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Fluorescent-Based Genetic Analysis for a Microfluidics Device

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Contract Scientific Authority: D. Bader

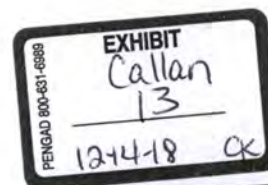
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Abstract for Part I

This work concentrated on transferring polymerase chain reaction (PCR) assays to a microfluidics format with fluorescent detection, with the ultimate goal of creating a rugged microchip for performing PCR in the field. Several parameters were explored with regards to transferring the assay to a microchip, including substrate suitability; effect of additives; and decreased volume on product formation and reproducibility. The phosphoglucose isomerase (PGI) gene from *Erwinia herbicola* was successfully cloned and characterized as the template for all subsequent PCR reactions. Additives such as DMSO and formamide appeared to be useful to reduce the denaturation and annealing temperatures of real time PCR assays. BSA interfered with SYBR Green I fluorescent detection in real time but was required for PCR success. Complete PCR master mixes including enzyme and template were found to be stable for up to 24 hours at room temperature and dried PCR reagents stored at room temperature retained their activity for 14 days; The plastic polydimethyl siloxane (PDMS) was found to be a suitable reaction medium for PCR and fluorescent detection using SYBR Green I dye.

The PCR reaction volume limited the amount of product obtained through PCR; reaction volumes of less than 10 μL required special handling to ensure product was reproducibly obtained. The variability of results in small volume PCR was attributed to local reagent concentration effects due to evaporation and condensation of water from the constant heating and cooling during cycling. This will impact any PCR assay in a microfluidics device, as evaporation becomes a key limiting factor.

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Executive summary for Part I

Introduction

This work concentrated on transferring polymerase chain reaction (PCR) assays to a microfluidics format with fluorescent detection, with the ultimate goal of creating a rugged microchip for performing PCR in the field. Several parameters were explored with regards to transferring the assay to microchip, including substrate suitability, effect of additives, and implications of PCR at the small volumes that would be required for a microfluidic format.

Results

To perform PCR on a microfluidics platform it is essential to use an established optimized standard PCR assay with a well-defined DNA template and DNA PCR primers. The phosphoglucose isomerase (PGI) gene was successfully cloned from the target bacterium *Erwinia herbicola* using modified test primers. This gene was characterized and used as the template for all subsequent PCR reactions.

Complete PCR master mixes including enzyme and template were found to be stable for up to 24 hours at room temperature. PDMS was found to be a suitable reaction medium for PCR and fluorescent detection using SYBR Green I dye. Additives such as DMSO and formamide appeared to be useful to reduce the denaturation and annealing temperatures. BSA was found to interfere with fluorescent detection but was required for PCR success. Dried PCR reagents stored at room temperature appeared to retain their activity for 14 days; strict quality control measures need to be taken to prevent contamination of dried reagents.

The PCR reaction volume limited the amount of product obtained through PCR; reaction volumes of less than 10 μL required special handling to ensure product was reproducibly obtained. Sephadex G-50 beads as a water trap appeared to improve PCR reaction efficiency using small volumes.

Significance

The variability of results in small volume PCR was attributed to local reagent concentration effects due to evaporation and condensation of water from the constant heating and cooling during cycling. This will impact any PCR assay in a microfluidics device, as evaporation will become a limiting factor in reactions, both in terms of the amount of product obtained and in specificity of the test system for detecting the target. This consideration will be increasingly more important if multiplex PCR is planned for the microchip.

Future Plans

The variation in the amount of product obtained in small volume PCR assays appears to be an interesting avenue to explore in further studies. Controlling the surface area at which the reaction takes place, or changing the physical geometry of the PCR reaction (e.g. depth vs. thin film) may improve the results considerably. The effect of local reagent concentrations can readily be investigated by decreasing the amount used in small volume PCR reactions. The effect of svPCR on reaction stringency also remains to be investigated.

Marenco A, Crichton M, Jager S, Dickinson Laing T, Mah M, Mah D, Chan N and Tang T. 2008. Fluorescent-Based Genetic Analysis for a Microfluidics Device. CWBios Inc.

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Fluorescent-Based Genetic Analysis for a Microfluidics Device—Part One (Phases I and II). PCR Analyses for Microchip Development.

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Introduction

Identification of microorganisms, including viruses, is fundamentally determined by differences in their genomic sequences. The ideal method for identification of organisms is to obtain their entire unique genomic sequence if possible. However, genetic analysis is more typically based on the identification of unique stretches of DNA within the target genome by hybridization with complementary probes and/or restriction fragment length polymorphism (RFLP).

Detection methods based on probe hybridization or restriction enzyme mapping require relatively large amounts of target DNA to be effective. In situations of very low genomic copy numbers or in low numbers of target organisms, it is generally necessary for the target sequence to be amplified by PCR (polymerase chain-reaction) thermocycling. The size and DNA sequence of the resulting product is then used to positively identify the target organism.

In all genetic analysis methods, high degrees of sequence homology between the DNA of target organisms and that of other closely related non-target organisms, often limit target specificity. In closely related organisms, it may be necessary to assay for secondary and tertiary unique DNA target sequences to distinguish between target and non-target organisms. Hence judicious probe or primer selection represents the greatest obstacle to the rapid development of diagnostic PCR primer sets for a wide range of target organisms of medical, military or economic interest. Using more numerous specific DNA probes or primers ensures a higher degree of confidence in the positive identification of particular organisms.

This proposal aims to address several key issues in the development of genetic-based biodetection devices. The first is speed and sensitivity, i.e. the ability to rapidly detect very small amounts of DNA of target organisms. This is done using fluorescent-based

amplicon detection technology, which measures real-time PCR production of amplicon DNA using fluorescent intercalating DNA dyes (Cepheid SmartCycler®). The integration of DNA sequencing with amplicon detection will allow the definitive identification of the target organism based upon known DNA sequences. While this does not exclude or prevent amplification of homologous DNA from non-target organisms, it does provide a secondary step to positively identify the origin of the amplified material. Finally, this work supports the development of an economical and compact microfluidics-based thermocycling device designed for rapid detection under rugged field conditions.

1.1. Approach and Objectives

This work was divided into three phases, some of which overlapped. Phases I and II, which were related, are discussed in Section 1 of this report; Phase III is discussed in Section 2 that follows (Interaction of tethered-dye gene probes (TDP) with complementary target sequences).

Phase I. Development of fluorescent-based genetic analysis capability “off-chip.”

This work involved the following key points:

- **Target Preparation:** including foreground data for *E. herbicola* clone pPL6; characterization of pPL6 signature target DNA; isolation and cloning of the 380bp *E. herbicola* phosphoglucose isomerase (PGI) gene; and characterization of *E. herbicola* PCR targets (melting points and heterologous DNA preparation).
- **PCR Product Synthesis:** using the PGI gene fragment as the template and the 18CA14 primer set, optimizing PCR of the target DNA with respect to effects of reaction volume; denaturation steps (including gradients); annealing and extension steps; effects of additives such as BSA or Tween-20; analysis of technical and systematic errors (*e.g.* in the effect of small reaction volumes); minimizing reagent contamination during reagent preparation and

experimental protocols; integrated PCR amplification with sequencing; and fluorescent amplicon detection using real-time PCR.

- **PCR Product Analysis:** including PCR products from optimizations; fluorescent PCR amplicon detection; and PCR amplification/sequencing reactions.

Phase II. Genetic Analysis of “on-chip” cycling reactions.

This work involved the following key points:

- **Material compatibility:** including developments in polydimethyl sialoxane (PDMS) PCR vessels; and PCR amplification of *E. herbicola* PGI using PDMS reaction vessels.
- **Stabilization of PCR Reagents:** including effects of temperature and storage duration pre-mixed aqueous, pre-mixed freeze-dried, or pre-mixed dried, PCR assays containing template, primers and Qiagen HotStar™Taq polymerase.
- **Adsorption of PCR reagents and products onto container surfaces:** including effects of BSA and Tween-20 (and other assay additives) on PCR assays.
- **Small-volume PCR and evaporation effects:** including reductions of melting temperature and melting time during assays; minimization of the PCR cycling program; modification of amplicon size.

Phase III. Interaction of tether-dye polynucleotide (TDP) gene probes with complementary target sequences.

This work (discussed in Section 2 of this report) involved the following key points:

- Thiazole orange (TO) fluorescent properties.
- Thiazole orange (TO) probe binding constants
- Real-time PCR by TDP
- TDP-PCR primers

Materials and Methods

1. Cloning of *Erwinia herbicola* phosphoglucose isomerase (PGI)

1.1. PCR Primer Design for Cloning

Erwinia herbicola (also known as *Pantoea agglomerans*) is a ubiquitous Gram negative soil bacterium that is used as a bacterial bio-threat simulant for testing detection and assay methods at DRDC Suffield. The phosphoglucose isomerase (PGI) gene was cloned from *E. herbicola* using modified PCR primers that are routinely used by DRDC Suffield in PCR assays. The parental 18CA14 forward and reverse primers for PGI were kindly provided by D. Bader of DRDC Suffield. Sensitivity and specificity of the primer set 18CA14 for *E. herbicola* PGI was first confirmed in PCR assays using genomic DNA from other related *Erwinia* species (*E. ananas*, *E. carotovora* and *E. uredovora*). The primers were modified for use in cloning *E. herbicola* PGI by adding a *Sph* I restriction site to the 18CA14 forward primer, and an *EcoR* I restriction site to the reverse primer (Figure 1-1). The parental and modified 18CA14 primers were synthesized by Operon Technologies (Huntsville AL) or by the University of Calgary Core DNA Synthesis Laboratory (Calgary AB) and purified by HPLC or polyacrylamide gel electrophoresis respectively. PCR was performed using the PCR Core Kit by Roche (Penzberg Germany).

Forward Primer

Actual sequence	TTACGACCAGTACATGCACC GTTTGGCCGCCTATTTC
<i>Sph</i> I	GCATGC
New primer	CGACCAGTGCATGCACC GTTTGGCCGCCTATTTC

Reverse Primer

Actual sequence	GAGTCTGTAGCCCATATCGTG CCGTTCAAG
Reverse Complementary	CTTGAA CGG CACGATATGGGCTACAGACTC
<i>EcoR</i> I	GAATTC
New Primer	TGAA CGG CACGATATGGGCTACAGACTC

Figure 1-1. Modifications to 18CA14 PCR Primers for Cloning *E. herbicola* PGI.

Legend: Bold region = original 18ca14 primer
Blue region = extra bases (region where the new restriction site must be present)
Red region = site of the restriction site
Green box = modifications to the new primer based on original sequence

1.2. Touchdown PCR Conditions

All touchdown experiments were conducted in an Eppendorf MasterCycler gradient thermocycler (Eppendorf, Hamburg Germany) or a DNA Engine Gradient Cyclers PTC-200 (MJ Research/Bio-Rad Laboratories, Waltham MA). Touchdown PCR conditions were as follows: initial denaturation/HotStar™ Taq activation, 95 °C for 15 min; denaturation, 94 °C for 1 min; touchdown annealing, starting at 72 °C for 1 min then reducing the annealing temperature by 2 °C each following cycle to 66 °C; extension for 2 min at 72 °C, for 3 cycles. After this, non-touchdown PCR conditions for up to 40 cycles used 94 °C for 1 min, 66 °C for 1 min and 72 °C for 2 min with a final extension at 72 °C for 7 min. Products were stored at 4 °C in the PCR instrument until recovered.

1.3. Non-Touchdown PCR Conditions

Non-touchdown PCR conditions used 94 °C for 1 min, 66 °C for 1 min and 72 °C for 2 min for up to 45 cycles as described in the experiments, ending with a final extension at 72 °C for 7 min. Products were stored at 4 °C in the PCR instrument until recovered.

1.4. Cloning of PGI

The PGI sequence was obtained from *E. herbicola* genomic DNA (10^6 molecules) using touchdown PCR conditions and modified primers as described. The resulting product was separated on a 1% agarose gel in TAE buffer. The PCR product (expected length 380 bp) was excised from the gel and purified using the QIAquick gel extraction kit (Qiagen, Mississauga ON) according to the manufacturer's directions. The fragment was digested with *Sph*I and *Eco*RI in OnePhorAll Buffer Plus (Amersham Bioscience, Baie d'Urfé, PQ) for one hour at 37 °C according to the manufacturer's directions. The digested fragment was gel-purified as described above, then ligated into *Sph*I/*Eco*RI-digested pDrive vector (Qiagen) using T4 DNA ligase (Gibco-BRL/Invitrogen, Mississauga ON) and a 3:1 or a 10:1 fragment: vector ratio. Ligations were used to transform 50 µL of chemically competent *E. coli* (One Shot Max Efficiency DH5α-T1 Competent Cells, Invitrogen), using a modified transformation protocol supplied by the manufacturer. Briefly, after the plasmid construct was added to the competent cells, they were incubated for 30 min on ice, then heat-shocked for 90 sec at 42 °C and immediately transferred to ice for 2 min. SOC media (250 µL) was added to each vial followed by incubation at 37 °C for 1 hour at 225 rpm. Finally, 10 µL of each reaction was plated onto LB-agar plates containing 100 µg/µL ampicillin and incubated overnight at 37 °C. Twenty colonies from each plate were picked using a pipette tip, inoculated in 25 µL sterile dH₂O in a 0.5 ml tube and re-plated onto a master plate. The colonies were labeled clones 1 to 20 for 3:1 ratio and clones 21 to 40 for 10:1 ratio. For the other two plates (the negative and positive control), two colonies of each were picked, inoculated into sterile distilled water and plated. The plates were stored at 37 °C overnight. Each inoculated clone was screened by PCR using the PCR conditions described above. Products were confirmed by agarose gel electrophoresis. Twelve positive clones were obtained

for the 3:1 fragment: vector ligation, and 16 colonies were obtained from the 10:1 fragment: vector ligation (not shown).

Clones 1, 16, 30 and 34 were picked from the master plate and streaked on agar/Amp₁₀₀ plates. These plates were incubated overnight at 37 °C. The following day, these colonies were inoculated into 3 ml LB broth/Amp₁₀₀ media. Also, since the experiment produced numerous clones, we inoculated 3 ml of LB broth/Amp₁₀₀ with clones 18&19, and 37&38, directly from the master plate. The inoculated media was incubated for 8 hours at 37 °C and 225 rpm. Then, one ml of inoculation was added to 500 ml LB broth/Amp₁₀₀. Because the two batches 18&19 and 37&38 were not as cloudy as the other inoculates, we combined 1.5 ml of each batch into 500 ml LB broth/Amp₁₀₀ ("Combined"). All cell mixtures were incubated overnight at 37 °C and 225 rpm for large-scale plasmid purification.

Plasmids were purified using the Qiagen Plasmid Mega Kit and samples were analyzed by *EcoRI* digestion and agarose gel electrophoresis in TBE buffer. Most samples showed evidence of bacterial chromosomal contamination. Plasmids were ethanol-precipitated and dried for long-term storage at room temperature. These plasmids were used for PCR product amplification in all subsequent studies.

1.5. Agarose Gel Electrophoresis

Samples were analyzed on a 1% agarose gel (1 g agarose per 100 mL fluid including 0.5 µg/µL EtBr) in either TAE (Tris-acetate-EDTA) or TBE (Tris-borate-EDTA) buffers. Samples (5 µL) were made to 10 µL using 4 µL distilled water and 10x gel loading buffer. Gels were run at 90-110 V for 1-1.5 hours.

1.6 Real-Time PCR Assay Conditions and Melting Point Determinations

PCR was performed in a Cepheid SmartCycler® using the following conditions for PGI plasmid/18CA14 primers: 1x PCR buffer (Qiagen), 2.5 mM MgCl₂, 0.2 mM dNTPs, 1:25000 dilution of SYBR Green I (Molecular Probes, Eugene OR), 1 mg/mL BSA and 0.025 U/μL Hotstar™ Taq. The standard thermocycling program used began with inactivation at 95 °C for 15 min followed by 40 cycles, each consisting of denaturation at 94 °C for 45 seconds; annealing at 66 °C for 75 seconds; extension at 72 °C for 75 seconds. A final extension was at 72 °C for 10 minutes with a decrease to ambient temperature. For product DNA melting curves, temperature was started at 40 °C, increasing to 95 °C at 0.2 °C/second using the Optic 1 channel. The temperature at which the fluorescent signal was lost, was deemed the melting temperature of the PCR product.

2. Drying of PCR Reagents

PCR reagent samples (buffer, dNTPs, magnesium, *Taq*, primers and Q-solution) were prepared in a master tube without adding water, then dispensed into 20 separate tubes. Samples (10) were either stored at 4 °C until use, or frozen at -70 °C for 30 minutes, followed by drying for up to 3 hours in an Automatic Environmental SpeedVac with Vapornet AES 1000 (Savant). Of these samples, 5 were used immediately after drying, while 5 were stored at room temperature for 24 hours before use. On use, water and PGI template were added under non-sterile conditions then subjected to PCR as described above under non-touchdown conditions.

2.1. Short-Term Stability of Pre-Mixed PCR Reagents

One complete PCR assay mixture was prepared containing 0.5 ng pPGI per 50 μL, 18CA14 primers, and HotStar Taq and left at room temperature. Aliquots (50 μL each) were stored at -20 °C, 10 °C or room temperature for up to 24 hours. One set of

samples was immediately subjected to PCR as a control time point for storing all samples at -70 °C. At different time points, aliquots were removed and stored at -70 °C until analysis.

2.2. Long-Term Stability of Pre-Mixed PCR Reagents

Master mix (without template) was prepared and 49 µL aliquots were transferred into 0.5 mL autoclaved thin walled Certified PCR Clean Eppendorf tubes. Samples were dried down in the Speed-Vac for 2 hours then stored at room temperature until the indicated time point. At each time point (0, 1, 2, 3, 7, 14, 21 and 28 days) 7 samples (5 assay samples and 2 negative controls) were reconstituted with 43 µL Nanopure water and 1 µL template, then subjected to regular PCR conditions as described above. All products were analyzed on 1% agarose/EtBr gel.

3. Small Volume PCR with Sephadex G-50

3.1. Small Volume PCR (sv)

Small volume PCR experiments were conducted in one of three different machines: The Idaho LightCycler, the Eppendorf MasterCycler and the MJ ThermoCycler. Master mixes including pPGI template and 18CA14 primers, were prepared and aliquoted in volumes down to 1 µL for each of the PCR machines. Quantization was performed by SYBR Green I fluorimetry using a Quantech digital fluorimeter.

Validation of the Roche and Eppendorf PCR reagent kits for small volume (sv) PCR was carried out varying the amounts of Roche Faststart Taq polymerase, BSA, Tween-20 and use of GC-rich solution. For the MJ ThermoCycler and Eppendorf MasterCycler, the PCR conditions used: 95 °C 5 minute activation; 34 cycles of (1 minute denaturation at 94 °C, 1 minute annealing at 55 °C, and 2 minute extension at 72 °C); lid temperature: 105 -110 °C; and volumes down to 1 µL. The Idaho

LightCycler used: 95 °C 5 minute activation; 34 cycles of 0 seconds denaturation at 94 °C, 5 seconds annealing at 55 °C, 20 seconds extension at 72 °C); and volumes down to 1 µL. The amounts of product visualized on a 1% agarose gel were rated on a scale of (-) up to (+++).

3.2. Sephadex G-50

Complete PCR master mixes (including DNA template and Faststart Taq polymerase) were prepared and used to rehydrate dried Sephadex G-50 beads at room temperature for 15-20 minutes. After thermocycling, the assay solution was recovered by centrifugation of the mixture through a microtube frit insert (Pharmacia Bioscience) into a 1.5 mL microfuge tube. The DNA recovered in the flow-through was then subjected to agarose gel electrophoresis and quantified using SYBR Green I fluorescence.

Results and Discussion

1. Cloning and Characterization of *E. herbicola* Signature Targets (PL and PGI genes).

We initially cloned the *E. herbicola* tyrosine phenol-lyase gene (TPL) from genomic DNA, intending to use this gene as the reporter construct for PCR assays. Previous work establishing a cycling probe technology (CPT) assay for *E. herbicola* (PWGSC Contract # W7702-97-R646/001/EDM) used TPL as the target because of unique sequence elements found within this gene. PCR cloning primers were designed from the *E. herbicola* TPL gene sequence (GENBank accession no. D13714) (Figure 1-2) and used to clone the gene into the DNA vector pRSETB (Invitrogen).

Name	Length	Sequence
EHTPL2 (+) primer*	29 nt	AT TAG AGC TCA ATG AAC TAT CCT GCC GAG
EHTPL2 (-) primer*	30 nt	GG GGA AGC TTG TTA AAT AAA GTC AAA ACG C

Figure 1.2. TPL Gene Primers for Cloning and PCR

A 1600 bp PCR amplified product was obtained using purified pL6 plasmid DNA. We found the pL6 product was readily amplified using *Pfu* DNA polymerase (Promega) but not by *Taq* DNA polymerase (Invitrogen/BRL). It was possible that the pL6 gene fragment contained GC-rich regions or hairpins that were only resolved for PCR amplification using the stringent conditions provided in the *Pfu* reaction buffer (which contains Triton X-100) (Figure 1-3).

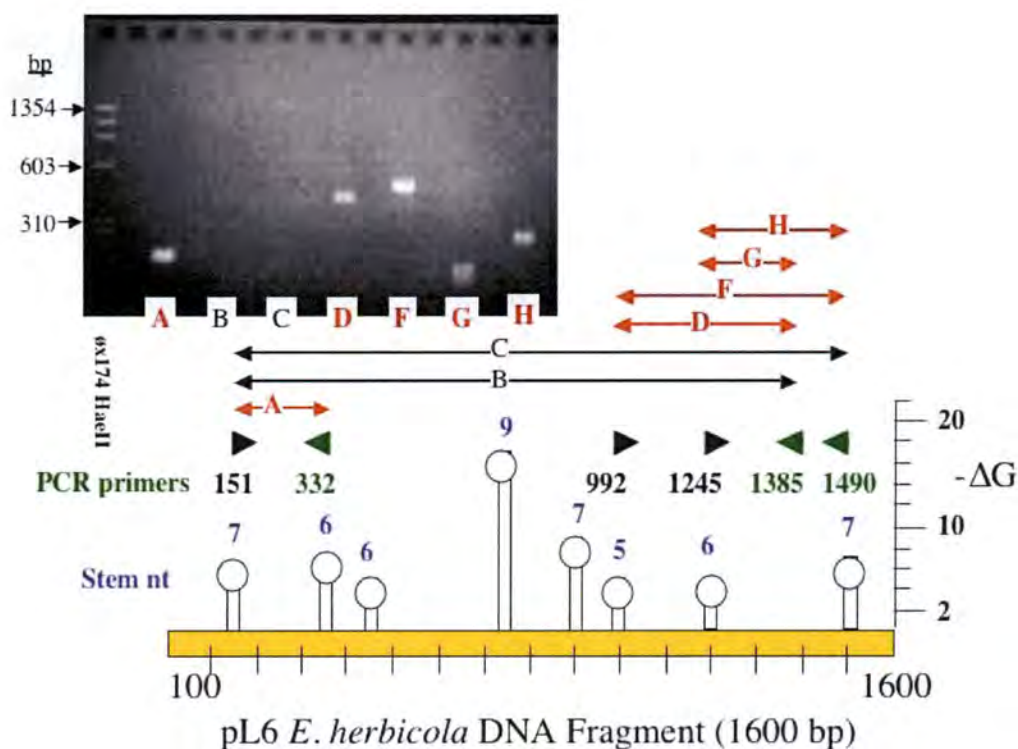


Figure 1-3. PCR Amplification of pL6.

Both the full-length cloned product (1600 bp), primer locations, and subsequences are shown. Scale to the right indicates the amount of energy required to resolve the hairpin structures within the sequence.

The 1600 bp size of the pL6 PCR product was too large for rapid PCR amplification (i.e. short PCR cycles). We thus sequenced the pL6 gene and designed unique primers for a smaller amplicon (100-200 bp) within the gene (Figure 1-3). The new amplicon was designed to work optimally with both *Pfu* and *Taq* polymerases. We also initiated work using the SmartCycler® (Cepheid, Sunnyvale CA) with this amplicon target/primer set. Using three different primer sets designed from the PL6 sequence, it was determined that this alternate cloned DNA fragment was unique to *E.*

herbicola. No PCR products were obtained using any of the three primer sets when tested on *E. ananas*, *E. uredovora*, *E. carotovora* or *E. coli* genomic template DNA.

On consultation with the contract scientific authority (D. Bader) it was decided to use a primer/target system developed at DRDC Suffield as the signature PCR target system for this study, in order to maintain congruity between our results and PCR results routinely obtained at DRDC. The DRDC 18CA14 primers are specific to the *E. herbicola* phosphoglucose isomerase (PGI) gene and have been previously well characterized. Although initial characterization of PL6 was completed, we discontinued using this clone and concentrated on the PGI/18CA14 system.

E. herbicola PGI was successfully cloned from genomic DNA using *SphI/EcoR* I-modified 18CA14 primers. Figure 1-4 shows a colony screen of various pPGI clones obtained using the pDrive vector (Qiagen). Restriction enzyme analysis and sequencing of the product verified that the 380 bp product obtained was correct, and that the parental 18CA14 primers amplified the product.

GeneE Thermocycler). PCR conditions were kept as similar as possible across the different platforms for consistency; samples were all derived from the same master mix containing DNA template, Hotstar polymerase (Qiagen) and 30 amplification cycles. The SmartCycler® used 25 µL assay volume while the others used 50 µL.

The SmartCycler® and Mastercycler showed production of PCR product down to 1 pg of plasmid template whereas the GeneE Thermocycler did not show any significant production of product amplicon at less than 1ng of plasmid template. The Mastercycler was able to amplify 1ng of genomic template under the conditions and primer set established for amplification of the genomic DNA and plasmid DNA; the SmartCycler and the Thermocycler were not able to amplify any genomic DNA. These results showed that detection limits of target DNA using PCR were greatly affected by the technical performance of the thermocycler (Figure 1-5). The detection limits for touchdown and non-touchdown PCR are 1pg/µl of pPGI plasmid DNA and 1ng/µl of genomic DNA. The detection limit of the PCR product is observed to be template concentration dependent.

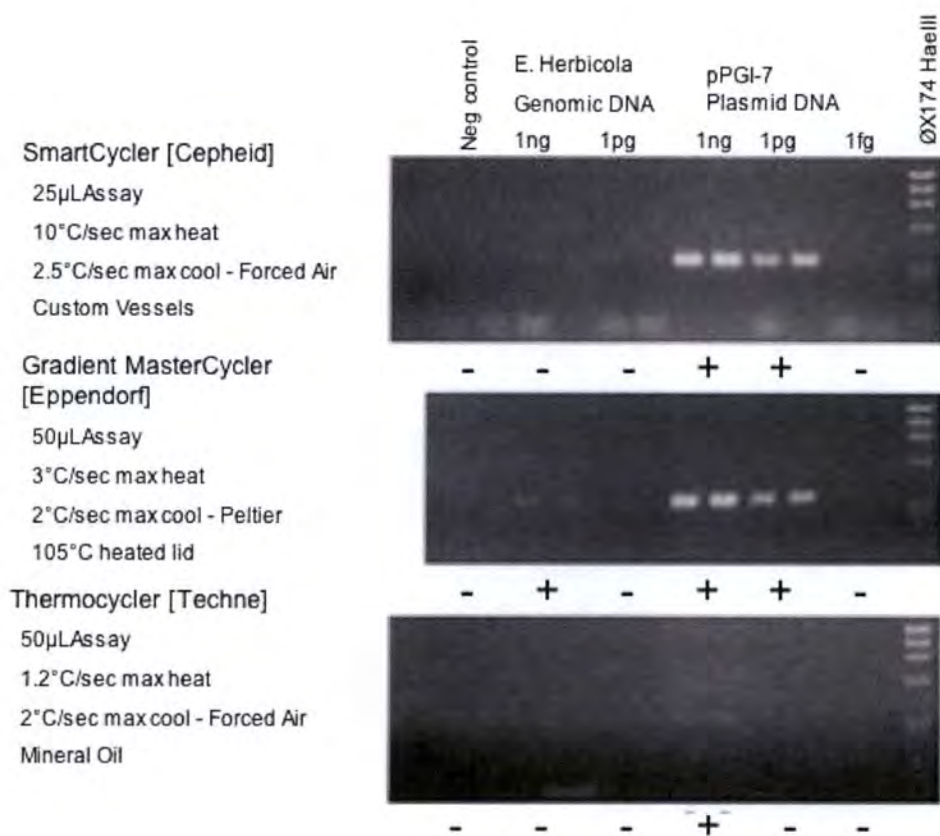


Figure 1-5. Detection limits of *E. herbicola* DNA in three different PCR machines.

Master mixes containing either 1µl genomic or plasmid template were prepared and run in the machines described under the conditions described in the figure. Samples were analyzed by gel electrophoresis with EtBr (staining as described in the Materials and Methods text).

1.2. Characterization of Signature PGI Plasmid Product: Melting Point Determination

Real-time PCR was performed on the SmartCycler® using PGI and pPL6 as target DNA. In all assays, fluorescence typically appeared between 20 to 30 cycles of amplification. The melting temperature (T_m) for PGI-derived product was 89.50 °C;

compared to a T_m of 88.92, 89.95 and 90.69 °C for products obtained from pPL6 (Figure 1-6). These results confirmed the unique melting point for the PGI fragment amplified from 18CA14 primers. Further studies into T_m are discussed in Section 4 below.

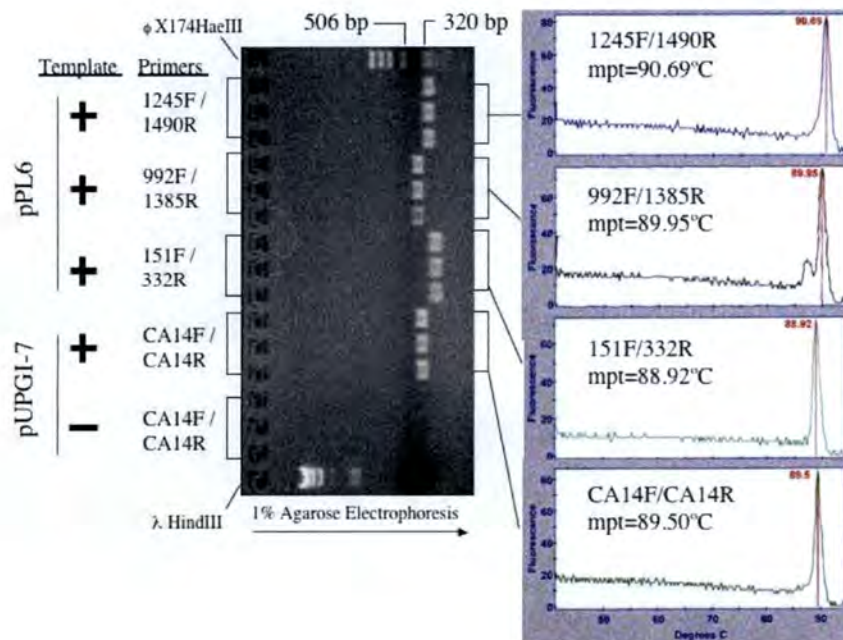


Figure 1-6. Melting Point Determination of *E. herbicola* PCR Products Using Cepheid SmartCycler SYBR Green I Fluorescence Assay.

After PCR amplification, products were subjected to melting point determination as described in the Materials and Methods text. Primers and products are as marked.

2. Testing PCR Reagents and Additives for Microchip Use

For these studies, several different reagents and additives were assessed for their effect on PCR reactions and/or fluorescent detection. Certain additives such as BSA, Tween-20, glycerol, DMSO and formamide reduced surface adsorption of reagents and products to the vessel wall; acted to increase reaction stringency; and reduced the

temperatures for denaturation and annealing. Reducing the denaturation and annealing temperatures is useful in microfluidic devices to control the amount of heating required for the system.

2.1. PDMS

One of the potting compounds considered in the manufacture of the microchip-based PCR device was polydimethyl siloxane (PDMS; Sylgard 184™, Dow Corning and DuPont). This silicone polymer is very commonly used in microchip design; it is readily available, inexpensive, and moldable; it is easily handled and cured; and it generates a non-adherent surface, which is important in biochemical assays to prevent reagent sticking to the reaction vessel wall.

We first determined whether cured PDMS would be compatible with common molecular biology assays. PCR amplification of pGUV10B5 plasmid was assessed using *Pfu* and *Taq* polymerases. Ligation experiments were carried out using λ HindIII fragments and T4 DNA ligase. A standard *EcoRI/HindIII* double restriction digest was carried out on pdRSB12F3-3 plasmid. All reactions were carried out as described in Figure 1-7, on vinyl (cut from a Costar vinyl 96-well plate), nitrocellulose (5 x 5 mm piece) or PDMS (3 x 3 x 3 mm piece) within the reaction vessel. Figure 1-7 shows that PDMS did not interfere with these standard molecular biology assays, compared to vinyl which inhibited *Taq*-mediated PCR, or nitrocellulose which inhibited *Pfu* and *Taq* PCR and to some degree the *EcoR/HindIII* digestion reaction.

Additives	<u>Guv10B5 PCR</u> <u>(<i>Pfu</i>)</u>				<u>Guv10B5 PCR</u> <u>(<i>Taq</i>)</u>				<u>Hind III</u> <u>Ligation</u>	<u>pdR SB12F3-3</u> <u>EcoRI/Hind III</u> <u>Digest</u>			
None	+	-	-	-	+	-	-	-	+	-	-	-	-
Sylgard 184	-	+	-	-	-	+	-	-	-	+	-	-	-
Vinyl	-	-	+	-	-	-	-	-	-	+	-	-	-
Nitrocellulose	-	-	-	-	-	-	-	-	-	+	-	-	-



Figure 1-7. Effects of 3 polymers on common enzymes used for DNA analysis.

Sylgard 184 (PDMS), vinyl and nitrocellulose were assayed to determine their effects on enzymes commonly used in the manipulation and analysis of DNA (*Pfu* polymerase, *Taq* polymerase, T4 DNA ligase, *EcoRI* and *HindIII* restriction endonucleases). *Pfu* and *Taq* polymerases were assayed for their ability to amplify a 734 bp product from pGUV10B5 in a standard PCR reaction. T4 DNA ligase was assayed for its ability to ligate 500 ng of λ /HindIII marker DNA in a standard ligation reaction. *EcoRI* and *HindIII* restriction endonucleases were assayed for their ability to liberate 2936 bp and 827 bp fragments from pdRSB12F3-3 in a standard double restriction digest. All reactions were carried out in the presence of vinyl (one well from a vinyl Costar 96-well plate), nitrocellulose (5 mm x 5 mm piece per reaction) or PDMS (3 x 3 x 3 mm piece per reaction).

We next tested the performance of PDMS as a reaction vessel for the pL6 PCR assay, to compare assay efficiency in the plastic compared to customary PCR vessels. Initial PDMS molds were prepared by using 96 multi-well PVC plates and heating manifolds of the MJ Thermocycler (DRDC) as templates. However we found that the PDMS molds adhered very tightly to the template material and were subject to tearing upon dislodging from the molding template. We discontinued using the MJ Thermocycler (DRDC) for PCR work using the "96 multi-well" PVC plates and PDMS molds, as the temperature controls on this device were poor, resulting in inadequate heating of

the sample DNA and molds. The Techne Thermocycler (CWBios), which had much better temperature control, was used to continue this study; the tube slots in the heating block were of adequate size and the spacing was convenient for pouring PDMS molds.

PDMS molds were prepared directly in the tube holding block of the Techne Thermocycler. Liquid PDMS was poured into the tube slots of the heating block and an aluminum block holding a series of pins to displace liquid PDMS was applied (Top panel, Figure 1-8). Using the hardened PDMS reaction vessels (70 μ L maximum volume), PCR amplification of pL6 992/1385 DNA fragment was carried out under standard conditions; product production was compared with commercially available polypropylene PCR tubes (Eppendorf) (Bottom panel, Figure 1-8). No significant difference in product was noted between PDMS reaction vessels and standard PCR tubes.

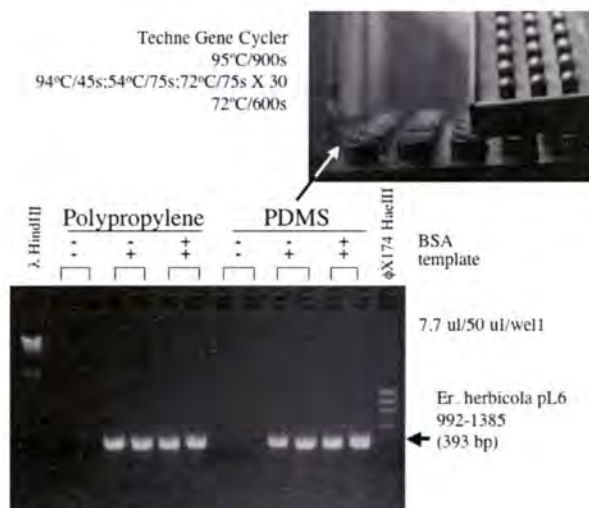


Figure 1-8. PCR amplification of *E. herbicola* pL6 992/1385 DNA fragment using molded PDMS (polydimethylsiloxane) reaction vessels and commercial (Eppendorf) polypropylene tubes.

Standard 50 μ L PCR assays were performed using one ng of pL6; 0.2 μ M each of 992(+) primer and 1385 (-) primer; and Qiagen Hotstar™ Taq Polymerase. Reactions were overlaid with 50 μ L of mineral oil to prevent evaporation. PCR products were separated on a 1.5% TAE agarose gel and stained with ethidium bromide.

From the above results, reaction vessels molded from PDMS can be successfully used to amplify DNA fragments in PCR using *Taq* polymerase. Successful amplification was also achieved using similar experimental conditions with the commercially available polypropylene PCR microtubes. These results indicated that PDMS was compatible with the biochemical and biophysical aspects of PCR, and had suitable thermoconductive properties that supported efficient thermocycling.

Tests were also conducted to determine whether SYBR Green I dye adsorbed onto the PDMS; sticking of fluorescent dye in the analysis channel would prevent multiple uses of the same chip. No visible absorption of SYBR Green I dye into PDMS was noted over one hour using a fluorescence microscope (not shown). PDMS has generally a non-adherent surface, which would prevent sticking of reagents. PDMS is thus a compatible plastic to use as a reaction vessel for the PCR assay with fluorescent detection of products using SYBR Green I dye.

These PCR studies used mineral oil to prevent evaporation, however mineral oil is not suited for small volume PCR work because of the possibility of losing samples on transfer. Alternative sealing methods (*e.g.* PDMS plugs or sheets to seal and encapsulate reaction solutions) may be required to prevent evaporation of PCR reaction solutions.

2.2. BSA

We measured the effect of increasing BSA concentrations (from 0 to 50 µg/mL per reaction) on amplifications of pL6 plasmid. A complete master mix with all reagents (except BSA) was prepared and aliquoted in 10 µL volumes in Eppendorf PCR tubes. One µL of BSA from each dilution was added to a positive control (with template) and negative control (without the template) in duplicate. This concentration range of BSA in PCR did not affect the amplification efficiency. (Figure 1-9)

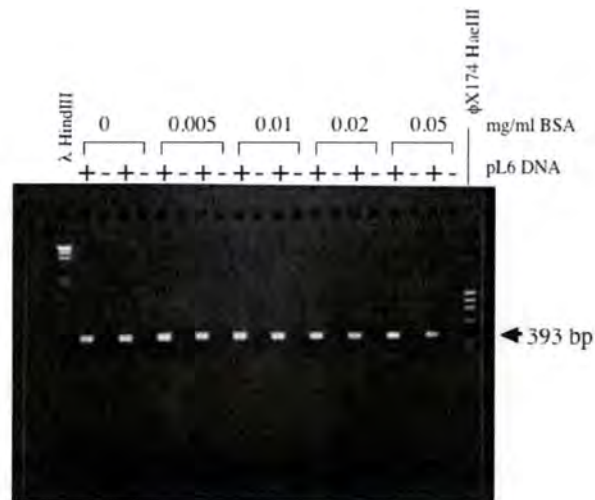


Figure 1-9. PCR of pL6 in 10 μ L reactions using different concentrations of BSA.

BSA was added to PCR master mixes containing enzyme, primers and pL6 template at the indicated concentrations, and PCR was performed as described in the text. Samples were run on 1% agarose gels with EtBr staining.

2.3. Other Additives in PCR Reactions

The addition of BSA, Tween-20 and glycerol to a PCR reaction using HotStar Taq Polymerase requiring the use of Q-solution (Qiagen) with pPGI plasmid template, resulted in more product being formed than compared to assays not containing these additives (Figure 1-10). This observation was noted by three separate operators at different sites, suggesting that this was a true observation and not an assay artifact. Assays may suffer from relative lack of sensitivity of product detection when additives are used, but the additives may be required to compensate for other assay issues (*e.g.* adsorption to the vessel wall, or reducing denaturation or annealing

temperatures to overcome evaporation). Increasing the cycle number in the presence of these additives may increase overall product yield, but at the risk of contamination with extraneous DNA (not shown). In real-time PCR assays, addition of either DMSO or formamide reduced the melting temperature (T_m) of the PGI PCR product by almost 4 °C (from 90 to below 87 °C), while addition of glycerol reduced the T_m by 1.5 °C (not shown). These additives may thus be useful for reducing the working temperatures of the microchip if required (e.g. to prevent evaporation). Pre-qualification of the expected primer/template set may be required to determine the most appropriate conditions for PCR amplification.

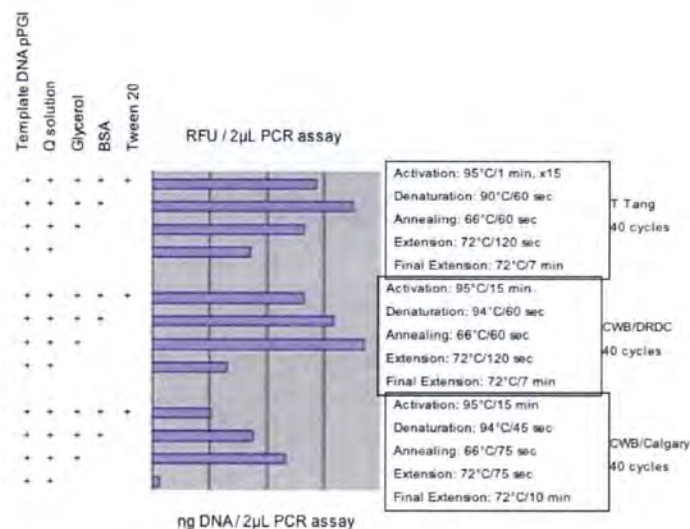


Figure 1-10. Effect of Additives and Thermocycling Conditions on *E. herbicola* pPGI PCR using 18CA14F/R primers.

Additives were added as shown in the figure to each PCR reaction containing pPGI template and primers. Samples were run by PCR at three separate sites as shown. Samples were visualized on agarose gels with EtBr staining, and were also analyzed by fluorescence based on SYBR Green I staining. Amounts of product produced were quantified as shown.

2.4. Additives in Measuring Product Fluorescence

2.4.1. Addition of SYBR Green I reduces PCR assay reliability.

In our hands, replicate PCR assays supplemented with SYBR Green I were more prone to assay failure; 3 of 10 replicate reactions containing SYBR Green I did not show product, compared to 0 of 10 replicates not containing the dye (Figure 1-11). The reason for the assay failure was unknown; it may have been due to a combined effect with BSA as described in the section below.

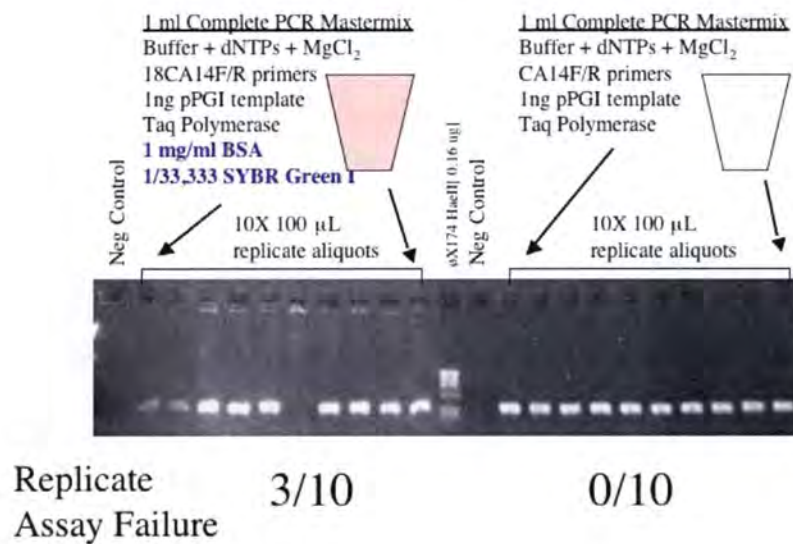


Figure 1-11. Addition of SYBR Green I dye to PCR reduces assay reproducibility.

Dye and BSA were added to one master mix and subjected to PCR (n = 10). Samples were compared to a second master mix that did not contain dye. Samples run on agarose gels and stained with EtBr.

2.4.2. Addition of BSA reduces fluorescence but generally stabilizes PCR assays.

The effects of BSA on the raw fluorescence of 100 μ L and 10 μ L PCR reactions containing SYBR Green I dye were measured. PCR products from these samples, each containing 0.1 mg/ml BSA, were diluted to a final concentration of 1:800 (with and without BSA). A 1:10,000 dilution of SYBR Green I was added to each 200 μ L sample in a cuvette and analyzed using the Quantech digital fluorometer. A standard of 350 ng/ml DNA fragment from Gibco BRL was prepared in TAE containing 1:10,000 dilution of SYBR Green I without BSA to provide an arbitrary measurement of raw fluorescence of 8,000 Fluorescence intensity units (FIU). The blank sample was prepared containing 200 μ L of TAE and 1:10,000 dilution of SYBR Green I. BSA was not added to the blank and the standard to subtract for background fluorescence.

A decrease in raw fluorescence in samples was noted in samples containing BSA, suggesting that BSA might interfere with SYBR Green I fluorescence (Table 1). SYBR Green I intercalates with dsDNA; reduced fluorescence in samples with BSA hence likely can be attributed to non-specific binding of the dye to BSA.

Table 1-1. SYBR Green I Fluorescence is Quenched by BSA

Sample	FIU (Raw Fluorescence)*
100 μ L +	5364.16
100 μ L + with BSA	2770.12
10 μ L +	2197.995
10 μ L + with BSA	236.10

*averaged over 5 readings

BSA is often included in PCR assays to reduce adsorption of reagents (mainly *Taq* enzyme) to the vessel wall. In our experience, PCR assays can be performed without addition of BSA, but the samples must be run immediately after preparation or the assays fail. SYBR Green I fluorescence in replicate samples was generally higher in the absence of BSA but the signal was more variable; the assay itself was more prone to failure if samples were not processed immediately. This was most likely due to decreased availability of *Taq* because of enzyme adsorption to the vessel wall. Samples containing BSA had decreased fluorescence but were less prone to variable fluorescence and assay failure if delays in thermocycling occurred; this appears to support the idea of enzyme adsorption. We noted that thermal denaturation of BSA with the formation of a particulate substance occurred during PCR cycling; this may have contributed to variability in fluorescent signals and melting curves. PCR assays supplemented with thermo-stable BSA may give better and reliable fluorescence measurements, but we did not test this.

Instructions for the Idaho LightCycler strongly recommend the use of both BSA (0.05 µg/µL) and 1% Tween-20 as PCR additives in assay reactions, to prevent adsorption of products onto the glass capillary. Standard reactions omitting either BSA or Tween-20 confirmed that no product was obtained in the LightCycler, while omitting either reagent from the reaction did not make any difference in the amount of product obtained for the other two machines (not shown).

3. Stability of Pre-Mixed PCR Assays

Setting up an experimental device such as the PCR microchip can often involve unavoidable delays caused by unexpected technical difficulties (for example, positioning sealing caps, aligning detectors, computer software issues, *etc.*). Hence we needed to determine how stable PCR assays would be during short-term (up to 24-hour) storage. Complete pre-mixed PCR assays using the Hotstar™ system (complete master mix solutions containing primers, template and enzyme) were stored up to 24

hours at -20 °C, 10 °C (refrigerator temperature) or 23 °C (room temperature); samples at different time points (0, 0.25, 0.5, 1, 3, 6, 12, 24 hours) were frozen at -70 °C until PCR amplification and agarose gel analysis. Results suggested that complete pre-mixed assays were stable over 24 hours at ambient temperature (Figure 1-12); the amount and quality of the PCR products were comparable among all the different time points. This implies that PCR assay solutions already placed on the chip will not be adversely affected by short (up to 24-hour) delays created by equipment issues. This assay was carried out under clean conditions using pure template with no contaminants; the comparable stability of impure templates (*i.e.* real world samples) was not assessed.

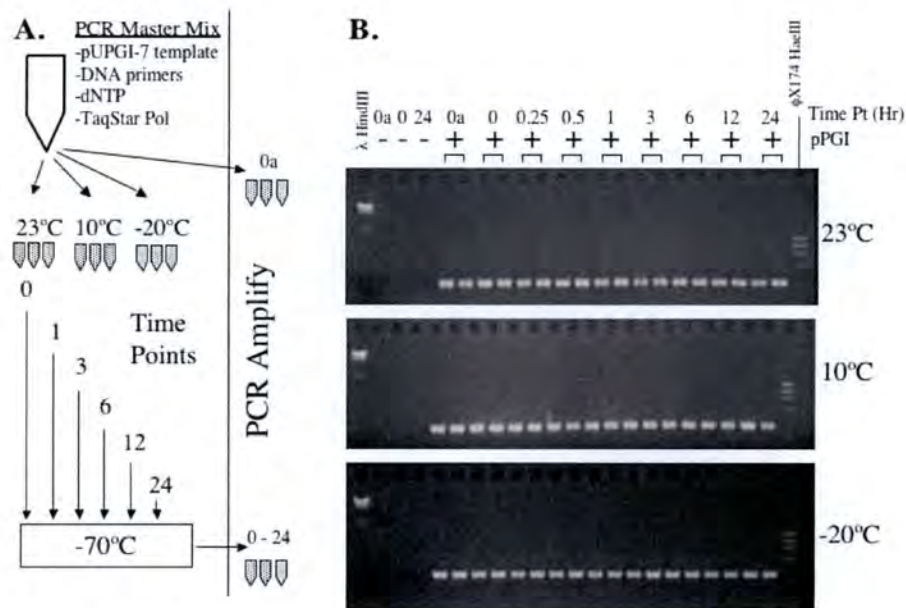


Figure 1. Temperature / Time Stability of Complete PCR Master Mix Solutions.

Figure 1-12. Short-Term Stability of PCR Master Mixes.

Complete master mixes (containing 0.5 ng plasmid pPGI (10^8 copies) per reaction, enzyme and primers) were dispensed in 50 µL aliquots in Eppendorf thin-wall PCR tubes and stored at different temperatures (-20, 10 or 23 °C) for up to 24 hours prior to analysis. PCR was performed on Eppendorf Gradient MasterCycler using a "Touchdown" PCR program as described in Materials and Methods. At the indicated time points, PCR was performed; samples were then stored at -70 °C until gel analysis.

3.1. Testing of Dried PCR Reagents

PCR reagents that were combined and dried down, were either reconstituted and used immediately after drying, or stored for 24 hours at room temperature before being reconstituted and used. These were compared to reagents that had been combined but stored for 24 hours at 4 °C. Template addition and reconstitution of dried reagents were performed under non-sterile conditions, similar as to what might be expected in the field. The volume after reconstitution and template addition was not 50 µL as expected but 60 µL, suggesting that Speed-Vac evaporation for 1.45 hours was not efficient at removing all water. For samples that had been dried for 3 hours, 43 µL was required to rehydrate the dried reagents. The dried reagents themselves would take up some volume, however residual water cannot be ruled out.

Figure 1-13 (top panel; days 0 and 1) shows representative gels of products obtained from using dried reagents. Significant contamination of the negative control samples was noted; this was thought to be due to the non-sterile water source used for reconstitution, however contamination was still noticed when water sources were controlled. As extra precautions, all PCR tubes used were autoclaved and new reagents were obtained in order to repeat the experiment. The source of contamination was eventually traced to residual DNA contamination of the Speed-Vac rotor or chamber (see next section).

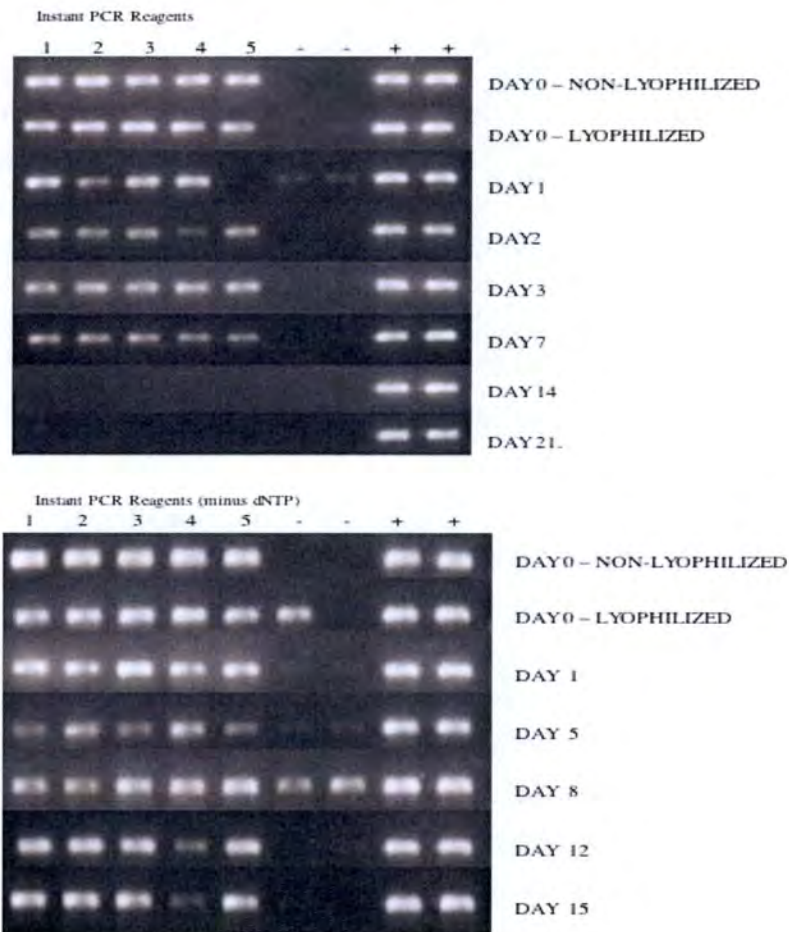


Figure 1-13. PCR with Lyophilized Reagents.

Top panel: Compilation of lyophilized PCR experiments. In this figure (lanes 1-5) 1 pg pPGI, (-) negative controls containing no template and (+) positive control containing 1pg pPGI template. At Day 14 the PCR reagent activity was lost. **Bottom panel:** Compilation of lyophilized PCR experiments without dNTPs. In this figure (lanes 1-5) 1pg pPGI, (-) negative controls containing no template and (+) positive controls containing 1pg pPGI template. There are some negative controls showing a band at ~340 bp which are most likely due to contaminated at the drying step in the Speed Vac Savant

3.2. Stability of Dried PCR Reagents After Long-Term Storage

Dried PCR reagents were stored for up to one month at room temperature and assayed at various time points (0, 1, 2, 3, 7, 14, 21 and 28 days) to determine their activity over long periods. Figure 1-13 shows a representative gel of the PCR products

obtained when assayed at each time point. The day 7 samples showed a significant drop in product compared to the previous time points. Activity was completely lost after 14 days of storage at room temperature; this was likely due to degradation of the *Taq* polymerase either during the freezing process or during storage at room temperature. The loss of activity could also be due to the degradation of the nucleotides during storage at room temperature. Bands appeared sporadically in the negative control samples, suggesting contamination occurred at some point; possible sources of the contamination were investigated as described below.

This experiment was repeated using dried PCR reagents except for the nucleotides, to rule out degradation of nucleotides during storage; the nucleotides were added along with the template after reconstitution of the sample with water. In this experiment, PCR product was produced up to 15 days post-drying, suggesting that degradation of nucleotides may have been at least partially responsible for the lack of PCR product at day 14 in the previous experiment; however band intensities varied, possibly due to pipetting error; negative controls still exhibited extraneous bands.

The drying step was thought to be the source of the contamination, for it is at this step that the reagents are exposed to ambient air for significant lengths of time. Negative controls manipulated under controlled, sterile conditions did not exhibit extraneous bands, suggesting that an outside step was the contamination source. Various surfaces on the Speed-Vac (including the inner rotor, wall and lid) were swabbed and the swab placed in water to capture any contaminants from the surfaces within the Speed-Vac prior to drying the reagents. PCR reagents were mixed then dried in the Speed-Vac. The Speed-Vac was then cleaned with a commercial detergent solution (MicroSol3™, Anachem, Luton UK) and the PCR reagent drying process was completed. The Speed-Vac was also swabbed after cleaning to see whether and by how much contamination was reduced. All samples were reconstituted with water and template

was added (for swab samples, 1 µL of swabbing solution was used as a template) for PCR.

Both swab samples showed strong PCR product bands suggestive of *E. herbicola* DNA, similar to results using 1 pg template, thereby showing that the contamination of the dried reagents most likely occurred during the drying process. The product obtained from the swab sample post-cleaning was much lower compared to before cleaning, suggesting that cleaning removed a significant amount of contamination but did not remove all of it. There were several limitations to this experiment: swabs were not sterile; a swab/sterile water only control was not included; and different sections of the Speed-Vac were not swabbed separately, but rather were combined, so the area of contamination within the instrument could not be determined.

These findings were significant because of the possibility of false positive results in the field due to reagent contamination by ambient air or equipment. Field conditions are not expected to be controlled or even sterile, hence the chance of false positives is likely much higher than in a controlled laboratory situation. Laboratory procedural changes that would reduce contamination (including autoclaving, frequent changing of gloves, separate freezer storage of template and reagents, and availability of sterile biological safety cabinets) may not be available or practical in the field. Also, strict quality control measures will be required for each dried reagent batch to confirm lack of contamination of dried reagents before use in the field.

3.3. Minimization of reagent contamination in reagent preparation and experimental protocols

Assay reproducibility is crucial to the widespread acceptance of PCR for detection. In our experience, avoiding possible contamination of PCR reagents by environmental DNA can be minimized by protocol control and meticulous preparation of PCR reagents. For example, when at all possible, all reagents should be prepared under

sterile conditions in a bio-safety cabinet. Assay samples and templates should be stored apart from reagents wherever feasible. Exposure of reagents and template to air should be minimized to prevent contamination from ambient DNA. If manipulations must be carried out under non-sterile conditions, areas and equipment should be maintained in as clean a condition as is reasonably possible. Gloves should be worn if available.

3.3.1. Reagent and Material Preparation : To autoclave or not?

Most autoclaves are used to sterilize garbage, biological samples and media as well as lab ware and liquids for experimental work. This process presumably denatures, vaporizes and aerosolizes biomolecules (including DNA) throughout the autoclave chamber. For some applications, sterilization is crucial (*e.g.* cell culture) or is preferred to prevent unwanted growth in lab reagents (*e.g.* buffers for biochemical work). For many other applications (*e.g.* enzyme assays or binding assays), contamination by residual biomolecules is inconsequential. However, in PCR assays, any contaminating DNA in either reagents or plastic ware can be detrimental to the assay and result in false positives.

Use of an autoclave for sterilizing PCR reagents may be acceptable if the autoclave has been dedicated to the sterilization of lab plastic ware, inorganic buffer solutions or lab ware dedicated to inorganic sample handling only. However, strict quality controls would be required to confirm the lack of contaminating DNA.

Most disposable plastic materials such as pipette tips and PCR microtubes are prepared free of contaminants although they are not sterilized; nevertheless they are usable without further autoclaving. Pipette tips for PCR should be purchased guaranteed sterile from the manufacturer with barrier plugs, in packages of 96 tips per box. Microtubes can be dispensed and used directly from manufacturers' packaging as long as they are handled with gloves or clean forceps to avoid contamination.

Water prepared by distillation or reverse osmosis/filtration is sufficiently free of contaminating biomolecules to be used directly. Water can be further sterilized by filtration (0.22 µm) without potential problems associated with autoclaving. The next section discusses the importance of maintaining these quality control procedures in the context of reagent preparation.

4. Real-time PCR - Fluorescent Amplicon Detection

Real-time PCR studies of pPGI template were performed using the Cepheid SmartCycler®. In real-time PCR, as the amount of DNA amplified increases, the fluorescent dye SYBR Green I™ (Molecular Probes) that is included in the assay reaction intercalates with the ds DNA to increase fluorescence in a quantitative manner. This allows real-time monitoring of the PCR without interruption of the assay to take sample aliquots for gel analysis of the amplified product. Once a particular PCR template/primer set has been optimized, the increasing fluorescence of the product is a reliable and practical indicator of the presence of target template. Upon completion of the assay, a temperature vs fluorescence curve is performed to determine the melting temperature (T_m) of the resulting PCR product. Defined ds DNA products have a distinct T_m, detected by the sudden loss of fluorescence as the ds DNA products melt to ssDNA and thereby release the intercalated dye into the surrounding solution. A distinct T_m is further evidence of defined ds DNA fragments. Relatively large DNA fragments (≥ 300 bp) often have similar T_m (90°C). However, smaller PCR products (50-200 bp) have lower T_m (between 70-90°C) which are distinctive and more reliable indicators of specific PCR products.

The SmartCycler® accepts fixed assay volumes of either 25 µL or 100 µL. Standard 25 µL assays were performed in all subsequent experiments described using the assay formulation shown in Table 1.

4.1. Real-Time PCR Optimization of pPGI Product

Real-time PCR parameters were optimized for reproducible production of the PGI product. Figure 1-6 shows that the T_m for pPGI product was 89.50 °C; this T_m was used as a diagnostic parameter in all following assays.

4.1.1. Replicate assays are necessary in reliable use of real-time PCR.

An important technical achievement of the SmartCycler® is that the 16 sample positions of the device can all function as independent thermocycling units. Replicate samples generally give similar real-time fluorescent curves but sometimes replicates can look very different. Figure 1-14 shows replicate assays for pPGI and for pL6 product. The melting curves for pPGI were very reproducible, almost super-imposable (Figure 1-14, top panel); however the melting curves for pL6 varied by up to 50% (Figure 1-14 bottom panel).

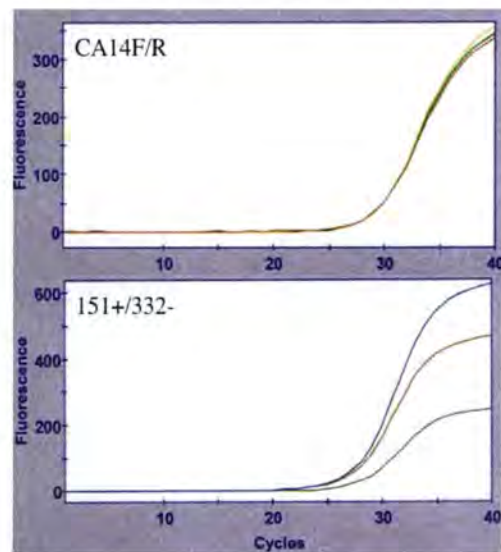


Figure 1-14. Triplicate Assay Reproducibility in Real-Time PCR Assays

Three samples from two different templates (pPGI and pL6) were run in real-time PCR assays as described in the text. Conditions: 0.25 ng plasmid, 1:25000 of SYBR Green I, 0.1 mg/mL BSA in 25 μ L reaction. Top panel: pPGI amplification with 18CA14 primers. Bottom panel: pL6 amplification with 151+/332- primers.

4.1.2. Optimization of Denaturation Time

The denaturation time was decreased from 75 sec/cycle down to 1 sec/cycle. At 45 sec, fluorescent signal was still 100% of that obtained at 75 sec; however the signal decreased by 50% using a 30 sec denaturation time, and signal was absent when denaturation times were shortened from 20 sec to 1 sec (Figure 1-15).

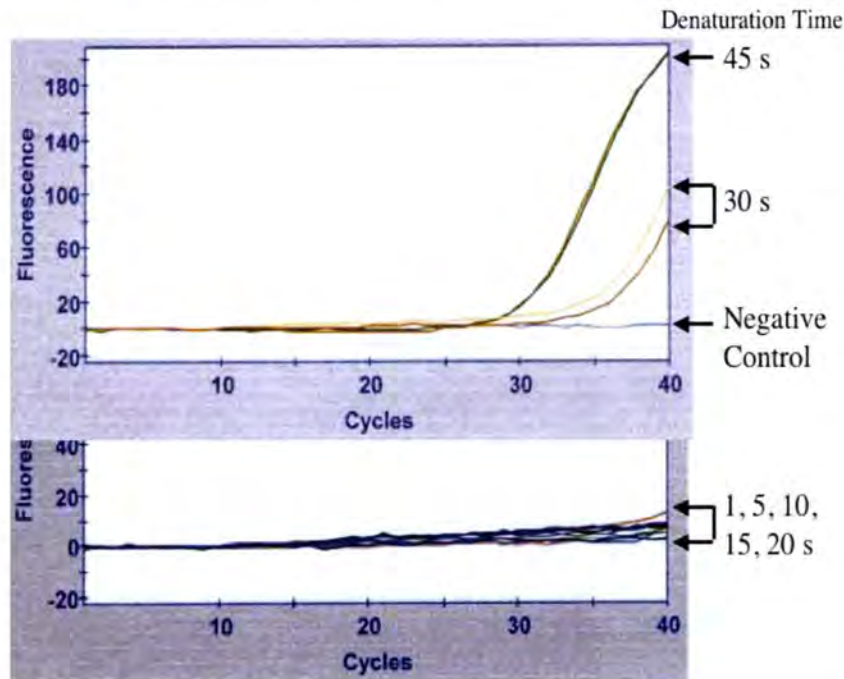


Figure 1-15. Reduced Target DNA Amplification by Shortening of Denaturation Step in Real Time PCR Using the Smartcycler™.

Denaturation time of each cycle was shortened as indicated in the figure, using pPgl template and 18CA14 primers. T_m determinations were performed at the end of the assay as described. Reactions performed in duplicate.

4.1.3. Optimization of Annealing Time

The annealing time was decreased from 75 sec/cycle to 1 sec/cycle. At 50 sec, the fluorescent signal decreased by 25%; the signal decreased by 90% using a 25 sec annealing time, and was almost non-existent when annealing times were reduced below 25 sec/cycle (Figure 1-16).

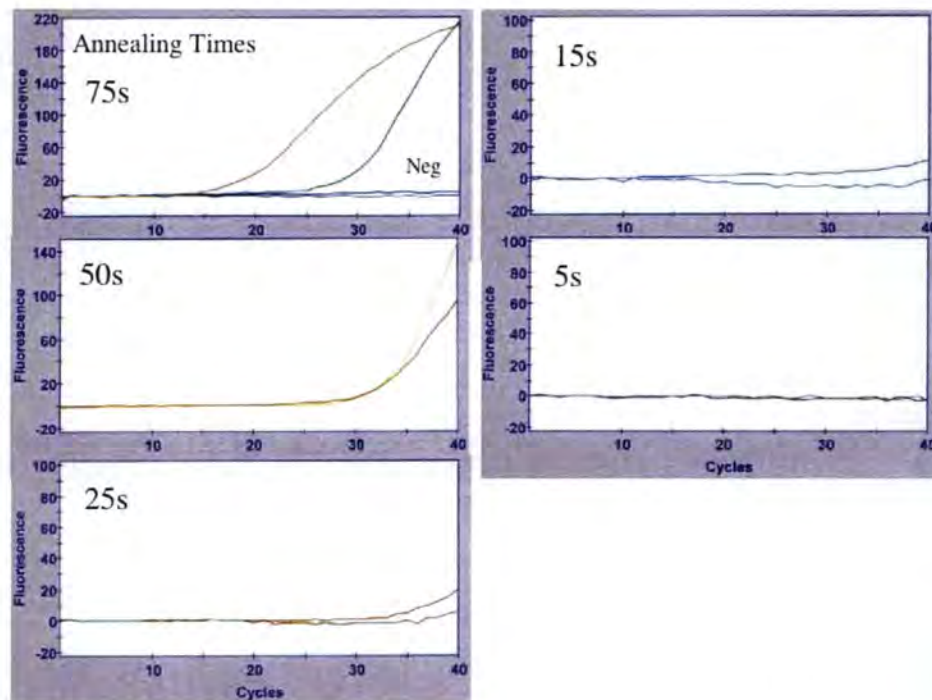


Figure 1-16. Reduced Target DNA Amplification by Shortening of Annealing Step in Real Time PCR Using the Smartcycler™.

Annealing time of each cycle was shortened as indicated in the figure, using pPGI template and 18CA14 primers. T_m determinations were performed at the end of the assay as described. Ten and one sec time points not shown. Positive and negative reactions performed in duplicate.

4.1.4. Optimization of Extension Time

The extension step was decreased from 75 sec/cycle to 1 sec/cycle. At 50 sec/cycle, the fluorescent signal was still 100% compared to 75 sec/cycle; the signal then decreased by 60% using a 25 sec/cycle extension and decreased by 80% at 15 sec/cycle extension. Signal was almost non-existent at extension times below 15 sec (Figure 1-17). However, even though the same fluorescence maximum is achieved by 50sec/cycle the cycle time required to achieve this maximum is increased. As the extension step decreases in time per cycle the cycle time in which fluorescence is observed increases and therefore maximum fluorescence is not visible in a 40-cycle limit.

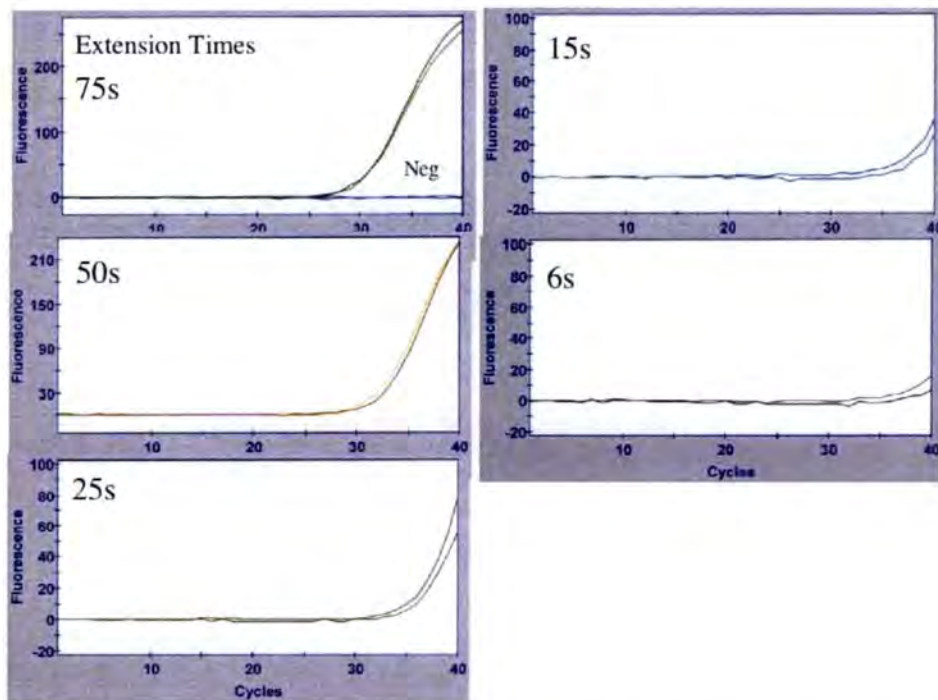


Figure 1-17. Reduced Target DNA Amplification by Shortening of Extension Step in Real Time PCR Using the Smartcycler™.

Extension time of each cycle was shortened as indicated in the figure, using pPGI template and 18CA14 primers. T_m determinations were performed at the end of the assay as described. Ten and one sec time points not shown. Positive and negative reactions performed in duplicate.

The above studies confirmed that a denaturation time of 45 sec, annealing time of 75 sec, and extension for 75 sec for 40 cycles provided the optimal amount of product for analysis in the SmartCycler® (Figure 1-18), although these results also suggested that a 50 sec extension step would still provide adequate signal for detection in 40 cycles. At a threshold of detection (zero cycles), a total fluorescence signal was visible in only 10 synthesis cycles for a 50 sec extension in comparison to 14 cycles for a 75 sec extension. This confirms that maximum fluorescence will be reached with 50 sec/cycle but at a later cycle, therefore increasing the detection time for confirmation of product in real time.

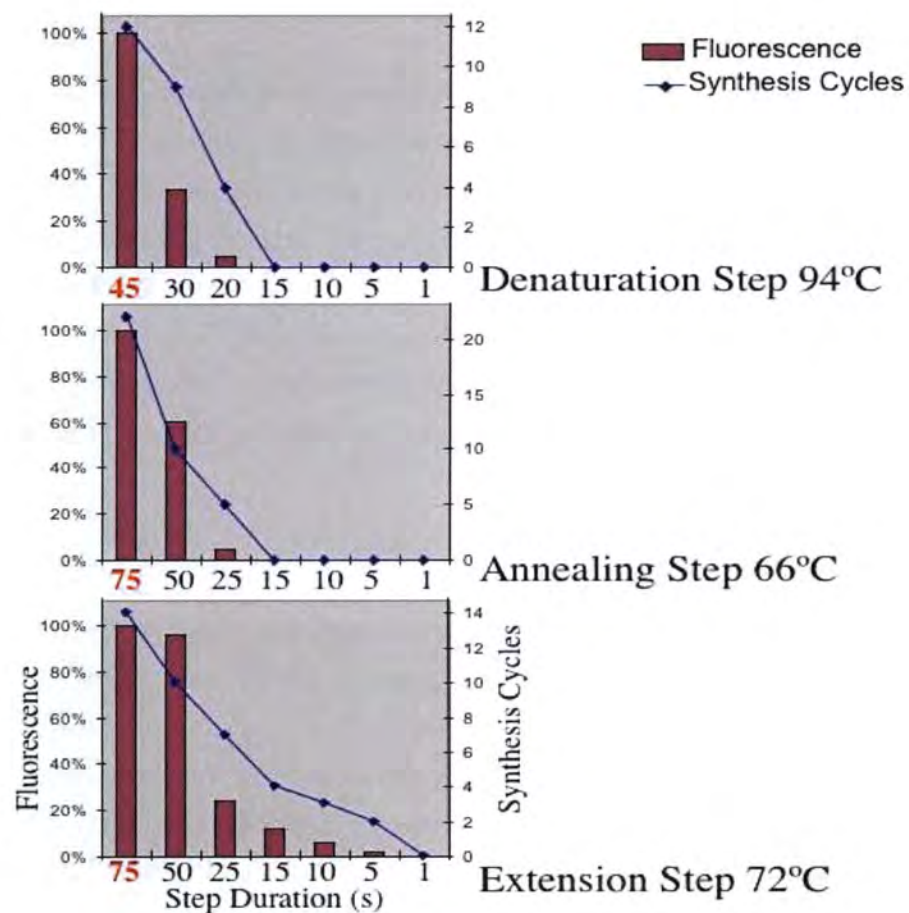


Figure 1-18. PCR Program Optimization Using Cepheid Smartcycler.

Twenty-five μL assays were performed in duplicate using 18CA14F/R primers and *E. herbicola* template pPGL using Qiagen Hotstar™ enzyme and reagents. The standard program included 1) Initial denaturation at 95°C/150 s, 2) PCR program (40 cycles) 94°C/45 s; 66°C/75s; 72°C/75s, and 3) final extension at 72°C/150 s. Synthesis cycles reflect fluorescence measured after crossing the background threshold (cycle zero).

4.2. Sample evaporation is significant and can alter the melting point determination.

Typically real-time PCR assays require 1-3 hours to complete; by the end of the assay, the condensation collected inside the top cap of the vessel may comprise as much as 20% of the total assay volume (e.g. 5 µL from a 25 µL assay). As a result, the melting temperature program that follows the PCR program will occur in a reduced assay volume with a coordinately higher ion concentration. We noted that the T_m obtained was usually slightly higher than that of the same assay sample where the condensation was displaced back into the main assay volume by centrifugation. In this event, either using a means to prevent evaporation, or running the melting temperature program after centrifuging the samples, is required.

5. Small-volume (sv) PCR Assays

Initial studies examined the effect of assay volume on the production of PCR product from pRSD12GUV plasmid DNA. Samples derived from the same master mix including template and polymerase, were aliquoted at different volumes (100, 50 or 10 µL) and underwent PCR amplification. These results showed that production of PCR product was dependent on assay volume. Fluorimetry of SYBR Green I-stained PCR products showed at least a 3-fold difference in the amount of product obtained when using a 10 µL assay volume (Table 1-2). This variation in the amount of product obtained was expected to be an important issue in reducing PCR volumes for the microchip; hence this avenue was explored further.

Table 1-2. Analysis of PCR Products of pGFPuv

Sample (µL)	Concentration 1 (ng/mL)	Concentration 2 (ng/mL)	Raw Fluorescence (FIU)
100	9.42	9.98	228.22
50	6.61	7.08	161.96
10	4.14	3.32	76.07
10	2.85	1.18	26.99

To explore sv PCR, three different PCR instruments were compared: the Idaho LightCycler (8 μ L volume), the MJ ThermoCycler (25 μ L volume) and the Eppendorf MasterCycler (25 μ L volume). All samples tested were derived from the same master mix, and then dispensed to each machine for PCR.

SvPCR assays were successfully performed using a 3 μ L assay volume in the LightCycler, and 5 μ L volumes in the ThermoCycler and MasterCycler (Table 1-3). In each case the amounts of product obtained were similar down to 3-5 μ L.

Table 1-3. 18CA14E. herbicola PGI Product Yield from sv PCR

Assay volume	8* or 25** μ L std	5* or 10** μ L sv	3* or 5** μ L sv
LightCycler*	9.9 ng/ μ L	21.8 ng/ μ L	12.2 ng/ μ L
ThermoCycler**	84.7 ng/ μ L	86.9 ng/ μ L	68.2 ng/ μ L
MasterCycler**	60.5 ng/ μ L	142.4 ng/ μ L	44.8 ng/ μ L

However, PCR assays at volumes smaller than 3 μ L (LightCycler) or 5 μ L (ThermoCycler and MasterCycler) were unsuccessful at producing PCR product. This was thought to be due to excessive evaporation and condensation of liquid on the lid of the reaction tube during cycling, resulting in altering the effective reagent concentrations in the reaction. *Taq* polymerase is inhibited by high concentrations of dNTPs; it was likely that the overall reduced volume caused by evaporation, may have increased the available dNTP concentration to an inhibitory level. Hence a means to retain the assay volume during the sv PCR process was investigated.

5.1. Reducing Systematic Errors in Fluorescence Measurements in Small-Volume PCR

Experiments varying the duration of the PCR denaturation step were performed on small volume PCR assays (10 μ L). Initial assays with shorter denaturation periods (95

°C / 5 sec) produced detectably more PCR product than assays with longer denaturation times (95 °C/ 15 sec or 30 sec) (data not shown). However the differences in product yield between samples were marginal enough for us to suspect systematic errors in the assay that most likely occurred during fluorescence measurements. Extensive work was done to investigate these variables. We examined the effects of background lighting, sample volume, and cuvette differences as sources of systematic equipment errors. We corrected for these errors by employing the following strategies:

1. Performing fluorescent readings in the absence of background fluorescent room lighting. Fluorescent room light increased the background readings by 1000 fold vs. samples read in the dark, thus subsequent readings were taken with the fluorescent lights turned off using natural light for visualization.
2. Increasing sample volumes from 100µL to 400 µL per cuvette. We also raised the cuvette position to a sufficient height to reach the PMT detector.
3. Uniformly positioning and matching cuvettes.

Once these corrective strategies were applied, RFU measurements were more reproducible; replicate error bars from same-sample RFU readings were significantly reduced (Figure 1-19).

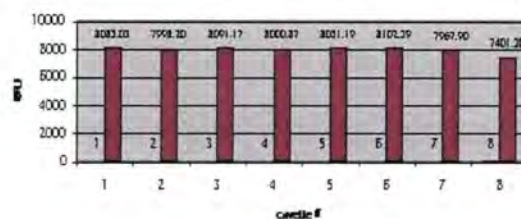


Figure 1-19. Controlling for Systemic Error During Fluorescence Measurements.

After undertaking the measures outlined in Section 5.1 in the Results to control for systemic error in denaturation experiments, fluorescence was measured in PCR samples. Eight replicates are shown.

5.2. Denaturation Step Effects

Having ruled out systematic errors arising from the equipment we examined specific trends in denaturation times, in order to optimize the conditions for amplification using small volumes.

Ten μL PCR reactions with and without BSA (five samples from each set) were carried out using the MasterCycler. Samples were denatured for 30 seconds, 15 seconds or 5 seconds per cycle. Each sample was analyzed by agarose gel electrophoresis and the fluorescence was quantified. Fluorescence readings were carried out five times per sample.

The yield of amplified PCR product was maximized by denaturation at 95 °C/5 sec per cycle, compared to denaturation at 95 °C/30 or 15 sec when using a 10 μL reaction (Table 1-4).

Table 1-4. RFU and corresponding Concentrations of 10 μL Samples of *E. herbicola* denaturing at different times.

Times (sec)	10 μL Rx		10 μL Rx +BSA	
	RFU	Concentration (ng/ml)	RFU	Concentration (ng/ml)
30	1585.241	78.012	1727.774	84.558
15	1494.135	73.828	1394.8733	69.269
5	1732.423	84.772	1861.1184	90.682

The variable amount of PCR product obtained at small volumes may be attributed to evaporation effects which are exaggerated at small volumes. In an effort to minimize evaporation we investigated the effects of further reducing the length of denaturation and annealing times in small-volume reactions (> 5 sec/cycle). pL6 and pPGI (360-380 bp) clones were aliquoted from master mixes (5 assays per sample) and amplified in 10 μL volumes, reducing the denaturation times from 5 to 1 sec/cycle at 94 °C [annealing at 54 °C/75 sec and extension at 72 °C/75 sec]. PCR product was produced with denaturation steps as short as 1 second; however the yield was reduced by 50% as determined by fluorescence of the sample (Figure 1-20). For these experiments samples with BSA demonstrated lower fluorescence than samples without BSA, though this difference was not as high in the samples denatured for 1 second. pL6 had a greater reduction in RFU for the sample denatured for 5 seconds with BSA compared to that of pPGI with BSA. The difference in yields was not exponential but

was significant when other systematic errors were controlled. The effect of denaturation time may be more important at PCR volumes of 1 μ L or less (i.e. volumes to be used on the microchip) and therefore must be considered in microchip protocol design.

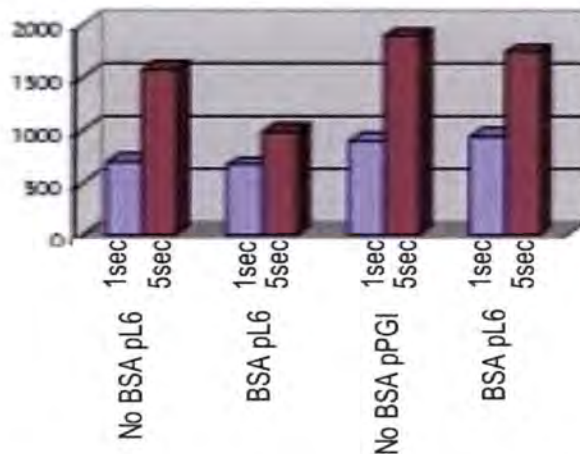


Figure 1-20. Comparison of 5 second and 1 second Denaturation Times for pL6 and pPGI Products in Small-Volume PCR.

Templates (pL6 and pPGI) were set up with respective primers and subjected to PCR analysis in the presence or absence of BSA, and reducing the denaturation time from 5 seconds/cycle to 1 second/cycle. Raw fluorescence measurements of products are shown.

5.3. Optimizing for Denaturation Temperature Using Gradient PCR

Results from the above denaturation experiments suggested that pL6 and pPGI had similar efficiency when amplified for 1 second. The effect of denaturation temperature at 1 second was measured on the Mastercycler across a denaturation temperature gradient using pPGI template.

10 μ L reactions with and without BSA and corresponding negative controls (total of 44 samples) were thermocycled using the MasterCycler programmed with a temperature gradient of $\pm 3^{\circ}\text{C}$. The positions in the block will give the following temperatures based on the gradient program:

1	2	3	4	5	6	7	8	9	10	11
90.1	90.3	90.8	91.5	92.2	93.1	93.9	94.7	95.4	95.9	96.3

Samples were analyzed in 1% EB gel for correct inserts and presence of contamination. Raw fluorescence readings were measured with Quantech digital Fluorometer using the same dilution as in previous experiments with Sybergreen I.

The optimal PCR denaturation temperature of the PGI product was 93.9 °C (+BSA) to 94.7 °C (no BSA).

5.4. Validation of Roche and Eppendorf PCR Reagents for sv PCR

To ensure reproducibility of performance of PCR reagents on the different instrument platforms, both Roche FastStart and Eppendorf HotMaster reagents were tested for their performance in sv PCR assays. Master mixes were prepared, then split amongst the different platforms. The amount of DNA produced was both assessed visually on gels and quantified using SYBR Green I fluorescence.

Tables 1-5 through 1-13 show the results of validation of both Roche and Eppendorf PCR reagents for sv PCR. Both Roche and Eppendorf reagents were suitable for standard volume PCR (Table 1-5). While 2x Taq gave a higher frequency of primer dimers, at low volumes it was required for detectable product formation (Table 1-6). The addition of BSA and Tween were absolutely required for the LightCycler, while they did not make any significant difference in the amount of product obtained from the ThermoCycler or MasterCycler (Table 1-7). Using both Roche and Eppendorf reagents, at intermediate volumes (5-10 µL), the lower volume did not result in less amplified product, although the LightCycler consistently produced much lower amounts of product compared to the other two thermocyclers (Table 1-8). Also, primer dimers appeared to be significantly increased in the negative controls at smaller volumes (Table 1-8). At volumes below 5 µL, the amount of product

significantly decreased with volume in the ThermoCycler and the MasterCycler (Table 1-9 and Table 1-10); this effect was noted at volumes of less than 3 μL in the LightCycler.

Table 1-5. Verification of Standard PCR Reagents –Basic ROCHE PCR Kit with no Additives.

		Idaho LightCycler (8ul)	MJ ThermoCycler (25ul)	Eppendorf MasterCycler (25ul)
1X Taq + GC Rich Solution 1X Taq does not always produce	Product Rating	none	+	++
	Product Amount (ng/ul)	N/A	N/A	43.58
	Background Rating	none	no lane smearing, weak primer-dimer evidence	no lane smearing, weak primer-dimer evidence
	Negative Amount (ng/ul)	N/A	N/A	4.616
	Corrected Amount (ng/ul)	N/A	N/A	38.964
2X Taq + GC Rich Solution 2X Taq produces more background than 1X Taq	Product Rating	none	+++	+++
	Product Amount (ng/ul)	N/A	148.63	149.98
	Background Rating	none	Less Background Observed in the MasterCycler than in the MJ ThermoCycler because of the stronger primer dimer evidence in the 2X samples weak smearing, + for primer-dimer evidence	
	Negative Amount (ng/ul)	N/A	26.74	8.829
	Corrected Amount (ng/ul)	N/A	121.89	141.151

1X Taq + No GC Rich Solution 1X Taq does not always produce	Product Rating	none	++	++
	Product Amount (ng/ul)	N/A	104.489	83.114
	Background Rating	none	no smearing, + primer-dimer	no smearing, ++ primer-dimer
	Negative Amount (ng/ul)	N/A	2.733	9.835
	Corrected Amount (ng/ul)	N/A	101.756	73.279
2X Taq + No GC Rich Solution GC Rich Solution decreases primer-dimer evidence and decreases the denaturing temperature	Product Rating	none	++	+++
	Product Amount (ng/ul)	N/A	127.26	153.34
	Background Rating	none	Smearing greater with MJ ThermoCycler, but primer- dimer evidence is equal in both the ThermoCycler and the MasterCycler Weak smearing, + for primer-dimer evidence	
	Negative Amount (ng/ul)	N/A	21.0576	12.97
	Corrected Amount (ng/ul)	N/A	106.2	140.37

Table 1-6. Verification of Common PCR Reaction Mixtures.

		Idaho LightCycler (8 μ L)	MJ ThermoCycler (25 μ L)	Eppendorf MasterCycler (25 μ L)
BSA ONLY (0.05 μ g/ μ L)	Product Rating	none	+++	+++
	Product Amount (ng/ μ L)	5.05	124.85	108.454
	Background Rating	Weak Primer-dimers	+ for smearing	++ for smearing, primer-dimers in negative control
	Negative Amount (ng/ μ L)	5.74	52.74	42.92
	Corrected Amount (ng/ μ L)	(N/A)	72.11	65.534
Tween ONLY (1%)	Product Rating	none	+++	+++
	Product Amount (ng/ μ L)	0.91	125.635	123.25
	Background Rating	none	+ for smearing	++ for smearing, primer-dimers in negative control
	Negative Amount (ng/ μ L)	0.884	61.226	50.62
	Corrected Amount (ng/ μ L)	(N/A)	64.409	72.63
BSA and Tween (0.05 μ g/ μ L and 1%) <i>With Respect to the PCR Program the LightCycler requires BSA and Tween to amplify any product</i>	Product Rating	+++	+++	+++
	Product Amount (ng/ μ L)	19.72	144.76	124.724
	Background Rating	Weak evidence of Primer-Dimers	+ for smearing	++ for smearing, primer-dimers in negative control
	Negative Amount (ng/ μ L)	9.78	60.058	64.272
	Corrected Amount (ng/ μ L)	9.94	84.702	60.452

Table 1-7. Product Comparison between the MJ ThermoCycler and the Eppendorf MasterCycler

		MJ ThermoCycler (25 μ L)	Eppendorf MasterCycler (25 μ L)
2X Taq + GC Rich Solution –NO BSA or Tween Additives	Product Rating	+++	+++
	Product Amount (ng/ μ L)	148.63	149.93
	Background Rating	Less Observed Background noticed in the MasterCycler than in the MJ ThermoCycler weak smearing, + for primer-dimer evidence	
	Negative Amount (ng/ μ L)	14.71	10.52
	Corrected Amount (ng/ μ L)	133.92	139.6
BSA ONLY (0.05 μ g/ μ L) –2X Taq + GC Rich Solution	Product Rating	+++	+++
	Product Amount (ng/ μ L)	124.85	108.454
	Background Rating	Less background observed in the MasterCycler in relation to smearing, however product and primer-dimers seem to have appeared in the Negative Control of the MasterCycler	
	Negative Amount (ng/ μ L)	52.74	42.92
	Corrected Amount (ng/ μ L)	72.11	65.534
Tween ONLY (1%) –2X Taq + GC Rich solution	Product Rating	+++	+++
	Product Amount (ng/ μ L)	125.635	123.25
	Background Rating	Less background observed in the MasterCycler in relation to smearing, however product and primer-dimers seem to have appeared in the Negative Control of the MasterCycler	
	Negative Amount (ng/ μ L)	61.226	50.62
	Corrected Amount (ng/ μ L)	64.409	72.63
BSA and Tween (0.05 μ g/ μ L and 1%) –2X Taq + GC Rich Solution	Product Rating	+++	+++
	Product Amount (ng/ μ L)	144.76	124.724
	Background Rating	Less background observed in the MasterCycler in relation to smearing, however product and primer-dimers seem to have appeared in the Negative Control of the MasterCycler	
	Negative Amount (ng/ μ L)	60.058	64.272
	Corrected Amount (ng/ μ L)	84.702	60.452

Table 1-8. Low Volume PCR Reactions Using the Roche PCR Kit.

Note: samples used 2X Taq, GC Rich Solution, 0.05µg/µL BSA, and 1% Tween

25µL PCR Reaction divided into 5µL and 10µL Reactions		Idaho LightCycler (5µL)	MJ ThermoCycler (10µL)	Eppendorf MasterCycler (10µL)
<i>Excess Product in ThermoCycler Negative Control</i>	Product Rating	+++	+++	+++
	Product Amount (ng/µL)	29.29	159.60	175.8
	Background Rating	Weak primer-dimers	Primer-dimers on Negative Control only, ++ smearing	Primer-dimers on Negative Control only, + smearing
	Negative Amount (ng/µL)	7.54	(product in Negative Control) (over-estimated) 72.7	33.4
	Corrected Amount (ng/µL)	21.75	86.9	142.4

Table 1-9. Low Volume PCR Reaction using the ROCHE PCR Kit.

Note: Samples used 2X Taq, GC Rich Solution, 0.05µg/µL BSA, and 1% Tween

25µL PCR Reaction divided into 3µL and 5µL Reactions		Idaho LightCycler (3µL)	MJ ThermoCycler (5µL)	Eppendorf MasterCycler (5µL)
	Product Rating	+++	+++	+++
	Product Amount (ng/µL)	17.92	83.34	69.35
	Background Rating	+ primer dimers on Negative Control	+ for negative control primer-dimer smearing	Weak evidence of primer-dimers in the negative Control Only, No smearing
	Negative Amount (ng/µL)	5.548	15.16	24.6
	Corrected Amount (ng/µL)	12.37	68.18	44.75

Table 1-10. Comparison of Roche and Eppendorf Kits for Small Volume PCR.

Note: Low volume conditions used 2X Taq, GC Rich Solution, 0.05µg/µL BSA, and 1% Tween with respect to the ROCHE KIT but only 1X Taq, 0.05µg/µL BSA and 1% Tween with respect to the EPPENDORF KIT.

25ul PCR Reaction divided into 2µL and 1µL PCR Reactions		Idaho LightCycler	MJ ThermoCycler	Eppendorf MasterCycler
2µL. ROCHE KIT -increased to 40 cycles (LightCycler)	Product Rating	N/A	++	++
	Product Amount (ng/µL)	none	53.42	43.83
	Background Rating	N/A	Weak evidence of smearing and primer dimers	Weak evidence of smearing and primer dimers
	Negative Amount (ng/µL)	none	14.52	9.3
	Corrected Amount (ng/µL)	N/A	38.9	34.53
1µL. ROCHE KIT	Product Rating	N/A	N/A	N/A
	Product Amount (ng/µL)	none	none	none
	Background Rating	N/A	N/A	N/A
	Negative Amount (ng/µL)	none	none	none
	Corrected Amount (ng/µL)	N/A	N/A	N/A
2µL. EPPENDORF KIT -increased to 40 cycles (LightCycler)	Product Rating	+	++	++
	Product Amount (ng/µL)	17.5	57.74	56.10
	Background Rating	No significant Background appearance	No significant Background appearance	No significant Background appearance
	Negative Amount (ng/µL)	6.52	18.815	16.19
	Corrected Amount (ng/µL)	10.92	33.93	39.94
1µL. EPPENDORF KIT	Product Rating	15.06		
	Product Amount (ng/µL)			
	Background Rating	5.75		
	Negative Amount (ng/µL)			
	Corrected Amount (ng/µL)			

Table 1-11. Low Volume PCR Reactions using the ROCHE PCR Kit

Note: Samples used 2X Taq, GC Rich Solution, 0.05µg/µL BSA, and 1% Tween

25µL PCR Reaction divided into 4µL, 3µL, 2µL and 1µL PCR Reactions		Idaho LightCycler	MJ ThermoCycler	Eppendorf MasterCycler
4µL	Product Rating	Begins at 3µL	++	++
	Product Amount (ng/µL)		54.765	57.76
	Background Rating		Weak evidence of smearing and primer dimers	Weak evidence of smearing and primer dimers
	Negative Amount (ng/µL)		23.82	33.675
	Corrected Amount (ng/µL)		30.945	24.085
3µL increased to 40 cycles (LightCycler)	Product Rating	+	++	++
	Product Amount (ng/µL)	14.3	49.48	47.87
	Background Rating	Weak evidence of smearing and primer dimers	Weak evidence of smearing and primer dimers	Weak evidence of smearing and primer dimers
	Negative Amount (ng/µL)	5.52	26.75	25.70
	Corrected Amount (ng/µL)	8.78	22.73	22.17
2µL without M.O. on the Thermo and MasterCycler	Product Rating	N/A – not visible on gel	++	++
	Product Amount (ng/µL)	6.66	58.24	69.14
	Background Rating	N/A – not visible on gel	No significant Background appearance	No significant Background appearance
	Negative Amount (ng/µL)	5.57	16.84	23.46
	Corrected Amount (ng/µL)	1.09	41.4	45.68

Table 1-12. Small Volume PCR with Roche Kit Reagents.

Note: conditions used 2X Taq, GC Rich Solution, 0.05µg/µL BSA, and 1% Tween.

25ul PCR Reaction divided into 2ul and 1µL PCR Reactions		Idaho LightCycler	MJ ThermoCycler	Eppendorf MasterCycler
2µL ROCHE KIT -increased to 40 cycles	Product Rating	N/A – not visible on gel	++	++
	Product Amount (ng/µL)	6.66	58.24	69.14
	Background Rating	N/A – not visible on gel	Weak evidence of smearing and primer dimers	Weak evidence of smearing and primer dimers
	Negative Amount (ng/µL)	5.57	16.84	12.17
	Corrected Amount (ng/µL)	1.09	41.4	56.97
1µL ROCHE KIT -increased to 40 cycles	Product Rating	N/A	N/A – not visible on gel	N/A – not visible on gel
	Product Amount (ng/µL)	none	none	none
	Background Rating	N/A	N/A – not visible on gel	N/A – not visible on gel
	Negative Amount (ng/µL)	none	none	none
	Corrected Amount (ng/µL)	N/A	N/A	N/A
1µL ROCHE KIT -with 2X DNA Template -increased to 40 cycles -0.5µg/µL BSA	Product Rating	N/A	+	+
	Product Amount (ng/µL)	none	2.57	4.09
	Background Rating	N/A	N/A	N/A
	Negative Amount (ng/µL)	none	1.175	2.356
	Corrected Amount (ng/µL)	N/A	1.395	1.734

Table 1-13. Small Volume PCR With Eppendorf Kit Reagents.

Note: conditions used 1X Taq, 0.5µg/µL BSA and 1% Tween.

25µL PCR Reaction divided into 2µL and 1µL PCR Reactions		Idaho LightCycler	MJ ThermoCycler	Eppendorf MasterCycler
2µL EPPENDORF KIT -increased to 40 cycles	Product Rating	+	++	++
	Product Amount (ng/µL)	4.23	52.74	56.10
	Background Rating		No significant Background appearance –weak smearing	No significant Background appearance –weak smearing
	Negative Amount (ng/µL)	3.39	18.8	16.19
	Corrected Amount (ng/µL)		33.94	39.91
1µL EPPENDORF KIT -increased to 40 cycles	Product Rating	+	+	+
	Product Amount (ng/µL)	7.87	2.94	6.10
	Background Rating		No significant Background appearance	No significant Background appearance
	Negative Amount (ng/µL)	2.99	2.16	3.98
	Corrected Amount (ng/µL)	4.88	0.78	2.12
1µL EPPENDORF KIT -increased to 40 cycles -with 2X DNA Template	Product Rating	+	+	+
	Product Amount (ng/µL)	8.315	3.23	7.36
	Background Rating		No significant Background appearance	No significant Background appearance
	Negative Amount (ng/µL)	2.62	2.09	3.0
	Corrected Amount (ng/µL)	5.695	1.14	4.36

Product bands were not visible on gels when using volumes less than 3 μL on the LightCycler (Table 1-11) although DNA product was detectable using SYBR Green I fluorescence. At very low volumes (less than 2 μL), mineral oil was required to prevent evaporation in all 3 instruments. However this may pose a problem with sample loss when pipetting through the oil to retrieve the sample for analysis.

These results suggested that 1 μL was the minimal PCR reaction volume that could reliably produce detectable product in all 3 thermocyclers, if the amount of DNA template was increased to double the concentration in larger reactions and the cycle number was increased from 35 to 40 (Tables 1-12 and 1-13). For the Roche kit, increasing the BSA concentration from 0.05 to 0.5 $\mu\text{g}/\mu\text{L}$ also helped improve product yield at low volumes. Both the Roche and the Eppendorf PCR reagents appeared to work equally well; no significant differences in product yield appeared when compared at low ($< 3 \mu\text{L}$) reaction volumes.

Because of the problems encountered by sample evaporation at such small volumes, and because mineral oil is impractical to use as a sealant on microchip, we investigated other methods to reduce sample evaporation during PCR.

5.5. Evaporation in Small-Volume PCR

Reducing or preventing evaporation at the volumes required for the microchip, will be critical in obtaining a detectable amount of specific PCR product. Evaporation may decrease the overall stringency of the reaction as the local buffer salt concentration is increased, thereby possibly resulting in the production of non-specific products; the increased amount of primer dimer formation was noted at smaller volumes during reagent validation for sv PCR. Lack of stringency may become an issue when genomic DNA from closely related species are present in a particular sample, or when performing multiplex reactions in the same sample.

5.5.1. PCR and sv PCR in the Presence of Sephadex G-50 Beads

Sephadex G-50 beads are commonly used for desalting and gel exclusion chromatography. In solution, these beads swell and trap water both within the bead, and on the surface of the bead in a microfilm, when placed in liquid (Figure 1-21). We theorized that the affinity for, and the trapping of water on and within the beads may help reduce evaporation and maintain effective local reagent concentrations during PCR thermocycling and improve overall PCR efficiency.

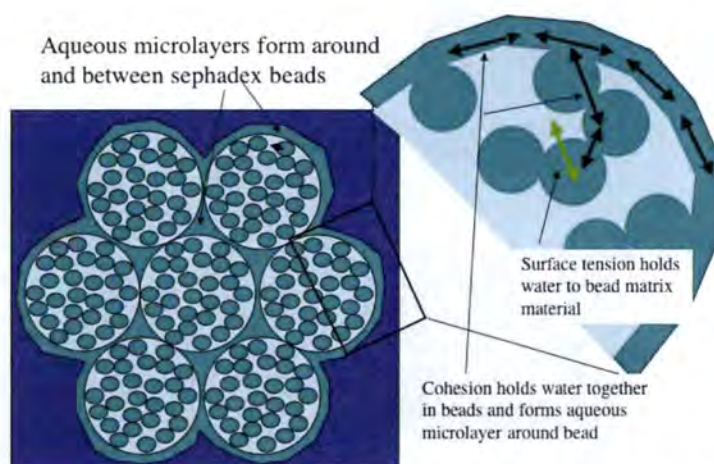


Figure 1-21. Physics of Aqueous Microlayer Formation in Sephadex G-50 Beads.

Initial experiments showed that PCR of the PGI plasmid template was successful in the presence of Sephadex G-50 when using volumes between 6 and 50 μL reaction slurry (beads plus master mix) (Figure 1-22). However, it needs to be noted that the control PCR in which the Sephadex beads were not used with the PCR product became contaminated as indicated by product amplicon in the no template lanes of the 1% agarose gel. Contamination, as observed, did not affect the results that PCR was successful with the use of Sephadex G-50 beads. To estimate the volume of required

PCR master mix solution required to rehydrate G-50 beads, various volumes of solution were added to dry beads and the total wet volume was measured. One volume of dry beads was rehydrated by approximately eight volumes of master mix (Figure 1-23, top panel). Although, approximately only one half of the fluid (i.e. 25 μL) was recovered after centrifugation (Figure 1-23, bottom panel).

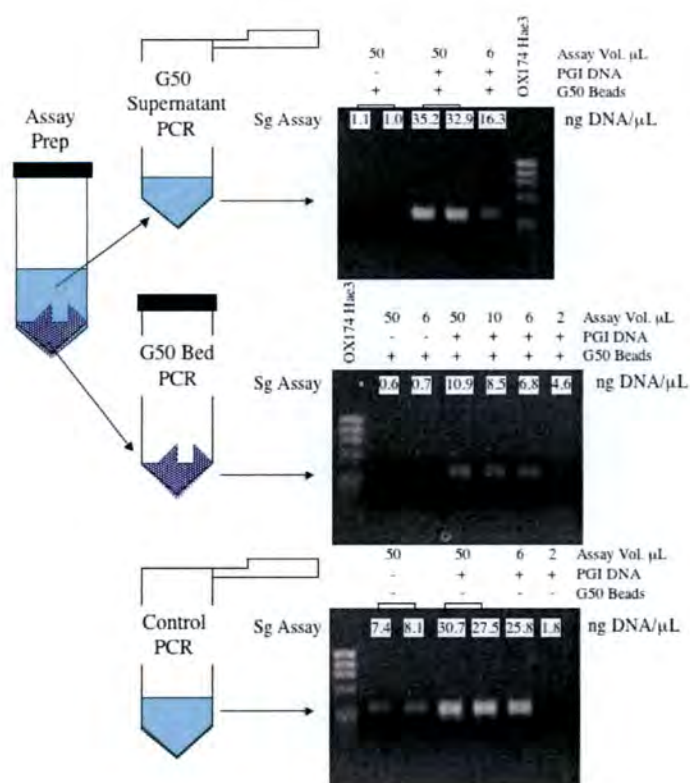
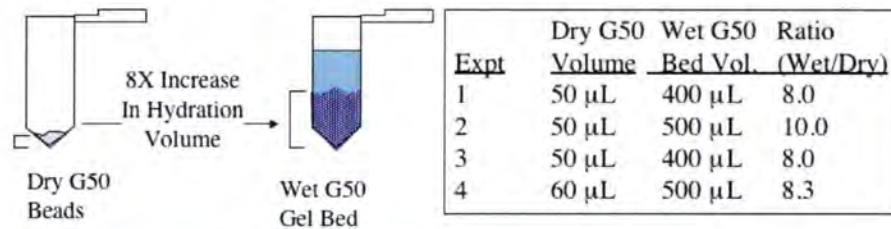


Figure 1-22. Sephadex G-50 aqueous microlayers support PCR assays with pPGI template.

Figures show PCR products obtained from supernatants and G-50 beds. Quantitative values for product were obtained from SYBR Green I (Sg) assays.

Sephadex G50 Hydration Volume



Flow-Through Liquid Recovered from Wet G50 Gel Bed

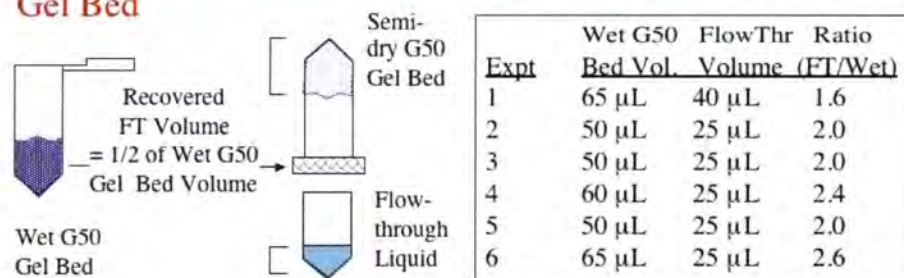


Figure 1-23. Sephadex G-50 Rehydration.

Top panel: Dry Sephadex G-50 beads were rehydrated with an excess of PCR Master Mix. Overlying supernatant was removed, and the volume of wet beads was measured. Bottom panel: Wet G-50 beads were centrifuged through a membrane filtration unit to remove as much liquid as possible; the "flow through" was collected and measured to obtain the amount of fluid recovered.

5.5.2. Sephadex G-50 aqueous microlayers support sv PCR volumes down to 2 μ L.

Figures 1-24 through 1-26 show the results of PCR and sv PCR using small volumes in the presence of Sephadex G-50. At 4 μ L PCR volumes, more product was obtained when assays were performed in the presence of G-50 beads than when not (Figure 1-24). However, at 2.5 and 1 μ L volumes, G-50 appeared to be unable to support PCR (Figures 1-25 and 1-26). Because previous experiments had already established that sv PCR can successfully be performed in volumes as low as 1 μ L, we theorized that the observed lack of product may have been due to lack of recovery of product from the beads rather than assay failure. Upon centrifugation of the G-50 slurry after assay and

capture of the residual liquid through a filtration membrane, we observed recovered product (Figure 1-27); this product was located in the microlayer of fluid surrounding the wet beads. Once again it must be noted that during the experiments with Sephadex G-50 beads the control PCR in which no template was added showed product amplicon. Contamination of the control does not affect the observed results that the sv PCR product was being trapped in the Sephadex beads. These results led to investigating how to standardize collecting sv PCR samples with G-50 beads after thermocycling.

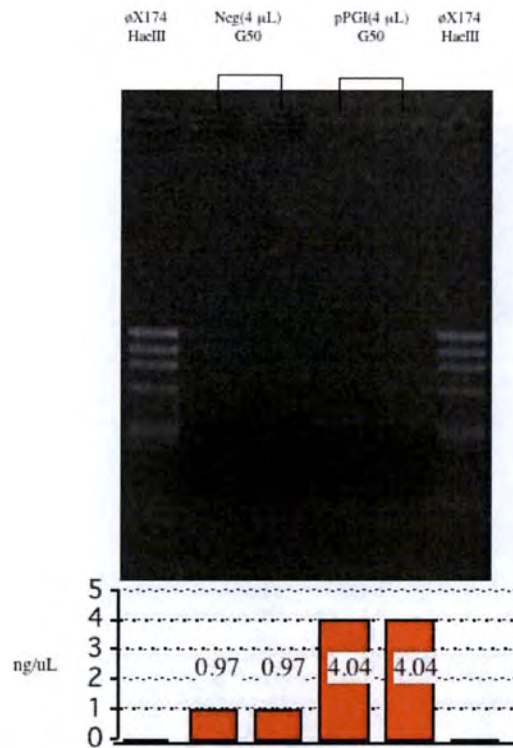


Figure 1-24. PCR amplification of pPGI plasmid DNA occurs in 4 µL Sephadex G-50 assay volumes.

Samples were set up using pPGI template and G-50 slurry. Both the stained gel and quantitative results from SYBR Green I assays are shown.

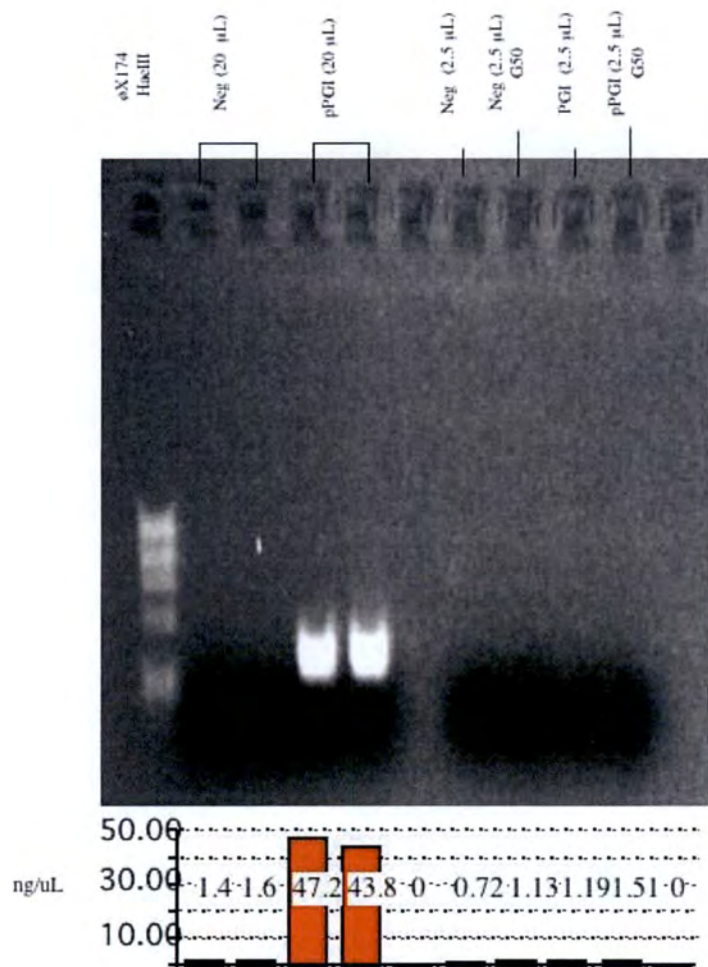


Figure 1-25. PCR amplification of pPGI plasmid DNA does not occur in 2.5 µL Sephadex G-50 assay volumes.

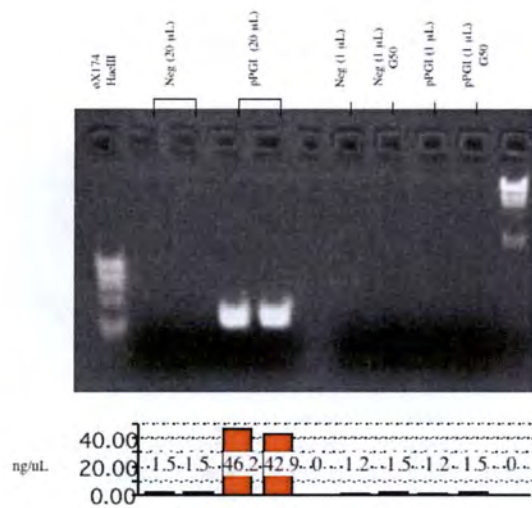


Figure 1-26. PCR amplification of pPGI plasmid DNA does not occur in 1 µL Sephadex G-50 assay volumes.

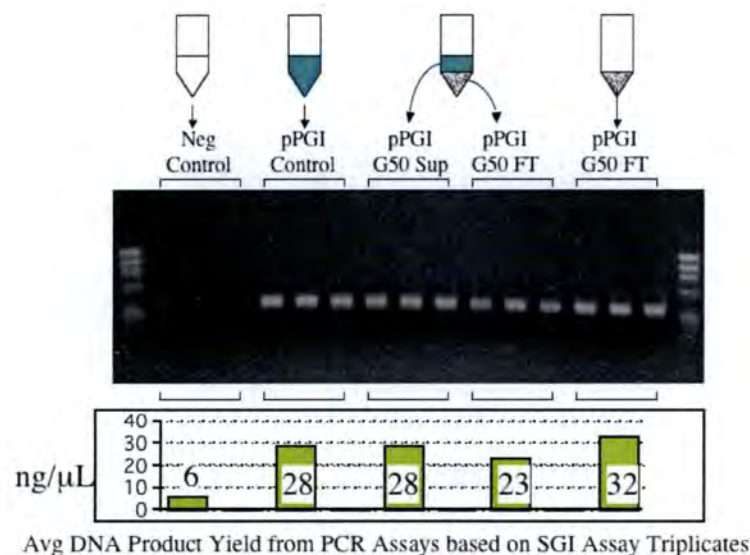


Figure 1-27. PCR amplification of pPGI plasmid DNA occurs in aqueous microlayers surrounding Sephadex G-50 beads.

Supernatants and residual fluid after spinning down wet G-50 beads were collected and analyzed by gel electrophoresis and SYBR Green I assay. All sites showed presence of PCR product.

5.5.3. Replicate Analysis Strategies with sv PCR

When sv PCR assays of 1-2 μL are performed, analysis is difficult if the sample must be divided for analysis by different assays (e.g. agarose gel electrophoresis, SYBR Green I fluorescence). There are significant problems with evaporation and adsorption of sample to container surfaces, or loss of sample in pipette tips during sample transfer. The technical difficulties in handling and analyzing small volume samples can be overcome if the following technical assumptions are accepted:

1. Pooled replicates give comparable results to a single small volume assay. However this would need to assume that sample-to-sample variability is minimized, which may or may not be the case.

2. Dilution of small volume assays (e.g. 2 μL) to larger volumes (10 μL) can minimize liquid carry-over loss of the sample in the pipette tip during sample transfer. The loss of sample is more significant at small volumes than larger volumes.
3. Carry-over sample loss due to adsorption of DNA or sticking of media to surfaces, can be minimized by reducing the number of contact surfaces (*i.e.* the number of transfers and containers) the sample encounters prior to analysis. Ideally, amplification and analysis would be conducted in the same vessel.

As noted in Figure 1-27 centrifugation of the G-50 beads resulted in recovery of the PCR product therefore we examined three different methods to recover svPCR samples from the G-50 beads (Figure 1-28). Multiple single centrifugations of individual sv PCR samples prior to pooling (Method A) resulted in significant volume for gel visualization but increased sample loss due to surface contact and evaporation. Rinsing the reaction vessels and the beads and then pooling the samples prior to centrifugation (Methods B and C) showed a significant increase in sample recovery. Pooling multiple sv PCR samples allowed for increased visualization of product on gels (Figure 1-29). As noted in the previous section, the use of G-50 beads in svPCR required centrifugation of the slurry to obtain product amplicon. For svPCR, it was observed that with the use G-50 beads, there was more product for reaction volumes of 2 μL in comparison to less product for reaction volumes of 6 μL . We did not examine direct transfer of G-50 assay samples (without prior centrifugation) to agarose gels after dilution with loading buffer; it is possible that the G-50 could interfere with SYBR Green I analysis, but we did not test this theory.

20 tube Replicates X 1 μ L G50 Assays		Sample Rinse & Pooling	Rounds of Centrifugation	Time	Loss to Surface Contact	Loss to Evaporation	Sample Conc.	Sample Recovery	Method
		None	20	++	++	++	++	+	A
			1	-	+	-	-	++	B
			4	+	-	-	-	++	C

Figure 1-28. Strategies for Recovering svPCR Assay Samples for Analysis

Three methods (A, B and C) are shown, along with a summary of results of sample recovery using each method.

7	20	# pooled replicate assays
6	2	Volume (μ L)/replicate pre-PCR
2.9	0.9	Avg. μ L/replicate post-PCR
6.8	4.6	ng DNA/ μ L [sg Assay]



svPCR Assay
+ G50

7	18	# pooled replicate assays
6	2	Volume (μ L)/replicate pre-PCR
4.3	1.4	Avg. μ L/replicate post-PCR
25.8	1.8	ng DNA/ μ L [sg Assay]



svPCR Assay

Figure 1-29. Pooled Replicates from sv PCR/G-50 Give Sufficient Signal to be Detectable on Gels.

Samples from svPCR with and without G-50 beads were collected and then run on agarose gels and quantified by SYBR Green I fluorescence. The top panel shows a comparison of pooled replicates used with Sephadex G-50 beads of 6 μ l (high volume) PCR reactions versus 2 μ l (low volume) PCR reactions. The higher PCR reaction volume showed a greater amount of product with fewer pooled replicates than an increased number of replicates using the lower reaction volume. The bottom panel is a comparison of pooled replicates without the use of Sephadex G-50 beads of 6 μ l (high volume) PCR reactions versus 2 μ l (low volume) PCR reactions. The higher PCR reaction volume showed a greater amount of product with fewer replicates and in comparison to no product in the absence of Sephadex G-50 beads at the lower reaction volume.

5.6. Other Strategies to Improve PCR Product Yield in sv PCR

During the validation experiments it was noted that adjustment of template and BSA concentration was required for sv PCR to obtain detectable product. Hence this may infer that adjustment of the current concentrations of reagents in the PCR reaction to accommodate small volumes may be required. Of note, removal of water from the reaction by evaporation effectively raises the local concentration of all PCR reagents; all of these have an effect on *Taq* enzyme proficiency. Hence for sv PCR, either reducing the amount of dNTPs or increasing the magnesium in the reaction may improve overall reaction yield. *Taq* requires free magnesium for activity; however, higher local concentrations of dNTPs, primers and template after evaporation, may sequester all the free available magnesium thereby inhibiting the *Taq*. Increasing the overall Mg^{2+} concentration also appears to improve product formation in the presence of high buffer salt concentrations; however concentrations above 10.8 mM Mg^{2+} inhibit the reaction. In this case, adjusting the Mg^{2+} concentration would be worthwhile to account for possible decreased stringency. Other additives, such as DMSO, may also inhibit the *Taq* under conditions of evaporation; DMSO concentrations >10% are known to inhibit the enzyme. None of these were addressed in these studies, but would form important avenues of investigation in future work.

Conclusions and Recommendations

PDMS was found to be a suitable medium to construct PCR reaction vessels for microchips. Inclusion of BSA had mixed effects: it was necessary for PCR amplification but interfered with fluorescent detection. PCR reagents were stable for at least 24 hours when fully reconstituted; dried reagents were active for up to 7 days after drying, and up to 15 days when nucleotides were added after reagent reconstitution. Small-volume PCR was technically difficult; water evaporation was significant at very small volumes, resulting in large variability of product obtained between samples. Addition of Sephadex G-50 beads as a water trap enhanced PCR product synthesis at small volumes (less than 2 μ L), and hence could be added in the microchip reaction chamber to ensure production of PCR product in situations where evaporation would be a concern. Special care must be taken at each step to ensure strict quality control of reagents and prevent any contamination especially when working at the very small sample volumes used in microfluidics.

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Fluorescent-Based Genetic Analysis for a Microfluidics Device—Part Two (Phase III). Interaction of tethered-dye gene probes with complementary target sequences.

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N. Chan, Independent Research Services

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Abstract for Part II

Polymerase chain reaction (PCR) is commonly used for biodetection, especially for low levels of biomaterials. Typically, the identity of a PCR product is confirmed on agarose gel electrophoresis and through DNA sequencing. This time-consuming detection-confirmation process can be eliminated through the incorporation of simultaneous gene probe hybridization and fluorescence detection. A proposed tethered-dye gene probe (TDP) was evaluated as a biosensor for use in real-time detection in PCR. Fluorescence characterization of untethered- vs. tethered- dye, and of unbound vs. bound TDP to target dsDNA, was measured in a fluorescence spectrophotometer. Two different dyes, thiazole orange (TO) and reactive SYBR® 101 (SYBR) were compared to the commonly used dsDNA binding dye, SYBR Green I (SGI). Gene probes were designed and prepared to target the *Erwinia herbicola* phosphoglucoisomerase (Eh PGI) gene. Real-time PCR was measured in a LightCycler (Idaho Technologies Inc.) thermal cycling instrument.

Executive summary for Part II

Introduction

The polymerase chain reaction (PCR) is commonly used in biodetection platforms because of its ability to amplify a gene of interest to detectable levels. Biodetection strategies require reagents with high sensitivity, specificity and stability to withstand the rigors of high throughput screening assays. DNA hybridization provides selectivity in PCR biodetection due to specific interaction of gene probes with complementary DNA. Fluorescence detection provides high sensitivity with a low detection limit. Real-time detection during PCR cycling allows fast identification of biomaterials, compared to otherwise time-consuming technologies such as agarose gel electrophoresis or DNA sequencing. Therefore, tethered-dye gene probes (TDP) are important as biosensors as they combine both PCR amplification and DNA hybridization for added specificity and fluorescence detection in real-time.

Erwinia herbicola (Eh) is used as a biological agent simulant for vegetative bacterial cells such as *Yersinia pestis* (bubonic plague). A PCR protocol was previously developed using the phosphoglucose isomerase gene (PGI) in *E. herbicola* as a template for identification. This PCR protocol was used to test the usefulness of TDP detection in real-time.

DNA probe hybridization provides specificity for recognition and confirmation of PCR product. A DNA probe hybridizes to its complementary sequence in the PCR product to form double-stranded DNA (dsDNA). The hybridized probe will then separate from its complement when the temperature of the solution exceeds a specific melt temperature, (T_m). Thus, a melt curve (fluorescence against temperature) can be used to obtain the T_m after PCR for confirmation provided that a suitable fluorescent binding dye is used in the reaction.

Fluorescent dye molecules such as TO or SYBR are specifically designed to interact with dsDNA and produce strong fluorescent signals. Due to their ability to exhibit exceptional affinity for dsDNA and a large fluorescence enhancement upon binding to dsDNA, the

background fluorescent signal is low. Thus, when TDP “melt” apart from complementary DNA, the fluorescent signal will drop drastically to reveal the specific melt temperature of the probe.

Results

A total of 3 gene probe sequences specific to Eh PGI were selected as candidates for TDPs. Two fluorescent dyes were chosen, thiazole orange (TO) and SYBR® 101 (SYBR), to chemically attach to the 5' end of a selected gene probe. Chemical synthesis of gene probes tethered to TO was done at the University of Toronto in Prof. U. Krull's laboratory. SYBR-tethered gene probes were prepared through a condensation reaction between a reactive SYBR reagent and a 5'-amine modified gene probe. Both fluorescent dyes were evaluated, based on thermal stability and sensitivity in fluorescence signals, for their utility in real-time PCR.

Results from fluorescence titration and melt curve experiments suggested TO was not a desirable dye to use in TDPs due to a high intrinsic fluorescence background, only minimal increase in fluorescence upon binding to dsDNA, and a failure to interact with dsDNA. The alternate dye, SYBR, showed some promising results in sufficient increase in fluorescence upon binding to dsDNA, and ability to bind complementary ssDNA up to 90 nucleotides. An accurate T_m was recorded for hybridization of SYBR-probe to complementary ssDNA with less than 0.2mM SYBR-probe.

Significance

The SYBR-probe has potential as a biosensor for use in real-time PCR experiments.

Introduction

Tethered-dye gene probes are important as biosensors for use in real-time PCR detection. Gene probe molecules are used as biosensors to detect DNA hybridization because of added selectivity. Many detection technologies are available for DNA hybridization, though fluorescence provides high sensitivity and a low detection limit. Real-time DNA target detection during amplification provides a fast, efficient method for sensitive and quantitative sequence detection and identification.

Fluorescent dyes, such as SYBR Green I (SGI), associate with double stranded (ds) DNA and exhibit a large increase in quantum yield resulting in an increase in fluorescent emission. This kind of detection is not selective because SGI will interact with the structure of dsDNA at non-specific locations. Other detection technologies such as fluorescence resonance energy transfer (FRET) [1, 2] with two singly-labeled oligonucleotide probes, and TaqMan [3] or Molecular Beacon [4, 5] with one doubly-labeled oligonucleotide probe, provide selectivity upon hybridization of the probe to target sequence. Though effective, the above-mentioned technologies can be costly due to the use of two different dyes and thus the requirement for different excitation and detection sources. In recent years, a new gene probe consisting of a peptide nucleic acid oligomer (PNA) was also used to specifically detect DNA hybridization with thiazole orange (TO) as the fluorescent tag [6]. An alternative detection method, using a tethered-dye DNA gene probe with one fluorescent label (TO or SYBR dye molecules) is described in this report.

Previous work on TDPs was done by tethering the fluorescent dye to polydeoxythymine (poly-dT) at the University of Toronto (Prof. U. Krull's group). TO was selected because of several factors including working wavelengths for fluorescence measurements, availability of starting material, suitable linking chemistries, and thermal stability in PCR conditions. TO tethered probes were

prepared by Prof. Krull's group [7]. An alternative dye, SYBR® 101, was also used to tether a gene probe as a comparison. The reactive SYBR® 101 molecule (Molecular Probes Inc.) contained a succinimidyl ester group that enabled formation of covalent bonds with primary amines. If a gene probe is modified with a terminal amine group, it will react readily with the SYBR-succinimidyl ester to form a stable covalent linkage.

In a conventional PCR procedure (Figure 2-1), an optimal amount of dsDNA is mixed with an excess of deoxynucleotides tri-phosphates (dNTPs), and DNA Taq polymerase in the presence of a PCR buffer containing MgCl₂. The target dsDNA is first denatured at 95°C to separate into single stranded DNA (ssDNA). PCR primers then anneal to specific locations along target DNA, followed by the extension of the DNA strand by Taq polymerase. This denature-anneal-extend cycle is repeated to replicate target dsDNA exponentially.

In a real-time PCR detection system using ds DNA binding dyes such as SGI, an excess of SGI dye molecules are added to the PCR mixture prior to amplification in a thermal cycler that has detection capability such as a LightCycler (Idaho Technologies Inc.). SGI dye interacts with dsDNA and emits fluorescence upon excitation by a light source. Fluorescence excitation and detection occur during the extension step. Fluorescence intensity increases exponentially as dsDNA is replicated, ultimately reaching a plateau when a limiting reagent, typically dNTPs, is completely utilized in the reaction.

The strategy of real-time PCR detection proposed under this contract was to use a single gene probe tethered with a single fluorescent dye molecule (Figure 2-2). Three nucleotide probe sequences were designed for the purpose of finding an appropriate TDP for real-time PCR detection. For this contract, the *Erwinia herbicola* (Eh) PCR of phosphoglucose isomerase gene (*pgi*) was used as the model. The constructs of the

plasmid (pPGI) containing Eh PGI, partial gene sequence of *pgi*, and resulting PCR amplicon can be found in Annex A. Fluorescence was detected during the annealing step in the PCR cycles. Fluorescence was observed when thiazole orange was excited at λ_{ex} 480nm or SYBR® 101 at λ_{ex} 485nm. All TDPs were blocked at the 3' end with a phosphate group or terminated with a dideoxynucleotide to prevent DNA Taq polymerase from using the TDP as “primers” to produce undesirable products.

The following criteria were established for designing tethered-dye probes: length of 18-20 nucleotides, <50% GC, low number of blast hits (false positives), does not readily form complexes with itself or with PCR primers, and has a higher melting temperature (T_m) than both PCR primers.

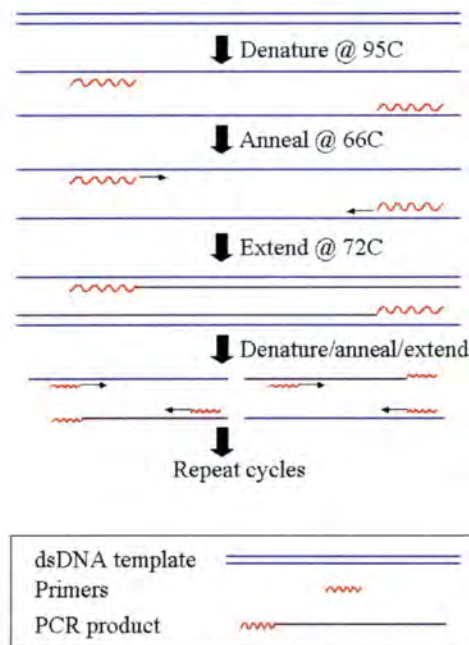


Figure 2-1. Scheme of a conventional PCR procedure.

Double stranded DNA (double blue lines) was first denatured at 95°C to separate into single stranded DNA (single blue line). PCR primers (red squiggles) were annealed to specific locations along target ssDNA prior to extension of DNA strand (purple lines) by DNA Taq polymerase. This denature-anneal-extend cycle is repeated to replicate target DNA exponentially.

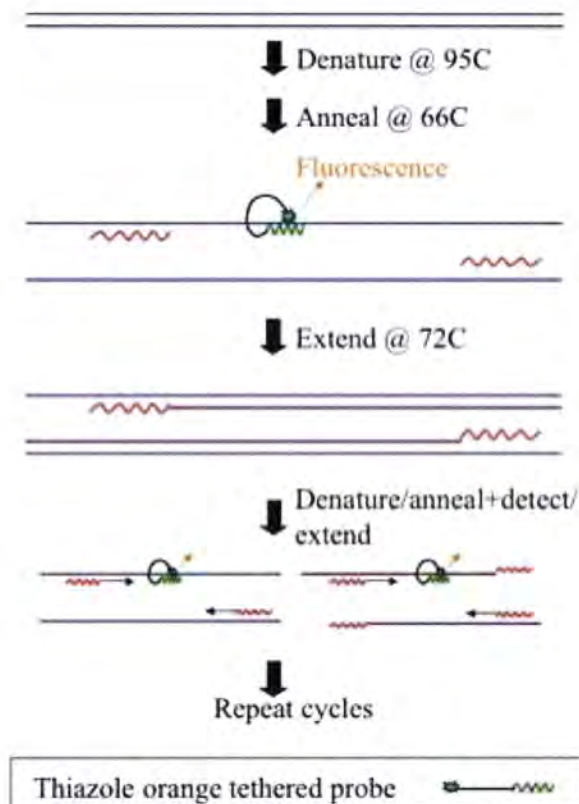


Figure 2-2. Scheme for the proposed strategy for one singly-labeled gene probe.

The above diagram shows a PCR procedure with tethered-dye probe (TDP) included for detection during the extension step (after annealing) in PCR. There are two possible approaches of using a TDP in a real-time PCR detection. One approach is to use primer tethered with fluorescent thiazole orange (red squiggle with green dye molecule), while the other approach is to design an oligonucleotide sequence (green squiggle with green dye molecule) within the sequence of DNA target to provide selectivity in detection. Fluorescence detection occurs during annealing step in each PCR cycle.

Materials and Methods

1. Chemicals

Thiazole orange (free dye) was purchased from Sigma-Aldrich, reactive SYBR® 101 and SGI were purchased from Molecular Probes Inc., electrophoresis grade agarose was obtained from Life Technologies Inc., all other chemicals were reagent grade quality from Sigma-Aldrich, and all solutions were prepared in nanopure, filtered water. Phosphate-buffered saline (PBS) solution contained 10mM sodium phosphate, pH7.4, 120mM NaCl, 2.7mM KCl. Tris/borate/EDTA electrophoresis buffer (1x TBE) contained 89mM Tris-HCl, 89mM borate, 2mM EDTA, pH8.0. 50mL Steriflip® sterile disposable vacuum filtration systems (Millipore Corp.), fitted with 0.22µm Durapore® PVDF membrane, were used to filter all aqueous solutions.

2. Probe Design

A large set of potential probe gene sequences based on *E. herbicola* *pgi* was created using DNAsis™ (Hitachi Software Engineering Company, Ltd.) and Oligo 4.1 (Molecular Biology Insights, Inc.). A set of specific criteria was established for designing tethered dye probes: length of 18-20 nucleotides; ≤50% GC content; low number of hits (false positives) when screened using the Basic Local Alignment Sequence Tool program (BLAST; NIH); does not readily form complexes with itself or with PCR primers; and has a higher melting temperature (T_m) than both primers. Special care was taken when selecting changes in the second and third codon between *E. herbicola* and *E. amylovora*, since both species share significant similarity in their *pgi* sequences (see Annex B for comparison of *pgi* sequences from both *E. herbicola* and *E. amylovora*).

3. Fluorescence Measurements

Fluorescence spectroscopy and melt temperature measurements were done in a Varian Cary Eclipse fluorescence spectrophotometer equipped with a temperature controller capable of ramping temperatures (between -10°C to $+100^{\circ}\text{C}$) at specific transition rates. Real-time PCR reactions were measured in a thermocycler equipped with both excitation and emission detections (LightCycler, Idaho Technologies Inc.)

Fluorescence titration of DNA into a solution of TDP in PBS was done in the Cary Eclipse fluorescence spectrophotometer using the dye's excitation and emission wavelengths (TO: 495/530nm, and SYBR: 485/520nm with a 5 nm slit in both wavelengths). Temperature ramping rate was at $0.5^{\circ}\text{C}/\text{min}$ between 30°C and 80°C , and samples were taken every 0.5-1min during all melt temperature experiments. Emission spectra were collected by scanning fluorescent signals between 505-555nm with a 5 nm slit.

4. PCR

PCR was done with 3 different kits: HotStar Taq polymerase from Qiagen, FastStart Taq polymerase from Roche Diagnostics, and HotMaster Taq polymerase from Brinkman/Eppendorf. The specific PCR conditions for each 25 μL reaction mixture were as follows:

0.625 μU HotStarTaq polymerase (in 20mM Tris-HCl, 100mM KCl, 1mM DTT, 0.1mM EDTA, 0.5% (v/v) Nonidet® P-40, 0.5% (v/v) Tween® 20, 50% (v/v) glycerol, stabilizer, pH 9.0) in the presence of 1x PCR buffer (Tris-HCl, KCl, $(\text{NH}_4)_2\text{SO}_4$, 1.5mM MgCl_2) supplemented with 2.0 mM MgCl_2 , 0.2 mM dNTPs, 0.6 mM forward primer (18CA14F), 0.6 mM reverse primer (18CA14R), 1x Q-solution, 0.05 $\mu\text{g}/\mu\text{L}$ BSA, and 1% Tween® 20.

0.625 µU or 1.25 µU FastStart Taq polymerase (in 20 mM Tris-HCl, pH 9.0, 100 mM KCl, 0.1 mM EDTA, 1.0 mM DTT, 0.2% Tween® 20 (v/v), 50% glycerol (v/v)) in the presence of PCR reaction buffer (50 mM Tris-HCl, 10 mM KCl, 5 mM (NH₄)₂SO₄, pH8.3), supplemented with 3.5 mM MgCl₂, 0.2 mM dNTPs, 0.6 mM 18CA14F, 0.6 mM 18CA14R, 1x GC-rich solution, 0.05 µg/µL BSA, and 1% Tween® 20.

0.625 µU HotMaster Taq polymerase (in 25 mM Tris-HCl pH8.0, 35 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween 20, 0.5% Igepal® CA-630 and stabilizers) in the presence of 1x HotMaster Taq buffer with 25 mM Mg²⁺ supplemented with 0.2 mM dNTPs, 0.6 mM 18CA14F, 0.6 mM 18CA14R, 1x GC-rich solution, 0.05 µg/µL BSA, and 1% Tween® 20.

PCR product was subjected to gel electrophoresis in 1% agarose in 1x TBE buffer/0.5µg/mL ethidium bromide (EtBr) with a Horizon 11-14 electrophoresis apparatus from Gibco BRL and a BioRad Power Pac 300 power supply.

Real-time PCR amplification was performed in a LightCycler with an enzyme activation step at 95°C for the length of time recommended by the manufacturer, followed by 45 cycles of denaturation at 94°C for a duration of 5sec., followed by annealing at 55°C for 5sec., and an extension at 72°C for 20sec at a temperature ramp rate of 2°C/sec. After PCR cycling, a melt experiment was done by denaturing dsDNA at 94°C for 0 sec, then decreasing the temperature rapidly to 50°C for a length of 20 sec followed by an increase in temperature slowly up to 100°C at a rate of 0.2°C/sec. All other temperature ramp rates were 20°C/sec.

Results and Discussion

1. Design of gene probes

Three gene probes were designed to be used as tethered dye probes (TDP). A search of the entire *E. herbicola* *pgi* sequence (see Annex C for complete sequence) using DNAsisTM and Oligo 4.1 resulted in a cluster of 20-nucleotide sequences suitable for use as gene probes. Table 2-1 shows melt temperatures calculated using equation 1, sequence length, location on *E. herbicola* *pgi* partial sequence for both primers. Table 2-2 shows the probe sequences (location on *pgi* partial sequence), and differences in T_m from primers determined by DNAsisTM and Oligo 4.1 with restrictions of 20 nucleotides and $\leq 50\%$ GC content. Further analyses of probe sequences to determine possibilities of false priming (blast search for matches to other DNA sequence in database, self-dimerization or forming complexes with either primers or PCR target) is recorded in Table 2-3.

Table 2-1. Analysis of forward and reverse primers for *E. herbicola* *pgi* gene PCR reaction.

T_m was calculated using equation 1 at 50mM salt concentration.

Eh primer in PCR reaction

Kind	Start	End	Length	T_m	GC%	Sequence
Forward	128	145	18	58.16	50	5'-GTTTTGCCGCCTATTTCC-3'
Reverse	451	471	21	57.311	52.381	5'-CACGATATGGGCTACAGACTC-3'
Product	128	471	344			

$$T_m = 81.5 + 16.6 (\log [Na+]) + 0.41 (GC\%) - 600/Length$$

Eq. 1

Table 2-2. Three probe sequences determined by DNAsisTM and Oligo 4.1.

T_m value was estimated with equation 1 and the difference in T_m (ΔT_m) from both primers were determined.

Start position	End position	Length	ΔT_m (°C) from primer	GC%	Sequence
293	312	20	+5.5	50	5'-TTCCGTGCGACTTTATTGCC-3'
319	338	20	+4.9	50	5'-ATTACCCATAATGCGCTCGC-3'
341	360	20	+2.3	50	5'-ATCACCATCCGAAACTGCTC-3'

Table 2-3. Results from BLAST search and Oligo 4.1 of the eight new sequences.

Start	End	Total blast hits	# of mismatches						ΔG (complex formation)			Possible false priming site
			zero	1	2	3	4	>5	Self-dimer	Upper primer	Lower primer	
293	312	1	0	0	0	0	0	1	-3.1	-6.7	-6.8	1
319	338	6	0	0	0	0	1	5	-9.8	-3.6	-10.4	nil
341	360	10	0	0	0	0	0	10	-3.1	-5.4	-6.5	nil

We decided to prepare the following DNA sequences (Annex D):

EhPGI 341-360 (5'-ATCACCATCCGAAACTGCTC-3')

EhPGI 319-338 (5'-ATTACCCATAATGCGCTCGC-3')

EhPGI 293-312 (5'-TTCCGTGCGACTTTATTGCC-3')

These sequences followed the criteria for ideal probe design as set out in the Materials and Methods. Please refer to Annex E for structures depicting formation of some possible complexes. The forward primer (18Cal4F (5'-GTTTGGCCGCCTATTTCC-3')) in the *E. herbicola* PCR reaction was also chosen for tethering as a comparison to using probes.

The above 4 sequences were forwarded to Prof. U. Krull for synthesis with thiazole orange dye tethered at the 5' end. Probe synthesis utilized a dideoxynucleotide at the 3' end to prevent elongation of the probe by DNA Taq polymerase. TDPs were purified from un-reacted TO dye and gene probes to prevent competition of binding with unlabeled probe. Two preparations of TDP were received, TO-EhPGI 319-338 and TO-EhPGI 341-360, in which TO-EhPGI 319-338 was contaminated with un-reacted EhPGI 319-338 and thus not useful for experiments. A measured amount of 3.8 nmol of TO-EhPGI 341-360 probe was used in experiments for evaluation of the usefulness of a TDP for hybridization to target DNA and in real-time PCR.

Additionally, 3 TO-tethered poly-dT probes were provided by Prof. U. Krull for preliminary experiments prior to receiving the above-mentioned TDP. These 3 TO-poly-dT probes were (Annex D):

5' TO-TTCTCTCTTTTTTTT-3' (TO-mix 15-mer);

5' TO-TTTTTTTTTTTTTTTTTT-3' (TO-dT₂₀);

5' TO-TTTTTTTTTTTTTTTTTT-NH₂ 3'.

A modified *E. herbicola* *pgi* plasmid was prepared in order to use the probes listed above. The new pPGI[pA1] contained a poly-A (pA1) linker prepared by annealing two synthetic oligonucleotides, CA14A20[+] linker and CA14A20[-] linker. The linker design was based on the plasmid, pPGI, a tract of A₂₀ (pA or poly-A) and (AG)₃ which served as a target sequence for the fluorescent tethered-dye probes, TO-dT₂₀ and TO-mix 15-mer, and *E. herbicola* PGI target gene probes. Details of the pPGI[pA1] sequence can be found in Annex F.

2. Excitation and emission spectra of TO and SGI

Both excitation and emission spectra of fluorescent dyes were observed separately on the fluorescence spectrophotometer. A 100 µL quartz cuvette was used in all experiments with a special cuvette holder (Annex G). A 10 µM solution of the tosylate salt of TO was mixed with chicken genomic DNA and scanned for both excitation (Ex) and emission (Em) spectra (Figure 2-3). The interference peaks in Figure 2-3, with high intensities were due to reflection of corresponding excitation and emission bands. The emission spectra of a solution of 0.3x SGI alone in the presence of dsDNA were obtained with an excitation wavelength set at 485 nm (Figure 2-4). The optimal excitation and emission wavelengths of TO were 495 nm and 530 nm, respectively. Optimal Ex and Em wavelengths for SGI were confirmed as 485 nm and 520 nm as reported by the manufacturer.

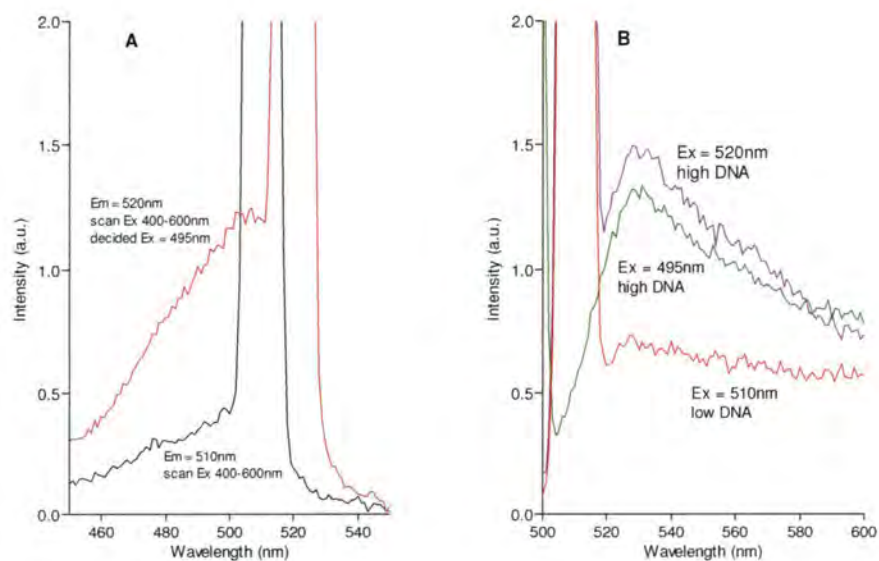


Figure 2-3. Excitation and emission spectra of thiazole orange (TO).

A) excitation scans were collected over a range of 400-600nm, with emission set at 510nm (black trace) and at 520nm (red trace). Big peaks (off scale) were both a reflection of the emission wavelength collected, 510nm and 520nm respectively. B) emission scans were collected over a range of 500-600nm, with excitation wavelength set at 510nm (red trace), 520nm (purple trace) and 495nm (green trace). Big peaks were a reflection of the excitation wavelengths of 510nm and 520nm, respectively.

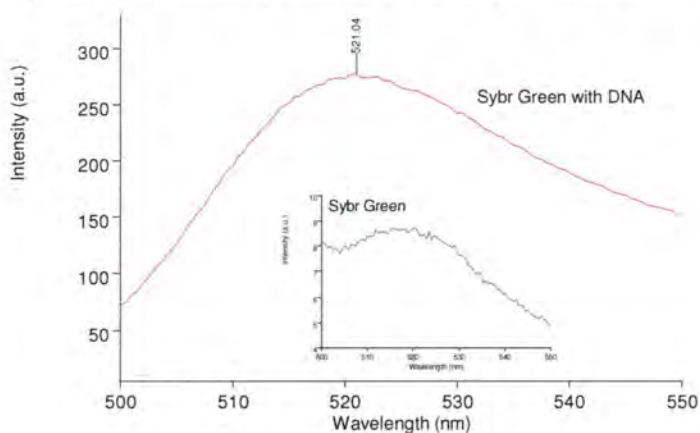


Figure 2-4. Emission spectra of SYBR Green I (SGI) with 20ng chicken genomic DNA, and SYBR Green I alone (insert).

Excitation wavelength was set at 485nm, PMT voltage at 800V. The increase in fluorescence in the presence of dsDNA was about 30 times.

3. Comparison of fluorescent sensitivity between TO and SGI

Chicken genomic DNA was used as a test sample for DNA titration using the Cary Eclipse fluorescence spectrometer. Both dyes were used for comparison in a 100 μ L volume, SGI at a final concentration of 3.33x and TO at 10 μ M. Chicken genomic DNA was added to the dye solution in aliquots of 5ng and 10ng, SGI and TO respectively (Figures 2-5 and 2-6). From the slopes of linear regressions fitted to both figures, sensitivity for DNA was 10-times higher with SGI than TO (2.24 and 0.220 respectively). This difference was likely due to less efficient intercalation with monomer TO.

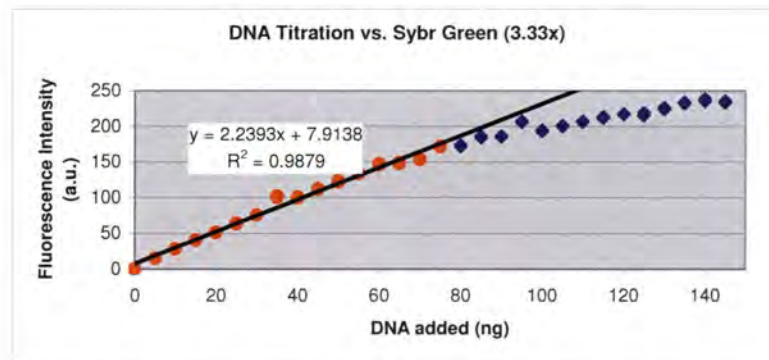


Figure 2-5. Chicken genomic DNA titrated against a 3.33x concentrated SGI solution.

DNA was added in aliquots of 5ng. Fluorescence was collected with Ex = 485nm, and Em = 520nm using Cary Eclipse fluorescence spectrometer. A linear regression was fitted to the linear portion of this titration curve. Slope of this linear regression was 2.24.

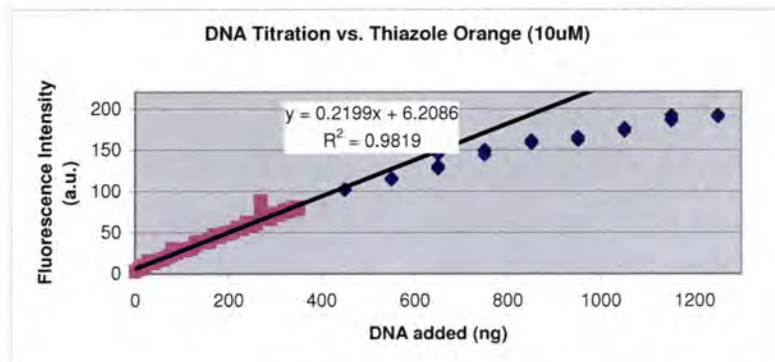


Figure 2-6. Chicken genomic DNA titrated against a 10 μ M TO solution.

DNA was added in aliquots of 10ng. A linear regression was fitted to the linear portion of this titration curve. Slope of this linear regression was 0.220.

4. Titration of EhPGI 319-338 dsDNA and free thiazole orange

Free TO was used to titrate dsDNA (EhPGI 319-338 and its complement, rEhPGI 319-338), also referred to as “DNA pair” in some figures, using a Cary Eclipse fluorescence spectrophotometer. Thiazole orange (TO) was added in aliquots of 5 pmol to a solution containing EhPGI 319-338 dsDNA (0.5 μ M and 0.1 μ M) in PBS (Figure 2-7).

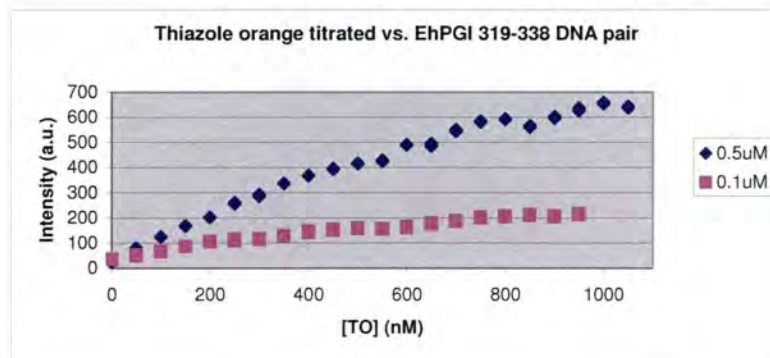


Figure 2-7. TO titrated against EhPGI 319-338 dsDNA in PBS.

Aliquots of TO were added to a solution containing 0.5 μ M (blue diamonds) or 0.1 μ M (pink squares) of the dsDNA. Both titration curves remained linear at low concentrations of free dye.

Alternatively, the dsDNA was also titrated against different fixed concentrations of free TO (Figure 2-8). Aliquots of 1 or 2 pmole of dsDNA were added to free TO concentrations of 0.1 μM , 0.5 μM , and 10 μM . The titration experiment was repeated with 10 μM TO and showed reproducible results. As expected, the higher the concentration of TO, the higher the fluorescence signal observed. In this case, 0.5 μM TO was sufficient for observing changes in fluorescence, compared to using high concentration such as 10 μM .

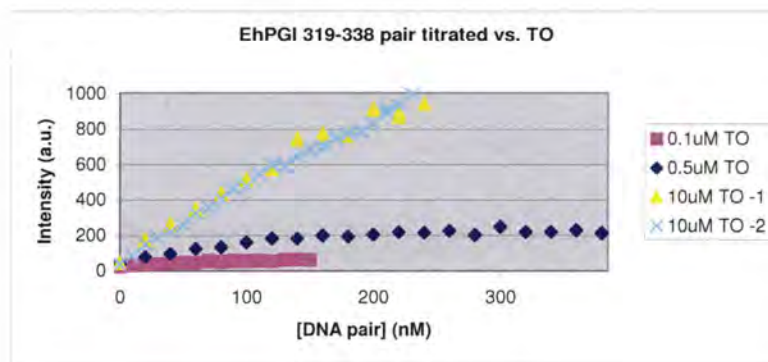


Figure 2-8. EhPGI 319-338 dsDNA titrated against free TO.

Aliquots of dsDNA were added to different concentrations of TO dye, 0.1 μM , 0.5 μM and 10 μM (duplicate). Increase in fluorescence signals were observed in all cases, though plateau was reached much faster in 0.1 μM and 0.5 μM .

5. Reproducible melt analysis in Cary Eclipse

Reproducible melt analysis was achieved with the Cary Eclipse using a large quartz cuvette with a 1 mL sample volume. When a 100 μL sample was used with the small quartz cuvette, the solution's out-gassing and evaporation caused problems in obtaining any T_m . Therefore, 1 mL samples were used. The temperature ramp rate was also decreased to 0.5 $^{\circ}\text{C}/\text{min}$ instead of 6 $^{\circ}\text{C}/\text{min}$. This decrease in ramp rate provided a slow increase in block temperature and better heat transfer to the solution inside the cuvette. Note that sample stirring was not available to introduce convection in any titration mixtures.

6. Comparison of melt temperatures obtained in Cary Eclipse to those in LightCycler

Once a reproducible melt analysis method was developed on the Cary Eclipse, a series of experiments were done to obtain Tms for all DNA probes designed for use as tethered dye probes. The conditions were the same in all cases where 0.1 μ M of dsDNA target was mixed with 0.333x SYBR Green I in PBS. A 1 mL volume was used in the Cary Eclipse, and a 10 μ L volume was used in the LightCycler. All Cary Eclipse experiments were done in triplicate, whereas 6 data points were collected using the LightCycler. Table 4 shows the tabulated results for comparison. Average Tm and standard deviations were calculated in all cases. These results showed that the Tms obtained by either instrument were comparable.

Table 2-4. Melt temperature comparison between Cary Eclipse and LightCycler for 4 different dsDNA targets.

dsDNA	Tm (Eclipse)	Tm (Light Cycler)
EhPGI 341-360	65.6°C \pm 0.231°C	65.9°C \pm 0.408°C
EhPGI 319-338	66.0°C \pm 0.755°C	65.1°C \pm 0.324°C
EhPGI 293-312	67.4°C \pm 0.245°C	66.3°C \pm 0.245°C
EhPGI 128-145	60.5°C \pm 0°C	60.1°C \pm 0.0676°C

7. Transient appearance of a non-specific Tm peak

In an experiment to determine the optimal annealing temperature for PCR, *pgi* was used as template with primers, 18CA14F and 18CA14R. Two PCR experiments using the Eppendorf MasterCycler, Hotstar taq polymerase (Qiagen) and its associated reagents were done simultaneously; one run used an annealing temperature (Ta) of 55°C, and the other used a Ta of 66°C. After PCR, the reaction mixtures were mixed with SGI (0.333x) for Tm determination in the LightCycler. The results suggested more than one PCR product per reaction based on the appearance of two distinct Tm values. The non-specific Tm peak generated different Tms at different annealing temperatures (see Figure 2-9). In order to visualize the products, PCR reaction

products for each annealing temperature were separated by agarose gel electrophoresis (1% agarose, 1x TBE, 60 minutes). The gel data showed only a single DNA band at 344 bp. Longer gel separation time (90 min) still showed only one band in both samples (Figure 2-10). Gel electrophoresis did not identify a PCR product that may have been associated with the transient T_m peak. Other factors were investigated to determine the possible source of the transient non-specific T_m peak as described in the following sections.

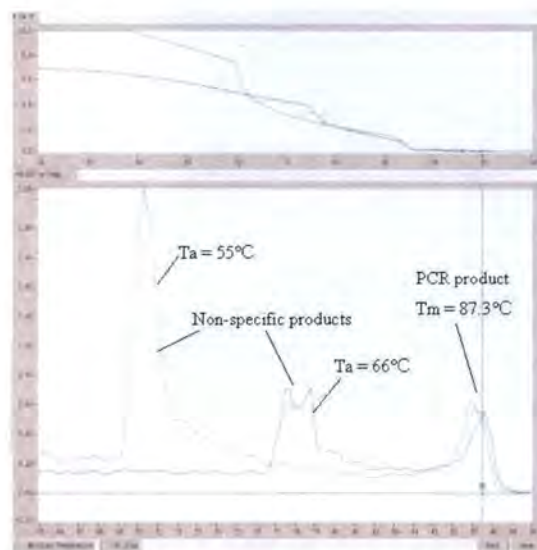


Figure 2-9. Melt analyses of PCR reaction mixtures.

Top panel shows a change in fluorescence over temperature (F vs T), bottom panel shows the negative derivative of fluorescence over temperature vs temperature (-dF/dT vs T). Two identical reaction mixtures were prepared and run simultaneously in the MasterCycler. Both were programmed to activate HotStar Taq polymerase at 95°C for 15min, then 40 PCR cycles of denaturation at 94°C for 1min, annealing at 55°C (green trace) or 66°C (blue trace) for 1min, extension at 72°C for 2min, with a final extension at 72°C for 7min. After PCR, a 10µL aliquot from each reaction was removed for melt analysis in the LightCycler and mixed with 0.333x SGI for detection. PCR product with a $T_m = 87.3^\circ\text{C}$ was observed in both reaction mixtures, non-specific products were observed at both annealing temperatures except with very different melt temperatures.



Figure 2-10. Agarose gel electrophoresis separation of EhPGI PCR products at different annealing temperatures (T_a).

Two different PCR product mixes were loaded onto a 1% agarose gel and separated in 1xTBE buffer containing 0.5µg/mL ethidium bromide (EtBr) at 90V constant voltage. Lane 1 and 2 showed the EhPGI DNA band at 344bp with HotStar Taq polymerase at a T_a of 55°C and 66°C, respectively. Lane 3 was loaded with Hae III fragments of $\phi\chi 174$ DNA as standards, each band size was labeled (in base pairs, bp) next to the corresponding DNA band.

7.1 Testing a different source of the forward primer

The forward primer EhPGI 128-145 which is the same sequence as 18CA14F made by Qiagen (purified by HPLC separation) was synthesized by U of C DNA synthesis services but purified by gel purification. These two primer sequences were compared in PCR reaction using the Eppendorf MasterCycler and Hotstar Taq polymerase. Aliquots were mixed with SGI and melt temperatures were obtained using the LightCycler. In this experiment, 18CA14F (Qiagen) produced only one specific

product Tm for a Ta of 55°C or 66°C, while EhPGI 128-145 (U of C) showed two product Tms for a Ta of 55°C, and only one Tm for Ta of 66°C. These reaction mixtures were then separated by agarose gel electrophoresis, which showed only one band in all samples (data not shown). The newly synthesized primer, EhPGI 128-145, produced multiple Tms at Ta of 55°C, even though it was the same sequence as 18CA14F. The new primer did not resolve the original problem and in fact created a new one, so this primer was not used further.

7.2 Testing salt and DNA contents

The non-specific product Tm peak disappeared upon dilution of the sample with water (1:1). Thus, buffer and salt contents were tested to determine if these were factors for this non-specific Tm peak. Samples previously shown having two melt temperatures were diluted at a 1:1 ratio with 10X buffer (500 mM Tris-HCl, 100 mM KCl, 50 mM (NH₄)₂SO₄, and 35 mM MgCl₂, pH 8.7), 1X buffer, and 1X buffer mixed with different amounts of water. All samples showed one melt temperature peak, except those samples diluted with 10X buffer which showed two melt temperature peaks. Therefore, the presence of the non-specific product Tm was buffer dependent (data not shown).

7.3 Testing Qiagen buffer mix, Roche buffer mix, and home-made 1X buffer mix

E. herbicola was used as a template for PCR with different buffers from two different manufacturers and a home-made buffer recipe. Qiagen's buffer mix contained 1X PCR buffer, 1X Q-solution, 3.5 mM MgCl₂, 0.05 mg/mL BSA, and 1% Tween 20. The Qiagen buffer mix without Q-solution contained the same buffer contents except 1X Q-solution. Roche's buffer mix contained 1X buffer (50 mM Tris-HCl, 10 mM KCl, 5 mM (NH₄)₂SO₄, and 3.5 mM MgCl₂, pH 8.7), 1X GC-rich solution, 3.5 mM MgCl₂, 0.05 mg/mL BSA, and 1% Tween 20. Again, the Roche buffer mix without GC-rich solution contained all components except 1X GC-rich solution. The home-

made recipe contained the same components as the Roche buffer mix without GC-rich solution. Betaine (1 M) was added to the home-made recipe.

Figure 2-11 showed that Q-solution, a reagent associated with Qiagen's HotStar Taq Polymerase, was responsible for the appearance of the non-specific transient peak. In the presence of Q-solution in the Qiagen mix, a melt temperature of 78 °C was observed, but disappeared when the PCR sample was prepared with Qiagen's buffer mix without Q-solution (Figures 2-11A and 2-11B).

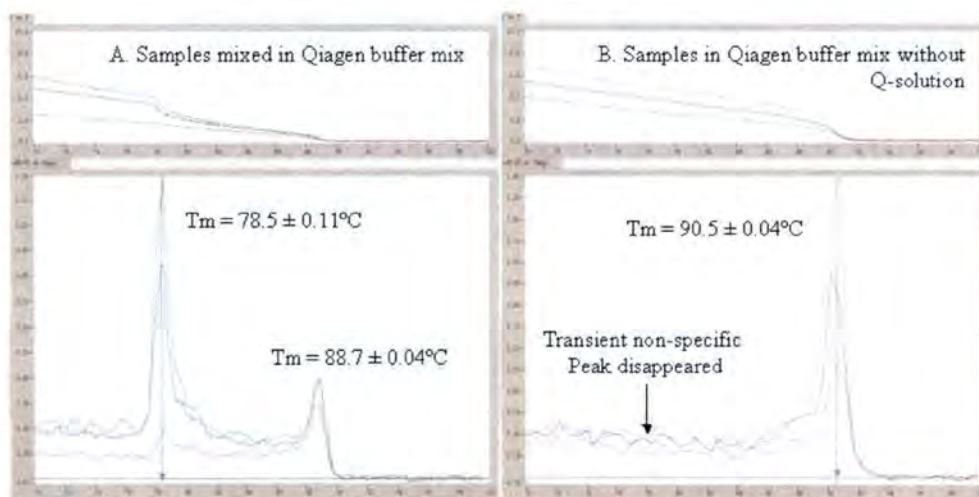


Figure 2-11. EhPGI PCR product mixed with Qiagen buffer with (A) and without (B) Q-solution. Note the appearance and disappearance of the non-specific transient T_m peak (78°C).

The Roche GC-rich solution generated a single T_m peak around 86 °C and the Roche buffer without GC-rich solution showed a single T_m of approximately 90 °C (Figure 2-12). It appeared that the Roche buffer did not produce a transient non-specific product peak, which suggested that the non-specific transient T_m peak was due to the Q-solution additive which may or may not be betaine. (Figure 2-12B). As expected, similar results to figure 2-11 (Q-solution within the buffer mix) were observed when

using the home-made buffer mix with and without 1 M betaine (Figures 2-12A and 2-12B). Betaine was added to the home-made buffer mix to mimic the effects of Q-solution.

The reason for the appearance of a non-specific melt temperature peak in the presence of Q-solution (or betaine) was investigated by consulting technical support from both Roche Diagnostics and Qiagen. Neither could explain the anomaly. Thus, we eliminated the use of Qiagen's Q-solution when the LightCycler was used for PCR and melt analysis. The Q-solution did not affect the production of PCR amplicon as observed by the single band in Figure 2-10.

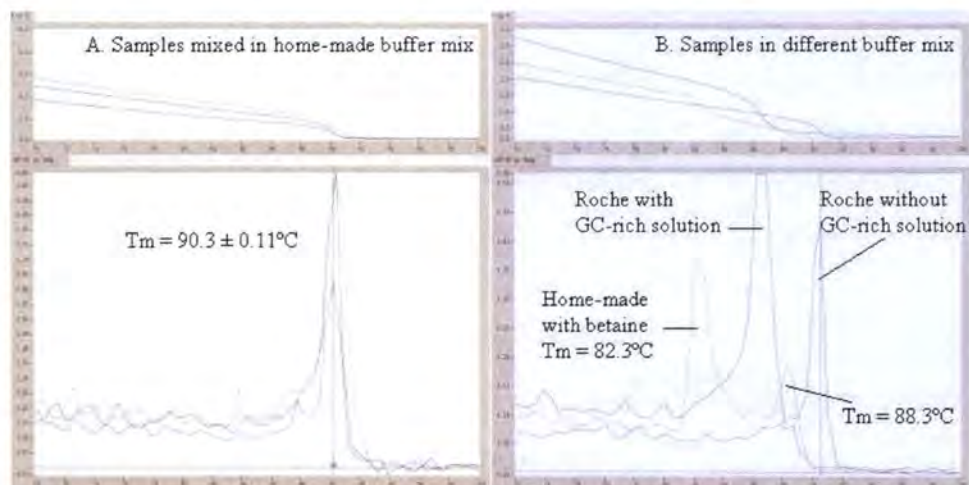


Figure 2-12. EhPGI PCR product mixed with different buffer mix.

In panel A, PCR product was mixed with home-made buffer without 1M betaine and showed 1 melt temperature of 90°C . The same sample in home-made buffer containing 1M betaine (red trace in panel B) showed 2 melt temperatures similar to those reported in figure 11A and 11B. Roche buffer mixed with and without GC-rich solution (grey traces with labels) did not result in the appearance of the second, non-specific transient T_m peak.

8. Testing Qiagen and Roche buffer mix for real-time PCR in the LightCycler

Figures 2-13A and 2-13B show the PCR reaction run profile and melt analysis respectively, using Roche FastStart Taq polymerase mix including the GC rich solution. Product was observed in the positive control, while some product was also observed in the negative control suggesting minor template contamination. Further investigation of the Roche FastStart enzyme suggested the initial heat activation step could be reduced to 4 min, instead of the manufacturer's recommendation of 5 min without visible difference in performance.

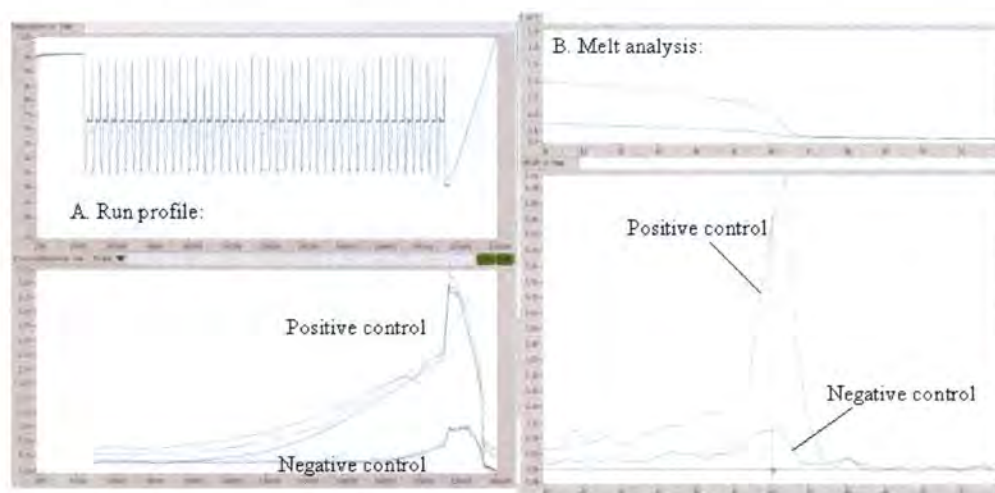


Figure 2-13. Real-time PCR of *E. herbicola* pgi with Roche FastStart Taq polymerase kit.

Run profile (A) showing initial heat activation (5min), followed by 45 cycles and a temperature ramp melt analysis in the top panel, while showing fluorescence collected at the end of each extension cycle vs. time in the bottom panel. Fluorescence started to increase after about 20 cycles in the positive control. A slight increase in fluorescence was observed in the negative control at the end of 45 cycles. Melt analysis (B) showed only 1 product with an average T_m of 86.2°C in the positive control and an almost undetectable amount in the negative control. Product in the negative control is a result of minor contamination.

Figures 2-14A and 2-14B show the PCR reaction run profile and melt analysis results respectively, using the Qiagen HotStar Taq polymerase kit with and without the addition of Q-solution. These results were much less desirable because they resulted

in no amplification in the absence of Q-solution but multiple T_m peaks (non-specific and specific products) in the presence of Q-solution.

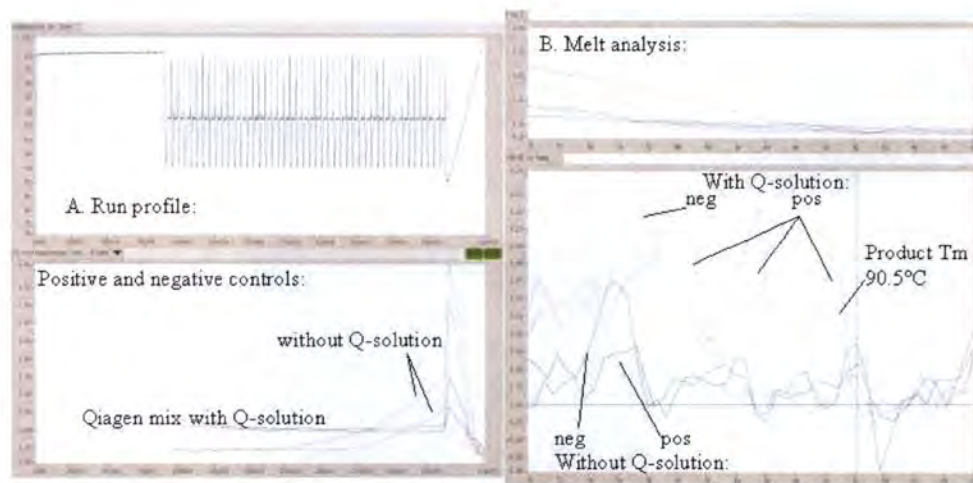


Figure 2-14. Real-time PCR of *E. herbicola pgi* with Qiagen HotStar Taq polymerase kit.

Run profile (A) showing initial heat activation (15min), followed by 45 cycles and a temperature ramp melt analysis in the top panel, while showing fluorescence collected at the end of each extension cycle vs. time in the bottom panel. No amplification was observed in the samples lacking Q-solution in buffer (neither positive nor negative template controls), while samples in the buffer containing Q-solution showed some amplification and resulted in multiple peaks within the melt analysis (B).

8.1. Additional Comparison Studies Between FastStart Taq polymerase and Qiagen HotStar Taq for *E. herbicola pgi*

The following experiments were conducted to determine the best PCR reaction buffer system to use for real time PCR of *E. herbicola* in the LightCycler for the tethered dye probe project. The Roche FastStart Taq polymerase kit was compared to the Qiagen HotStar kit under identical PCR conditions using the MasterCycler. The PCR protocol used involved initial heat activation of Taq polymerase at 95 °C (15 min for HotStar and 5 min for FastStart), followed by 40 PCR cycles of denaturation (94 °C) for 1 min, annealing (55 °C) for 1 min, and extension (72 °C) for 2 min, with a final

extension of 7 min at 72 °C. PCR products were separated on a 1% agarose gel in 1x TBE/EtBr buffer with electrophoresis at 90 V. The resulting products were equivalent in intensity when detected on an agarose gel with ethidium bromide (Figure 2-15). PCR products of 344 bp were observed in both positive controls with FastStart and HotStar Taq polymerases. In both cases, only 1 DNA band was observed. Some amplification of *pgi* was observed in a no-template negative control with FastStart Taq polymerase as observed in the previous real time PCR experiment suggesting minor template contamination.



Figure 2-15. Agarose gel electrophoresis separation of EhPGI PCR products with different Taq polymerases using the MasterCycler.

Three different PCR product mixes were loaded onto a 1% agarose gel and separated in 1xTBE buffer containing 0.5µg/mL ethidium bromide (EtBr) at 90V constant voltage. Lanes 1 and 2 showed the EhPGI DNA band at 344bp with FastStart Taq polymerase in the negative control (no template) and positive control, respectively. Lane 3 showed the same product band from the positive control with HotStar Taq polymerase. Lane 4 was loaded with Hae III digested fragments of $\phi\chi 174$ DNA as standards, each band size was labeled (in base pairs, bp) next to the corresponding DNA band.

Similar experiments were done using the LightCycler. Roche buffer mix contained the GC-rich solution, while Qiagen mix was tested with and without the Q-solution, otherwise all other components were the same between the Roche and Qiagen mixes. The PCR reaction was first heat activated at 95 °C (5 min for Roche kit and 15 min for Qiagen kit), followed by 45 cycles of denaturation (94 °C) for 0 sec, annealing (55 °C) for 5 sec, and extension (72 °C) for 20 sec with fluorescence acquisition at the end of each extension step. Immediately following the last PCR cycle, melt analysis was performed using an initial temperature of 94 °C, then lowered to 50 °C for 20 sec, and ramped up to 100 °C at a rate of 0.2°C/sec while collecting fluorescence signal continuously. Two concentrations of FastStart Taq polymerase were used (0.025 U/μL and 0.05 U/μL (2x)) in otherwise identical reaction mixtures. After real-time PCR experiments, all PCR reactions were separated on an agarose gel and DNA bands were identified (Figure 2-16). EhPGI PCR product was not observed in any reaction mix using HotStar Taq polymerase (lanes 1-4 in Figure 2-16) even though products were observed in those samples with the same PCR conditions but using the MasterCycler (typical volume of 25 μL). DNA products were observed in real-time PCR using FastStart Taq polymerase for the positive reactions containing template but also intermittently and to a lesser extent for no template controls (lane 6), indicating again minor template contamination (Figure 2-16). Higher amounts of the 344 bp product were observed with increased amounts of FastStart Taq polymerase. Due to the poor amplification results obtained using Qiagen Hotstar PCR reaction mix, it was not selected for further investigation. Instead, the Roche FastStart Taq system was used under the following general conditions: 4 min activation, 35 cycles, 0.05 U/μL FastStart Taq polymerase.



Figure 2-16. Agarose gel electrophoretic separations of EhPGI PCR products, with different Taq polymerases, from real-time PCR.

This figure shows a combination of 2 gel images from different separations. Qiagen HotStar Taq polymerase was used in real-time PCR; lane 1 is positive control with Q-solution, while lane 2 is the negative control of the same condition; lanes 3 and 4 are those without Q-solution of positive and negative controls, respectively. Roche FastStart Taq polymerase was also used in real-time PCR experiments; lanes 5 and 8 are both positive controls with 0.025 U/μL Taq polymerase, and lanes 6 and 9 are no-template, negative controls. Lane 10 shows PCR product resulting from 2x amount of FastStart Taq polymerase (0.05 U/μL). Lanes 7 and 11 are loaded with Hae III digested fragments of ϕ X174 DNA as standards; each band size is labeled (in base pairs, bp) next to the corresponding DNA band.

8.2. Optimizing real-time PCR detection using thiazole orange (TO) in LightCycler

Free TO (thiazole orange) was used in real-time PCR experiments and compared to SGI (SYBR Green I). Recommended SGI concentration was 0.333x in the PCR mixture, while 1 μ M and 10 μ M TO were used. All PCRs were performed using optimized Roche buffer system described above. Detection of melt temperature after real-time PCR was not possible when 1 μ M TO was used (data not shown). A melt temperature was detected when 10 μ M TO was used, but the fluorescence intensity was much lower than that obtained with SGI (Figure 2-17). The low fluorescence intensity was due to a rapid drop in fluorescent signal with respect to increasing temperature. This observed phenomenon was explained by Nygren et al. [9] in that the intrinsic quantum yield of thiazole orange decreases with higher temperature.

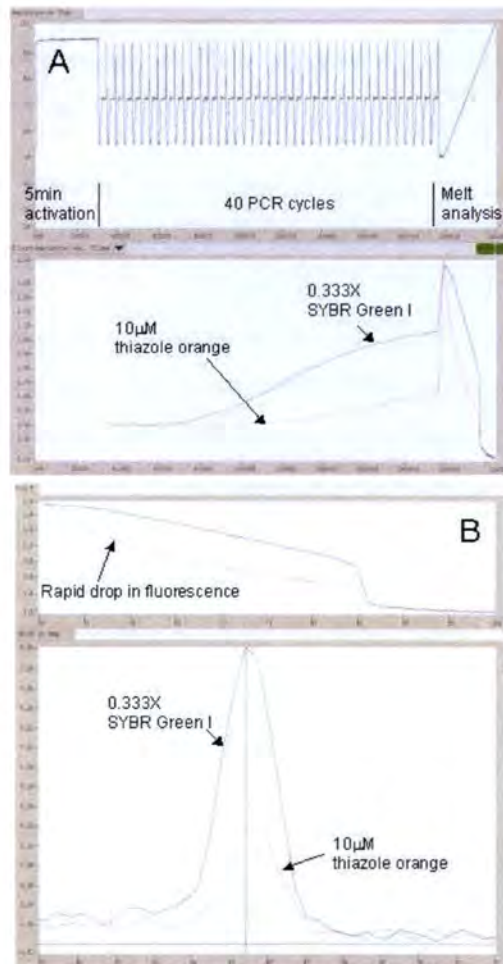


Figure 2-17. Real-time PCR in LightCycler using both thiazole orange (TO) and SYBR Green I (SGI) for detection.

In (A), the run profile and the fluorescence detection of the real-time PCR experiment are shown. Top panel (run profile) shows temperature changes over time with initial Taq polymerase activation, then 40 PCR cycles, and finally melt analysis. Bottom panel (fluorescence detection) shows fluorescence acquired during the PCR experiment. Fluorescence data shows much higher fluorescence intensity with SGI than with TO. In (B), the expanded view of the melt analysis was shown with both fluorescence vs. temperature and the derivative plot ($-dF/dT$) in the top and bottom panel, respectively. In the F vs. T plot, it was very obvious that fluorescence of both dyes started at the same level, while TO dropped off rapidly as the temperature increased whereas SGI remained quite stable over the temperature range. Thus, T_m in the derivative plot (bottom) has a lower intensity for TO than SGI.

9. Characterization of TO-tethered probes

Two TO-tethered probes (TO-mix 15-mer and TO-EhPGI 341-360) were characterized by UV spectroscopy. Optical densities (OD) at 260 nm, 280 nm, and 510 nm were measured for each probe. OD₂₆₀ allows the calculation of the amount of DNA in sample, while the 260/280 ratio estimates the purity of DNA sample from protein or phenol contamination. OD₅₁₀ is the maximum absorbance wavelength for TO, thus allowing the calculation of the amount of dye incorporated into DNA. All OD values were obtained using a Unicam UV530 spectrophotometer.

For TO-mix 15-mer (W20752):

$$OD_{260} = 0.403$$

$$OD_{280} = 0.370 \Rightarrow \text{ratio of } 260/280 = 1.09 \text{ (1.8 in pure DNA)}$$

$$OD_{510} = 0.208 \Rightarrow \text{ratio of } 260/510 = 1.94$$

$$[\text{TO-mix 15-mer}] = 0.403 \times 33 \mu\text{g} / \text{mL} \times \frac{1OD}{15(330 \mu\text{g} / \mu\text{mol})} \times 1000 \text{mL} / L = 2.7 \mu\text{M}$$

Amount of TO-mix 15-mer is $2.7 \mu\text{M} \times 200 \mu\text{L} \times 50$ (dilution factor) = 27 nmol.

Concentration of TO = $OD_{510}/\epsilon = 0.208/65,000 = 3.2 \mu\text{M}$ ($\approx 2.7 \mu\text{M}$ DNA)

For TO-EhPGI 341-360 (W21511):

$$OD_{260} = 0.757$$

$$OD_{280} = 0.626 \Rightarrow \text{ratio of } 260/280 = 1.21 \text{ (1.8 in pure DNA)}$$

$$OD_{510} = 0.263 \Rightarrow \text{ratio of } 260/510 = 2.88$$

$$[\text{TO-EhPGI 341-360}] = 0.757 \times 33 \mu\text{g} / \text{mL} \times \frac{1OD}{20(330 \mu\text{g} / \mu\text{mol})} \times 1000 \text{mL} / L = 3.785 \mu\text{M}$$

Amount of TO-EhPGI 341-360 is $3.785 \mu\text{M} \times 100 \mu\text{L} \times 10$ (dilution factor) = 3.785 nmol.

Concentration of TO = $OD_{510}/\epsilon = 0.263/65,000 = 4.05 \mu\text{M}$ ($\approx 3.785 \mu\text{M}$ DNA)

For TO-dT₂₀ (W20751):

$$OD_{260} = 0.409$$

$$OD_{280} = 0.274 \Rightarrow \text{ratio of } 260/280 = 1.49 \text{ (1.8 in pure DNA)}$$

$$OD_{510} = 0.053 \Rightarrow \text{ratio of } 260/510 = 7.72$$

$$[\text{TO-EhPGI 341-360}] = 0.409 \times 33 \mu\text{g} / \text{mL} \times \frac{1OD}{20(330 \mu\text{g} / \mu\text{mol})} \times 1000 \text{mL} / L = 2.05 \mu\text{M}$$

Amount of TO-EhPGI 341-360 is $2.05 \mu\text{M} \times 100 \mu\text{L} \times 10$ (dilution factor) = 2.05 nmol.

Concentration of TO = $OD_{510}/\epsilon = 0.053/65,000 = 0.815 \mu\text{M}$ ($\approx 40\%$ of $2.05 \mu\text{M}$ DNA)

The results showed that the two probes, TO-mix 15-mer and TO-EhPGI 341-360 contained a 1:1 ratio of DNA:TO, while the TO-dT₂₀ sample had 40% of DNA the containing TO. TO-EhPGI 341-360 also smelled strongly of solvents, most likely due to incomplete cleanup after HPLC. Therefore, solvent was evaporated from TO-EhPGI 341-360 in a Speed Vac rotary evaporator. The resulting stock concentrations of TO-dT₂₀, TO-mix 15-mer, and TO-EhPGI 341-360 were 20.5 μ M, 100 μ M, and 150 μ M respectively.

Figure 2-18 shows the UV absorption spectrum of TO-EhPGI 341-360 (a) and HPLC separation of the tethered probe from the untethered probe and the untethered probe modified with phosphate group (b).

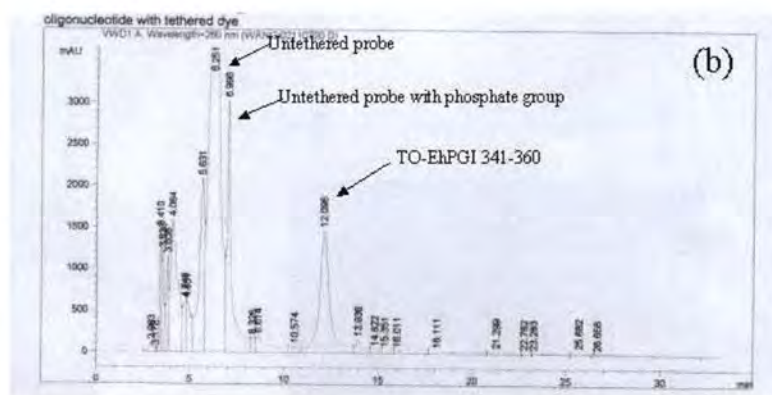
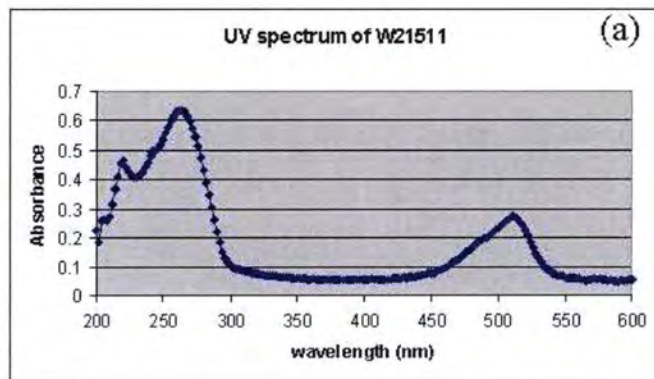


Figure 2-18. UV spectrum of TO-EhPGI 341-360 (a) and HPLC separation of TO-EhPGI 341-360 from untethered sequences (b).

In the UV spectrum (a), the tethered probe showed a strong absorption at 260nm and an another absorption maximum at 510nm corresponding to DNA and thiazole orange absorbance, respectively. In the HPLC separation trace with 260nm absorbance detection (b), TO-EhPGI 341-360 was eluted at 12min, clearly separated from contaminating probes at 6.25min and 6.99min corresponding to the untethered probe and the untethered probe modified with a phosphate group, respectively. Incomplete cleanup after HPLC resulted in the tethered probe sample that smelled strongly of solvents. Solvent was removed by vacuum in a speedvac. Courtesy of Kris Wang at University of Toronto.

9.1. Characterization of new poly-A plasmid (pPGI[pA1])

The new poly-A plasmid, pPGI[pA1], contained fewer DNA base pairs than the original pPGI plasmid (2888 bp and 2942 bp respectively; see annexes A and F for comparison). The resulting PCR products of those two plasmids also reflected the shortened sequence in pPGI[pA1] due to a replacement piece that was 58 bp shorter. Figure 2-19 shows the PCR products of pPGI[pA1] and pPGI separated by electrophoresis on an agarose gel. The shorter pPGI[pA1] PCR amplicon (286 bp) migrated faster than the longer pPGI amplicon (344 bp). Figure 2-20 shows the HindIII/EcoR I restriction enzyme digests of pPGI[pA1] (3 samples picked from different *E. coli* colonies). Using only λ Hind III should result in one band reflecting the size of the plasmid (2888 bp), while using both λ Hind III and EcoR1 should result in two bands, 2578 bp and 310 bp (size of DNA insert).



Figure 2-19. Electrophoretic separation of PCR amplicons.

Lane 1 and 12 are DNA markers of low and high molecular weights, respectively. Sizes of each band (in base pairs, bp) in lane 1 are labeled next to the DNA band. Lane 2 is empty. Lanes 3-5 are PCR products of pPGI[pA1] plasmids (286 bp). Lanes 6-11 represent pPGI amplicons (344 bp) that ran slower than the 310 bp marker and pPGI[pA1] amplicons.

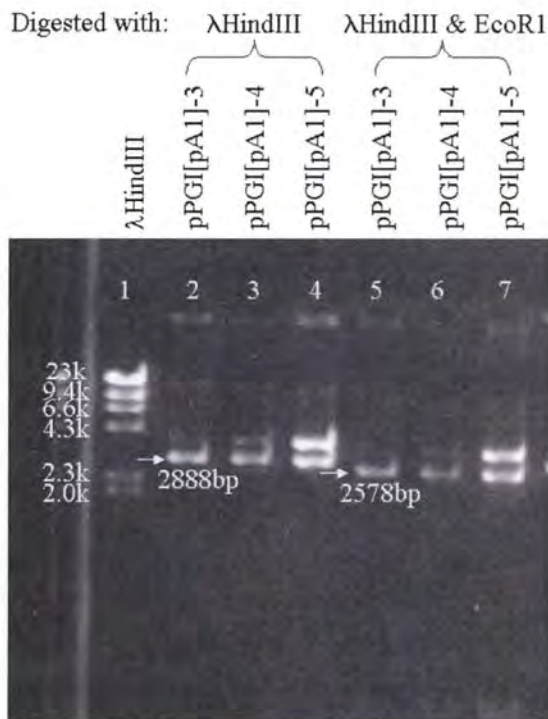


Figure 2-20. Electrophoretic separation of restriction enzyme digests of pPGI[pA1].

Lane 1 is high MW marker with corresponding sizes (in bp) labeled next to each band. Lanes 2-4 are three different samples of pPGI[pA1] digested with only λ HindIII. Lanes 5-7 are the same samples digested with both λ HindIII and EcoR1. The arrows indicate the size of bands 2888 bp and 2578 bp (minus 310 bp) respectively.

Results in the characterization of product post-PCR using agarose gel electrophoresis showed that the plasmid, pPGI[pA1], produced the correct size amplicon as expected (Figure 2-19). However, the restriction enzyme digestion experiments (Figure 2-20) showed that only fraction 3, pPGI[pA1]-3 (lanes 2 and 5), produced one band as expected (indicated by an arrow). The other two samples showed an unidentified larger band migrating just above the expected plasmid band. Therefore only fraction

3, pPGI[pA1]-3, was used for all subsequent PCR amplicon preparations, and its PCR products were used in all fluorescence titration and temperature effect experiments.

PCR amplicons from the three different clones of pPGI[pA1] and pPGI were mixed with SYBR Green I dye and melt analyses were done to obtain the Tms. Melt analyses were done in the LightCycler from 50°C to 100°C as previously described. Table 2-5 shows the tabulated results of the experimental Tm values and the change in Tm from that of the pPGI. Lower Tm values in all poly-A PCR amplicons suggested the presence of a shorter sequence that was different from that of the pPGI product.

Table 2-5. Melt temperatures, Tm, of PCR products from different plasmid template and the deviation from the original, pPGI.

PCR products	Tm (mean \pm std. dev.)	Δ Tm (pPGI - pPGI[pA1])
pPGI	88.18°C \pm 0.033°C	-
pPGI[pA1]-3	87.15°C \pm 0.00°C	1.03°C
pPGI[pA1]-4	87.28°C \pm 0.066°C	0.90°C
pPGI[pA1]-5	87.15°C \pm 0.13°C	1.03°C

9.2. Melt analyses of TO-probes on the LightCycler

PCR amplicons generated from pPGI and pPGI[pA1] were used as template for recognition by TO tethered probes. pPGI[pA1] amplicon contains sequences that will be recognized by TO-mix 15-mer, TO-dT₂₀, and TO-EhPGI 341-360, while the pPGI amplicon only contains a sequence recognized by TO-EhPGI 341-360. Therefore the pPGI amplicon can be used as a negative control in binding experiments where TO-mix 15-mer or TO-dT₂₀ were used.

Melt analysis results from Figure 2-21 showed that there were no interactions between TO-probes and template. Theoretically, TO-mix 15-mer and TO-dT₂₀ will bind to pPGI[pA1] amplicon and a peak indicating the melt temperature should be observed

in the $-dF/dT$ plots; however these probes did not interact with pPGI amplicon, thereby resulting in no peak in the derivative plots. Moreover, TO-EhPGI 341-360 should bind to both amplicons theoretically, but this probe did not show any binding interaction or a peak representing T_m in the $-dF/dT$ plot.

It was suspected that background was too high in the first derivative plot in all cases, especially in the region where melt temperature was expected, 40°C - 50°C. The starting temperature (35°C) was also higher than experimental requirements (lower than room temp.). Therefore, these experiments needed to be repeated in 1 ml cuvettes in a spectrofluorimeter equipped with a Peltier temperature controller of -10 °C to 100 °C. More PCR amplicon is needed for experiments of such scale (1 mL of dsDNA at 0.1 μ M equals 0.1 nmole or 20 μ g). Excess PCR amplicon was prepared and purified.

Salt (NaCl) was gradually added to the mixtures in Figure 2-21, in order to increase the affinity of probe for template. Results showed that increasing the amount of NaCl did not increase the binding interaction or T_m value (data not shown).

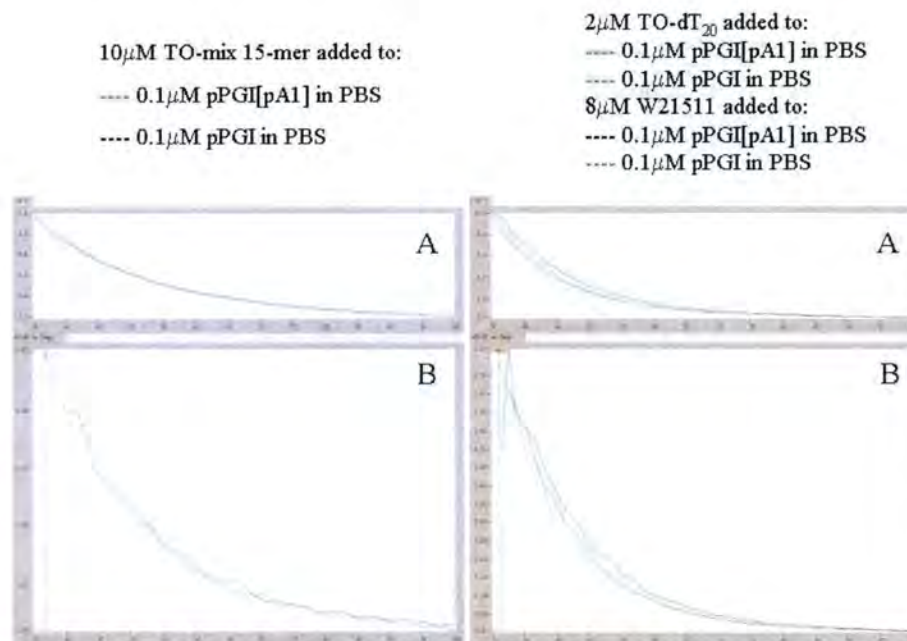


Figure 2-21. Melt analyses of TO-mix 15-mer (left panel), TO-dT₂₀ and TO-EhPGI 341-360 (or W21511) (right panel) with PCR amplicons from pPGI[pA1] and pPGI.

Temperature increased from 35 °C to 100 °C, at a rate of 0.2 °C/sec. Top panels (A) fluorescence plotted against temperature (F vs. T) of TO-probes in presence of templates. Bottom panels (B) first derivative of fluorescence against temperature (-dF/dT) of the corresponding F vs T plots. Peaks were not observed in first derivatives, showing no interaction between probes and templates.

9.3. Titration of TO-probes into PCR products in PBS solution

TO-probes were titrated into PCR amplicon solutions in 100 μ L cuvettes and fluorescence was detected in a spectrofluorimeter at an excitation of 495 nm and an emission wavelength of 530 nm. A solution of PCR product (pPGI or pPGI[pA1]) typically at 1.5 μ M, was mixed with PBS at equal volume. Aliquots of TO-probes were then added to the amplicon solution, typically at 10 pmol increments. Excess TO-probes were added to mimic conditions in real-time PCR experiments where probes are in excess of amplicon. Thiazole orange however showed high background fluorescence, thus causing problems in differentiating between positive and negative interactions. (Figures 2-22 and 2-23). In figures 2-22 and 2-23 it is shown that TO-

mix 15-mer and TO-dT₂₀ fluoresce in the presence of pPGL amplicon which is a result of background fluorescence as neither of these probes should bind to pPGL. At the same time, the fluorescence increase expected for the TO probes in the presence of positive control (pA1) was not observed.

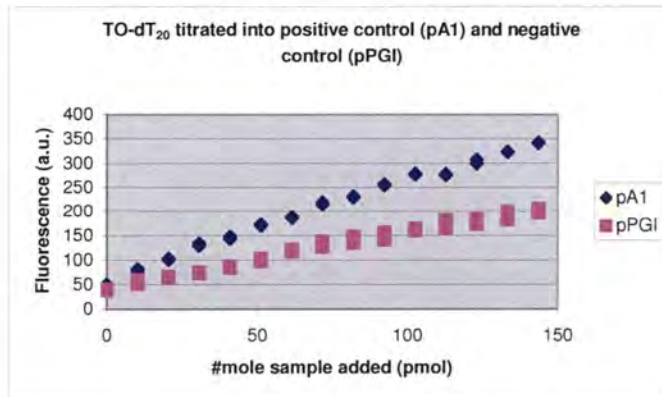


Figure 2-22. Titration curve of TO-dT₂₀ with PCR amplicon from pPGL[pA1] (positive control) and that from pPGL (negative control).

Notice fluorescence signal increased as more probe was added to the negative control indicating background fluorescence from thiazole orange.

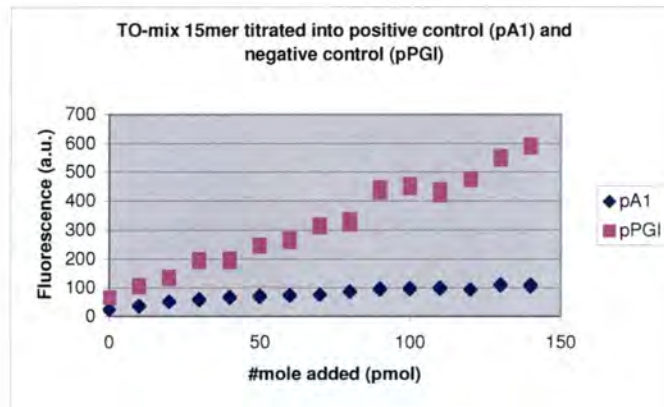


Figure 2-23. Titration curve of TO-mix 15-mer with PCR amplicon from pPGI[pA1] (positive control) and that from pPGI (negative control).

Once again, fluorescence signal increased as more probe was added to the negative control indicating background fluorescence from thiazole orange. The strong background in the negative control exhibited higher fluorescence signal than the positive control.

In Figure 2-24 , the difference in fluorescence signal gained from TO-EhPGI 341-360 binding to pPGI and pA1 was not significant. Thus, it was concluded there was no interaction between TO-EhPGI 341-360 with any specific complementary sequence of pA1 or pPGI, due to a lack of significant difference. This was confirmed in Figure 2-25 when the TO-EhPGI 341-360 probe was titrated against its complementary ssDNA sequence rEhPGI 341-360 and PBS. Strong binding was expected between the probe and the DNA however no significant difference in fluorescence was observed between the positive control and the negative PBS control. Once again supporting the conclusion of a lack of interaction of the probe with the target DNA.

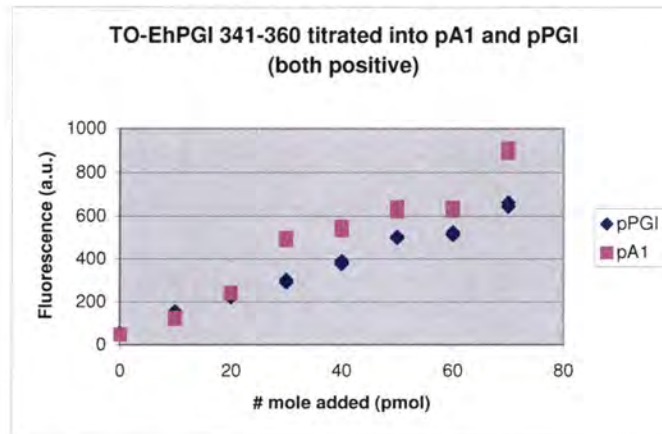


Figure 2-24. Titration curve of TO-EhPGL 341-360 with PCR amplicon from pPGL[pA1] and pPGL.

Both amplicons should interact with the TO-probe. The level of fluorescence in both cases is similar.

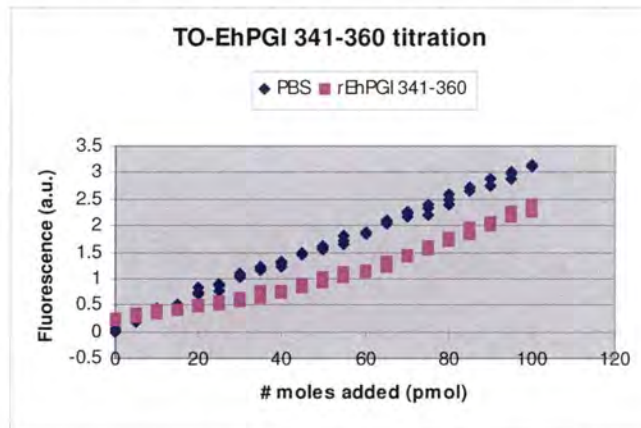


Figure 2-25. Titration curve of TO-EhPGL 341-360 with PBS and ssDNA target rEhPGL 341-360.

Both PBS (negative control; blue diamonds) and ssDNA target (pink squares) showed increase in fluorescence signals indicating a lack of hybridization of TO-probe to its complementary sequence.

9.4. Emission spectra of TO-probes in presence of PCR products and temperature effects on fluorescence of TO

Samples from the titration experiments were subjected to changes in temperature in the cuvette holder equipped with a Peltier temperature controller. The emission

spectra (505 nm to 555 nm) was collected over a range of temperature changes. Figures 2-26 to 2-29 show emission spectra collected with respect to increasing temperature in the peltier block. Positive interactions will show a decrease in fluorescence intensity with respect to an increase in temperature, while negative interactions will show fluctuations in fluorescence signals but not dramatic decreases.

Results from the temperature effects on the TO-probe emission spectra showed a drastic drop in fluorescence intensity in mixtures with positive interactions (Figures 2-26A, 2-27B, and 2-28), whereas fluorescent signals from mixtures of the TO-probe with the negative control (2-26B, 2-27A) remained roughly the same with only small fluctuations. These results indicated binding interactions between TO-mix 15-mer, and TO-dT₂₀ to amplicon from pPGI[pA1] plasmid, and not to that from pPGI plasmid, whereas TO-EhPGI 341-360 interacted with both amplicons as expected.

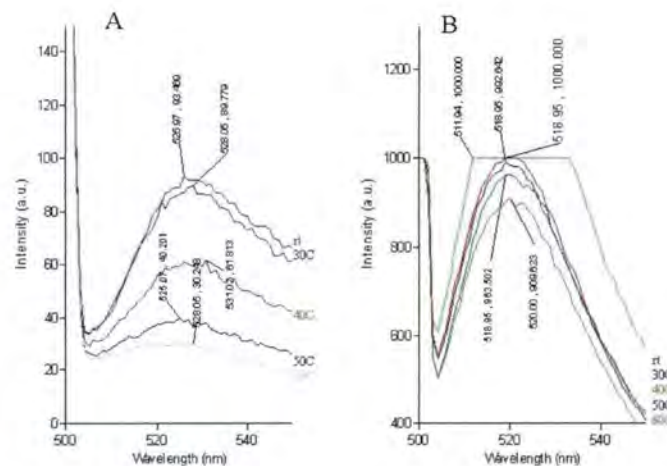


Figure 2-26. Emission spectra of TO-mix 15-mer mixed with pPGI[pA1] amplicon (A) and pPGI amplicon (B).

Fluorescence intensity dropped as temperature increased from rt to 60 °C in (A) indicating a positive interaction, while fluorescence fluctuated but did not drop dramatically in (B) over the same range of temperatures indicating a negative interaction.

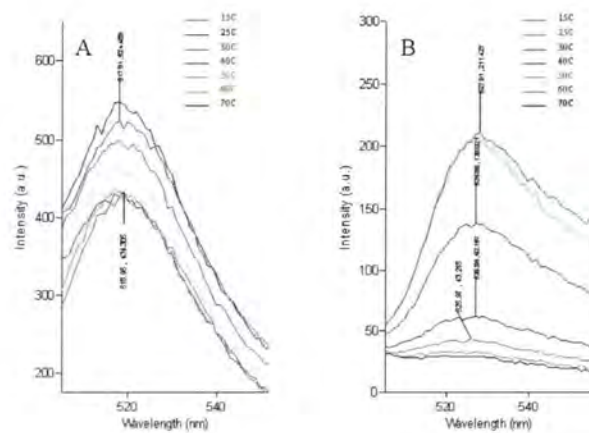


Figure 2-27. Emission spectra of TO-dT₂₀ mixed with pPGL amplicon (A) and pPGL[pA1] amplicon (B).

Fluorescence intensity dropped as temperature increased from room emperature to 60°C in (B), while fluorescence fluctuated but did not drop dramatically in (A) over the same range of temperatures.

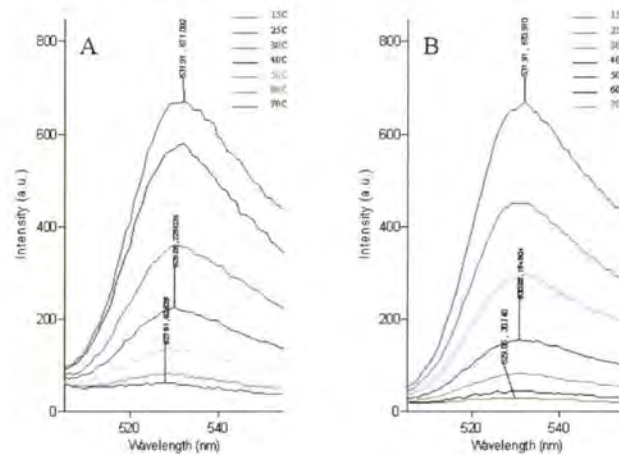


Figure 2-28. Emission spectra of TO-EhPGL 341-360 mixed with pPGL amplicon (A) and pPGL[pA1] amplicon (B).

Fluorescence intensity dropped as temperature increased from room emperature to 60 °C in both (A) and (B).

Results from Figure 2-29 also showed a decrease in fluorescent signals with respect to temperature increase but no template was included in the reaction. It was suspected that TO-EhPGI 341-360 was interacting with itself (dimerization with another probe molecule), since there was no DNA template for TO-EhPGI 341-360 to bind to in the buffer solution yet a positive emission profile was observed. This result is not ideal because some form of background subtraction will then have to be included when running a real-time PCR detection.

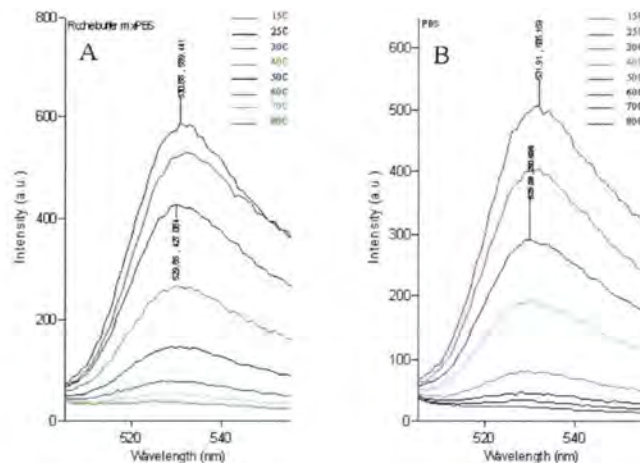


Figure 2-29. Emission spectra of TO-EhPGI 341-360 mixed with 1:1 of Roche PCR buffer 9mix:PBS (A) and PBS (B).

Fluorescence intensity dropped as temperature increased from room temperature to 60°C in both (A) and (B).

9.4. T_m estimates by curve fitting experimental data of fluorescence with respect to temperature change

Fluorescent intensities at 530 nm of the TO-probes interacting with DNA amplicons, with respect to increased temperature were recorded. A plot of fluorescent intensity against Log₁₀ temperature was created. A sigmoidal curve was fitted to each set of

data and the inflection point of the curve, i.e. T_m , was determined. Figure 2-30 shows a plot of fluorescence against Log_{10} temperature containing 4 sets of data. Table 2-6 shows the tabulated T_m results from the sigmoidal curves.

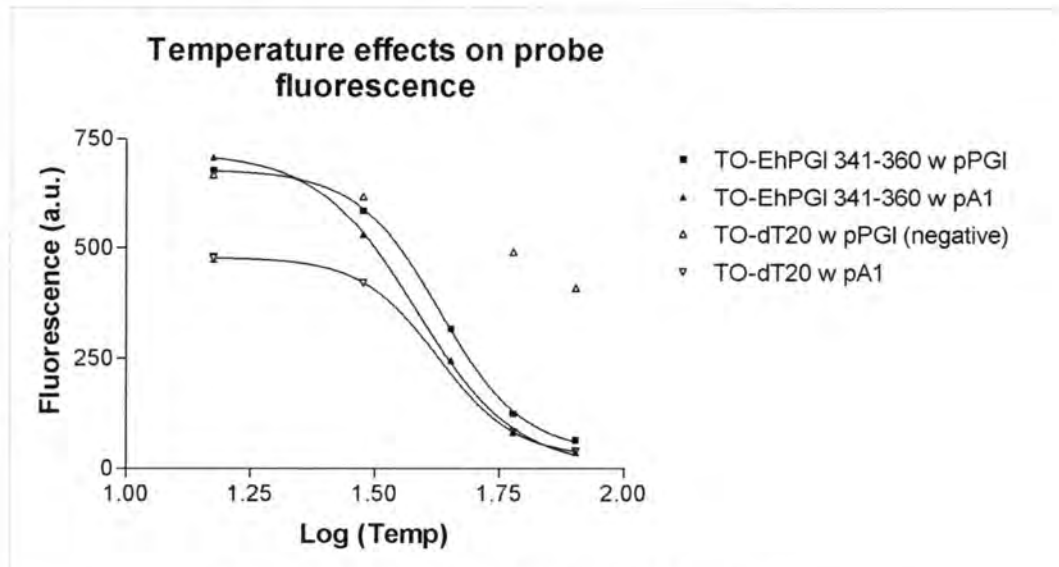


Figure 2-30. Temperature effects on probe fluorescence

There are 3 sets of positive interactions between probe and amplicon, but the negative control containing TO-dT₂₀ and pPGI amplicon was negative as expected. A sigmoidal curve (dose-response curve with variable slopes) was fitted to each of the 3 positive interactions. An inflection point of the sigmoidal curve, i.e. melt temperature of the probe, was determined.

Table 2-6. Inflection points determined from each sigmoidal curves in Figure 2-29.

	T_m
TO-EhPGI 341-360 + pPGI	$42.61^{\circ}\text{C} \pm 0.61^{\circ}\text{C}$
TO-EhPGI 341-360 + pPGI[pA1]	$38.22^{\circ}\text{C} \pm 0.763^{\circ}\text{C}$
TO-dT ₂₀ + pPGI[pA1]	$42.42^{\circ}\text{C} \pm 2.02^{\circ}\text{C}$

The T_m s obtained from curve fitting were significantly lower than expected for each probe (60°C to 65°C). It was unclear whether these temperature values reflected those of T_m values of each positive interaction between TO-probe and target. The temperature estimated in Table 2-6 might have represented a reflection of the drastic

drop in fluorescence between 35 °C and 50 °C in Figure 2-21, or the effect of other unknown interactions.

Due to the uncertainty of any interaction between the TO-probes and target DNA from the titration and T_m results, an alternative dye molecule was considered for use instead of TO. SYBR-101™ is a commercially available dye specific for dsDNA, and was examined in the following sections.

10. SYBR-tethered probe synthesis

Reactive SYBR 101™ (0.33 μmol) was dissolved in DMSO and reacted with 5'-amino modified EhPGI 341-360 probe sequence (33 nmol). The reactive SYBR dye was in 10-fold mole excess of the probe sequence. The condensation reaction took place overnight in the presence of 0.1 M sodium tetraborate, pH 8.5 at room temperature. After reaction, DNA was precipitated in the presence of 1/10 equivalent volume of 3 M sodium acetate pH 7.0 and 2.5 equivalent volume of 95% EtOH. The DNA pellet was collected by centrifugation at 4 °C and 12,000x g for 30 min in an Eppendorf 5417R centrifuge. The pellet was rinsed with cold 80% EtOH and centrifuged repeatedly to collect DNA before subjecting the pellet to SDS-PAGE to separate un-reacted EhPGI 341-360 probe from SYBR-labeled EhPGI 341-360 probe. Approximately 60 μg SYBR-EhPGI 341-360 (9.1 nmol) was retrieved from 200 μg starting material, resulting in a final yield of about 30% labeled probe.

10.1. Titration of complement 20-mer ssDNA into SYBR-EhPGI 341-360 in PBS solution

Aliquots of rEhPGI 341-360 (20-mer complement) were added to a PBS solution containing 0.2 μM SYBR-EhPGI 341-360. Fluorescence emission was monitored on a Cary Eclipse fluorescence spectrophotometer with excitation and emission wavelengths set at 485 nm and 520 nm respectively and a PMT voltage set at 700 V. Figure 2-31 shows the comparison between the addition of complement ssDNA (blue

diamonds) and PBS (negative control, pink squares) to solutions containing the SYBR-probe. Fluorescence signals increased as aliquots of ssDNA target were added to SYBR-EhPGI 341-360 in PBS, while fluorescence remained the same with addition of PBS, thereby indicating binding between target ssDNA and the SYBR-probe.

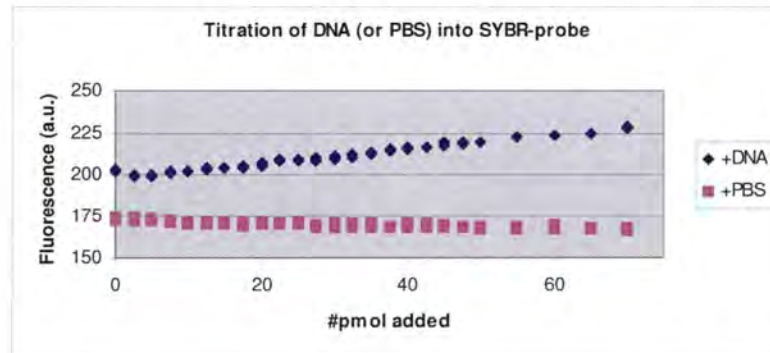


Figure 2-31. Complement ssDNA titrated into SYBR-tethered EhPGI 341-360 probe.

Aliquots (2.5 pmol or 5 pmol) of complement ssDNA (rEhPGI 341-360) were added to a solution containing SYBR-EhPGI 341-360 in PBS (blue diamonds). A negative control included the addition of an equal volume of PBS added to another cuvette containing SYBR-EhPGI 341-360 (pink squares). Both solutions were monitored simultaneously in Cary Eclipse fluorescence spectrophotometer.

10.2. Characteristics of SYBR-probe binding to complement (20-mer) events

10.2.1. Blue shift in emission maximum when SYBR-tethered probe was bound

Emission spectra at different temperatures were monitored for SYBR-EhPGI 341-360 in the presence of complement and PBS (negative control), in the Cary Eclipse fluorescence spectrophotometer. Selected spectra are displayed in Figure 2-32 to show the change of maximum emission of the SYBR-tethered probe in the negative control with PBS (Figure 2-32A) and the positive control with rEhPGI 341-360 (Figure 2-32B). The results indicated a blue shift in the $\lambda_{Em, max}$ from unbound to bound state in the positive control as the temperature was raised (Figure 2-32B). A similar shift was not observed in the negative control. Thus, these results indicated both a binding

interaction between the probe and target ssDNA, and SYBR 101™ dye with the resulting dsDNA.

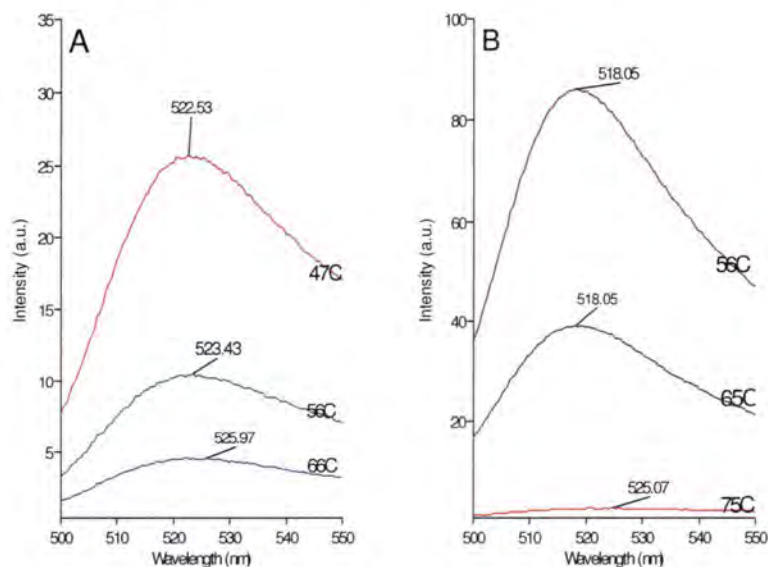


Figure 2-32. Emission spectra of SYBR-EhPGI 341-360 in the negative control with PBS (A), and a positive control with the complement rEhPGI 341-360 (B).

Fluorescence intensity dropped as temperature increased in both (A) and (B). Emission maximum was recorded and remained roughly the same around 524nm in negative control (A), while it changed from 518nm to 525nm when solution temperature was higher than theoretical T_m (66 °C) for EhPGI 341-360 DNA pair (B).

10.2.2 Melt analysis

Fluorescence intensity changes were monitored with respect to changes in temperature in solutions containing bound and unbound SYBR-tethered EhPGI 341-360 in the Cary Eclipse fluorescence spectrophotometer. In Figure 2-33A, fluorescence signal dropped significantly in the unbound sample of SYBR-EhPGI 341-360 (red trace) before solution temperature reached 40°C. In the same diagram, bound SYBR-EhPGI 341-360 (in duplicates) also showed a drop in fluorescence signal with a second distinctive drop after 60 °C. The T_m was determined to be 66 °C for bound SYBR-EhPGI 341-360, and none for the unbound species from the derivative plot (B) of $-dF/dT$ vs.

temperature. The melt analysis data aligned with the T_m results obtained using SGI in either the Cary Eclipse or the LightCycler in Table 2-4.

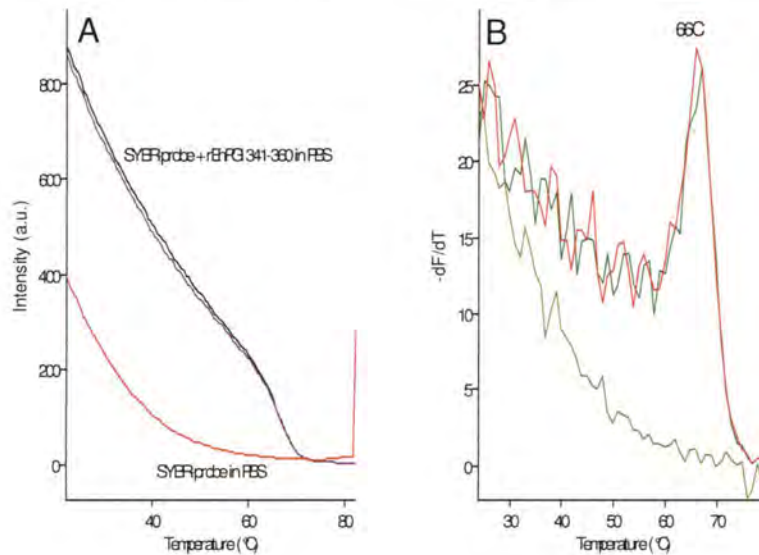


Figure 2-33. Melt analysis of bound and unbound SYBR-tethered probe.

Panel A (F vs. T) showed the change in SYBR-probe fluorescence with respect to temperature, bound with complement (duplicates) and unbound in PBS. The negative of the first derivative of fluorescence with respect to temperature was plotted against temperature ($-dF/dT$ vs. T) to show the melt temperature of SYBR-EhPGI 341-360 DNA pair at 66°C, panel B. Similar experiment with temperature ramp in reverse, from 95 °C dropped to 25 °C, was done and results were same as above (data not shown).

10.3. Characteristics of SYBR-probe binding to complement (>20-mer) events

Titration of synthetic ssDNA of varying sizes from 20-mer to 90-mer against SYBR-EhPGI 341-360 in PBS was recorded in the Cary Eclipse fluorescence spectrophotometer. These synthetic ssDNA templates were prepared using rEhPGI 341-360 as the base with expanding lengths (+5/+5, +15/+15, +35/+35) on either side (both 5'- and 3'-ends) to create shortened complementary sequences of *E. herbicola* PGI gene (see Annex H). Aliquots of 10 pmol shortened complementary sequences were added to a solution of 0.2 μ M SYBR-EhPGI 341-360 in 1mL PBS. Fluorescence

intensities were recorded and shown in Figure 2-34. With complementary ssDNA of 20 nts and 30 nts, the increase in fluorescence intensity was similar with a plateau in signal at about 200 pmol of targets (equivalent to amount of SYBR-tethered probe). Signals for 50-mer and 90-mer targets were much lower, at 35% and 10% respectively, compared to those obtained with 20-mer and 30-mer targets. Thus, the results indicated a decrease in fluorescence signal as target DNA length increased.

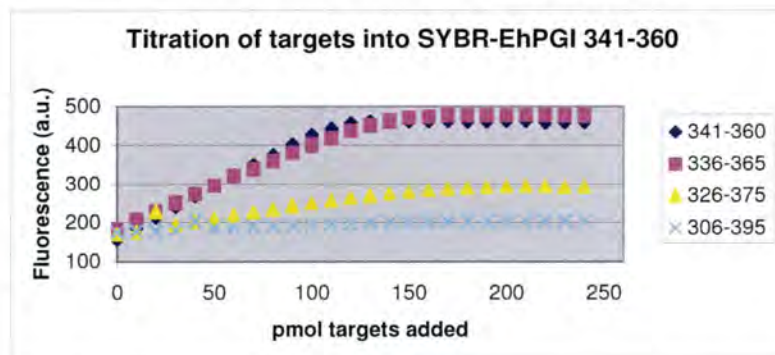


Figure 2-34. Titration of various lengths of targets (shortened sequences of *E. herbicola* pgi) into 200 pmol of SYBR-EhPGI 341-360 in PBS.

Aliquots of 10pmol targets added to each of 4 cuvettes containing SYBR-probe in PBS. Targets are ssDNA of different lengths: 20-mer (rEhPGI 341-360, blue diamonds), 30-mer (rEhPGI 336-365, pink squares), 50-mer (rEhPGI 326-375, yellow triangles), and 90-mer (rEhPGI 306-395, light blue crosses). Fluorescence intensity, measured in Cary Eclipse fluorescence spectrophotometer, increased in a linear fashion in all samples and reached a plateau when the amount of SYBR-probe was equivalent to that of target ssDNA.

Melt analysis of SYBR-probe solutions mixed with various lengths of targets showed Tms at 70 °C (Figure 2-35). Results suggested hybridization of the SYBR-probe to all targets occurred regardless of lengths; SYBR dye molecule interacted with dsDNA in all cases to produce fluorescence signals. The reason for the decrease in fluorescence signals as target lengths increased as observed in Figure 2-34, is unknown to the authors. This decrease in fluorescence signal may potentially interfere with obtaining measurable signals when SYBR-probe is mixed with EhPGI PCR product (344 bp); this was investigated further in the following section.

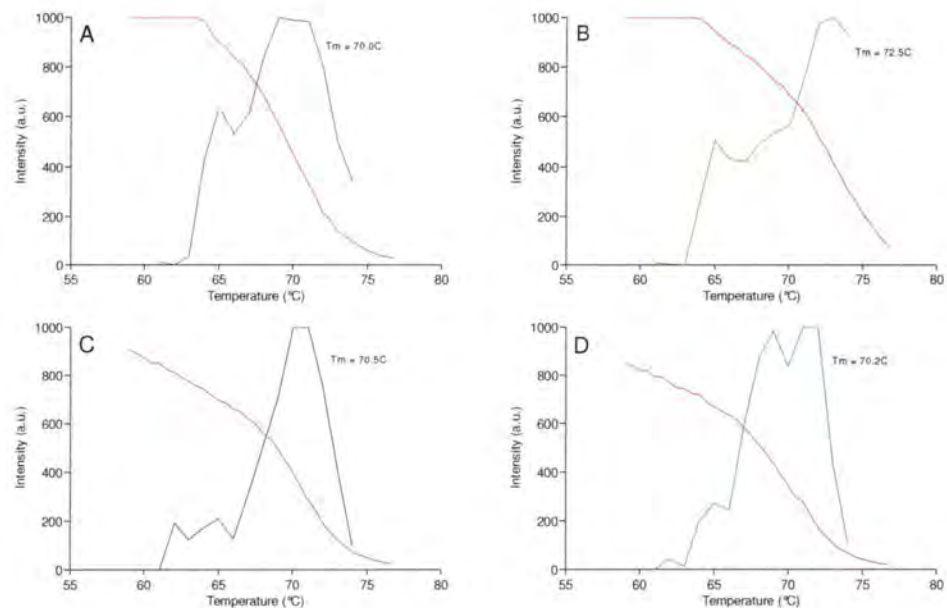


Figure 2-35. Melt analyses of SYBR-EhPGI 341-360 mixed with targets of various lengths.

In panel A, target size 20-mer, rEhPGI 341-360, was used as exact complement to SYBR-probe, and targets of sizes: 30-mer (panel B), 50-mer (panel C), and 90-mer (panel D) were also used to measure DNA hybridization and fluorescence signal strengths of SYBR-probe.

10.4. Characteristics of SYBR-probe binding to *E. herbicola* pgi PCR products (dsDNA and ssDNA)

SYBR-EhPGI 341-360 probe was used to check for hybridization against the PCR product of *E. herbicola* PGI gene. Different PCR products were produced: ssDNA was generated with a PCR mixture containing either 18CA14F (forward primer) or 18CA14R (reverse primer), and dsDNA was produced from a PCR mix containing both primers. The ssDNA containing the forward sequence (Black letters, 5' → 3' sequence in Annex C) was a negative control for SYBR-EhPGI 341-360 because the ssDNA did not contain a complementary sequence to the SYBR-probe. The other ssDNA containing the reverse sequence (blue letters, 3' → 5' sequence in Annex C) and the dsDNA PCR product were positive controls because they both contained a sequence complementary to the SYBR-probe (rEhPGI 341-360). ssDNA was prepared

by mixing Eppendorf HotMaster Taq polymerase and its respective PCR reagents with either 18CA14F or 18CA14R as the only primer in the reaction along with pPGI plasmid (Annex A). Thus, the resulting ssDNA was multiplied linearly. The dsDNA PCR product was also prepared with Eppendorf HotMaster Taq, its PCR Reagents and both 18CA14F and 18CA14R primers, along with the pPGI[pA1] plasmid template (Annex F).

Aliquots of target DNA (ssDNA and dsDNA) were titrated into a PBS solution containing 200 pmol SYBR-EhPGI 341-360. Change in fluorescence during titration (Figure 2-36) was monitored on a fluorescence spectrophotometer with λ_{Ex} at 485nm, and λ_{Em} at 520nm. In the negative control, an equal volume of PBS was added. Melt analysis was done to monitor changes in fluorescence due to a temperature increase for the same solutions as tested in the titration experiment (Figure 2-37). Neither experiment showed positive identification of SYBR-probe interaction with the positive control targets.

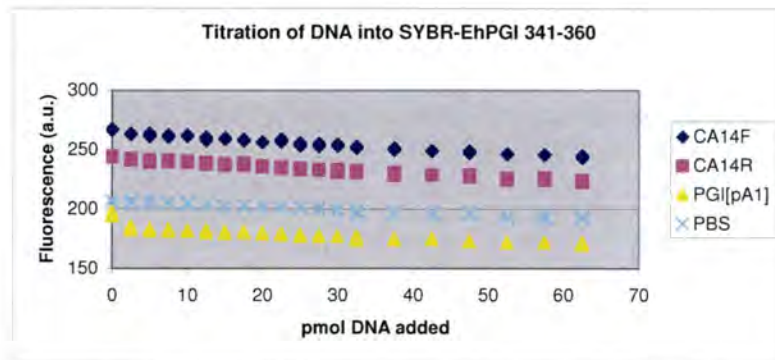


Figure 2-36. Titration of targets, ssDNA and dsDNA, into SYBR-EhPGI 341-360.

Aliquots of the forward sequence ssDNA (blue diamonds, CA14F), the reverse sequence ssDNA (pink squares, CA14R), the dsDNA PCR product from pPGI[pA1] plasmid (yellow triangles, PGI[pA1]), and PBS; added as a no-DNA, negative control (light blue crosses, PBS).

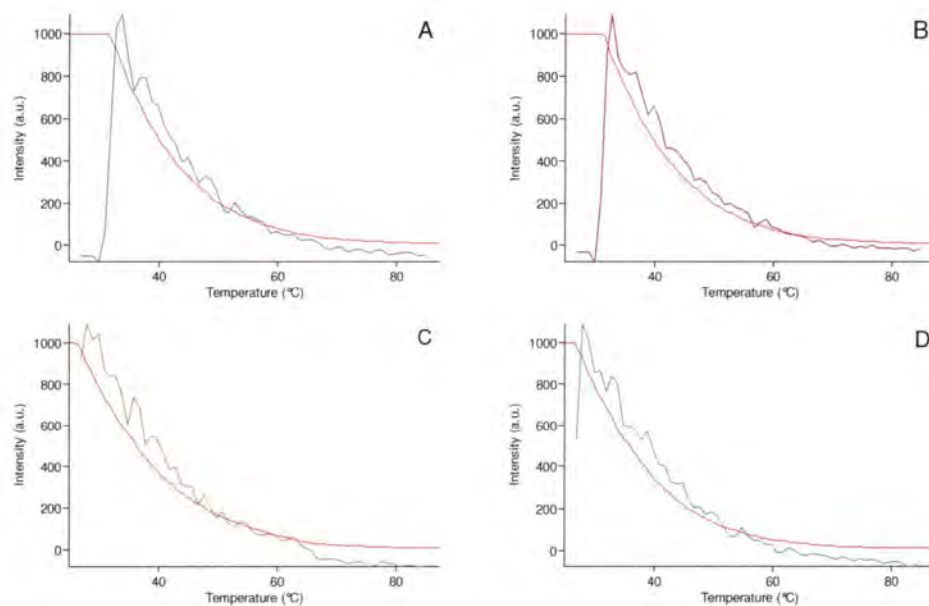


Figure 2-37. Melt analyses of SYBR-EhPGI 341-360 mixed with ssDNA, and dsDNA targets.

In diagram A, SYBR-probe was mixed with the forward sequence ssDNA (negative control), while the reverse sequence ssDNA (B) and dsDNA product of pPGI[pA1] (C) were used as positive controls, along with PBS as no-DNA control (D). Red traces in all 4 diagrams showed the fluorescence signals with respect to temperature, while the other trace (different colour) in each diagram represented the derivative plot ($-dF/dT$ vs T). None showed any probe interaction with complement in positive controls.

11. Conclusions and Recommendations

Roche FastStart Taq polymerase was used for all applications where small volume polymerase chain reaction (PCR) was conducted using the Lightcycler. A 40-cycle PCR with twice the normal amount (0.05 U/ μ L) of Taq polymerase was used to reproducibly produce PCR product. Contamination occurred during the preparation of the FastStart PCR reactions as seen throughout the results section in which positive results were detected in the negative controls. Contamination did not, however, interfere with the outcomes of the experiments. Qiagen HotStar Taq polymerase was only used for 25 μ L reactions in the Mastercycler because amplification was not observed in lower volume reactions. As well as, the Q-solution from the Qiagen HotStar kit introduced a transient melt temperature peak resulting in a false sense that an undesired PCR product was produced. Therefore Qiagen HotStar was deemed an unacceptable Taq polymerase for further study.

Thiazole orange-tagged DNA probes proved difficult to prepare, as only two of the four TO-tagged probe sequences were provided from U of T. Of these, only one was successfully purified for experimental use. The other TO-tagged probe contained excess contaminating DNA sequences that were not tagged with TO and therefore would compete for target in binding experiments.

Experimental results showed a high concentration of TO was required for detection in the LightCycler or Cary Eclipse fluorescence spectrophotometer. A comparison between TO and SYBR Green I (SGI) showed a 10 μ M solution of TO in a real-time PCR had a third of the fluorescent signal of that with only 0.33x SGI. Another intrinsic problem with using TO was higher background fluorescence from the dye even in the absence of dsDNA. This was evident in cases where TO-probe was added to a solution of PBS, which was used as a negative control, and fluorescence signal increased in increments as aliquots of TO-probe were added. Furthermore, the

fluorescence signal from TO was temperature-sensitive. As the temperature increased, the fluorescence signal dropped significantly between 30 °C to 60 °C resulting in low levels of fluorescence signal able to detect the T_m of the probe, even if hybridization occurred. This temperature sensitivity was due to a drop in the molecule's quantum yield, which was directly proportional to fluorescence signal.

In a series of experiments, different TO-probes (TO-mix 15-mer, TO-dT₂₀, and TO-EhPGI 341-360) were added to their complementary target sequences (single-stranded 20-mer, and 344 bp dsDNA from PCR). These experiments included titrations, melt analyses, and emission spectra scans. Results from all three experiments showed a lack of sufficient evidence to prove hybridization occurred between the TO-probe and target sequences.

Alternatively, SYBR 101TM was covalently linked to the 5' end of EhPGI 341-360 for comparison. The coupling reaction was a simple condensation reaction between the reactive SYBR 101TM containing a succinimydyl ester group and the primary amine group on the modified 5' end of EhPGI 341-360. The resulting SYBR-probe was purified from un-reacted probe sequences using SDS-polyacrylamide gel electrophoresis (PAGE) before use in any experiments.

SYBR-EhPGI 341-360 probe was hybridized against its complementary 20-mer ssDNA, and various lengths (30-mer, 50-mer, and 90-mer) of ssDNA containing the complementary sequence, rEhPGI 341-360. In all cases, titration and melt analysis results showed hybridization of SYBR-probe to the complementary sequence with the correct melt temperature. It was evident the fluorescence signal decreased as target DNA lengths increased, from 100% for the 20-mer and 30-mer, to 35% for the 50-mer, and 10% for the 90-mer. SYBR-probe was also tested for hybridization against 344-mer ssDNA and 344 bp dsDNA containing the complementary sequence. Hybridization was not observed in either of these cases as observed from titration and

melt analysis results. The reason for the drop in fluorescence signal level and lack of hybridization (which maybe a direct result of low fluorescence level) was unknown to the authors.

The authors would like to recommend the use of SYBR 101TM dye instead of thiazole orange for future tethered dye probe (TDP) experiments. This is due to the ease of the use of reactive SYBR 101TM reagent, the low level of background noise, and the relative stability of the dye with respect to temperature changes. Further investigation of fluorescence signal decrease with respect to target length up to 90 nt and the lack of hybridization to longer PCR products should be done using different concentrations of SYBR-probe. If correct conditions for hybridization of SYBR-probe to target are found, use of SYBR-probes as tools for real-time PCR detection and identification could be pursued.

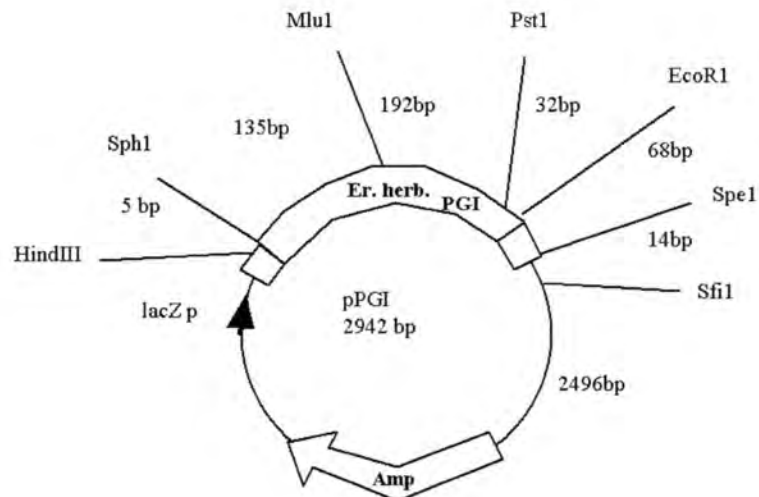
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Annexes

Annex A—Construction of pPGI

The following figure shows the plasmid used for PCR, pPGI, is a pUC19 plasmid containing the *Erwinia herbicola* phosphoglucose isomerase gene (*pgi*). To clone the *Er. herbicola* phosphoglucose isomerase (PGI) gene fragment, restriction sites (SphI and EcoRI) were introduced into specific PCR primers CA14F and CA14R. The PCR PGI gene fragment was obtained by PCR amplification of *Er. herbicola* genomic DNA using the two PCR cloning primers, Sp18CA14F (SphI) [CGACCAGTGCATG CACCGTTTGGCGCCTATTCC - 35mer] & 2Ec18CA14R (EcoRI) [TGAATTCCACGATATGGGCTAC AGACTC - 28 mer]. The PGI gene fragment was then gel purified prior to restriction digested with SphI and EcoRI.



Sequence of *Erwinia herbicola* phosphoglucose isomerase (*pgi*) mRNA gene, partial cds (identification from Blast search: U54764, and gi 1314760). Blue letters denote sequence of PCR product (pPGI amplicon) of 344 base pairs (bp). The amplicon contains 54% GC content, at an approximate molecular weight of 261474Da. Underlined sequences are forward primer (18CA14F), and complement to reverse primer (18CA14R).

```

1  CAG CAC TTC GCC TCT GCG CCG GCT GAG CAG AAC CTG CCG GTG CTG
46 CTG GCG CTG ATT GGC ATC TGG TAC AAC AAT TTC TTT GGT GCT GAA
91 ACC GAA GCG ATT CTG CCT TAC GAC CAG TAC ATG CAC CGT TTT GCC
136 GCC TAT TTC CAG CAG GGC AAT ATG GAG TCA AAC GGC AAG TAT GTG

```

181 GAT CGT GCC GGT CAT CCG GTG GAT TAC CAG ACT GGC CCA ATC ATC
226 TGG GGC GAA CCT GGC ACC AGC GGT CAG CAC GCG TTT TAT CAG CTG
271 ATC CAC CAG GGT ACC AAG CTG GTT CCG TGC GAC TTT ATT GCC CCG
316 GCC ATT ACC CAT AAT GCG CTC GCG GAT CAC CAT CCG AAA CTG CTC
361 TCT AAC TTC TTT GCC CAG ACT GAA GCG CTG GCG TTT GGT AAG TCA
406 CGC GAC GTC GTA GAG AAA GAG TTT ACT GAT GCC GGT AAA TCT GCA
451 GAG TCT GTA GCC CAT ATC GTG CCG TTC AAG GTG TTT GAA GGT AAC
496 CGC CCG

Compare phosphoglucose isomerase gene sequence similarity shared between *E. herbicola* (black sequence) and *E. amylovora* (blue sequence). Red asterisks (*) represent mis-matched nucleotides between *E. herbicola* and *E. amylovora* (most differences occur in 2nd and 3rd codon).

E. herbicola 361 TCT AAC TTC TTT GCC CAG ACT GAA GCG CTG GCG TTT GGT AAG TCA
E. amylovora TCG AAC TTC TTT GCC CAG ACT GAA GCG CTG GCG TTT GGT AAA TCG

E. herbicola ⁴⁰⁶CGC GAC GTC GTA GAG AAA GAG TTT ACT GAT GCC GGT AAA TCT GCA
E. amylovora CGC GAG GCG GTG GAA CAA GAG TTT GCC GAT GCA GGC AAG GAT GCG
* * *

E. herbicola ⁴⁵¹GAG TCT GTA GCC CAT ATC GTG CCG TTC AAG GTG TTT GAA GGT AAC
E. amylovora AAA TCA GTC GAA CAC ATT GTG CCT TAC AAG GTA TTC GAG GGT AAT
* * *

E. herbicola ⁴⁹⁶CGC CCG
E. amylovora CGT CCT
* *

Annex C—Sequence of *E. herbicola* PGI product

Sequence of *Erwinia herbicola* PGI gene product from PCR using 18CA14F and 18CA14R primers (underlined sequences). The numbering used to identified nucleotide location started with 128, the start of 18CA14F (forward primer) within the sequence of *Erwinia herbicola* phosphoglucose isomerase (*pgi*) mRNA gene, partial cds.

18CA14F

```
5' 128 GTT TTG CCG CCT ATT TCC AGC AGG GCA ATA TGG AGT CAA ACG GCA
3'   CAA AAC GGC GGA TAA AGG TCG TCC CGT TAT ACC TCA GTT TGC CGT

173 AGT ATG TGG ATC GTG CCG GTC ATC CGG TGG ATT ACC AGA CTG GCC
    TCA TAC ACC TAG CAC GGC CAG TAG GCC ACC TAA TGG TCT GAC CGG

218 CAA TCA TCT GGG GCG AAC CTG GCA CCA GCG GTC AGC ACG CGT TTT
    GTT AGT AGA CCC CGC TTG GAC CGT GGT CGC CAG TCG TGC GCA AAA

263 ATC AGC TGA TCC ACC AGG GTA CCA AGC TGG TTC CGT GCG ACT TTA
    TAG TCG ACT AGG TGG TCC CAT GGT TCG ACC AAG GCA CGC TGA AAT

308 TTG CCC CGG CCA TTA CCC ATA ATG CGC TCG CGG ATC ACC ATC CGA
    AAC GGG GCC GGT AAT GGG TAT TAC GCG AGC GCC TAG TGG TAG GCT

353 AAC TGC TCT CTA ACT TCT TTG CCC AGA CTG AAG CGC TGG CGT TTG
    TTG ACG AGA GAT TGA AGA AAC GGG TCT GAC TTC GCG ACC GCA AAC

398 GTA AGT CAC GCG ACG TCG TAG AGA AAG AGT TTA CTG ATG CCG GTA
    CAT TCA GTG CGC TGC AGC ATC TCT TTC TCA AAT GAC TAC GGC CAT

443 AAT CTG CAG AGT CTG TAG CCC ATA TCG TG      3'
    TTA GAC GTC TCA GAC ATC GGG TAT AGC AC      5'
                                18CA14R
```

Annex D—Proposed Tethered-dye Probe Sequences for *E. herbicola* PGI

Proposed tethered-dye probe sequences (blue), and corresponding complementary sequence (red). EhPGI denotes *Erwinia herbicola* phosphoglucose isomerase, the numbers denote start-end position of sequence within *pgi*. 18CA4F is the designation of the PCR forward primer.

EhPGI 341-360	5' TO-ATCACCATCCGAAACTGCT-ddC 3'
rEhPGI 341-360	3' TAGTGGTAGGCTTTGACGAG 5'
EhPGI 319-338	5' TO-ATTACCCATAATGCGCTCG-ddC 3'
rEhPGI 319-338	3' TAATGGGTATTACGCGAGCG 5'
EhPGI 293-312	5' TO-TTCCGTGCGACTTTATTGC-ddC 3'
rEhPGI 293-312	3' AAGGCACGCTGAAATAACGG 5'
18Ca14F	5' TO-GTTTTGCCGCCTATTTC-ddC 3'
rEhPGI 128-145	3' CAAAACGGCGGATAAAGG 5'

Thiazole orange (TO) tethered probes available from Prof. U. Krull's laboratory:

W20752	5' TO-TTCTCTCTTTTTTTTT-3' (TO-mix 15-mer)
W20751	5' TO-TTTTTTTTTTTTTTTTTTTTTT-3' (TO-dT ₂₀)
W21094	5' TO-TTTTTTTTTTTTTTTTTTTTTT-NH ₂ 3'
W21511 (TO-EhPGI 341-360)	5' TO-ATCACCATCCGAAACTGCT-ddC 3'
W2118 (TO-EhPGI 319-338, estimated only <5% incorporation of TO to probe)	5' TO-ATTACCCATAATGCGCTCG-ddC 3'

Annex E—Binding Data for EhPGI Probes

EhPGI 293-312:

Binding to itself:

1. $\Delta G = -3.6$

```

5' TTCCGTGCGACTTTATTGCC 3'
      ||  ||
3' CCGTTATTTACGCGTGCCTT 5'

```
2. $\Delta G = -3.1$

```

5' TTCCGTGCGACTTTATTGCC 3'
                        ||
3' CCGTTATTTACGCGTGCCTT 5'

```
3. $\Delta G = -3.1$

```

5' TTCCGTGCGACTTTATTGCC 3'
      || |      | ||
3' CCGTTATTTACGCGTGCCTT 5'

```

Binding to Lower *Erwinia herbicola* (Eh) primer (18CA14R):

1. $\Delta G = -6.8$

```

5' TTCCGTGCGACTTTATTGCC 3'
      |  ||||
3' CTCAGACATCGGGTATAGCAC 5'

```
2. $\Delta G = -6.2$

```

5' TTCCGTGCGACTTTATTGCC 3'
      || | | | |||
3' CTCAGACATCGGGTATAGCAC 5'

```

Binding to Upper Eh primer (18CA14F):

1. $\Delta G = -6.7$

```

5' TTCCGTGCGACTTTATTGCC 3'
      ||| |  |
3' CCTTTATCCGCCGTTTIG 5'

```

EhPGI 319-338:

Binding to itself:

1. $\Delta G = -9.8$

```

5' ATTACCCATAATGCGCTCGC 3'
      ||||
3' CGCTCGCGTAATACCCATTA 5'

```
2. $\Delta G = -6.7$

```

5' ATTACCCATAATGCGCTCGC 3'
      ||| |||
3' CGCTCGCGTAATACCCATTA 5'

```
3. $\Delta G = -4.4$

```

5' ATTACCCATAATGCGCTCGC 3'
      |||      |||
3' CGCTCGCGTAATACCCATTA 5'

```

4. $\Delta G = -3.4$

```
5' ATTACCCATAATGCGCTCGC 3'
      | | | | | | |
3' CGCTCGCGTAATACCCATTA 5'
```

Binding to Lower Eh primer (18CA14R):

1. $\Delta G = -10.6$

```
5' ATTACCCATAATGCGCTCGC 3'
      | | | | | | |
3' CTCAGACATCGGGTATAGCAC 5'
```

2. $\Delta G = -5.2$

```
5' ATTACCCATAATGCGCTCGC 3'
      | | |
3' CTCAGACATCGGGTATAGCAC 5'
```

Binding to Upper Eh primer (18CA14F):

1. $\Delta G = -6.7$

```
5' ATTACCCATAATGCGCTCGC 3'
      | | |
3' CCTTTATCCGCCGTTTGTG 5'
```

2. $\Delta G = -3.4$

```
5' ATTACCCATAATGCGCTCGC 3'
      | | |
3' CCTTTATCCGCCGTTTGTG 5'
```

EhPGI 341-360:

Binding to itself:

1. $\Delta G = -3.1$

```
5' ATCACCATCCGAAACTGCTC 3'
      | |
3' CTCGTCAAAGCCTACCACTA 5'
```

Binding to Lower Eh primer (18CA14R):

1. $\Delta G = -6.5$

```
5' ATCACCATCCGAAACTGCTC 3'
      | | |
3' CTCAGACATCGGGTATAGCAC 5'
```

2. $\Delta G = -3.5$

```
5' ATCACCATCCGAAACTGCTC 3'
      | | |
3' CTCAGACATCGGGTATAGCAC 5'
```


Binding to Upper Eh primer (18CA14F):

1. $\Delta G = -5.4$

5' ATCACCATCCGAAACTGCTC 3'

|||||
3' CCTTTATCCGCCGTTTTG 5'

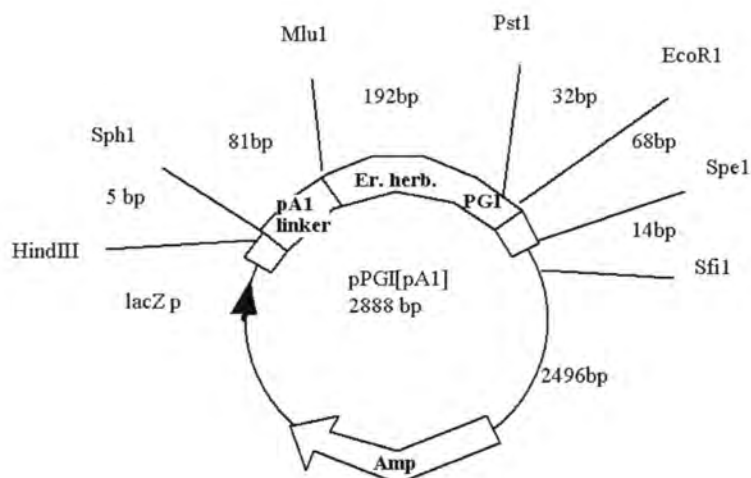
2. $\Delta G = -5.1$

5' ATCACCATCCGAAACTGCTC 3'

| | | ||||
3' CCTTTATCCGCCGTTTTG 5'

Annex F—Construction of pPGIp[A1]

The following figure shows new plasmid with poly-A inserted, pPGIp[A1]. The pA1 linker was prepared by annealing two synthetic oligonucleotides, CA14A20[+] linker [CACCGTTTTGCCGCC TATTTCCAGCAGGGCAATAAAAAAAAAAAAAAAAAAAGAGAGAACCAGCGGT CAGCA]& CA14A20[-] linker [CGCGTGCTGACCGCTGGTTCTCTCTTTTTTTTTTTTTTTTTTTTATTG CCCTGCTGGAAATAGGCGGCAAAACGGTGCATG]. The linker design was based on the phosphoglucose isomerase gene fragment of pPGL, a tract of A₂₀ (pA or poly-A) and (AG)₃ which will serve as a target sequence for currently available fluorescent tethered-dye probes (T20), (TCn), and *E. herbicola* PGI target sequences.



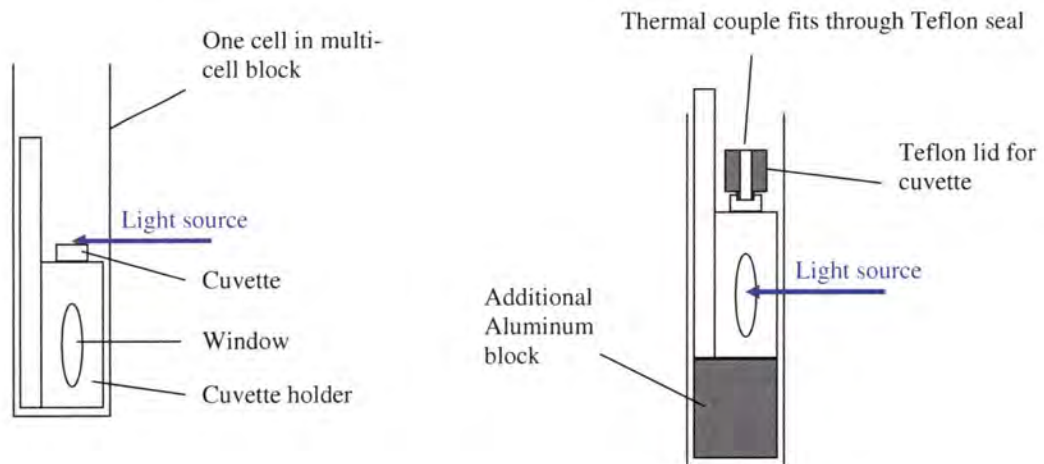
Sequence of *Erwinia herbicola* phosphoglucose isomerase with poly-A sequence (pPGIp[A1]) cloned into positions 157 to 202, replacing positions 157 to 260 in pPGL. This replacement results in a 58 nucleotides truncate. Red letters denote the new poly-A insert in pPGIp[A]. Blue letters denote remaining sequence of PCR product as in pPGL amplicon. The resulting PCR product, pPGIp[A] amplicon has 286bp. The amplicon contains 49% GC content, at an approximate molecular weight of 217349Da.

¹ CAG CAC TTC GCC TCT GCG CCG GCT GAG CAG AAC CTG CCG GTG CTG
⁴⁶ CTG GCG CTG ATT GGC ATC TGG TAC AAC AAT TTC TTT GGT GCT GAA
⁹¹ ACC GAA GCG ATT CTG CCT TAC GAC CAG TGC ATG CAC CGT TTT GCC
¹³⁶ GCC TAT TTC CAG CAG GGC AAT AAA AAA AAA AAA AAA AAA AAG AGA

181 GAA CCA GCG GTC AGC ACG CGT TTT ATC AGC TGA TCC ACC AGG GTA
226 CCA AGC TGG TTC CGT GCG ACT TTA TTG CCC CGG CCA TTA CCC ATA
271 ATG CGC TCG CGG ATC ACC ATC CGA AAC TGC TCT CTA ACT TCT TTG
316 CCC AGA CTG AAG CGC TGG CGT TTG GTA AGT CAC GCG ACG TCG TAG
361 AGA AAG AGT TTA CTG ATG CCG GTA AAT CTG CAG AGT CTG TAG CCC
406 ATA TCG TGC CGT TCA AGG TGT TTG AAG GTA ACC GCC CG

Annex G—Machining of Cuvette Holders

Machining work required to outfit aluminum holder for small volume cuvette (max. vol. 150 μL). An Aluminum block is required to bring the content of cuvette to height of light source. A Teflon lid is also required to seal cuvette to prevent evaporation and to provide support to fix thermal couple in place when running melt curve experiments.



Sequences of ssDNA used as templates in titration and melt temperature experiments with SYBR-Eh PGI 341-360 probe (in blue). These ssDNA (in red) were prepared to show the extent of TDP binding to template in single-stranded against double-stranded forms.

90-mer: +35 nucleotides

5' TATTGCCCCGGCCATTACCCATAATGCGCTCGCGGATCACCATCCGAAACTGCTC

3' ATAACGGGGCCGGTAATGGGTATTACGCGAGCGCCTAGTGGTAGGCTTTGACGAG

+35 nucleotides

TC TAACTTCTTTGCCAGACTGAAGCGCTGGCGTT 3'

AGA TTGAAGAAACGGGTCTGACTTCGCGACCGCAA 5' (rEhPGI 306-395)

List of symbols/abbreviations/acronyms/initialisms

18CA14F	forward primer for <i>pgi</i> PCR
18CA14R	reverse primer for <i>pgi</i> PCR
λ_{ex}	excitation wavelength
A	adenine
bp	base pairs
BSA	Bovine serum albumin
C	cytosine
Da	daltons
ddC	dideoxycytidine
dF/dT	first derivative of fluorescence signal with respect to temperature
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide 5'-triphosphate
DNA	Deoxyribonucleic acid
DRDC	Defence Research and Development Canada
ds	Double-stranded
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
Eh	<i>Erwinia herbicola</i>
<i>E. herbicola</i>	<i>Erwinia herbicola</i>
EtBr	Ethidium bromide
FRET	Fluorescent resonance energy transfer
G	guanosine
HPLC	High performance liquid chromatography

kb	kilobases
KCl	Potassium chloride
kDa	kiloDaltons
LB	Luria-Bertani
MgCl ₂	magnesium chloride
PCR	Polymerase chain reaction
PDMS	Polydimethyl sialoxane
<i>Pfu</i>	<i>Pyrococcus furiosus</i> polymerase
<i>pgi</i>	phosphoglucose isomerase gene
PGI	Phosphoglucose isomerase
PNA	peptide nucleic acid oligomer
poly-dT	polydeoxythymine
PVC	Polyvinyl chloride
PVDF	polyvinylidene fluoride
RFLP	Restriction fragment length polymorphism
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SGI	SYBR Green I
SOC	Bacterial growth media
ss	Single-stranded
sv	Small volume
T	Thymine
Ta	Annealing temperature
Taq	<i>Thermus aquaticus</i>
TDP	Tethered-dye probe

Tm	Melting temperature
TO	Thiazole orange

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Part 1: This work concentrated on transferring polymerase chain reaction (PCR) assays to a microfluidics format with fluorescent detection, with the ultimate goal of creating a rugged microchip for performing PCR in the field. Several parameters were explored with regards to transferring the assay to a microchip, including substrate suitability; effect of additives; and decreased volume on product formation and reproducibility. The phosphoglucose isomerase (PGI) gene from *Erwinia herbicola* was successfully cloned and characterized as the template for all subsequent PCR reactions. Additives such as DMSO and formamide appeared to be useful to reduce the denaturation and annealing temperatures of real time PCR assays. BSA interfered with SYBR Green I fluorescent detection in real time but was required for PCR success. Complete PCR master mixes including enzyme and template were found to be stable for up to 24 hours at room temperature and dried PCR reagents stored at room temperature retained their activity for 14 days; The plastic polydimethyl siloxane (PDMS) was found to be a suitable reaction medium for PCR and fluorescent detection using SYBR Green I dye. The PCR reaction volume limited the amount of product obtained through PCR; reaction volumes of less than 10 μ L required special handling to ensure product was reproducibly obtained. The variability of results in small volume PCR was attributed to local reagent concentration effects due to evaporation and condensation of water from the constant heating and cooling during cycling. This will impact any PCR assay in a microfluidics device, as evaporation becomes a key limiting factor.

Part 2: Polymerase chain reaction (PCR) is commonly used for biodetection, especially for low levels of biomaterials. Typically, the identity of a PCR product is confirmed on agarose gel electrophoresis and through DNA sequencing. This time-consuming detection-confirmation process can be eliminated through the incorporation of simultaneous gene probe hybridization and fluorescence detection. A proposed tethered-dye gene probe (TDP) was evaluated as a biosensor for use in real-time detection in PCR. Fluorescence characterization of untethered- vs. tethered- dye, and of unbound vs. bound TDP to target dsDNA, was measured in a fluorescence spectrophotometer. Two different dyes, thiazole orange (TO) and reactive SYBR® 101 (SYBR) were compared to the commonly used dsDNA binding dye, SYBR Green I (SGI). Gene probes were designed and prepared to target the *Erwinia herbicola* phosphoglucose isomerase (Eh PGI) gene. Real-time PCR was measured in a LightCycler (Idaho Technologies Inc.) thermal cycling instrument.

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PCR, polymerase chain reaction, microfluidics, tethered-dye gene probe, fluorescent detection, SYBR Green I

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