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ResonSense®: simple linear fluorescent probes for quantitative homogeneous rapid polymerase chain reaction☆

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Abstract

Here we report a strand-specific fluorescent homogeneous assay format for rapid polymerase chain reaction (PCR). A number of similar assays are commonly used for research applications and are an ideal solution for a closed tube quantitative PCR. These assays use fluorescent resonant energy transfer (FRET) between donor and acceptor fluorescent moieties as the reporting mechanism. However, for different reasons these assays do not report amplification when very rapid cycling times are used. This is because current assays, such as TaqMan®, are limited, in terms of assay speed, by the 5′–3′ exonuclease activity of Taq DNA polymerase. Other assays based on hybridisation require either a complex de-conformational event to occur, or require more than one probe to report amplification. Reducing the complexity of the experiment reduces costs in terms of design, optimisation and manufacture. Here, we describe ResonSense® chemistries that use a simple linear fluorescent-labelled probe and a DNA minor-groove binding dye as either donor or acceptor moieties in a homogeneous assay format on the LightCycler®. This assay format will provide for rapid analysis of samples and so it is particularly well suited to point-of-use testing.

Keywords: Rapid PCR; ResonSense®; Angler®; LightCycler®; Fluorescence; SYBR®Gold

1. Introduction

In this report we discuss the background, and development of a novel homogeneous reporting chemistry for rapid polymerase chain reaction (PCR). A number of commercially available assays are commonly used for research applications and are an ideal solution for a closed-tube quantitative PCR. These chemistries utilise fluorescence and have a number of common advantages over alternative approaches to detection of PCR products. Fluorimeters are the instrumentation used to detect fluorescence. These are relatively simple optical arrangements that collect light from the whole sample rather than at a specialised surface. The only requirement is that an optical window is available in the PCR chamber to allow the passage of visible light in and out of the reaction mixture. Instruments such as the ABI 7700 and Idaho LightCycler® combine thermal cycling technology with an integral fluorimeter. These homogeneous fluorescent assays have relatively low sensitivity compared with other detection technologies. The ability to detect extremely low numbers of nucleic acid species using these

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approaches is derived from the performance of the PCR.

Until the development of fluorimeter based instruments such as those mentioned the usefulness of PCR was limited by the small dynamic range of quantification that conventional analysis provided. The dynamic range problem arises from the fact that the reaction reaches a plateau that is largely independent of initial target copy number. This plateau is the result of a number of factors that include substrate depletion and product inhibition. The end point analysis techniques such as gel electrophoresis used previously were limited to a dynamic range of approximately three log units of initial target copy number. Some complicated approaches such as competitive PCR could achieve a better dynamic range. The development of kinetic or "real-time" analysis [1] using fluorescence reporting chemistries now provides a real-time kinetic closed-tube approach to quantification that covers the whole range of PCR amplification.

This real-time approach involves taking a measurement once per cycle, usually at the end of extension or annealing [2,3]. In this manner, the progress of individual reactions can be monitored. The cycle value where fluorescence rises above background noise is termed the "cycle threshold", or CT value. The number of cycles that an individual assay requires is logarithmically related to the initial target copy number. Control assays, with a known concentration of initial target copy number, can be used to generate a standard curve of cycle threshold number against initial target copy number. The initial target copy number of unknown samples can be determined by interpolation of their cycle threshold using this type of plot.

These homogeneous fluorescent reporting chemistries require additional fluorescent reagents in the assay. These reagents can be broadly grouped into two types. The first type of reagent is one that simply reports the generation of total nucleic acid amplification in the reaction. These reagents are intercalating dyes that bind to double stranded DNA (dsDNA) with enhanced fluorescence [4,1,5–8]. These include dyes such as ethidium bromide [1], used routinely for staining DNA electrophoresis gels. Other dyes such as the YO-PRO® dyes, YoYo® dyes [9], and the SYBR® dyes, can be used effectively in PCR because they exhibit significantly more fluorescence when bound to dsDNA than to single stranded DNA (ssDNA). Molecular Probe's SYBR® dyes, particularly SYBR®Green-1 and SYBR®Gold, are now used routinely because their fluorescent peak emission spectrum closely matches that of fluorescein. This is convenient because PCR fluorimeter designs are often optimised for fluorescein. These dyes are simply added as a reagent to the PCR cocktail of standard reactions. We term this type of monitoring of the reaction "non-strand specific" detection. DNA melting point analysis is a methodology that can be applied to this assay [10] and this allows DNA intercalaters to be used to generate quasi-strand specific data on amplification products. Specific PCR products and reaction artefacts melt at different temperatures that are dependent upon a number of factors including their GC content, length, secondary and tertiary structure, and the chemical formulation of the reaction chemistry. This methodology can be used instead of separation analysis such as agarose or capillary gel electrophoresis. However, the discriminatory power of melting point analysis is limited because it does not have the resolution of a separation analysis and subsequent molecular mass determination.

The second reagent type is nucleic acid probes that are specific for the non-amplimer sequence of the amplicon. They interact with the amplicon, "at the sequence level", generating a signal that is highly specific for the intended PCR amplification product. We term this type of monitoring of the reaction, "strand specific" detection. Strand specific analysis requires the design and synthesis of one or more bespoke fluorescent nucleic acid probes for each PCR assay. These probes interact with the product reporting an increase after each cycle of amplification. Probes may also be used in melting point analysis to provide additional identification of amplified product. These types of reporting system utilise fluorescent resonance energy transfer (FRET) [11] between two fluorescent dyes as the basis of detection. One dye, the donor, is excited with an incidence light source. When excited the donor emits light at longer fluorescent emission wavelengths. When a second dye, the acceptor is in close proximity, it will accept energy from the donor provided there is sufficient spectral overlap between donor emission and acceptor excitation. This interaction is termed fluorescent quenching. The mechanism by which this occurs involves a number of distance dependent and orientation factors. Therefore, FRET

efficiency is spatially constrained. The types of dyes used for these probes include fluorescein, rhodamine, and cyanine dyes. The chemistries for label incorporation into nucleic acid probes are well developed since they are used in other molecular biology procedures such as DNA sequencing. Other non-fluorescent dyes such as DABCYL and methyl-red, termed "dark quenchers" are also often used as the acceptor dye.

Probe systems fall into two categories based on how they report amplification. The first category is the hydrolysis probes. These are probes that are designed to be consumed during the course of the reaction, liberating the donor dye from proximity to acceptor. The second type of probes are hybridisation probes. These either liberate or bring together the acceptor and donor dyes by hybridisation events.

The most common hydrolysis probe is an oligonucleotide labelled terminally with an acceptor and donor dye at the 5′ and 3′ end [12–15]. The 3′ end is protected with a phosphate group so that it will not act as an amplimer in the PCR. The probe sequence is designed such that it hybridises in front of the nascent strand of the extending forward amplimer and is concomitantly hydrolysed by the 5′–3′ exonuclease activity of the Taq polymerase each cycle. The previously quenched acceptor is liberated from donor as the basis of detection. Each cycle the fluorescent signal accumulates. These types of probes are known commercially as TaqMan™ probes.

There are several types of hybridisation probes. They differ in the hybridisation mechanism that they use to report amplification. The main difference between hybridisation probes and hydrolysis probes is in their design. These types of probes are designed not to be consumed during the reaction. This signal does not accumulate. Each cycle probe must hybridise to products to report the increase in amplification. Dual-hybe probes are pairs of fluorescently labelled oligonucleotide probes that hybridise to the internal sequence of an amplification product [16,6]. One probe is labelled terminally at the 3′ end with either a donor or acceptor dye. The other probe is labelled terminally at the 5′ end with either a donor or acceptor dye. The probes are designed such that they hybridise adjacently on a natent complementary strand bringing the 3′ terminus of the first probe spatially close to the 5′ terminus of the second, thus, allowing efficient fluorescent quenching by FRET as the basis of detection. Molecular beacons are oligonucleotide probes that have hairpin secondary structures [17,18]. This is achieved by the addition of extra complementary sequences at the 5′ and 3′ termini. The molecular beacon has donor and acceptor dyes at the 5′ and 3′ ends. In the absence of target these terminal sequences hybridise bringing donor and acceptor together, and thus, efficient fluorescent quenching. In the presence of target the energetics favour a linear duplex structure and allows probe binding such that the donor is liberated from acceptor, reducing FRET, as the basis of detection. Sunrise® or Amplifluor® is a probe system that uses a molecular beacon attached to the 5′ end of an amplimer [19]. This molecular beacon has no specificity for the target PCR product and a generic molecular beacon may be used for all reactions. During the amplification the complement sequence of the molecular beacon is generated such that when product and probe anneal the beacon structure is no longer formed. The donor is liberated from the quencher as the basis of detection. Since the probe has no specificity for the reaction this is not a true strand specific approach. Scorpions® is also an oligonucleotide probe system that has a molecular beacon joined to the 5′ end of an amplimer. However, the probe has a linker between the amplimer and the molecular beacon that stops the enzyme from reading, and thus, generating the molecular beacon complement [20]. This molecular beacon does have specificity for the strand that is generated by the amplimer it is attached to. During the cooling of the PCR, when the energetics are favourable, the molecular beacon structure destabilises and anneals to the complementary strand. This hybridisation event liberates the acceptor from quencher with a resulting reduction in FRET as the basis of detection.

We have developed a FRET reporting mechanism for rapid PCR that utilises a linear oligonucleotide probe labelled with a single fluor. The oligonucleotide is designed to hybridise to non-amplimer encoding sequence of the amplicon and has a protective 3′ label to stop it initiating extension in the presence of Taq polymerase. An exonuclease deficient mutant Taq polymerase ensures that the probe is not consumed during the reaction. A DNA intercalater is used as either a donor or acceptor FRET moiety. In the experimental data described the donor moiety used is SYBR®Gold and the acceptor moiety is CY5 or CY5.5. By using a

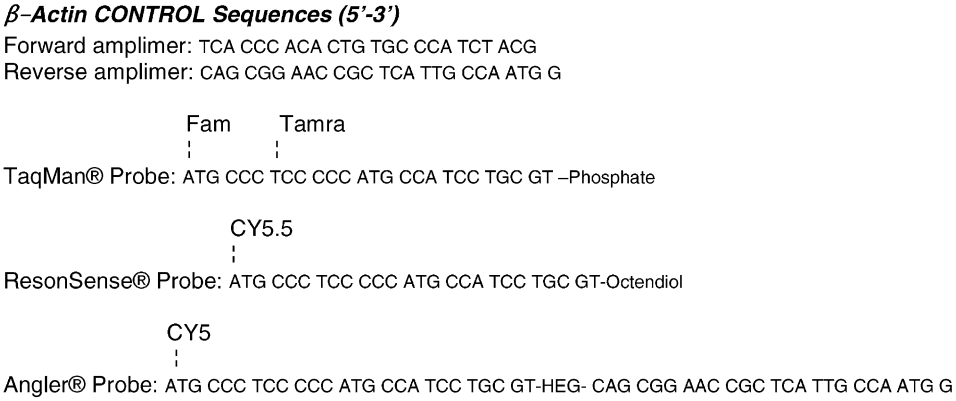


Fig. 1. The amplimer and probe sequences for the human β-actin gene.

colour compensation algorithm of the Idaho RAPID or ROCHE LightCycler® and monitoring more than one fluorescent signal both non-strand specific and specific amplification can be detected. The simple reporting mechanism based on a linear hybridisation probe offers a number of advantages over existing systems including assay speed, design and manufacturing costs.

2. Experimental

2.1. PCR reaction mixtures

PCR master mixes were prepared as a 2× concentrate. The final composition was: 50 mM Trizma pH 8.8 at 25 °C (T-5753 Sigma Chemicals, Fancy Road, Poole Dorset BH12 4QH), magnesium chloride 3mM (Sigma M-1028), glycerol 8% (w/v) (Sigma G-5516), non-acetylated bovine serum albumin 250 ng/μl (Sigma *B*-8667), dNTP's PCR nucleotides 200 μM (Nucleotide PLUS® Roche UK, Bell Lane, Lewes, East Sussex BN7 1LG, UK), uracil-*n*-glycosylase 0.01 units/μl (Sigma U-1257), Taq (exo 5′–3′ deficient) DNA polymerase 0.04 units/μl (Genesys Ltd., 40 Lynchford Road, Farnborough, Hanst GU14 6EF, UK), TaqStart® anti-Taq antibody 0.03 μM (Sigma T-4808). The Taq DNA polymerase and TaqStart® anti-Taq antibody were incubated together for 10 min before addition to the mixture. SYBR®Gold was included in the reactions to a final concentration of 1:20,000 to 1:200,000 dilution of the reference solution (S-11494, Cambridge Bioscience, 25 Signet Court, New Market Road, Cambridge CB5 8LA). Target template was male human placental DNA (Sigma D-3160).

Fig. 2. Showing a amplification plots and standard curve generated using both the ResonSense® ((a) and (b)) and Angler® chemistries ((c) and (d)) for the human β-actin target on the ROCHE LightCycler®, the mean values for 5 replicates are shown with error bars indicating 95% confidence limits (Student's *t*-test). (a) and (c) show the non-specific generated from SYBR®Gold in each type of reaction as observed in the F1 detector. (b) and (d) show the strand specific signal derived from the probe fluorescence in each type of reaction ((b) a ResonSense® Cy5.5 probe, (d) an Angler® Cy5 probe), as observed in the F3 Detector. (e) shows standard curves derived from (b) and (d) using the second derivative maximum algorithm of the LightCycler® ((- - -)ResonSense®, (—)Angler®). A correlation coefficient of −1 was obtained for both chemistries. The ResonSense® signal was determined using the colour compensation algorithms of the ROCHE LightCycler®. The drop in fluorescence observed in the no template controls (NTC) is an anomaly of the compensation and background subtraction of the LightCycler® analysis. The Angler® system did not require colour compensation. The excessive background noise may be attributed to the optical filter of the ROCHE LightCycler® F3 optical module missing the peak emission of the CY5 and CY5.5 dyes used. The amplification used the optimum temperature transition rates and hold times of the ROCHE LightCycler®. At later cycles the signal reduces due to the characteristic "hook effect", of product displacing probe that is also observed in dual-hybe probe reporting chemistries.

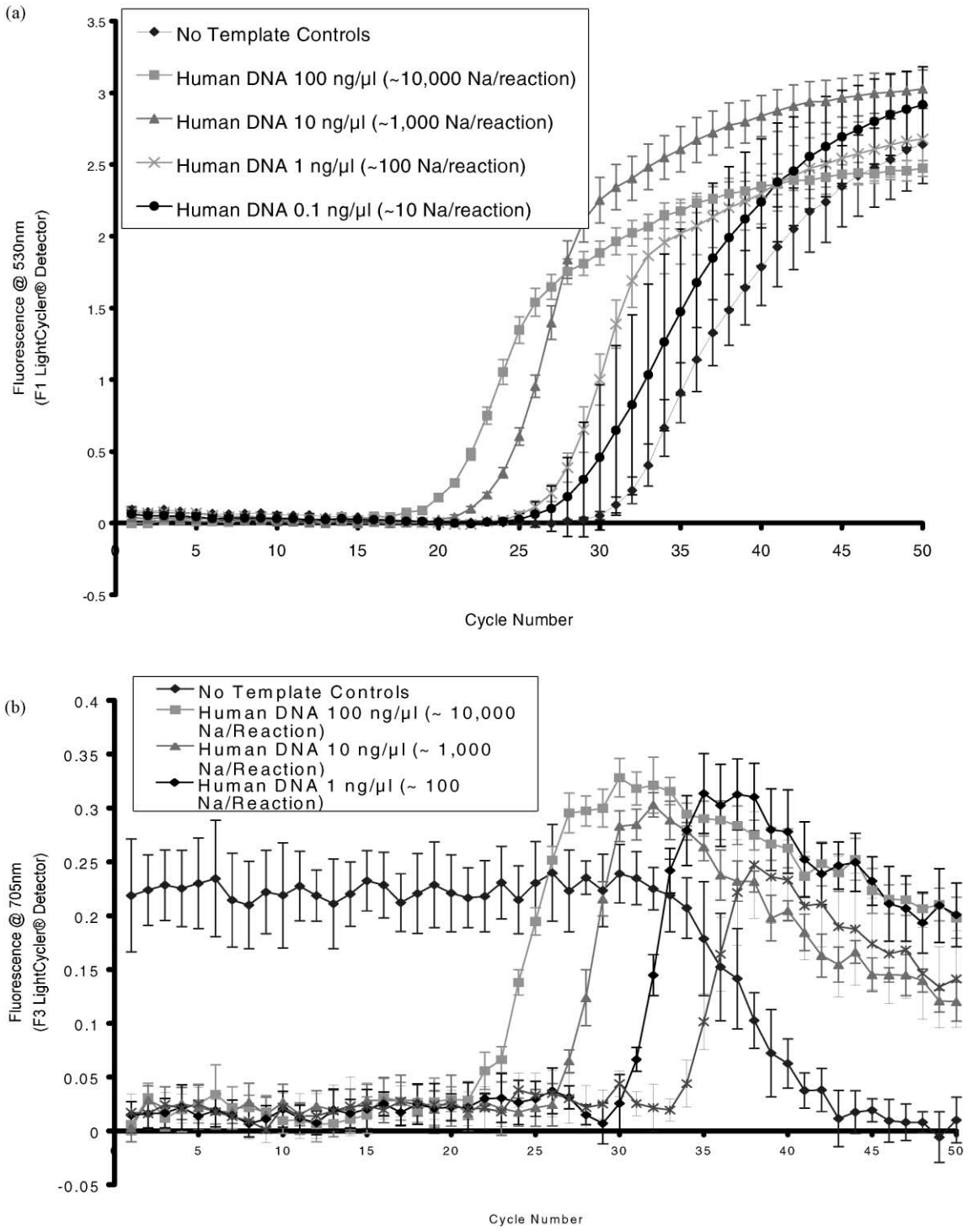
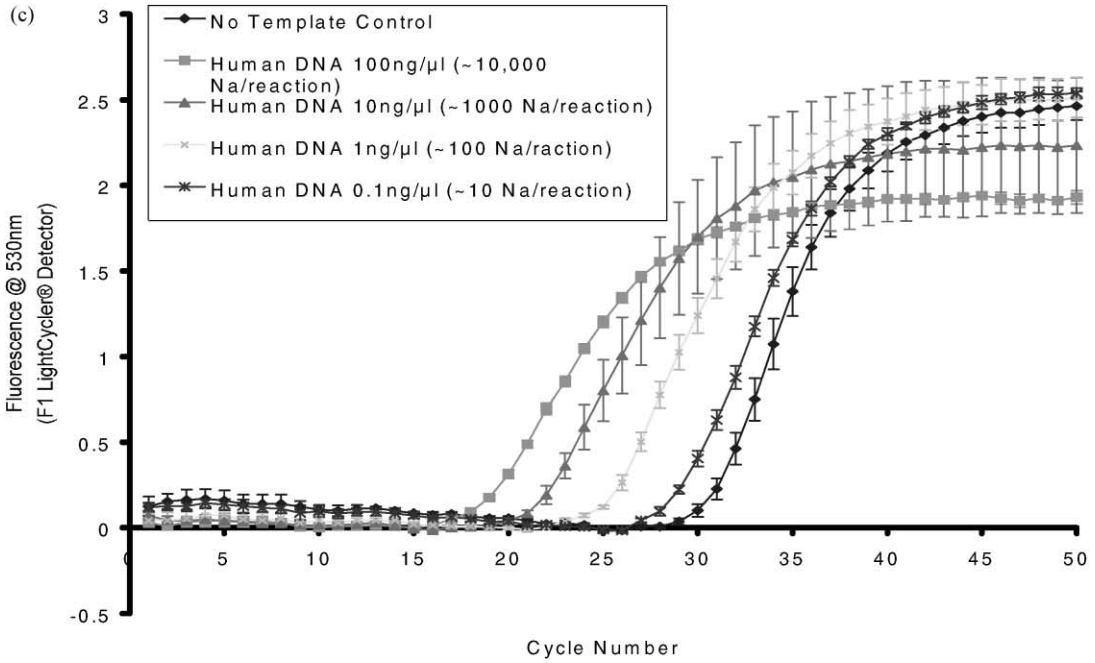

(a)
No Template Controls
Human DNA 100 ng/µl (~10,000 Na/reaction)
Human DNA 10 ng/µl (~1,000 Na/reaction)
Human DNA 1 ng/µl (~100 Na/reaction)
Human DNA 0.1 ng/µl (~10 Na/reaction)
Fluorescence @ 530nm (F1 LightCycler® Detector)
Cycle Number
(b)
No Template Controls
Human DNA 100 ng/µl (~ 10,000 Na/Reaction)
Human DNA 10 ng/µl (~ 1,000 Na/Reaction)
Human DNA 1 ng/µl (~ 100 Na/Reaction)
Fluorescence @ 705nm (F3 LightCycler® Detector)
Cycle Number

Fig. 2.



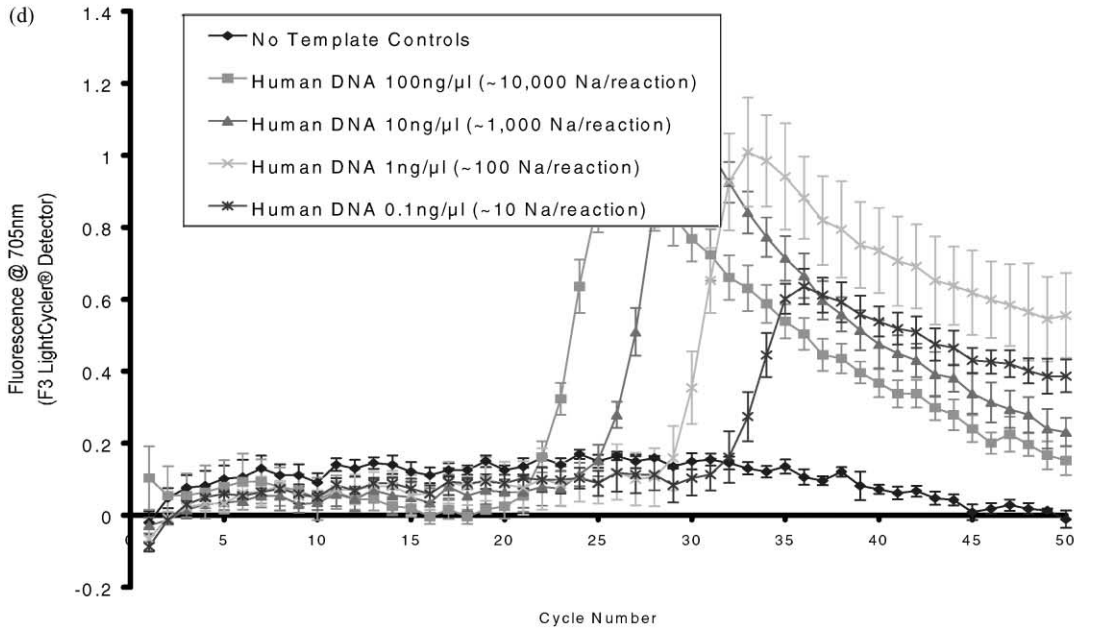


Fig. 2 (*Continued*).

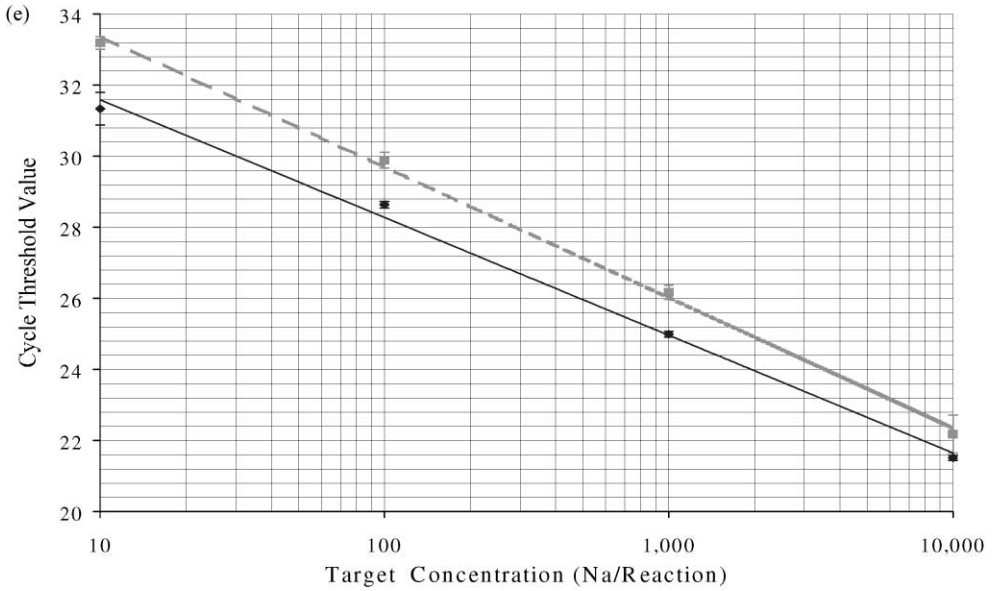


Fig. 2 (*Continued*).

2.2. Amplimers and probes

Custom oligonucleotide amplimers and custom fluorescent probes (see Fig. 1) for the ABI human β-actin amplicon were obtained from Oswel Research Products, Medical and Biological Sciences Building, University of Southampton, Boldrewood, Bassett, Crescent East, Southampton, SO16 7PX, UK.

2.3. Thermal cycler

The LightCycler® real-time PCR instrument and consumables were obtained from Roche. The colour calibration was set up using a modified protocol to allow analysis. This involved running the colour calibration programme using: Tube 1, Blank buffer only; Tube 2, Specific Product with SYBR®Gold; Tube 3, Tamra dye, and Tube 4 -Cy5. The thermal cycling protocols are: TaqMan® 50 °C hold for 1 min for carry over prevention, 95 °C hold for 1 min for initial denaturation, followed by 50 cycles of 95 °C 5 s denaturation, 60 °C 20–120 s annealing/extension, collecting fluorescence at the end of this step: SYBR®Gold/ResonSense®/Angler® 50 °C hold for 1 min for carry over prevention, 95 °C hold for 1 min for initial denaturation, followed by 50 cycles of 95 °C 5 s denaturation, 60 °C 5 s annealing, and 5 s extension at 74 °C, fluorescence was collected at the end of the annealing step.

For quantitative analysis cycle threshold values were determined using the second derivative maximum algorithm of the LightCycler® software.

3. Results and discussion

Fig. 2b shows amplification plot generated on the ROCHE LightCycler® using the ResonSense® reporting chemistry (Fig. 3). The target sequence was the human β-actin gene. The amplification was carried out using the ABI β-actin control amplimer sequences and the corresponding TaqMan® probe was used for comparing the performance of the assay. Male human placental DNA was used as the target template. The ResonSense® probe utilised a CY5.5 fluorescent Fluor as the acceptor fluorescent moiety, and SYBR®Gold DNA intercalating dye as the donor fluorescent moiety. The TaqMan® assay required an annealing hold step of at least 20 s, preferably 2 min at 60 °C, as is the

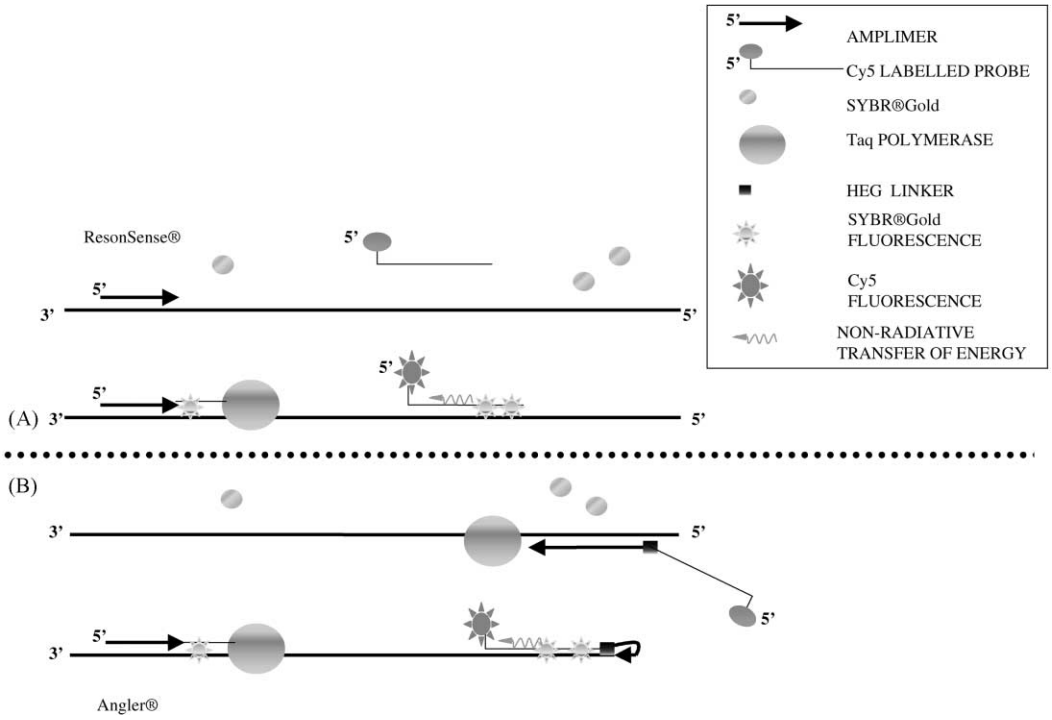


Fig. 3. Pictorial representation of ResonSense® and Angler® FRET reporting mechanism. (A) ResonSense®: during amplification free probe labelled with and acceptor moiety (Cy5.5) does not fluoresce because there is no donor fluorescence moiety within the distance required for energy transfer, however, when annealed to product SYBR®Gold DNA intercalater binds to product probe duplex and fluoresces behaving as a donor moiety in a FRET pair with the acceptor moiety on the probe. (B) Angler®: similar to (A) except that the probe is attached to the amplimer by a chemical linker brining it spatially close to the extended amplimer for efficient self-probing of product. In both embodiments both specific signal from the probe fluorescence and non-specific intercalater signal from all amplification products can be monitored. In the ResonSense® embodiment the 3′ of the probe is protected to prevent primer extension.

recommended cycling values for TaqMan® assays, to generate sufficient signal for accurate quantification. The ResonSense® probe produces optimum signal after 5 s at the annealing step. This is the minimal hold times recommended on the ROCHE LightCycler® which is the fastest commercial thermal cycler with an integral fluorimeter capable of analysing this type of assay.

Fig. 2d shows the same assay with a second embodiment of the probe system that we term Angler® (Fig. 3). The probe sequence was linked using established techniques to the reverse amplimer using a hex-ethylene glycol (HEG) linker [21,20]. The signal:noise ratio achieved in this embodiment is better. We believe this is due to more efficient hybridisation since the probe sequence is incorporated into the reverse strand of the amplimer. The "hook" effect observed at increasing cycle number is a result of the accumulating product strands hybridising, and thus, displacing, the probe from template (see Fig. 2b and d.

A 5′–3′ exonuclease deficient Taq DNA polymerase was used to ensure that the probes were not hydrolysed during the course of the reaction, although this was found not to be necessary because of the short hold times utilised. The F1 (520 nm per fluorescein) optical detector was used for detecting the non-strand specific amplification signal generated from the SYBR®Gold intercalating dye (Fig. 2a and c). The F3 (705 nm per C705 dye) optical detector was used for detecting the amplification of specific product using the signal generated by the probe. The probe system utilised CY5 fluorescent dye as the acceptor moiety, instead

of LC705, because of the better yield of incorporated dye during oligonucleotide synthesis. The peak emission of CY5 is 670 nm, so the detector does not measure the peak emission of the dye resulting in a lower signal-to-noise ratio than could be achieved with a bespoke optical unit for this assay. Melting experiments could be carried out showing that the probe melts from target at approximately 63 °C (data not shown).

Modified colour calibration protocols were required because of the discrete spectral capabilities of the ROCHE LightCycler®. The ResonSense® signal was determined using the colour compensation algorithms of the ROCHE LightCycler®. The drop in fluorescence observed in no template controls (NTC) is an anomaly of the colour compensation and background subtraction analysis of the ROCHE LightCycler®. The Angler® system did not require colour compensation because the amount of SYBR®Gold (1:200,000 of reference) required was below the threshold of the F3 detector. We believe that those instruments such as the ABI 7700 instrument with spectral optical detectors will be able to provide for a better method of analysis.

Using the rapid temperature transition times of the ROCHE LightCycler®, the ResonSense® and Angler® probe systems produced a considerable saving in cycling time over TaqMan® and other probe systems (data not shown).

The ability to monitor both intercalater (non-strand specific; Fig. 2a and c) and probe (strand specific; Fig. 2b–e) signal has advantages. In most probe formats it is difficult to control for the strand specific probe(s). Monitoring intercalater amplification improves the confidence of the assay because this will generate signal independent of the specific probe. In the second embodiment the probe is linked to the amplimer and therefore better controlled because reverse amplimer is required for amplification. The simple reporting mechanism means that the signal generated by these probe systems is generated from the probe binding to template and so is closely related to product concentration. The broad emission spectra of SYBR®Gold should make multiplexing the reaction possible with different coloured probes and/or melt profiles. This would be particularly useful for the inclusion of a competitive internal control [22] for controlling all assay components and reducing the risk of false positives. A second probe attached to the forward amplimer could be used to detect specific amplification of a control competitive mimic internal control using a second reporting dye.

The ability to decrease cycling times for strand specific PCR will improve the effectiveness to which PCR can be utilised in many application areas and on future faster thermal cyclers. The single fluor required on the reporting probe simplifies synthesis and purification, thus reducing costs. The simple mechanism of reporting amplification reduces the time needed for optimisation. ResonSense® probe systems therefore offer a more cost effective and a simple, faster method, for strand specific PCR.

We are currently optimising protocols for other targets and utilising different dye combinations for assay improvements.

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