

Analysis of gene expression in single oocytes and embryos by real-time rapid cycle fluorescence monitored RT-PCR

Nury Steuerwald^{1,2}, Jacques Cohen¹, Rene J.Herrera² and Carol A.Brenner^{1,3}

¹Gamete and Embryo Research Laboratory, Institute for Reproductive Medicine and Science of Saint Barnabas, West Orange, New Jersey, 07052, and ²Department of Biology, Florida International University, Miami, Florida, 33199, USA

³To whom correspondence should be addressed

Rapid cycle DNA amplification is a refinement of the polymerase chain reaction (PCR) method that permits increased product specificity while reducing amplification time by an order of magnitude. Combined with the use of micro volume capillaries, minute samples can be examined by this technique. Thus, this approach is ideally suited to the analysis of gene expression in individual cells. As the current understanding of early developmental processes is still rudimentary, further characterization of transcription in single oocytes and embryos may provide additional insight into the molecular mechanisms directing these events. In this study, we examined the suitability of fluorescence monitored reverse transcription (RT)-PCR for the study of gene expression during oogenesis and embryogenesis using transcripts of the housekeeping gene, β -actin, as an experimental model. Product accumulation was monitored by either the double-stranded DNA dye SYBR Green I or sequence-dependent hybridization of reporter molecules called molecular beacons. Dyes bind generically and are economical to use. However, both specific and non-specific products are labelled. Hybridization probes permit very specific and sensitive target recognition but they can be costly to manufacture. Once molecular markers indicative of optimal development are identified, this technology could be used in a clinical in-vitro fertilization laboratory as a diagnostic tool.

Key words: human oocytes/molecular beacons/preimplantation embryos/rapid cycling/RT-PCR

Introduction

Gaining knowledge about the physiological timetable of gene expression during oocyte maturation and preimplantation development is crucial for a better understanding of human development and refinement of assisted reproductive technology. It is necessary to understand what molecular markers are important for assessment of embryonic health, viability and genetic status of the mammalian oocyte. Once markers are chosen which reflect prognosis for development, techniques can be developed to extract minute amounts of cytoplasm or polar bodies for use as a diagnostic tool.

In order to pursue a detailed analysis of the processes of oocyte and embryo development, highly sensitive investigative methodologies are required. Ideally, the strategy selected would be sensitive enough for use in single cells to avoid analysis of pooled material that could otherwise confound interpretations. Analysis of gene expression from single cells has been hindered by the limitations of classical molecular techniques such as Northern blot analysis. The advent of reverse transcription-polymerase chain reaction (RT-PCR) provided the level of sensitivity necessary to study transcription despite a scarcity of material (Rappolee *et al.*, 1988; Brenner *et al.*, 1989). With further refinements such as rapid cycle DNA amplification, specificity and yield has been improved (Wittwer and Gerling, 1991) precluding the need for nested amplifications. This technique allowed the examination of extremely minute samples when used in conjunction with micro volume

capillaries (Wittwer *et al.*, 1997b). Coupled with radiolabelled probes, RT-PCR has permitted the analysis of expression from a small number of embryos (Rambhatla *et al.*, 1995). By the addition of fluorescent probes or double-stranded DNA (dsDNA) dyes, product accumulation can be monitored using a suitably equipped fluorescence temperature cyclers. Sequence-specific fluorescent probes allow for highly sensitive and specific target detection. With probe systems, fluorescence is typically achieved when a fluorophore and a quencher are separated either by distance or by hydrolysis. For example, hairpin primers (Nazarenko *et al.*, 1997) are designed in a manner to achieve fluorescence only when the primer, labelled with a fluorophore at one end and a quencher at the other, is made linear by incorporation into an amplification product. Hairpin probes or molecular beacons (Kramer and Tyagi, 1996) employ a similar scheme to obtain fluorescence. The beacon unfolds when it comes in contact with its complementary sequence within the PCR amplicon producing a fluorescent signal. Hydrolysis probes (Holland *et al.*, 1991) achieve fluorescence by separating the fluorophore and quencher through the 5' to 3' exonuclease activity of *Taq* polymerase during product extension. An alternate approach uses resonance energy transfer by adjacent hybridization probes (Wittwer *et al.*, 1997a). A pair of probes are designed such that the labelled ends are brought together during hybridization in order to obtain a fluorescent signal. In contrast, dsDNA specific dyes permit more generic product identification.

Early oocyte maturation in mammals is distinguished by active gene transcription that results in the production of vast amounts of various RNA species (Telford *et al.*, 1990). Some transcripts are required for oocyte-specific processes and metabolism while others are presumably stored for use during early embryonic development, prior to the activation of the embryonic genome at the 4–8-cell stage (Tesarik *et al.*, 1986; Braude *et al.*, 1988). The early embryo undergoes rapid cell proliferation prior to the emergence of two distinct cell populations, the inner cell mass and trophectoderm, at the blastocyst stage. Undoubtedly, this differentiation is the result of differential gene expression. Although striking progress in in-vitro techniques has permitted the study of oogenesis and embryogenesis at a molecular level, these processes still remain better described than understood. The specific transcripts of the oocyte and embryo have yet to be characterized. Clearly, further research is warranted in order to decipher the role that these messages play as well as to unravel the tapestry of responses that they mediate throughout development.

It is the aim of this investigation to employ rapid realtime RT-PCR fluorescent methods to analyse gene expression in oocytes and embryos. To demonstrate the suitability of fluorescence monitored RT-PCR for this purpose, transcripts of the housekeeping gene, β -actin, were used as an experimental model. Furthermore, in order to compare fluorescent probe technology, rapid cycle DNA amplification was monitored by two different techniques, one which uses the double-stranded DNA dye SYBR Green I (Molecular Probes, Eugene, OR,

USA), and the other which uses sequence-dependent hybridization of reporter molecules called molecular beacons.

Materials and methods

Oocytes and embryos

Spare human oocytes were obtained from patients undergoing assisted reproduction at The Institute for Reproductive Medicine and Science of Saint Barnabas following written consent and Institutional Review Board approval. Oocytes used in this study ($n = 21$) consisted of discarded immature oocytes (metaphase I) or mature oocytes (metaphase II) which failed to fertilize following insemination.

Mouse blastocysts were obtained from CB6F1 female mice stimulated for ovulation with 10 IU pregnant mare's serum (PMS; Sigma, St Louis, MO, USA) followed 49 h later with 10 IU human chorionic gonadotrophin (HCG; Sigma). The animals were immediately placed to mate with males. The females were killed by cervical dislocation upon detection of the copulation plug. Embryos were flushed from the excised oviducts and cultured for ~96 h in KSOM culture media (Cell and Molecular Technologies Inc, Lavallette, NJ, USA) to obtain blastocysts.

RNA isolation

Total RNA was isolated from individual oocytes and embryos using the Micro RNA Isolation Kit (Stratagene) as per the manufacturer's protocol except for the addition of 10 μ g glycogen (Boehringer Mannheim, Indianapolis, IN, USA) as a carrier, prior to precipitation with isopropanol.

Reverse transcription

First-strand complementary DNA was synthesized by priming with random hexamers. The lyophilized samples were resuspended in an 8.5 μ l solution consisting of 1 μ l 50 μ mol/l random hexamers, 0.2 μ l 0.1 mol/l dithiothreitol (DTT), 0.05 μ l RNase inhibitor (20 IU/ μ l) (RNasin[®]; Promega, Madison, WI, USA) and 7.25 μ l sterile nuclease free water. The hexamers were annealed by incubating the samples to 70°C for 6 min and immediately quenched on ice for 1 min. Reverse transcription was performed by the addition of 11.5 μ l containing 4 μ l 25 mmol/l MgCl₂, 2 μ l 10 \times PCR buffer II (Perkin Elmer, Foster City, CA, USA), 4 μ l dNTP 2 mmol/l, 1 μ l RNase inhibitor (20 IU/ μ l), and 0.5 μ l Maloney murine leukaemia virus (MMLV) reverse transcriptase (Gibco BRL, Grand Island, NY, USA) and incubated at 37°C for 60 min. The reaction was stopped by heating to 95°C for 5 min. The entire final product was used directly for PCR. Concurrently, commercially available liver total RNA, 1 μ g (Clonetech, Palo Alto, CA, USA) was processed as a positive control.

Table I. Polymerase chain reaction (PCR) primer and molecular beacon sequences

PCR primers	
Human β -actin	5'-GGCCACGGCTGCTTC-3' 5'-GTTGGCGTACAGGTCTTGC-3'
Mouse β -actin	5'-TGCGTGACATCAAAGAGAAG-3' 5'-GATGCCACAGGATTCCATA-3'
Molecular beacons	
Human β -actin	5'-6 FAM -GCTCGTCCATGCCAGGAAGGAACGAGC-DABCYL -3'
Mouse β -actin	5'-6FAM-GCGCGAGCCATCTCCTGCTCGAAGTCTAG TCGCGC-DABCYL-3'

Figure 1. Comparison of hybridization probes and dsDNA dyes for monitoring rapid cycle amplification. A 197 bp fragment of the murine β -actin gene was amplified from individual and pooled mouse blastocysts ($n = 100, 75, 50, 20$). Log fluorescence versus cycle number is plotted for each sample to permit simple visualization of the exponential phase of amplification. Molecular beacon hybridization probes (A) and SYBR Green I dsDNA dye (B) were used to monitor product accumulation. Both methods proved to be equally effective. Amplification was evident earlier from samples containing pooled embryos as expected. Nonetheless, product accumulation was observed from individual embryos following as few as 25 cycles. No product was detected from the negative control.

Figure 2. Fluorescence versus temperature: Melting curve profiles. (A) The preprogrammed melting protocol. During the melting segment, samples are slowly heated while monitoring fluorescence continuously in order to observe the loss of fluorescence that occurs at the denaturation temperature. The negative derivative of fluorescence with respect to temperature is plotted to generate a melting peak. The melting curve can be used to distinguish products since it is sequence specific. The melting peak observed for the negative control represents primer dimer formation whereas the peaks obtained from single oocytes and the positive control are consistent with the expected melting temperature of the specific polymerase chain reaction (PCR) product. (B) The derivative melting curve calculated for each of the samples corresponds with products identified by (C) gel electrophoresis (1 = liver positive control; 2–8 = 100, 75, 50, 20, 1, 1, 1 embryos; 9 = no template; 11 = 100 bp ladder).

Primer and probe design

Complementary DNA PCR primers for human β -actin were designed using Oligo primer analysis software (National Biosciences Inc, Plymouth, MN, USA) from DNA and RNA sequences obtained from GenBank (Benson *et al.*, 1998) for β -actin (Ponte *et al.*, 1984). The cDNA primers and probe for mouse β -actin were gifts from Ken Pierce (Hamilton Thorne Research, Beverly, MA, USA). Primer and molecular beacon hybridization probe (Midland; Midland, TX, USA)

sequences are presented in Table I. Secondary structure analysis was conducted to ensure template and probe compatibility using the Mfold server (Michael Zuker, Institute for Biomedical Computing, Washington University in St. Louis, St Louis, MO, USA).

PCR

PCR amplification was achieved using a Light-cycler (Wittwer *et al.*, 1997b), a combination micro volume fluorimeter and rapid

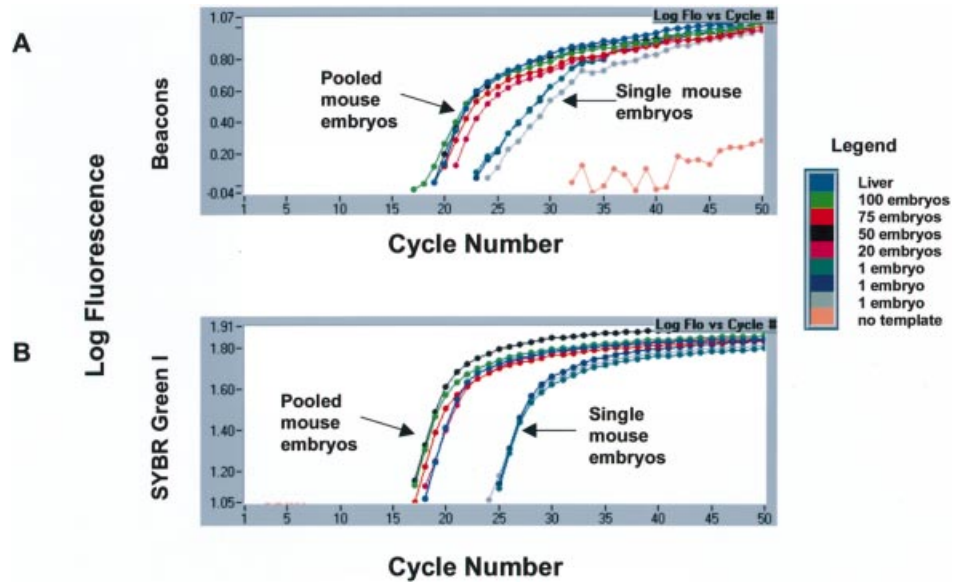


Figure 1.

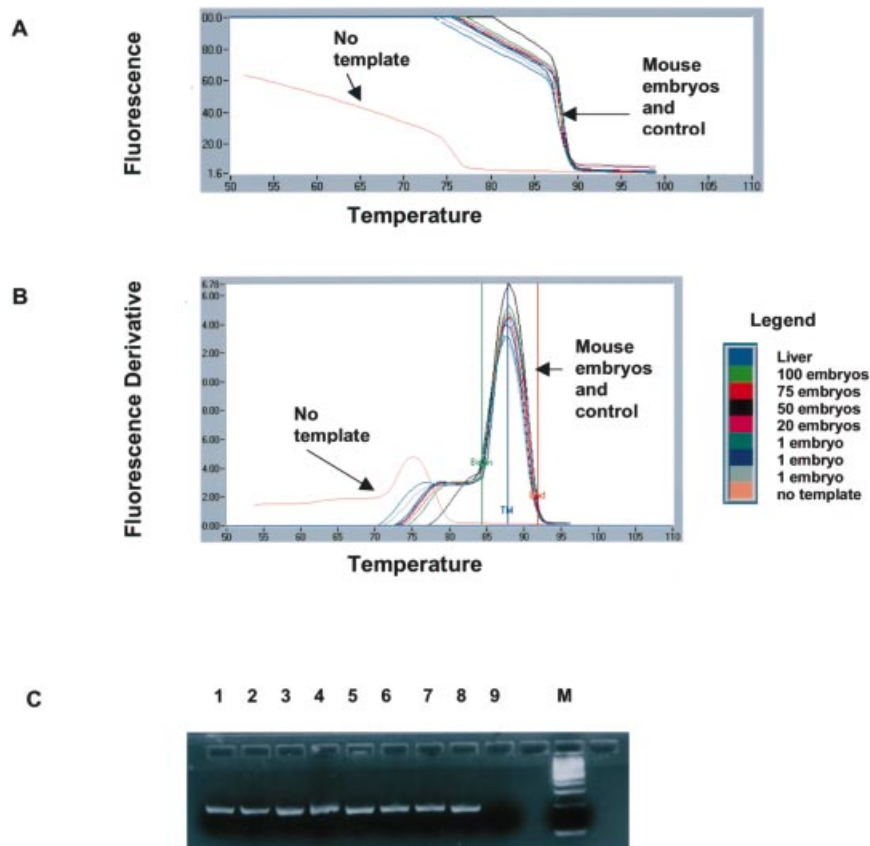


Figure 2.

temperature cycler (Idaho Technology Inc, Idaho Falls, ID, USA). The reaction mixture consisted of cDNA, 0.5 $\mu\text{mol/l}$ each primer, 200 $\mu\text{mol/l}$ each dNTP, 2–4 mmol/l MgCl_2 , 50 mmol/l Tris-HCl, 500 $\text{ng}/\mu\text{l}$ bovine serum albumin (BSA), 0.05 IU/ μl *Taq* DNA polymerase, and 11 $\text{ng}/\mu\text{l}$ TaqStart antibody (Clontech, Palo Alto, CA, USA). In addition, either the double-stranded DNA dye, SYBR Green I, 1:3000 of 10 000 \times stock solution (Molecular Probes, Eugene, OR, USA) or molecular beacons (1 $\mu\text{mol/l}$) were included in each reaction. A 7 μl volume was loaded into the glass microcapillary reaction vessels. The cDNA was denatured by heating to 96°C for 1 min. The template was amplified by 50 cycles of denaturation for 0 s at 95°C, annealing of primers (and molecular beacons, if applicable) at 60–65°C for 0 s and extension at 72°C for 10–20 s. Fluorescence data was acquired during annealing or extension for reactions containing molecular beacons or SYBR Green I, respectively. Thereafter, PCR products were identified by generating a melting curve. Since the melting curve of a product is sequence specific, it can be used to distinguish between them (Kirk *et al.*, 1997). Fluorescence was monitored continuously while slowly heating the samples in order to observe the loss of fluorescence at the denaturation temperature. The melting protocol consisted of heating the samples to 96°C followed by cooling to 50°C and slowly heating at 0.2°C per second to 97°C while monitoring fluorescence. The curve was then redrawn as the negative derivative of fluorescence with respect to temperature to generate a melting peak. Product identity was confirmed by ethidium bromide stained agarose gel electrophoresis and the sequence verified with the murine and human β -actin coding sequences.

Results

Results during amplification of a 197 bp fragment of the murine β -actin gene are presented in Figure 1. Reaction samples consisted of mouse liver (positive control), pooled mouse blastocysts ($n = 100, 75, 50, 20$), and individual mouse blastocysts. Amplification was evident from all samples while the negative control (no template) responded appropriately. However, additional amplification cycles (five for a total of 25) were required before fluorescence rose above background for samples consisting of individual embryos whereas only 20 cycles were required for pooled samples or the liver cDNA positive control. Both molecular beacon fluorogenic hybridization probes and SYBR Green I dsDNA dye were used to monitor fluorescence. Both methods were equally effective at detecting product accumulation. Nonetheless, higher overall fluorescence was observed with SYBR Green as compared to that obtained with molecular beacons. Amplification products

were identified by melting curve profile analysis and confirmed by gel electrophoresis (Figure 2).

A 208 bp fragment of the human β -actin gene was amplified from individual oocytes (Figure 3). Fluorescence was monitored by hybridization of molecular beacons as well as with SYBR Green I. Again, additional cycles of amplification (10) were required before the threshold cycle was reached for individual oocytes as compared with the positive control amplicons. However, the threshold cycle number varied among single oocytes which may be indicative of their quality and/or be due to their varying stages of maturation. Nonetheless, product accumulation was observed following as few as 30 cycles of amplification. As before, both fluorescence systems yielded comparable results. Sample fluorescence for the negative control was indistinguishable from background as expected.

In order to compare the sensitivity of fluorescent monitored product accumulation versus conventional product identification by gel electrophoresis, the minimum number of cycles required for product detection with each method was determined. The threshold cycle using SYBR Green I to detect murine β -actin amplicons is cycle 12. In contrast, a minimum of 20 cycles were required to detect a faint band corresponding to the PCR product produced from the same liver cDNA template by ethidium bromide stained gel electrophoresis (Figure 4).

Discussion

Fluorescence monitored rapid cycle RT-PCR from individual embryos and oocytes can be used as a tool for the study of early developmental processes. Increased sensitivity was illustrated by the ability to detect transcripts from individual cells during single round amplifications. In particular, SYBR Green I monitored product accumulation allows earlier product detection than possible by conventional gel electrophoresis. Rapid cycle thermal cyclers by design permit nearly instantaneous transition times. Specificity is improved in comparison with conventional block cyclers when minimal length anneal and denature segments are used (Wittwer and Gerling, 1991). This is especially beneficial for quantitative analysis as the need for two rounds of competitive PCR is obviated and the potential for introducing contamination in the process is minimized. Therefore, unambiguous quantification is feasible.

Figure 3. Fluorescence monitored polymerase chain reaction (PCR) amplification of β -actin in individual human oocytes. A 208 bp fragment of the human β -actin gene was amplified from single oocytes and positive control amplicons. Predictably, fluorescence was observed earlier for the control sample. Nonetheless, amplification was evident for single embryos following ~30–40 cycles. The variation may be due to sample quality and/or stage of maturation. Log fluorescence vs. cycle number is plotted using (A) molecular beacon hybridization probes and (B) SYBR Green I dsDNA dye. (C) The appropriate derivative melting curve corresponding to the amplified product and primer dimer formation.

Figure 4. Comparison of continuous fluorescence monitored polymerase chain reaction (PCR) sensitivity to conventional electrophoresis. A fragment of the murine β -actin gene was amplified from positive control liver cDNA. (A) Fluorescence was monitored continuously during amplification using SYBR Green I dsDNA dye. Fluorescence rose above background during cycle 12, the threshold cycle. (B) This fragment was also amplified from the same template in 11 separate reaction vessels for 15–25 cycles respectively. A faint product first becomes visible on an ethidium bromide stained agarose gel following 20 cycles of amplification. The intensity of the band increases with increasing cycle numbers, as expected. Fluorescence monitored PCR permits earlier detection of PCR products than possible by conventional gel electrophoresis. Product accumulation was apparent eight cycles earlier with this technique.

In our study, we selected fluorogenic hybridization probes called molecular beacons and the dsDNA dye SYBR Green I to monitor product accumulation. In our hands, both technologies

proved to be equally well suited to this purpose confirming the observations made by others (Wittwer *et al.*, 1997b). However, their differences must be taken into account during

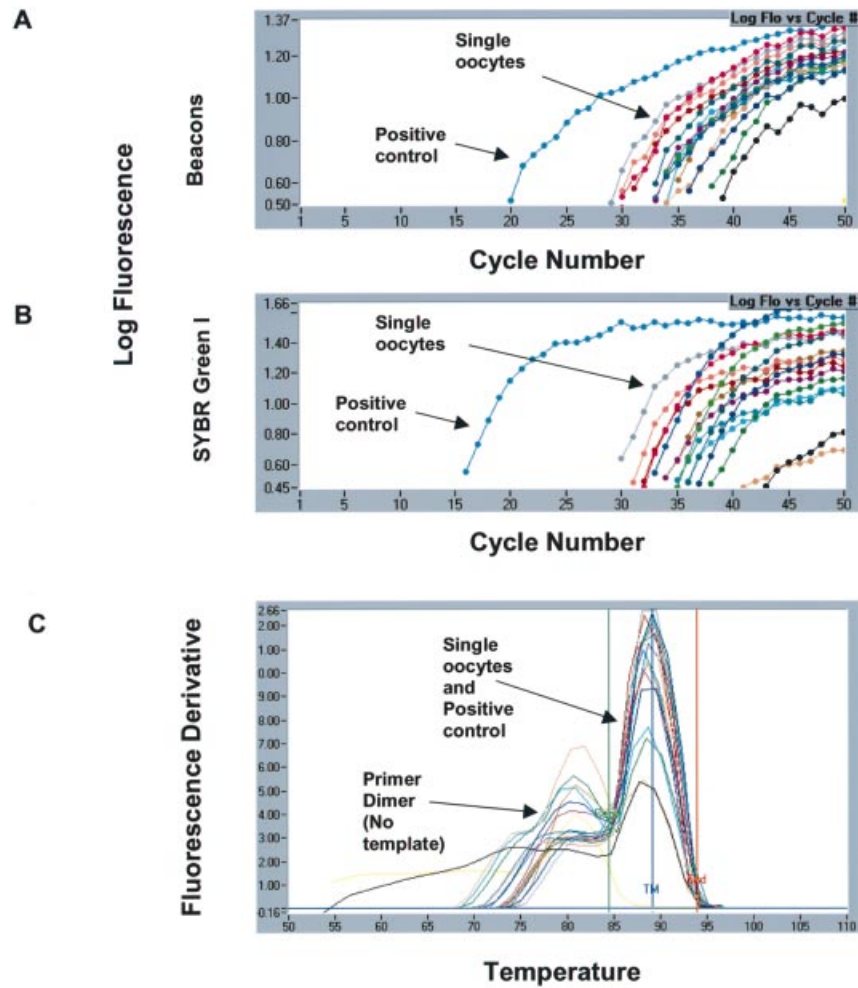


Figure 3.

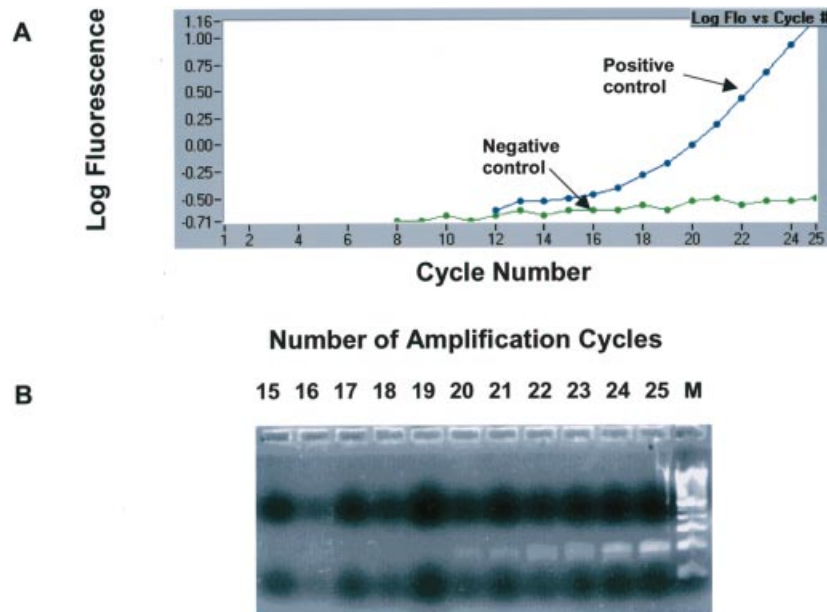


Figure 4.

experimental design and optimization in order to achieve ideal results. In particular, secondary structure is a prime consideration with molecular beacons whereas it appears not to be a crucial factor when using dsDNA dyes. Not surprisingly, template secondary structure appears to affect the efficiency if not the ability of the molecular beacon to bind to its target. Theoretically, higher anneal temperatures can alleviate this problem. However, if too high an annealing temperature is selected to make the template linear, then probe hybridization may be impeded as the beacon's melting temperature is approached. Consequently, primers must be designed to yield templates such that the formation of hairpins at the optimum anneal temperature of the beacon is minimized. We found that these complications can be more readily overcome with amplicons that are shorter in length. In fact, we were unable to attain hybridization when the amplicon length exceeded a few 100 bp in length (data not shown). Accordingly, the thermal profile of the beacon alone as well as in conjunction with target must be considered during their design and use. Such analysis can be conducted with modelling software such as Mfold server, available on the internet (<http://mfold.wustl.edu/~folder/dna/form1.cgi>) to ensure template and probe compatibility.

Double-stranded DNA dyes do not appear to be subject to difficulties stemming from template structure. However, reaction conditions do play a role in their use. Since dsDNA dyes bind generically by intercalation, it is not possible to discriminate between the accumulation of non-specific products and that of the product of interest. Therefore, the reaction must be optimized to precisely amplify the desired target. Alternately, fluorescence can be acquired at the specific amplicon's melting temperature if it can be distinguished from that of non-specific products (Morrison *et al.*, 1998). Hot-start PCR can further enhance the accuracy and sensitivity of the reaction (Morrison *et al.*, 1998). It should be noted that working solutions of the dye may only be stable for a few days especially if diluted in water. Therefore, these solutions should be prepared frequently and stored in a light-protected container in order to ensure maximal staining sensitivity. With these considerations in mind, SYBR Green I provides for a simple and inexpensive means of monitoring fluorescence during amplification.

By employing rapid real-time RT-PCR fluorescence capable of analysing expression at a single cell scale and likely on a sub-cellular level, we hope to gain a better insight into the molecular events directing early developmental processes. Thus, it would no longer be necessary to rely on morphological, chromosomal or metabolic criteria for oocyte and embryonic assessment if more reliable molecular markers indicative of enhanced prognosis can be identified (Gardner and Schoolcraft, 1998; Munné *et al.*, 1998). In addition, it may be possible to screen for genetic disorders based on quantitative expression rather than by gross aberrations alone. Furthermore, molecular beacons provide a convenient means to detect multiple mutations simultaneously (Kostrikis *et al.*, 1998). Ultimately, the goal is to apply this technology in a clinical in-vitro fertilization (IVF) setting by sampling cytoplasm

during intracytoplasmic sperm injection (ICSI) or polar bodies to provide a more in-depth preimplantation diagnostic tool.

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