also produces target quantity in fluorescence units. Conversion of fluorescence units into the number of target molecules substantially increases the utility of the LRE-based quantification, particularly for biomedical diagnostics in which accurate, verifiable quantification of the number of target molecules is paramount (e.g. for viral load and residual disease).

Optical calibration is a multi-faceted process in which fluorescence units are correlated to DNA mass through derivation of a calibration factor (CF). In addition to conversion of F_0 into the number of target molecules (N_0), optical calibration enables assessment of optical precision and accuracy of real-time instruments in an effective, accurate and timely manner. Furthermore, verification of accuracy-of-scale can be accomplished by comparing optical calibrations obtained by two independent methods, a capability lacking under currently applied methodologies.

The nucleic acid standard

Optical calibration requires a nucleic acid standard that preferably has the following characteristics:

- 5 1. Known size, preferably accurate to the base pair
2. Complete sequence available
3. Easily obtained in highly pure form and lacking contaminants that could act as an inhibitor of PCR; preferably available commercially

10 In some instances the nucleic acid will be DNA. In some cases it will be RNA. In some instances, the nucleic acid standard is preferably DNA when the target is DNA and RNA when the target is RNA.

Commercially available DNA from the bacteria phage lambda is an effective standard for optical calibration in many cases.

15 A quantified stock of the nucleic acid standard is prepared, from which diluted samples are made.

Two general methods for optical calibration

Optical calibration encompasses two distinct methods that are linked through the use of the same quantified stock of the nucleic acid standard. The first method uses PCR amplification of a target(s) within the nucleic acid standard, using a diluted sample containing an appropriate amount of the nucleic acid standard. The nucleic acid mass of the target (M_0) is determined by first calculating the number of nucleic acid molecules of the standard contained in the sample (N_0):

25

$$N_0 = (S_M \times 9.1 \times 10^{11}) / S_S \quad \text{Equation 9}$$

Where S_M is the mass of the nucleic acid standard in nanograms and S_S is the molecular size in base pairs (where the standard is DNA) of the nucleic acid standard. The nucleic acid mass of the target quantity (M_0) is then calculated:

30

$$M_0 = (N_0 \times A_S) / 9.1 \times 10^{11} \quad \text{Equation 10}$$

Where A_S is the size of the target amplicon in base pairs. Equations 9 and 10 can be combined and simplified:

5

$$M_0 = (S_M \times A_S) / S_S \quad \text{Equation 11}$$

Following PCR amplification of the target, the LRE process is used to determine the target quantity in fluorescence units (F_0) and the conversion factor (CF_0) determined:

10

$$CF_0 = M_0 / F_0 \quad \text{Equation 12}$$

Where CF_0 is the number nanograms per fluorescence unit (ng/FU) based upon real time PCR quantification of the nucleic acid standard.

15

The second method is called "direct calibration" and does not involve PCR amplification. Direct calibration can be conducted by preparing mock PCR reaction mixes identical to that used for PCR amplification, except that they contain the nucleic acid standard in an amount(s) similar to that generated within a typical amplification profile (approximately 10-50 ng for a 25 μ l reaction volume). Reaction mixes lacking the nucleic acid standard are also prepared for determining background fluorescence (F_b). Note also that is not necessary to use the same standard as that used for PCR-based calibration, such that any source of nucleic acid can be used assuming that it is accurately quantified.

25

The mock reaction mixes are placed into the real time thermocycler and the block temperature increased to the same temperature used to read reaction fluorescence during PCR amplification. This is necessary because reaction fluorescence is temperature dependent. Once that the selected temperature is reached, fluorescence reading(s) are taken (F_d). The conversion factor based upon direct calibration (CF_d) can then be calculated using the equation:

30

$$CF_d = S_M / (F_d - F_b) \quad \text{Equation 13}$$

Where CF_d is the "direct" calibration factor in units of ng/FU, S_M is the mass of the nucleic acid standard in nanograms, F_d is the fluorescence of the mock PCR reaction at the read temperature and F_b is the background fluorescence at the read temperature produced by a mock PCR reaction containing no DNA standard. It should be noted that a primary assumption upon which this approach is based, is that fluorescence readings produced by the real time thermocycle (i.e. the instrument optics) have a linear relationship to DNA mass. This is not to say however, that alternative analyses could be used to compensate for such a lack of linearity, and thus a lack of linearity between reaction fluorescence and DNA mass does not necessarily invalidate this process.

Evaluating quantitative accuracy via optical precision

In addition to enabling the conversion of F_0 into N_0 , optical calibration can also be used to evaluate optical precision, that in turn has a direct impact on quantitative accuracy. The most fundamental approach is assessment of the correlation between CF_d and CF_0 ; that is quantitative calibrations derived by two independent methods whose level of correlation provides an indication of the precision of the optical calibration process. Additionally, the level of optical stability over time can be evaluated through the correlation between multiple applications of the optical calibration process, conducted over time. This in turn provides an indication of how frequently a real time thermocycler needs be recalibrated in order to maintain a specified tolerance for accuracy-of-scale, and thus ultimately quantitative accuracy.

Assessment of quantitative accuracy can be extended further by evaluating optical precision produced by individual wells (both intra- and inter-run variation in absolute units of fluorescence generated by the instrument), in addition to assessing the correlation of the absolute units of fluorescence generated from each well (inter-well variation in fluorescence units). This is based upon the principle that the absolute value of the fluorescence readings

directly determines the quantitative value of the target. That is, fluorescence is the sole quantitative unit generated during PCR amplification, such that aberrancies in determination of reaction fluorescence impacts the resulting quantitative determination. Thus the overall quantitative accuracy produced by a real time thermocycler machine is directly dependent on the precision, stability, and accuracy of its optics.

Attributes of optical calibration

In some embodiments, optical calibration allows one or more specific advantages, such as:

- 10 1. Direct calibration enables optical calibration without PCR amplification; effective, easy to execute, and reliable
2. Enables verification of optical calibration by assessing the correlation between amplification of a quantified standard (CF_0) with that of direct calibration (CF_d)
- 15 3. Provides a single quantitative scale for all targets; prevailing threshold-based methods requires preparation of quantified standard for each individual target; this process eliminates this limitation through the application of a single DNA standard, greatly facilitating high throughput applications
- 20 4. Use of an easily accessible DNA standard facilitates standardization of optical calibration and thus for establishment of quantitative scale, a process that is also platform independent (i.e. not unique to any individual real time thermocycler machine, model or manufacture)
- 25 5. Direct calibration can be verified via application of multiple DNA sources; that is direct calibration is not limited to a single source of DNA standard
- 30 6. Verification of PCR-based optical calibration through amplification of multiple targets within the DNA standard; that is to conduct optical calibrations via amplification of multiple targets using the same DNA standard preparation

7. Verification of PCR-based calibration via direct calibration using the same DNA standard preparation; this provides a direct link between the two calibrations methods providing further verification of the accuracy of quantitative scale

5 8. Enables standardization by adoption of specified DNA sources for preparation of standards for optical calibration, based upon several desirable attributes such as accessibility, purity, known size and sequence

10 In an embodiment of the invention there is provided a method for identifying a patient having a condition of interest involving or identifiable by the abundance of a least one selected nucleic acid sequence, said method comprising:

- 15 a) identifying a target nucleic acid sequence of interest;
 b) amplifying the target sequence using fluorescence-based quantitative Polymerase Chain Reaction;
 c) determining the reaction fluorescence for each cycle
 d) determining the ratio-based estimate of efficiency (E_R), for each
 20 thermocycle wherein

$$E_R = \frac{F_C}{F_{C-1}} - 1$$

25 " F_C " is reaction fluorescence following a selected thermocycle and " F_{C-1} " is reaction fluorescence following the previous thermocycle;

- e) plotting E_R against F_C to produce a line as described by:

$$E_R = \Delta E \times F_C + E_{\max}$$

30

- f) determine the Y intercept and slope of the line from step e, where in the intercept is the maximal amplification efficiency

($E_{\max}$) and the slope is the rate-of-decay of the base amplification efficiency (ΔE);

g) determining $F_{\max}$ wherein

5
$$F_{\max} = \frac{E_{\max}}{-\Delta E}$$

h) generating a determination of target quantity (F_0) in fluorescence units (FU) for a given cycle (C), wherein

10
$$F_0 = \frac{F_{\max}}{1 + \left(\frac{F_{\max}}{F_c} - 1 \right) (E_{\max} + 1)^C}$$

i) determining the target quantity in polynucleic acid mass (M_0) using a conversion factor (CF), wherein

15
$$M_0 = F_0 \times CF$$

where CF is determined using a process called optical calibration, in which reaction fluorescence is correlated to polynucleic acid mass

20 j) calculating the number of polynucleic acid molecules based upon polynucleic acid mass of the target (M_0) based upon the size of the amplified region (A_s), wherein

25
$$N_0 = (M_0 \times 9.1 \times 10^{11}) / A_s$$

k) comparing the number of polynucleic acid molecules calculated above to a predetermined number or range of polynucleic acid molecules associated with a condition of interest.

In some instances it is useful to have a process for quantifying amplification efficiency for a nucleic acid. For instance, one may wish to optimize reaction conditions for a specific nucleic acid, and a ready measure of efficiency can assist in this.

5

In some instances it is useful to have a process for determining the extreme limit of a signal used in reporting nucleic acid incorporation during or as a result of the polymerase chain reaction. Knowledge of such a limit can be used in selecting a signaling/reporting system and well as a detection system.

10

In some instances it is useful to have a process for determining the rate of decay of amplification efficiency relating to the amplification of a nucleic acid in a sample. Knowledge of the decay rate of efficiency can be used in determining reaction conditions and duration.

15

In some instances it is useful to have a process for determining the quantity of the predict of amplification of a nucleic acid sequence after a selected number of thermo cycles in the polymerase chain reaction. Quantity may be determined based on signal output, or may be converted into actual copy number for the amplified sequence. Nucleic acid quantity can be used to assess the abundance of a nucleic acid sequence of interest from a sample, for instance to determine if it exceeds a threshold level which is considered "safe" or "normal" for the healthy population.

20

In some instances it is useful to have a machine readable media such as a read-only memory, a random access memory, an optical or magnetic disk or tape, or other device for storage of information, containing instructions for carrying out one or more of the processes listed above. Such media can be used together with a polymerase chain reaction apparatus to assess PCR results on an ongoing basis, or may be used on a separate processor to examine cycle-specific signal data obtained from a PCR apparatus.

25
30

It will be understood that the "signal" to be assessed relating to nucleic acid incorporation may be of any suitable type. At present, fluorescence is a popular signal method used to track PCR reactions. However, in the past radio-labeled nucleotide incorporation was used, and such methods may still
5 be preferred for particular applications. Additionally other methods will be useful for certain situations. So long as a method provides an assayable signal, it is contemplated by this invention. While the examples relate to signals which increase with the level of nucleotide incorporation, it will be understood that methods may also arise where signal decreases with the
10 level of nucleotide incorporation (for example due to quenching up on incorporation). It will be apparent to one skilled in the art, in light of the disclosure herein, to apply the methods and processes disclosed herein to such signal systems as well.

15

EXAMPLES**Detailed information relating to the Figures**

Figure 1 depicts sigmoidal modeling of the polymerase chain reaction reveals symmetry between amplicon DNA accumulation and loss in amplification rate.

5 (A) Plots of the sigmoid functions describing amplicon accumulation (equation 1; solid line) and cycle efficiency (equation 2; dashed line) illustrate a symmetrical relationship, where $C_{1/2}$ defines the fractional cycle when both reach precisely half of their respective maxima. F_0 is target quantity expressed in fluorescence units, E_{max} is the maximal amplification efficiency and F_{max} is

10 the maximal reaction fluorescence. (B) Plotting cycle efficiency against reaction fluorescence produces a line, indicating that the progressive loss in cycle efficiency is directly related to amplicon accumulation. This generates a linear representation of PCR amplification, where the Y intercept defines the maximal amplification efficiency (E_{max}) when $F_C=0$ and the X intercept defines

15 the end-point or plateau phase (F_{max}) when $E_C=0$, with the slope defining the rate-of-loss in cycle efficiency (ΔE) that occurs as amplification progresses.

Figure 2 Fundamentals of linear regression of efficiency (LRE). (A) Loss in amplification efficiency is directly related to amplicon DNA accumulation.

20 Ratio-derived amplification efficiency (E_R , equation 1) is plotted against reaction fluorescence (F_C), demonstrating a linear relationship between amplicon DNA mass and cycle efficiency. The intercepts define the starting or "maximal" efficiency (E_{max} ; $F_C=0$) and the end-point or plateau phase (F_{max} ; $E_R=0$), with the slope defining the rate-of-decay in cycle efficiency (ΔE). This

25 in turn allows two of three kinetic parameters to be determined using linear regression analysis. (B) Actual fluorescence readings (dots) plotted with predicted reaction fluorescence (F_P) calculated using equation 6 (circles) with E_{max} and ΔE values derived from (A). Arrows indicate the cycles that were included in the linear regression analysis shown in (A). (C) Ratio-derived

30 amplification efficiency, E_R (dots; equation 1) plotted with E_C predicted by equation 4 (circles) using E_{max} derived from (A). The sporadic fluctuation in E_R produced by cycles 10-14 reflect high error-of-measurement produced by low

reaction fluorescence. Arrows indicate the cycles that were included in the linear regression analysis conducted in (A).

Figure 3 Reaction volume is a determinant of $F_{\max}$. (A) Amplification profiles of reactions containing an identical quantity of target but of increasingly greater volume (20 μl ($\bullet$), 30 μl ($\blacktriangledown$), 40 μl ($\blacklozenge$), 50 μl ($\blacktriangle$) and 60 μl ($\blacksquare$)). (B) Ratio-derived estimates of cycle efficiency (E_c , equation 3) were determined from the fluorescence readings generated by each amplification reaction and plotted against the respective reaction fluorescence (F_c), producing a linear representation of each of the amplification profiles shown in (A) (Fig. 1B). This demonstrates that increasing reaction volume reduces the rate-of-loss in cycle efficiency (slope, ΔE) but has no impact on the maximal amplification efficiency (Y intercept, $E_{\max}$), producing a proportional increase in maximal reaction fluorescence (X intercept, $F_{\max}$) consistent with that predicted by equation 5. Table III below relates to Figure 3 and provides a summary of the linear regression analysis (LRE analysis, equation 4) of the plots shown in Figure 3 (B), which reveals that amplicon concentration at $F_{\max}$ (FU/ μl) is only modestly impacted by the three fold increase in reaction volume. Vol., reaction volume; r^2 , linear correlation coefficient; $F_{\max}$ FU/ μl , fluorescence units per μl of reaction at $F_{\max}$.

Vol. (μl)	r^2	$E_{\max}$	ΔE	$F_{\max}$	$F_{\max}$ FU/ μl
20	0.9984	92.2%	-1.18×10^{-4}	7,810	391
30	0.9995	93.5%	-8.19×10^{-5}	11,400	380
40	0.9996	93.1%	-6.47×10^{-5}	14,400	360
50	0.9994	91.8%	-5.09×10^{-5}	18,000	360
60	0.9996	93.4%	-4.70×10^{-5}	19,900	332

Table III relating to Figure 3.

Figure 4 Time of annealing and elongation is a determinant of maximal amplification efficiency. (A) Amplification profiles of reactions containing identical quantity of target but with different annealing and elongation (A&E)

times (4 min (▼), 2 min (▲), 1 min (■) and 0.5 min (●)). This reveals that both maximal reaction fluorescence and slope of the amplification profile decrease with decreasing A&E time, along with a progressive shift in profile position consistent with a reduced maximal amplification efficiency. (B) LRE plots show that reduced time of A&E produces a decreased maximal amplification efficiency (Y intercept, $E_{\max}$), but has minimal, if any impact on the rate-of-loss in cycle efficiency (slope, ΔE). Table IV relates to figure 4 and provides a summary of the linear regression analysis (LRE analysis; equation 4) of the plots shown in (B). Vol., reaction volume; r^2 , linear correlation coefficient.

10

A&E (min)	r^2	$E_{\max}$	ΔE	$F_{\max}$
4.0	0.9996	94.7%	-8.74×10^{-5}	10,800
2.0	0.9988	90.1%	-9.63×10^{-5}	9,360
1.0	0.9981	81.0%	-1.04×10^{-4}	7,790
0.5	0.9892	65.7%	-9.60×10^{-5}	6,840

Table IV relating to Figure 4.

15

Figure 5 Drifting plateau produced by low base amplification efficiencies. Actual (F_C) compared to predicted (F_P) reaction fluorescence of the amplifications presented in Figure 4A reveal a trend for amplification to continue within the plateau region, that becomes more pronounced as base amplification efficiency is reduced. Drift in the plateau region has a major impact on curving fitting to a sigmoid function whereas LRE is unaffected (Figure 4).

20

Figure 6 Optical calibration via two distinct processes. A quantified, lambda DNA stock solution was employed as a standard for optical calibration using both direct (A and B) and PCR-based (C) determination of the calibration factor (CF_d and CF_0 respectively) used for converting fluorescence units into DNA mass. A linear relationship of DNA mass with fluorescence is found over the range of 15-50 ng of lambda DNA when placed into mock PCR reactions

25

(25 μ l containing all of the components in a standard amplification reaction). The tubes were placed into the thermocycler, heated to the read temperature used for real time PCR (68 °C) and multiple optical reads taken. Following subtraction of the fluorescence produced in the absence of lambda DNA (i.e. 0 ng), the average of the fluorescence readings were plotted against the corresponding mass of lambda DNA, confirming a linear relationship of DNA mass with reaction fluorescence ($r^2=0.995$). (Table V below) A summary of the data shown in (A) in which CF_d (direct calibration factor; $CF_d = \text{DNA mass/fluor.}$) is calculated for each lambda DNA mass, from which an average is determined. (Table VI below) PCR-based optical calibration in which three targets were amplified from the same stock of lambda DNA standard used for direct calibration, with each amplification reaction containing 500 fg of this lambda DNA preparation (S_M). The predicted target quantity in DNA mass (M_0 ; nanograms) was calculated using equation 11 based upon the known size of the lambda DNA molecule (48,502 bp; S_S) and the amplicon size of each target (A_S). Following four replicate amplification for each target was conducted using the same thermocycling conditions and read temperature (68 °C), LRE was used to determine F_0 (target quantity in fluorescence units) from which a calibration factor (CF_0) was calculated for each target and an average taken. Both methods produced an average CF that agree within ~4%, providing verification of accuracy, at least based upon a shared DNA standard. Fluor., fluorescence.

C

DNA mass	Fluor.	CF _d	S _s (ng): 0.0005				
			S _m (bp): 48,502				
15	0.2851	52.6	Target	A _s	M ₀	F ₀	CF ₀
25	0.3480	71.8	K1/K2	178	1.83x10 ⁻⁵	2.90x10 ⁻⁵	63.4
35	0.4443	78.8	K3/K4	218	2.25x10 ⁻⁵	3.69x10 ⁻⁵	80.9
50	0.5732	87.2	K5/K8	228	2.35x10 ⁻⁵	2.84x10 ⁻⁵	82.7
Average: 72.6±20.3%			Average: 70.0±17.3%				

Tables V and VI relating to Figure 6.

- 5 **Figure 7 Mathematical modeling of PCR amplification and assessment of the quantitative precision provided by LRE analysis.** (A) Replicate amplifications were conducted on ten fold dilutions of lambda genomic DNA and LRE quantification conducted on each amplification profile. Actual (●) and predicted (○, equation 7) reaction fluorescence for each target quantity are
- 10 plotted against cycle number. The table below depicts a summary of the LRE analysis conducted on the fluorescence profiles presented in Figure 7A, along with the calculated target quantity (F₀, equation 6, Table II). R², nonlinear correlation coefficient (see Materials and Methods and related Figures)

# of Lambda Genomes	R ²	E _{max}	ΔE	F _{max}	F ₀
188,000	0.99996	95.6%	-9.51x10 ⁻⁵	10,100	4.30x10 ⁻²
18,800	0.99999	95.3%	-9.49x10 ⁻⁵	10,000	4.10x10 ⁻³
1,880	0.99999	94.9%	-9.25x10 ⁻⁵	10,300	4.41x10 ⁻⁴
188	0.99999	95.7%	-9.23x10 ⁻⁵	10,400	3.22x10 ⁻⁵
19	0.99997	96.1%	-9.86x10 ⁻⁵	9,750	3.55x10 ⁻⁶
	0.99998	95.5%	-9.47x10 ⁻⁵	10,100	
	+/-0.00001	+/-0.5%	+/-2.7%	+/-2.5%	

Table VII related to Figure 7.

Figure 8 Screen shot of the MS Excel worksheet used for the LRE quantification conducted on the 18,800 molecule (1 pg) lambda genome amplification presented in Fig. 7. Graphs of the LRE analysis and comparison of actual to predicted fluorescence profiles are presented, with arrows

5 denoting the range of cycles included in the analysis. Following background subtraction, reaction fluorescence readings from four replicate reactions were averaged (F_C), imported into MS Excel, and ratio-derived estimate of cycle efficiency (E_C) calculated using equation 1. Linear regression analysis was then conducted on the seven cycles within the central region of the

10 amplification profile (cycles 20-26), from which values for E_{max} (intercept) and ΔE (slope) were determined (equation 2), and F_{max} calculated using equation 3. These values were then inputted into equation 5 and a F_0 estimate calculated for each cycle using cycle number and the corresponding fluorescence reading, from which an average F_0 was determined (cycles 19-

15 26), along with the coefficient of variance (CV). Predicted reaction fluorescence (F_p) were then calculated for each cycle using equation 6.

Mathematical derivations

20 A significant advancement in quantitative PCR, is disclosed herein, based upon curve-fitting of fluorescence readings to the four-parametric sigmoid function:

$$F_C = \frac{F_{max}}{1 + e^{-\left(\frac{C - C_{1/2}}{k}\right)}} - F_b \quad \text{Equation 14}$$

25

where C is cycle number, F_C is reaction fluorescence at cycle C, F_{max} is the maximal reaction fluorescence at which amplification ceases, $C_{1/2}$ is the fractional cycle at which reaction fluorescence reaches half of F_{max} , k is the slope of the curve, and F_b is the background reaction fluorescence.

30

Although not commonly used for analysis of PCR datasets, nonlinear regression can provide values for these four parameters by fitting

fluorescence readings to equation 14. This in turn allows the kinetics within individual amplification reactions to be investigated, revealing previously unrecognized principles of PCR amplification such as that fact that amplification efficiency is never constant, but is highly dynamic as described
 5 by a second sigmoid function (Liu and Saint 2002):

$$E_C = \frac{1 + e^{-\left(\frac{C - 1 - C_{1/2}}{k}\right)}}{1 + e^{-\left(\frac{C - C_{1/2}}{k}\right)}} - 1 \quad \text{Equation 15}$$

where E_C is the amplification efficiency at cycle C , also referred to as
 10 "cycle efficiency". Under this model, amplification efficiency decreases continuously such that each cycle has a unique amplification efficiency. The principle of this and other insights can be illustrated by plots of these equations, the most notable being a striking symmetry between amplicon DNA accumulation and loss in amplification efficiency (Figure 1).
 15

Notwithstanding the complexity of these sigmoid functions, it is unnecessary to invoke nonlinear mathematics in order to examine amplification dynamics. An underutilized approach is based upon the classic
 20 definition of amplification efficiency, that is, the relative increase in amplicon DNA over a single thermocycle:

$$E_R = \frac{F_C}{F_{C-1}} - 1 \quad \text{Equation 1}$$

25 where F_{C-1} is the fluorescence of the preceding cycle and E_R the "ratio-derived" cycle efficiency. This allows the apparent interrelationship between amplicon DNA accumulation and loss in amplification rate to be examined directly, without having to resort to nonlinear regression. Indeed, a simple plot of E_R against F_C reveals a linear relation, indicating that loss in amplification
 30 efficiency is directly correlated to amplicon DNA accumulation (Figure 2A).

The dynamics of amplification efficiency can thus be described by the linear equation:

$$E_R = \Delta E \times F_C + E_B \quad \text{Equation 2}$$

5

where the slope (ΔE) defines the rate-of-decay in amplification efficiency and the intercept (E_B) is defined as the "base" amplification efficiency, that is, the apparent amplification efficiency in the absence of target DNA (when $F_C=0$). It is also evident that as PCR amplification enters the plateau phase, F_C approaches a maximum (F_{max}) as E_R approaches zero, so that:

10

$$0 = \Delta E \times F_{max} + E_B$$

15 such that:

$$F_{max} = \frac{E_B}{-\Delta E} \quad \text{Equation 3}$$

indicating that F_{max} is determined by the ratio of base amplification efficiency to its rate-of-decay. By analogy to cycle efficiency, E_R can be replaced by E_C :

20

$$E_C = \Delta E \times F_C + E_B \quad \text{Equation 4}$$

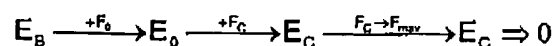
Equation 4 allows determination of cycle efficiency based solely upon reaction fluorescence. A subtle distinction between base amplification efficiency and the initial amplification efficiency as previously defined by Rutledge (2004) can be illustrated such that when $C=0$, $F_C=F_0$:

30

$$E_0 = \Delta E \times F_0 + E_B \quad \text{Equation 16}$$

Thus, although essentially identical at low target quantities, the difference between E_B and E_0 reflects the apparent reduction in amplification efficiency produced by addition of the target during reaction preparation. The dynamics of amplification efficiency could thus be illustrated as a series of

5 steps in which F_0 represents the target quantity and F_C represents amplicon DNA:



10 In addition to several practical implications, this allows for further elaboration of the sigmoid functions described by equations 14 and 15. For example, a direct relationship between E_B and k can be demonstrated by considering the scenario that if $C_{1/2}=0$ when $C=0$, then $E_C = E_B/2$ (Figure 1) so that equation 15 becomes:

$$E_C = \frac{E_B}{2} = \frac{1 + e^{\left(\frac{1}{k}\right)}}{1 + e^{\left(\frac{0}{k}\right)}} - 1$$

$$E_B = e^{\left(\frac{1}{k}\right)} - 1$$

or

$$k = \frac{1}{\ln(E_B + 1)} \quad \text{Equation 17}$$

Returning to the four-parametric sigmoid function, equation 14 can be modified by first substituting for k using equation 17, which simplifies to:

$$F_C = \frac{F_{max}}{1 + (E_B + 1)^{(C_{1/2}-C)}} \quad \text{Equation 18}$$

When $C=0$, F_C equals the target quantity such that:

$$F_0 = \frac{F_{\max}}{1 + (E_B + 1)^{C_{1/2}}} \quad \text{Equation 19}$$

where F_0 is target quantity in fluorescence units. Another fundamental
 5 aspect apparent from the linear equation 2, is that E_B and ΔE are the sole
 determinants of the shape of the amplification curve, that in turn is
 independent of target quantity, such that $C_{1/2}$ is the sole parameter defining
 target quantity.

These derivatives can be extended still further through rearrangement
 10 of equation 18:

$$C_{1/2} = \text{Log}_{(E_B+1)} \left(\frac{F_{\max}}{F_C} - 1 \right) + C \quad \text{Equation 20}$$

so that substituting for $C_{1/2}$ and simplifying, equation 19 becomes:

15

$$F_0 = \frac{F_{\max}}{1 + \left(\frac{F_{\max}}{F_C} - 1 \right) (E_B + 1)^C} \quad \text{Equation 5}$$

Importantly, equation 5 allows target quantity to be determined directly from
 reaction fluorescence, once estimates for E_B and ΔE are obtained via linear
 20 regression using equation 2, a process termed "linear regression of efficiency"
 or LRE. The effectiveness of LRE for target quantitative is illustrated in
 another example.

Similarly, rearranging equation 19:

$$25 \quad C_{1/2} = \text{Log}_{(E_B+1)} \left(\frac{F_{\max}}{F_0} - 1 \right) \quad \text{Equation 21}$$

so that substituting for $C_{1/2}$ and simplifying, equation 18 becomes:

$$F_P = \frac{F_{\max}}{1 + \left(\frac{F_{\max}}{F_0} - 1 \right) (E_B + 1)^{-C}} \quad \text{Equation 6}$$

which illustrates that the kinetics of PCR amplification can be traced back to just three fundamental parameters: target quantity (F_0), base
 5 amplification efficiency (E_B) and the rate-of-decay of amplification efficiency (ΔE), if it is remembered that $F_{\max}$ is determined by E_B and ΔE as described by equation 3.

LRE quantification

The general effectiveness of these derivatives for modeling real time
 10 PCR can be demonstrated the linear regression analysis presented in Figure 2A, and comparing the actual F_C readings to that predicted by equation 6 (F_P), which produces a nonlinear correlation coefficient of 0.99996 over the complete curve (Figure 2B). A similar level of correlation can also be shown for E_R from equation 2 with that predicted by equation 4 (E_C ; Figure 2C),
 15 which produces a nonlinear correlation coefficient of 0.99895 over the thermocycles included in Figure 2A.

Further verification that these LRE can effectively describe PCR amplification can be illustrated by examining the impact of increasing reaction volume, which was found to produce a proportional increase in $F_{\max}$ (Figure
 20 3A). Linear regression analysis reveals that while maximal efficiency is unaffected ($E_{\max}$; intercept), there is a progressive reduction in rate-of-decay in amplification efficiency (ΔE ; slope) with increasing reaction volume (Figure 3B and Table III).

Furthermore, reducing amplification efficiency by reducing the time of
 25 annealing and elongation greatly impacts $F_{\max}$, along with the shape and position of the amplification curve (Figure 4A). Linear regression analysis reveals that reducing time of annealing and elongation reduces maximal efficiency ($E_{\max}$; intercept) whilst having a modest if any impact on the rate-of-decay in amplification efficiency (ΔE ; slope) (Figure 4B and Table IV). Table
 30 IV).

These data also provide direct experimental support for the relationship of $F_{\max}$ to $E_{\max}$ and ΔE , as described by equation 3. The ability to investigate the kinetics of PCR amplification (i.e. mechanistic modeling) can also have important practical applications, as for example, in the evaluation and optimization of amplification efficacy. These encompass issues familiarity to most, but which to date have received little or no experimental support. With respect to reaction preparation, examples include the relative impact of primer and enzyme concentration, or of amplicon size on quantitative efficacy.

Of possibly greater importance is the ability to define in analytical terms, the impact of thermocycling parameters. As demonstrated here, the time of annealing and elongation has a major influence on amplification efficiency. Indeed, of all parameters examined to date, time of annealing and elongation has been found to have the greatest impact on amplification efficiency, significantly greater for example, than differences observed between primer pairs or produced by changes in the temperature of annealing and elongation (data not shown). Furthermore, establishment of a set of universal amplification conditions has been developed, in which high amplification efficiencies (95-100%) are routinely achieved, without having to resort to optimizing conditions unique to each individual primer-pair. Combined with the quantitative capabilities of LRE, these additional attributes significantly impact development of high-throughput applications.

Furthermore, it is evident that LRE will have a major impact on extending the quantitative capabilities and applications. Indeed, all of the quantitative attributes of sigmoidal curve-fitting using Boltzmann-based sigmoid functions, are directly applicable to an LRE-based approach. These include the ability to monitor amplification efficacy within individual reactions, and to conduct quantification without having to construct standard curves. A major advantage of LRE is however, circumventing the complexities of nonlinear regression (i.e. curve fitting), and in particular, the difficulties produced by a drifting plateau..

Drifting plateau

A major deficiency in the curving fitting approach as described by Liu and Saint (2002) is the inability to conducted accurate quantification under

conditions in which amplification does not complete cease in the plateau phase. Although not documented to date, this continued amplification within the plateau phase can be illustrated by comparing actual to predicted reaction fluorescence (Figure 5) in which actual fluorescence increase above that
5 predicted by sigmoidal curve-fitting. Also referred to a plateau drift this persistent increase in reaction fluorescence is inversely related to maximal efficiency, such that the rate of continued amplification increase as the predicted cycle efficiency approaches zero (Figure 5). A major deficiency for quantification via curve fitting is that a drifting plateau dramatically impacts the
10 quantitative effectiveness. LRE circumvents this deficiency (Figure 4).

Optical Calibration

A linear relationship between reaction fluorescence and DNA mass was demonstrated using a quantified stock of lambda DNA as a DNA standard (Figure 6A). Based upon equation 13, a calibration factor (CF_d) was
15 calculated from the average of the DNA mass to fluorescence ratio for each mock reaction (Table V). Verification of this CF_d value was then obtained by conducting PCR amplifications using the same lambda DNA preparation, based upon M_0 to F_0 ratios generated from three targets within the lambda DNA (equation 12). The average CF_0 was found to be nearly identical to that
20 obtained via direct calibration, differing from CF_d by only 4%.

Development of a protocol for large-scale qPCR applications

A major deficiency of existing qPCR applications is the need to optimize both primer design and cycling regime for each individual target. This requires construction of a standard curve for each target, that in turn requires
25 preparation of a quantified DNA standard unique to each target. Selection and preparation of these standards are technically challenging and time consuming, greatly hindering the design of large numbers of primer pairs as is required for large-scale applications, such as for example, that required for high-throughput gene expression analysis.

30 Combined with optical calibration, LRE analysis completely abrogates the requirement for standard curve construction, facilitating the simultaneous analysis of large numbers of targets. By virtue of allowing amplification

efficiency to be determined for each individual amplification reaction, LRE also facilitates testing and routine quality control of both primer design and cycling regime. Large-scale application of LRE analysis has subsequently led to development of a universal cycling protocol that generates both high
5 amplification efficiencies and a very low frequency of non-specific amplification products (i.e. produces high amplification specificity). This protocol preferably incorporates a two-step cycling regime consisting of a denaturation step at or about 95 °C for 10 seconds, and a combined annealing and elongation (A&E) step at 65 °C for 180 seconds. The selection
10 of temperature and time of the A&E step, influences amplification specificity and high amplification efficiency. It is within the skill of one in the art, in light of the disclosure herein, to select suitable annealing and elongation temperatures. Typical temperature ranges are 60 to 75 degrees Celsius, sometimes 65 to 70 degree Celsius. (These temperature ranges are driven
15 significantly by the activity and stability of the Taq enzyme. One skilled in the art in possession of a different polymerase would be able to select a suitable temperature in light of the disclosure herein.)

The application of LRE analysis was further utilized to examine the efficacy of primer design methodologies. To date, it is common practice to
20 utilize specialized software programs to design primers, based upon the objective of reducing the frequency of non-specific amplification products and promoting the general effectiveness of primer pair combinations. Despite the general perception that careful selection of the DNA sequence encompassed by a primer is required for a high rate of success, LRE analysis, in
25 combination with the cycle regime described above, led to the discovery that only a single parameter is important for effective primer design. This parameter is primer melting temperature (T_m), a parameter determined solely by primer length and base pair composition; the actual DNA sequence within a primer was generally found to have no substantive impact on amplification
30 efficiency, nor on the determination of amplification specificity.

In addition to the simplicity of this approach to facilitate large-scale primer design, this discovery greatly facilitates large-scale application of qPCR by allowing the position of the primer within the target to be selected

without restriction. In practice this involves selecting a 3 prime starting position, generally near to the stop codon for mRNA quantification, and to increase the length of the primer until the calculated T_m reaches 70 °C.

- Combined with the universal cycling regime described above, LRE analysis of a large number of primer pairs has demonstrated that >95% of all primer pairs produce high amplification specificity, with all primer pairs producing amplification efficiencies within the range of 95-100% as determined by LRE analysis.

10 **Materials and Methods**

Figure 8 Data Analysis

- SigmaPlot (Version 8) was used to generate the plots presented in Fig. 8 based upon sigmoidal curve-fitting of the fluorescence readings taken from the 4.0 min amplification reaction presented in Fig. 4, which produced: $k=1.51$, $C_{1/2}=21.02$, $F_{max}=10,837$, $F_b=0$. All other analyses were conducted using MS Excel.

Nonlinear correlation coefficients of predicted fluorescence profiles presented in Fig. 7 were calculated over the range of cycles included in the LRE analysis (Fig. 8), using the equation:

20

$$R^2 = 1 - \frac{\sum (F_C - F_P)^2}{\sum (F_C - F_{av})^2}$$

- where F_C is reaction fluorescence and F_P is the predicted reaction fluorescence at cycle C , with F_{av} being the average reaction fluorescence over the range of cycles used in the LRE analysis.

PCR amplification

- Bacteriophage lambda DNA (New England BioLabs) was used as template for all amplification reactions, which were conducted with a MX3000P spectrofluorometric thermal cycler (Stratagene) using QuantiTect™ SYBR® Green PCR Kit (Qiagen Inc.). Data presented in Fig. 3 and 4 were

generated using primer pair Lam K7-K8 (amplicon size 225 bp), and primer pair Lam K7-K12 (amplicon size 150 bp) was used to generate the data presented in Fig. 7.

Lam K7: CTGCTGGCCGGAATAATGAATTTATTGGT

5 Lam K8: ACCGAGTTCAGAAATAAATAACGCGTCGCCGGAA

Lam K12: ATGCCACGATGCCTCATCACTGTTG

Unless otherwise indicated, four replicate amplification reactions containing 10 pg lambda DNA and 500 nM of each primer were conducted, starting with a 15 min incubation at 95 °C, followed by a cycling regime of 95 °C-10 sec, 65 °C-180 sec, and reaction fluorescence determined by averaging
10 five fluorescent readings taken at the end of each cycle. Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimers. Following fluorescence background subtraction, fluorescence readings from the four replicates were averaged and
15 exported into MS Excel for analysis.

Table I. Impact of amplicon size on amplification kinetics.

Primer Pair	Amplicon Size (bp)	E_{max}	ΔE	F_{max}
K7-K10	100	95.4%	-5.79×10^{-5}	16,500
K7-K12	150	99.9%	-5.83×10^{-5}	17,100
K10-K19	200	94.9%	-6.51×10^{-5}	14,600
K7-K14	200	101.7%	-6.85×10^{-5}	17,400
K7-K18	300	102.7%	-6.13×10^{-5}	16,800
K10-K11	300	100.3%	-6.86×10^{-5}	14,600
K10-K13	400	95.8%	-6.15×10^{-5}	15,600
K10-K15	500	94.0%	-6.98×10^{-5}	13,500
K10-K17	800	95.9%	-6.11×10^{-5}	15,700
K10-K19	800	94.4%	-5.99×10^{-5}	15,800
K10-K21	1000	93.2%	-5.97×10^{-5}	15,600
Average:		97.1%	-6.20×10^{-5}	15,700
SD/CV:		+/-3.4%	+/-6.6%	+/-7.5%

5

Table II. Assessment of the precision of LRE-based quantification.

Lambda DNA Mass (fg)	# of Lambda Genomes	LRE F_0	F_0 Adjusted to 10 pg	% of Average
10,000	188,000	4.30×10^{-2}	4.30×10^{-2}	108.7%
1,000	18,800	4.10×10^{-3}	4.10×10^{-3}	103.7%
100	1,880	4.41×10^{-4}	4.41×10^{-4}	111.5%
10	188	3.22×10^{-5}	3.22×10^{-5}	81.6%
1	19	3.73×10^{-8}	3.73×10^{-8}	94.4%
Average: 3.95×10^{-2}				
+/-12.17%				

References

Inclusion of a reference is neither an admission nor a suggestion that it
5 is relevant to the patentability of anything disclosed herein.

- Bustin, S.A. (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol*, **29**, 23-39.
- 10 Higuchi, R., Fockler, C., Dollinger, G. and Watson, R. (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology*, **11**, 1026-1030.
- Liu, W. and Saint, D.A. (Jun 7, 2002) Validation of a quantitative method for real time PCR kinetics. *Biochem Biophys Res Commun*, **294**, 347-353.
- 15 Motulsky, H., A. Christopoulos, Fitting Models to Biological Data using Linear and Nonlinear Regression: A practical guide to curve fitting (GraphPad Software, Inc., 2003).
- Rasmussen, R. (2001) Quantification on the LightCycler. In Meuer, S., Wittwer, C. and Nakagawara, K. (eds.), *Rapid Cycle Real-Time PCR: Methods and Applications*. Springer Press, Heidelberg, pp. 21-34.
- 20 Rutledge, R.G. and Côté, C. (Aug 15, 2003) Mathematics of quantitative kinetic PCR and the application of standard curves. *Nucleic Acids Res*, **31**, e93.
- US 6,691,041 B2 Method for the efficiency-corrected real-time quantification of nucleic acids.
- 25 US 2002/0058262 A1 Method for determining the efficiency of nucleic acid amplifications.
- US 6,569,627 B2 Monitoring hybridization during PCR SYBR Green I.

I Claim:

1. A method for polymerase chain reaction (PCR) quantification comprising
5 the following steps:
- a) conducting real time PCR such that fluorescence are assayed at a selected temperature,
 - b) said real time PCR comprising thermocycles;
 - 10 c) measuring raw reaction fluorescence following each thermocycle at a selected temperature;
 - d) exporting said raw reaction fluorescence measure into a data file for analysis;
 - e) obtaining a background fluorescent based upon the average fluorescence
15 of cycles within a baseline region of the amplification profile;
 - f) subtracting said background fluorescent from said raw reaction fluorescence measure for each thermocycle obtaining the reaction fluorescence;
 - g) calculating amplification efficiency E_R for each thermocycle normalized from
20 the ratio of reaction fluorescence (F_C) to the reaction fluorescence from the previous thermocycle;
 - h) plotting the amplification efficiency E_R against the reaction fluorescence;
 - i) extrapolating the slope ΔE and the Y intercept $E_{\max}$ from said plot;
 - j) calculating the maximal fluorescence produced by the amplification using;

25

$$F_{\max} = \frac{E_{\max}}{-\Delta E}$$

- k) calculating the target quantity in fluorescent units (F_0) for each thermocycle
30 using;

$$F_0 = \frac{F_{\max}}{1 + \left(\frac{F_{\max}}{F_c} - 1 \right) (E_{\max} + 1)^c}$$

where c is the cycle number and F_c is the corresponding reaction fluorescent reading.

5

l) calculating an average F_0 value from the F_0 values generated by the cycles within the central region of the amplification profile

2. The method of claim 1 comprising the additional steps;

10 m) calculating DNA mass using:

$$M_0 = CF \times F_0$$

where CF is a conversion factor obtained from optical calibration;

n) calculating the number of target molecules using

15
$$N_0 = (M_0 \times 9.1 \times 10^{11}) / A_s$$

where A_s is the amplicon size.

3. The method of claim 2 where said optical calibration is performed using a direct calibration method comprising the steps;

20 a) preparing mock PCR reaction mixes that contain the DNA standard in an amount similar to that generated with a typical amplification profile;

b) preparing mock PCR reaction mixes lacking DNA standard;

c) placing said mock reaction mixes from step a into a real time thermocycler;

d) placing said mock reaction mixes from step b into a real time thermocycler;

25 e) increasing the block temperature in both thermocyclers to the selected temperature used in said real time PCR;

f) taking said fluorescent reading from thermocycler of step c to obtain (F_d);

g) taking said fluorescent reading from thermocycler of step d to obtain (F_b);

h) calculating said conversion factor using;

30
$$CF_d = \frac{S_m}{F_d - F_b}$$

4. The method of claim 2 where said optical calibration is performed using a PCR amplification method comprising steps;

- a) calculating the number of DNA mass of the standard contained in the
5 sample using ;

$$M_o = \frac{S_m \times A_t}{S_s}$$

Where S_m is the mass of the DNA standard in nanograms and S_s is the molecular size in base pairs of the DNA standard and A_s is the size of the
10 target amplicon in base pairs,

b) performing LRE method of claim 1 using a diluted sample containing DNA standard to obtain F_o

c) calculating the calibration standard using;

$$CF_o = \frac{M_o}{F_o}$$

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5. Evaluating the quantitative accuracy by assessing a correlation between the calculated CF_d from claim 3 and the CF_o from claim 4.

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6. A process for quantifying the amplification efficiency for a nucleic acid in a sample subjected to the polymerase chain reaction, said process comprising:

- 25 a) obtaining a nucleic acid sequence containing a forward primer binding site and a complement to a reverse primer binding site 3' to the forward primer binding site;
- b) obtaining a forward primer and a reverse primer;
- 30 c) combining the nucleic acid sequence from step (a) and the primers from step (b) and subjecting them to the polymerase chain reaction to amplify the nucleic acid sequence between the 5' end of the forward

- primer binding site and the 3' end of the complement to the reverse primer binding site;
- d) obtaining a signal related to nucleotide incorporation into a polynucleotide prior to amplification and following each thermo cycle;
- 5 e) determining the ratio of the signal from each cycle to the signal from the immediately preceding cycle, wherein prior to comparison each signal is corrected to remove background;
- f) employing linear regression analysis of the ratio from step (e) as a function of the signal obtained from step (d) for each thermo cycle to
- 10 identify the y-intercept of the line as maximal amplification efficiency for the reaction
7. The process of claim 6 further comprising identifying the slope of the
- 15 line as the rate of decay of amplification efficiency in the reaction.
8. The process of either claim 6 or 7 wherein the signal of step d is a fluorescence signal.
- 20 9. A machine readable media having stored thereon instructions for carrying out steps (e) to (f) of claim 6.
10. A process for determining, with respect to a nucleic acid in a sample subjected to the polymerase chain reaction wherein amplification is tracked by a signal related to the incorporation
- 25 of nucleotides into polynucleic acids, the extreme limit of that signal, said process comprising:
- a. obtaining a nucleic acid sequence containing a forward primer binding site and a complement to a reverse primer binding site 3' to the forward primer binding site;
- 30 b. obtaining a forward primer and a reverse primer;
- c. combining the nucleic acid sequence from step (a) and the primers from step (b) and subjecting them to the polymerase

- chain reaction to amplify the nucleic acid sequence between the 5' end of the forward primer binding site and the 3' end of the complement to the reverse primer binding site;
- 5 d. obtaining a signal related to nucleotide incorporation into a polynucleotide prior to each thermo cycle;
- e. determining the ratio of the signal from each cycle to the signal from the immediately preceding cycle, wherein prior to comparison each signal is corrected to remove background;
- 10 f. calculating the equation of a line formed by linear regression analysis of the ratio from step (e) as a function of the signal obtain from step (d) for each thermo cycle;
- 15 g. calculating the value of (-1) multiplied by the ratio of the y-intercept of the line from step (f) to the slope of the line from step (f) to obtain the extreme limit of observable signal following repeated amplification.
11. The process of claim 10 wherein the signal of step d is a fluorescence signal.
- 20 12. A machine readable media having stored thereon instructions for carrying out steps (e) to (g) of claim 10.
- 25 13. A process for quantifying the rate of decay of amplification efficiency for a nucleic acid in a sample subjected to the polymerase chain reaction, said process comprising:
- 30 a. obtaining a nucleic acid sequence containing a forward primer binding site and a complement to a reverse primer binding site 3' to the forward primer binding site;
- b. obtaining a forward primer and a reverse primer;
- c. combining the nucleic acid sequence from step (a) and the primers from step (b) and subjecting them to the polymerase chain reaction to amplify the nucleic acid sequence between

- the 5' end of the forward primer binding site and the 3' end of the complement to the reverse primer binding site;
- d. obtaining a signal related to nucleotide incorporation into a polynucleotide prior to amplification and following each thermo cycle;
- e. determining the ratio of the signal from each cycle to the signal from the immediately preceding cycle, wherein prior to comparison each signal is corrected to remove background;
- f. applying linear regression analysis of the ratio from step (e) as a function of the signal obtain from step (d) for each thermo cycle to identify the slope of the line as the rate of decay of amplification efficiency for the reaction.
14. The process of claim 6 further comprising solving the equation from step (f) to identify the slope of the line as in the reaction.
15. The process of either claim 6 or 7 wherein the signal of step d is a fluorescence signal.
16. A machine readable media having stored thereon instructions for carrying out steps (e) to (f) of claim 6.
17. A process for identifying the quantity of amplified nucleic acid sequences in a sample after a selected number of thermocycles, said process comprising:
- a. obtaining a nucleic acid sequence containing a forward primer binding site and a complement to a reverse primer binding site 3' to the forward primer binding site;
- b. obtaining a forward primer and a reverse primer;
- c. combining the nucleic acid sequence from step (a) and the primers from step (b) and subjecting them to the polymerase

- chain reaction to amplify the nucleic acid sequence between the 5' end of the forward primer binding site and the 3' end of the complement to the reverse primer binding site;
- 5 d. obtaining a signal related to nucleotide incorporation into a polynucleotide prior to amplification and following each thermo cycle;
- e. determining the ratio of the signal from each cycle to the signal from the immediately preceding cycle, wherein prior to comparison each signal is corrected to remove background;
- 10 f. employing linear regression analysis of the ratio from step (e) as a function of the signal obtained from step (d) for each thermo cycle to identify the y-intercept of the line as maximal amplification efficiency for the reaction
- 15 g. obtaining a nucleic acid sequence containing a forward primer binding site and a complement to a reverse primer binding site 3' to the forward primer binding site;
- h. obtaining a forward primer and a reverse primer;
- i. combining the nucleic acid sequence from step (a) and the primers from step (b) and subjecting them to the polymerase chain reaction to amplify the nucleic acid sequence between the 5' end of the forward primer binding site and the 3' end of the complement to the reverse primer binding site;
- 20 j. obtaining a signal related to nucleotide incorporation into a polynucleotide prior to each thermo cycle;
- 25 k. determining the ratio of the signal from each cycle to the signal from the immediately preceding cycle, wherein prior to comparison each signal is corrected to remove background;
- l. calculating the equation of a line formed by linear regression analysis of the ratio from step (e) as a function of the signal obtain from step (d) for each thermo cycle;
- 30 m. calculating the value of (-1) multiplied by the ratio of the y-intercept of the line from step (f) to the slope of the line from

step (f) to obtain the extreme limit of observable signal following repeated amplification;

- n. determining the number of amplified nucleic acid sequences in terms of fluorescence units for a selected cycle number by dividing (the maximal signal from step (m)) by (1 plus(((the maximum signal from step (m))/(observed fluorescence following the selected cycle))-1] multiplied by ((the maximal amplification efficiency of the reaction from step (f)) plus 1) to the (exponent -(cycle number))

18. The process of claim 17 wherein the signal of step d is a fluorescence signal.

19. A machine readable media having stored thereon instructions for carrying out steps (e) to (n) of claim 17.

20. An apparatus for amplification of polynucleic acid sequences by thermocycling and detection of a signal related to nucleic acid incorporation, said apparatus comprising: a thermocycling unit, processor means for control of thermocycle reactions, sensor mean for measuring the signal; output means for output of thermocycle information and signal; machine readable memory means containing information for carrying out the process of at least one of claim 1, 6, 10, 13 or 17.

21. A method for identifying a patient having a condition of interest involving or identifiable by the abundance of a least one selected nucleic acid sequence, said method comprising:

- a) identifying a target nucleic acid sequence of interest;

- b) amplifying the target sequence using fluorescence-based quantitative Polymerase Chain Reaction;
- c) determining the reaction fluorescence for each cycle
- d) determining the ratio-based estimate of efficiency (E_R), for each thermocycle wherein

$$E_R = \frac{F_C}{F_{C-1}} - 1$$

" F_C " is reaction fluorescence following a selected thermocycle and " F_{C-1} " is reaction fluorescence following the previous thermocycle;

- e) plotting E_R against F_C to produce a line as described by:

$$E_R = \Delta E \times F_C + E_{\max}$$

- f) determine the Y intercept and slope of the line from step e, where in the intercept is the maximal amplification efficiency ($E_{\max}$) and the slope is the rate-of-decay of the base amplification efficiency (ΔE);
- g) determining $F_{\max}$ wherein

$$F_{\max} = \frac{E_{\max}}{-\Delta E}$$

- h) generating a determination of target quantity (F_0) in fluorescence units (FU) for a given cycle (C), wherein

$$F_0 = \frac{F_{\max}}{1 + \left(\frac{F_{\max}}{F_C} - 1 \right) (E_{\max} + 1)^C}$$

- i) determining the target quantity in polynucleic acid mass (M_0) using a conversion factor (CF), wherein

$$M_0 = F_0 \times CF$$

5

where CF is determined using a process called optical calibration, in which reaction fluorescence is correlated to polynucleic acid mass

10

- j) calculating the number of polynucleic acid molecules based upon polynucleic acid mass of the target (M_0) based upon the size of the amplified region (A_S), wherein

$$N_0 = (M_0 \times 9.1 \times 10^{11}) / A_S$$

15

- k) comparing the number of polynucleic acid molecules calculated above to a predetermined number or range of polynucleic acid molecules associated with a condition of interest.

20

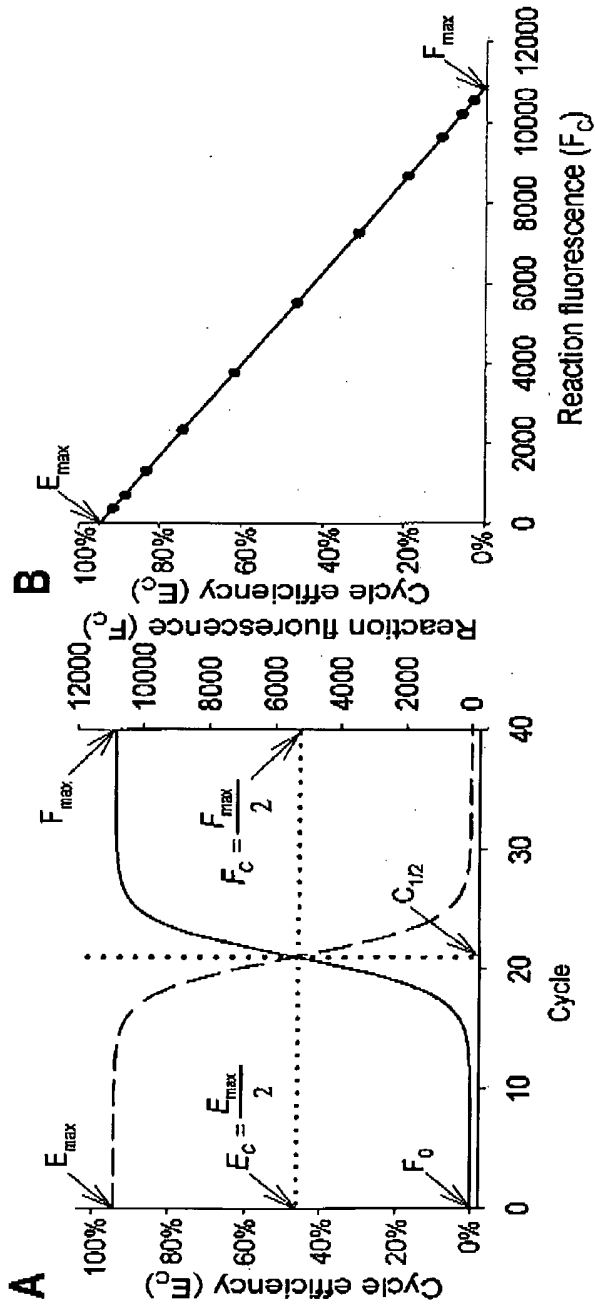


Figure 1

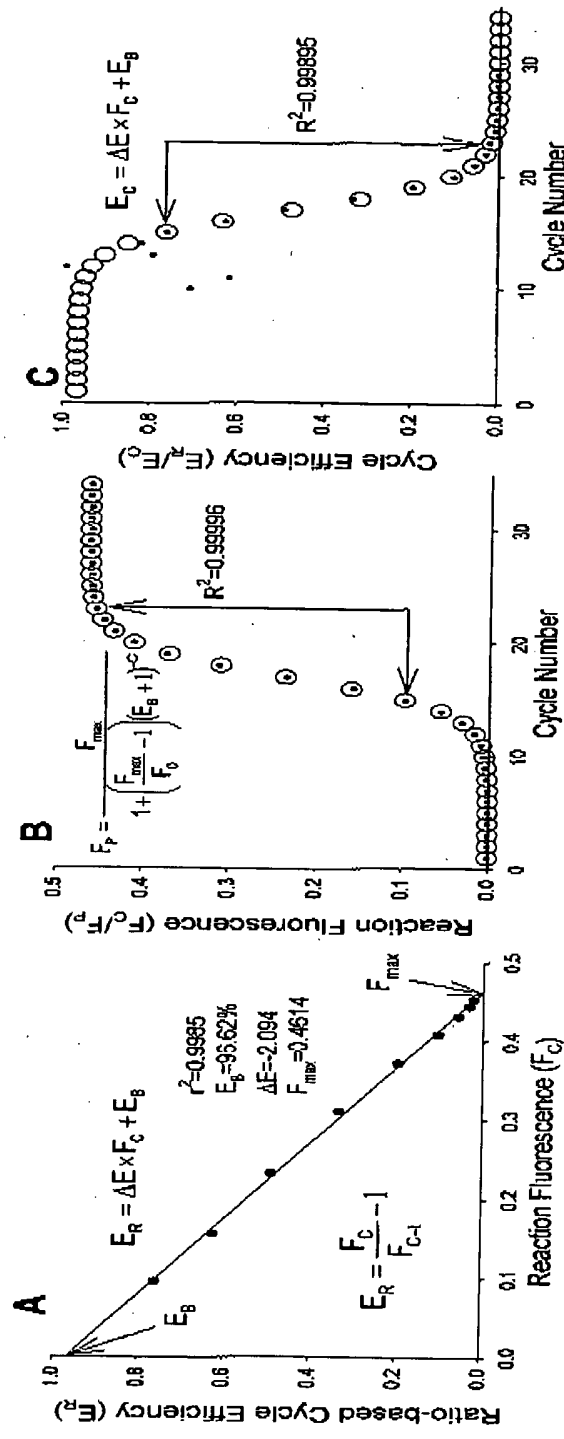


Figure 2. Arrows indicate the cycles that were included in the linear regression analysis conducted in (A).

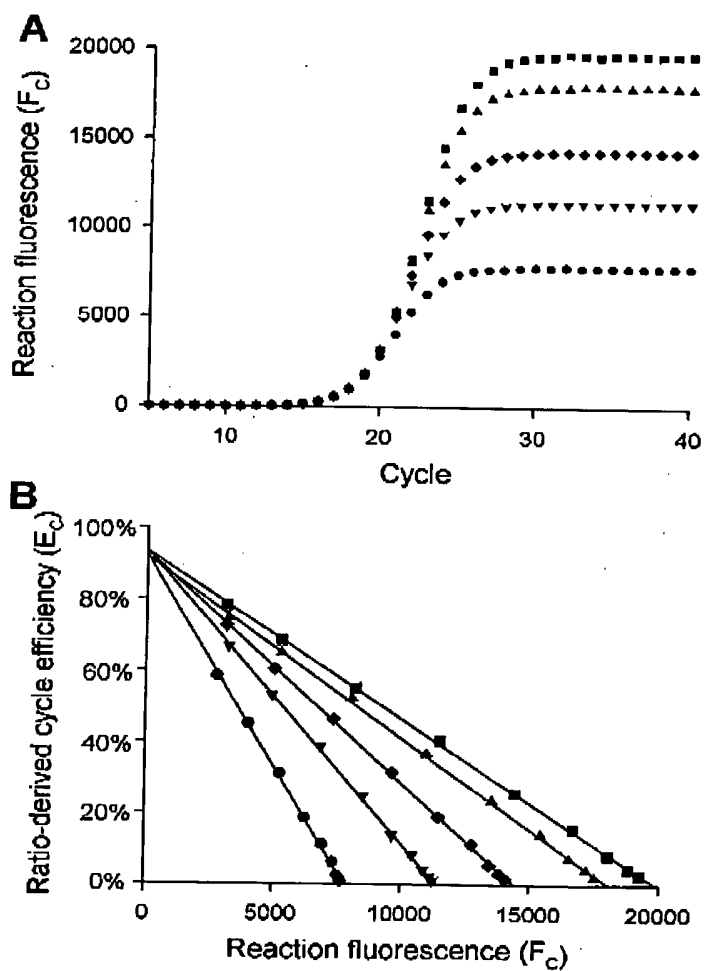


Fig. 3. Reaction volume is a determinant of F_{max} . (A) volume (20 μ l (●), 30 μ l (▼), 40 μ l (◆), 50 μ l (▲) and 60 μ l (■)).

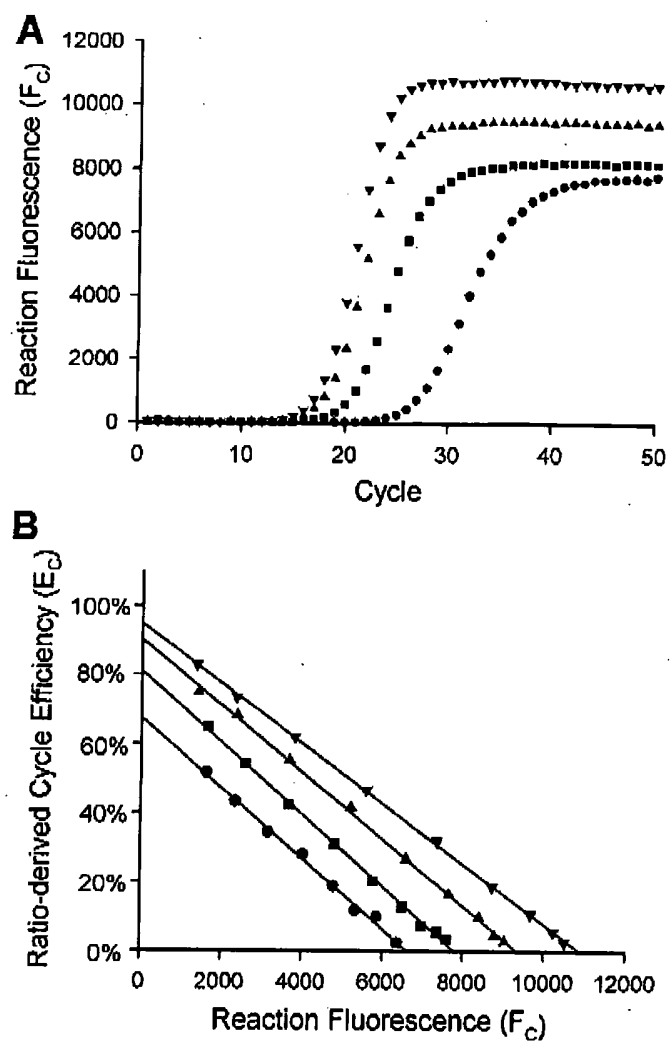


Figure 4.. (A) times (4 min (▼), 2 min (▲), 1 min (■) and 0.5 min (●))

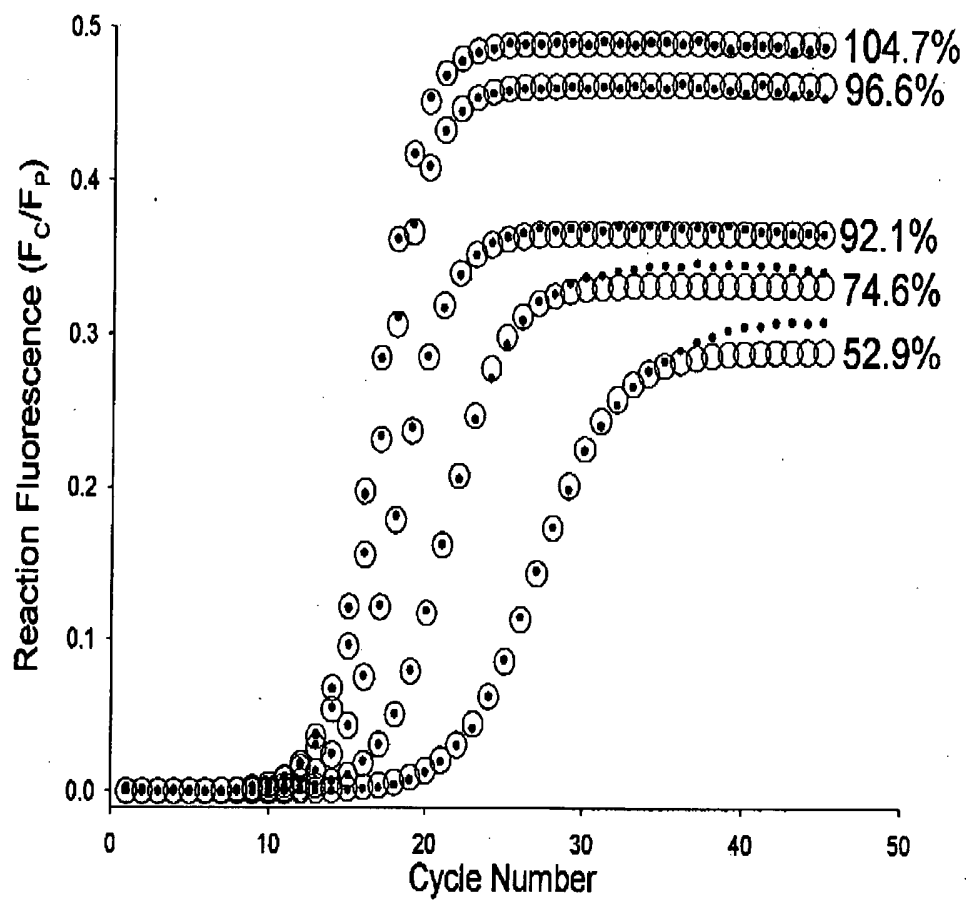


Figure 5. Drifting plateau produced by low base amplification efficiencies.

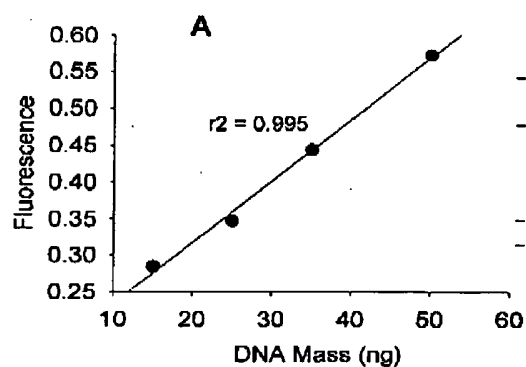
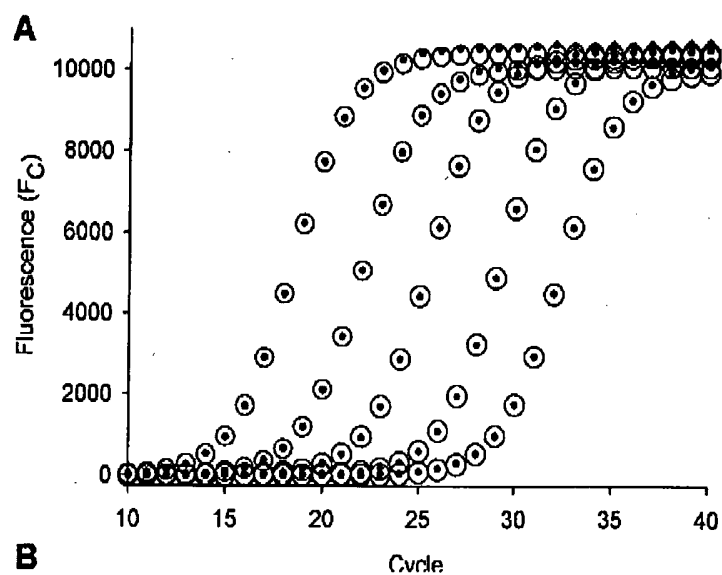
**Figure 6**

Figure 7. Actual (●) and predicted (○, equation 7) reaction fluorescence for each target quantity

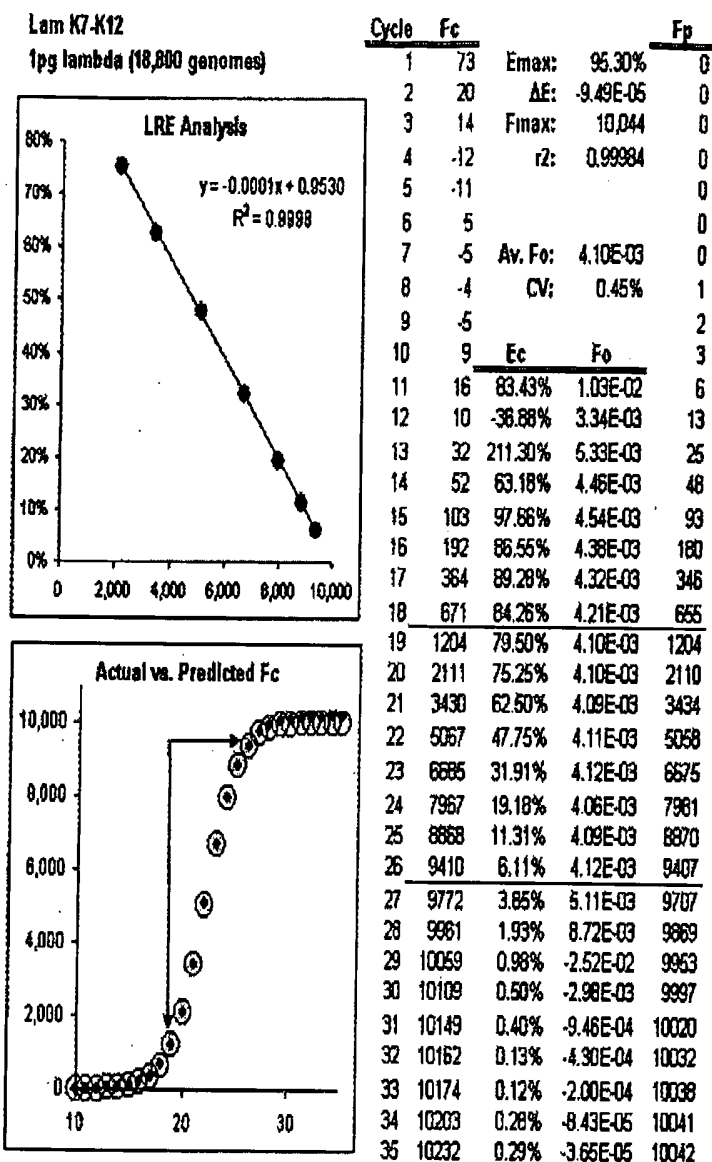


Figure 8.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/001354

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7): C12Q 1/68, C12M 1/40, G01N 21/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(7): C12Q 1/68, C12M 1/40, G01N 21/64

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Canadian patent database, USPTO, Delphion, Pubmed, Keywords: quantitative, real time, PCR, polymerase chain reaction, quantif*, analysis, amplif*, efficiency, kinetic*, fluorescence.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LIU, W. et al. Validation of a quantitative method for real-time PCR kinetics. Biochemical and Biophysical Research Communications. 2002, Vol.294, No.2, pages 347-53, ISSN 0006-291X.	1 to 20
Y	whole document	21
Y	BUSTIN, S.A. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. Journal of Molecular. Endocrinology. 2000, Vol.25, No.2, pages 169-193, ISSN 0952-5041.	21
	whole document	
A	PEIRSON, S.N. et al. Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. Nucleic Acids Research. 2003, Vol.31, No.14, 7 pages. ISSN 0305-1048.	1 to 21
	whole document	
A	TICHOPAD, A. et al. Standardized determination of real-time PCR efficiency from a single reaction set-up. Nucleic Acids Research. 2003, Vol.31, No.20, 6 pages, ISSN 0305-1048.	1 to 21
	whole document	

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

31 October 2005 (31-10-2005)

Date of mailing of the international search report

3 November 2005 (03-11-2005)

Name and mailing address of the ISA/CA
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/001354

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6303305 B1 (ROCHE DIAGNOSTICS; UNIVERSITY OF UTAH RESEARCH FOUNDATION) October 16, 2001. whole document	1 to 21
P, A	RUTLEDGE, R.G. Sigmoidal curve-fitting redefines quantitative real-time PCR with the prospective of developing automated high-throughput applications. Nucleic Acids Research. 2004, Vol.32, No.2, 8 pages, ISSN 0305-1048. whole document	1 to 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/001354

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. ☒ Claim Nos. : 1 to 21

because they relate to subject matter not required to be searched by this Authority, namely :

Claims 1 to 21 encompass analysis and interpretation of data using mathematical formulas, which are considered scientific and mathematical theories, which the Authority is not required to search under Rule 39.1 (i) of the PCT. In addition, claims 9, 12, 16 and 19 comprehend machine readable media comprising instructions, which is considered as mere presentation of information, and consequently, no search is required by the Authority under Rule 39.1(v) of the PCT. In addition, claim 20 encompasses computer programs, which the Authority is not required to search under Rule 39.1 (iv) of the PCT. The search has been carried out based on the methods for obtaining said data using quantitative real-time polymerase chain reaction as described in the description.

2. ☐ Claim Nos. :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. ☐ Claim Nos. :

because they are dependant claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/CA2005/001354

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
US6303305	16-10-2001	AU733255 B2	10-05-2001
		AU2257300 A	12-10-2000
		CA2302881 A1	30-09-2000
		EP1041158 A2	04-10-2000
		JP2000312600 A	14-11-2000
		US6503720 B2	07-01-2003
