

DEVELOPMENT OF A REAL-TIME IMMUNO-PCR ASSAY FOR THE QUANTIFICATION
OF ENVIRONMENTAL CONTAMINANTS

DANIEL GAUDET

BSc Biology, University of Lethbridge, 2012

A Thesis

Submitted to the School of Graduate Studies

of the University of Lethbridge

in Partial Fulfilment of the

Requirements for the Degree

MASTER OF SCIENCE

Department of Biological Sciences

University of Lethbridge

LETHBRIDGE, ALBERTA, CANADA

© Daniel Gaudet, 2014

THESIS EXAMINATION COMMITTEE MEMBERS

DEVELOPMENT OF AN IMMUNO-PCR ASSAY FOR THE QUANTIFICATION OF ENVIRONMENTAL CONTAMINANTS

DANIEL GAUDET

Date of Defence: December 16th 2014

Dr. C. Sheedy Supervisor	Adjunct Professor	Ph.D.
Dr. A. Hontela Co-Supervisor	Professor	Ph.D.
Dr. S. Rood Thesis Examination Committee Member	Professor	Ph.D.
Dr. B. Selinger Thesis Examination Committee Member	Professor	Ph.D.
Dr. U. Kothe Thesis Examination External Committee Member	Professor	Ph.D.
Dr. T. Burg Thesis Examination Chair	Professor	Ph.D.

ABSTRACT

DEVELOPMENT OF A REAL-TIME IMMUNO-PCR ASSAY FOR THE QUANTIFICATION OF ENVIRONMENTAL CONTAMINANTS

This thesis outlines the development of two universal real-time immuno-PCR (RT-iPCR) assays for application in agriculture. The first RT-iPCR was developed for the sensitive quantification of 17β -estradiol in water. Using a universal iPCR method and polyclonal antibodies, 17β -estradiol was accurately quantified at concentrations ranging from 1 pg mL^{-1} to $10 \text{ }\mu\text{g mL}^{-1}$, with a limit of detection of 0.7 pg mL^{-1} . The RT-iPCR assay provided an 800-fold increase in sensitivity as well as an expanded working range compared to the corresponding enzyme-linked immunosorbent assay. Antibodies were swapped with antibodies specific to *P. brassicae* for the quantification of clubroot resting spores using the same RT-iPCR assay. *P. brassicae* resting spores were quantified in the range of 50 to 10 000 spores, with a detection limit of 29 spores. Both RT-iPCR showed equal or improved sensitivity compared to other published analytical methods, with expanded linear working ranges and high-throughput capabilities.

ACKNOWLEDGMENTS

I would like to extend my sincerest appreciation for the guidance and constant support of my supervisor, Dr. Claudia Sheedy. Her encouragement and dedication made my experience as a Master's student all the more enjoyable, and contributed to my learning experience by allowing me to attend various meetings and scientific conferences in order to showcase my research. This thesis represents two unforgettable years with the University of Lethbridge and Agriculture and Agri-Food Canada.

I would also like to extend my gratitude to the other members of my committee, Dr. Alice Hontela, Dr. Stewart Rood and Dr. Brent Selinger. Special thanks to Dr. Alice Hontela for being my co-supervisor, and for assisting me with my travels. Thank you all for your time and expertise. My degree would not have been possible without your support and guidance.

Special thanks to Denise Nilsson for all her help and laboratory experience, I truly appreciate her dedication to this project. Also, I would like to thank Tara Vucurevich, and CO-OP student Tanner Lohr for their efforts and contributions to this project. Finally, I would like to thank Dr. Larry Kawchuk for his collaboration and providing us with *P. brassicae* spores.

I would also like to thank everyone at the University of Lethbridge who supported and encouraged me throughout my education.

I dedicate this thesis to my parents, and in particular to my father, Dr. Denis Gaudet, who has helped me more than he will ever know, in education and in life.

TABLE OF CONTENTS

THESIS EXAMINATION COMMITTEE MEMBERS.....	ii
ABSTRACT.....	iii
ACKNOWLEDGMENTS.....	iv
TABLE OF CONTENTS.....	v
LIST OF TABLES.....	ix
LIST OF FIGURES.....	x
LIST OF ABBREVIATIONS.....	xi
1. LITERATURE REVIEW.....	1
1.1 Introduction.....	1
1.2 Immuno-PCR.....	4
1.3 iPCR Formats.....	7
1.3.1 Antibody coupling with DNA.....	9
1.3.2 Chemical coupling.....	9
1.3.3 Supramolecular conjugates.....	10
1.4 Signal Generation and detection.....	11
1.5 Reporter DNA Design.....	13
1.6 Other iPCR Strategies.....	14
1.7 RT-iPCR Applications.....	16

1.8 Challenges	18
1.9 RT-iPCR Application in Agriculture	20
1.9.1 Manure-borne estrogens.....	20
1.9.2 Antibody swapping: <i>Plasmodiophora brassicae</i>	22
1.10 Conclusions	23
2. DEVELOPMENT OF A REAL-TIME IMMUNO-PCR ASSAY FOR THE QUANTIFICATION OF 17β-ESTRADIOL IN WATER	25
2.1 Abstract	25
2.2 Introduction	25
2.3 Materials and Methods	28
2.3.1 Reagents and chemicals.....	28
2.3.2 ELISA development.....	29
2.3.3 CI-ELISA characterization and cross-reactivity.....	30
2.3.4 Biotinylated reporter DNA.....	30
2.3.5 RT-iPCR.....	31
2.3.6 RT-iPCR characterization.....	33
2.3.7 Spiked water samples.....	33
2.4 Results and Discussion	34

2.4.1 ELISA characterization and cross-reactivity.....	34
2.4.2 RT-iPCR characterization.....	34
2.4.3 17 β -estradiol recovery from spiked samples.....	36
2.5 Conclusion.....	37
2.6 Figures and Tables.....	39
3. DEVELOPMENT OF A REAL-TIME IMMUNO-PCR ASSAY FOR THE QUANTIFICATION OF <i>PLASMODIOPHORA BRASSICAE</i> RESTING SPORES.....	43
3.1 Abstract.....	43
3.2 Introduction	43
3.3 Materials and Methods.....	46
3.3.1 Spore preparation.....	46
3.3.2 Immunization and preparation of antisera.....	47
3.3.3 ELISA development.....	48
3.3.4 RT-iPCR.....	48
3.3.5 RT-iPCR optimization and characterization.....	50
3.4 Results and Discussion.....	51
3.4.1 ELISA characterization.....	51
3.4.2 RT-iPCR optimization and characterization.....	51

3.5 Conclusion	52
3.6 Acknowledgments	53
3.7 Figures	54
4. DISCUSSION AND CONCLUSIONS.....	57
5. REFERENCES.....	60
6. SUPPLEMENTARY INFORMATION.....	71

LIST OF TABLES

Table

1.1	Application of iPCR for the detection of haptens.....	17
2.1	Cross-reactivities of structurally related compounds to 17β -estradiol.....	41
2.2	Recovery of 17β -estradiol from spiked water samples using RT-iPCR.....	42
6.1	Average Ct values of 17β -estradiol iPCR assay with urine samples.....	69

LIST OF FIGURES

Figure

1.1. Schematic representation of a variety of ELISA methodologies.....	3
1.2. Universal iPCR formats with streptavidin linker and biotinylated antibody and reporter DNA.....	8
2.1. Schematic representation of real-time immuno-PCR (RT-PCR).....	39
2.2 RT-iPCR 17 β -estradiol standard plot.....	40
3.1 Schematic diagram of the <i>P. brassicae</i> iPCR assay.....	53
3.2 RT-iPCR <i>P. brassicae</i> standard plot.....	54
3.3 RT-iPCR <i>S. subterranea</i> standard plot.....	56
6.1 RT-iPCR 17 β -estradiol standard plot.....	70

LIST OF ABBREVIATIONS

Abbreviation	Meaning
Ab	Antibody
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
Ag	Antigen
AuNP	Gold Nanoparticle
BC	Background Control
BSA	Bovine Serum Albumin
CEA	Carcinoembryonic antigen
CR	Cross reactivity
CT	Cycle Threshold
E1	Estrone
E2	17 β -estradiol
E3	Estriol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAM	Fluorescein amidite
FL	Fluoranthene
GC	Gas Chromatography
HBsAg	Hepatitis B Surface Antigen
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HRPO	Horseradish Peroxidase
IC ₅₀	Inhibition Concentration 50%
IgG	Immunoglobulin G

iPCR	immuno-PCR
LB	Luria-Berthani Broth
LC	Liquid Chromatography
LOD	Limit of Detection
mAbs	Monoclonal Antibodies
MDL	Method detection Limit
MMP	Magnetic Microparticle
MS	Mass Spectrometer
NA-iPCR	Nanoparticle Amplified-Immuno-PCR
NC	Negative Control
OVA	Ovalbumin
pABs	Polyclonal Antibodies
PAH	Polycyclic Aromatic Hydrocarbon
PBS	Phosphate Buffered Saline
PCB	Polychlorinated Biphenyls
PCR	Polymerase Chain Reaction
PH	Phenanthrene
qPCR	Quantitative PCR
RSD	Relative Standard Deviation
RIA	Radioimmunoassay
RSV	Respiratory Syncytial Virus
RT-iPCR	Real-time-immuno-PCR
STN-PCR	Single-tube Nested PCR
STV	Streptavidin

1. LITERATURE REVIEW

1.1 Introduction

Since the 1960s, immunoassays have played an important role as rapid diagnostic tools with a wide range of applications in biomedical research and environmental monitoring. As antibody-based assay technology continues to expand, they now incorporate more recent technologies such as molecular biology and nanoparticles to further improve existing techniques. This literature review examines recent developments in antibody-based diagnostic methods for the quantification of environmental contaminants with a focus on immuno-PCR.

Medical diagnostics, environmental monitoring and research all rely on the rapid and specific quantification of analytes of interest. An important contribution to the field of diagnostic research has been the development of the immunoassay; a biochemical technique based on the specific interaction between antigen (Ag) and antibody (Ab) [1]. Early pioneers Rosalyn Yalow and Solomon Berson were recipients of the Nobel Prize in Physiology and Medicine in 1959 for their initial research on immunodiagnostic technology [2]. Since then, a wide array of proteins and other compounds have been successfully quantified using immunoassays, which have become powerful tools in diagnostic analyses [1, 3, 4]. Examples such as infectious diseases, including hepatitis B [5] and human immunodeficiency virus (HIV) [6], allergens [7] and other human and animal disease-specific antigens have been detected and quantified using immunoassay technology [1]. Immunodiagnostic methods have also been extensively employed by toxicologists for rapid detection and quantification of environmental contaminants [8-11]. The expansion of antibody-based diagnostic technologies can be attributed to the specificity and selectivity conferred by the Ab-Ag interaction, as well as the simplicity and ease of assay performance. Immunoassays rely on antibodies which can bind specifically to a protein, hapten

or any other antigen of interest [1]. They typically consist of a solid support onto which reactants, including analyte, antibodies and detection reagents, are sequentially added in order to quantify the analyte via a colorimetric or radiolabel detection [1]. Variations of immunoassays include immunofluorescence assays [12] and radioimmunoassays (RIA) [13], as well as enzyme linked immunosorbent assays (ELISA).

RIAs, which are among the earliest developed immunodiagnostic methods, rely on antibody or antigen labelling with radioisotopes which permit, in some cases, sensitivity as low as pg mL^{-1} level [2, 14]. While RIAs continue to be employed in a variety of diagnostic applications, drawbacks include rapid decay of isotopes with short half-life, the inherent dangers of radioisotopes and cost associated with handling and disposing of radioactive material [3, 14]. ELISAs, initially developed by Engvall and Perlmann [4] in the early 1970's, offered an alluring alternative to radioisotope labeling. Enzyme labels, such as horseradish peroxidase (HRPO) or alkaline phosphatase were employed for signal generation, with the absorbance being quantified using spectrophotometry. Signal generation is therefore accomplished through enzymatic activity acting upon a chemical substrate, producing a colored or fluorescent product relative to the amount of bound reactants [1]. A variety of ELISA methodologies exist, four of which are summarized in Figure 1.1.

While ELISAs offer a simple and effective way to quantify an analyte, assay sensitivity is limited by the quality of antibodies being used and the ability of the spectrophotometer to quantify color change. The limit of detection (LOD) can vary among the different assays but typically does not go beyond the ng mL^{-1} levels [9]. The LOD in this context represents the limit at which a positive signal can be distinguished from a blank (negative control) [1, 3].

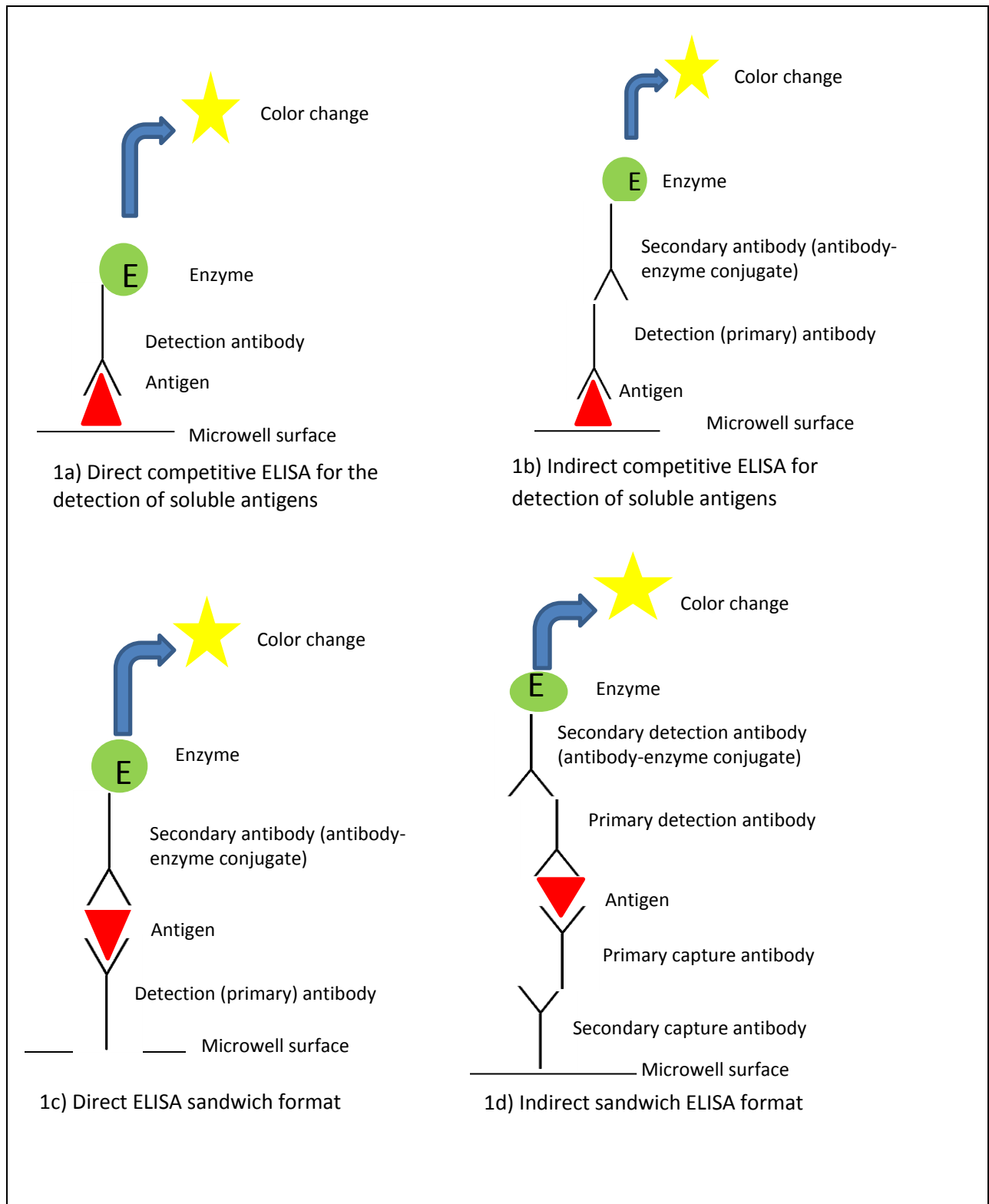


Figure 1.1. Schematic representation of a variety of ELISA methodologies.

ELISAs may lack sensitivity in certain applications, for example in medical diagnostics where antigenic determinants from pathogens may be present in low concentrations and patient survival is dependent on rapid and accurate diagnosis. Environmental monitoring also relies on ultrasensitive detection, particularly with contaminants that cause ecotoxicological harm at concentrations below ng mL^{-1} [15, 16].

The detection of antibody-antigen interactions is no longer restricted to enzymatic activity. Other macromolecular compounds, notably DNA and its associated technologies, are increasingly being employed for detection and quantification of analytes [17-19]. The exponential amplification of DNA through Polymerase Chain Reaction (PCR) takes advantage of the intrinsic properties of DNA to be used as a template for signal generation. Even a single DNA molecule can be amplified leading to detection levels well beyond the sensitivity of conventional ELISAs. Immuno-PCR (iPCR) employs a short length of DNA (referred to as reporter DNA) which replaces the chromophore typically used in ELISAs. When the reporter DNA is amplified by PCR, the resulting number of DNA amplicons is proportional to the amount of antigen detected by the antibodies [18, 19]. The combination of antibody specificity with the amplification power of PCR has opened up a range of innovative analytical techniques for ultrasensitive detection [18, 19]. This literature review will examine the history, development and variations of iPCR assays used for antigen detection in both fundamental and applied research.

1.2 Immuno-PCR

The concept for employing DNA as an amplifiable template in immunodiagnostics was first demonstrated by Sano *et al.* (1992) [20]. In order to facilitate the specific detection of

antigens present at low concentrations, they utilized PCR to amplify a unique DNA template through the use of specific primers, with the DNA products being detected through a variety of methods. The specific primers to the DNA template permitted the amplification of only the desired sequence, thereby reducing false signals originating from foreign nucleic acids present in the sample.

In practice, the antibodies used in iPCR must be coupled to a reporter DNA to be amplified by PCR, similar to the coupling of antibodies with an enzyme as in ELISAs. The reporter DNA can either be coupled to an antibody covalently or through linker molecules, such as streptavidin [21]. The original iPCR assay employed a streptavidin-protein A (STV-A) chimera as the linker between biotinylated DNA and monoclonal antibodies (mAbs) specific to bovine serum albumin (BSA) [20]. In the original protocol by Sano *et al.*, the chimera provided two independent binding sites, one to biotin through the streptavidin moiety, and the other to the fragment crystallizable domain of an immunoglobulin G (IgG) through protein A. Their reporter DNA was a biotinylated fragment amplified from plasmid pUC19. The detection procedure is similar to that of an ELISA, with the exception that the enzyme conjugated secondary antibody has been replaced with the biotinylated DNA-STV A chimera [20]. Following the formation of antigen-antibody-DNA complexes, a 260-bp DNA product was amplified by PCR and analyzed by gel electrophoresis. Using this technique, Sano *et al.* [20] achieved signal generation with a BSA concentration of 9.6×10^{-22} mol, corresponding to 580 molecules of BSA. This represented a 10^5 -fold enhancement in sensitivity over the corresponding ELISA [20].

While this iPCR methodology achieved a significant improvement over ELISA, the STV-A linker presented a number of disadvantages: firstly, this type of linker can only be used in the direct iPCR format. This limitation arises because it is impossible to fully suppress STV-A's

binding to capture antibodies immobilized on the microwell surface that would occur in an indirect format due to the generic binding of protein A to any antibody subtype [18]. Secondly, protein A has widely different affinities for antibodies from different classes and subclasses from various species, thereby limiting its versatility [22]. Despite these limitations however, the STV-A chimera has been used in several iPCR assays [23, 24]. For example, Ren *et al.* (2001) employed a bispecific fusion protein made of the carcinoembryonic antigen (CEA) binding protein and STV for the detection of circulating CEA in serum using iPCR [24].

Other early iPCR-based assays employed avidin as the linker; which also presented technical challenges: the tetrameric nature of avidin can result in the formation of a variety of conjugates, leading to reduced sensitivity and reproducibility from high background noise [17]. Additionally, streptavidin presents numerous advantages over avidin: streptavidin does not possess a carbohydrate moiety and has a much lower isoelectric point (pI 5), compared to avidin (pI 10), ultimately leading to less non-specific binding [17]. Finally, streptavidin can also be conjugated to enzymes such as peroxidase, thereby permitting ELISA and iPCR to be performed simultaneously [17].

While the STV-A chimera used by Sano *et al.* [20] provided significant detection improvements over the corresponding ELISA, there was a need for a more versatile methodology without antibody source or subclass restriction while still retaining the same sensitivity and specificity. By substituting STV-A chimera with biotinylated secondary antibodies and free streptavidin, Zhou *et al.* [25] developed a universal iPCR methodology which retained the original iPCR's sensitivity and specificity yet could be applied universally without restrictions to certain classes of antibodies.

Using full-length recombinant protein ETS1 as the target antigen, ETS1-specific mAbs, biotinylated sheep anti-mouse secondary antibody, free streptavidin and biotinylated DNA, they were able to detect ETS1 at a concentration of 9.6×10^{-21} moles, corresponding to approximately 5.78×10^3 ETS1 molecules [25]. This detection level corresponded to a 10^5 -fold increase in sensitivity over the corresponding ELISA. In addition to the universal nature of the assay, this method also reduced the number of washing steps required, and assay completion time. The universal iPCR format is more flexible since it is compatible with direct, sandwich and indirect assays. Schematic representations of universal iPCR formats are outlined in Figure 1.2.

Because of their flexibility, universal iPCRs have since been widely used in research [18]. Bacterial antigens [26], toxins [27], prion proteins [28], and viral antigens [29] have all been successfully detected using the universal iPCR format [17, 18, 29]. Despite the gains in sensitivity and reproducibility achieved through universal iPCR, certain disadvantages accompany the *in situ* assembly of the complex. For example, to ensure quantitative binding, each coupling step must achieve thermodynamic equilibrium [30]. With most immunoassay incubation times being one hour, the heterogeneous system may not achieve equilibrium, resulting in fewer signal-generating complexes and ultimately weaker amplification. The following section outlines variations of the iPCR assay which address some of these challenges and limitations.

1.3 iPCR formats

Due to its inherent flexibility, employing DNA for signal amplification in immunoassays permitted the development of numerous formats. From a practical standpoint, the iPCR format selected must be compatible with the reagents and equipment available in the laboratory. Each

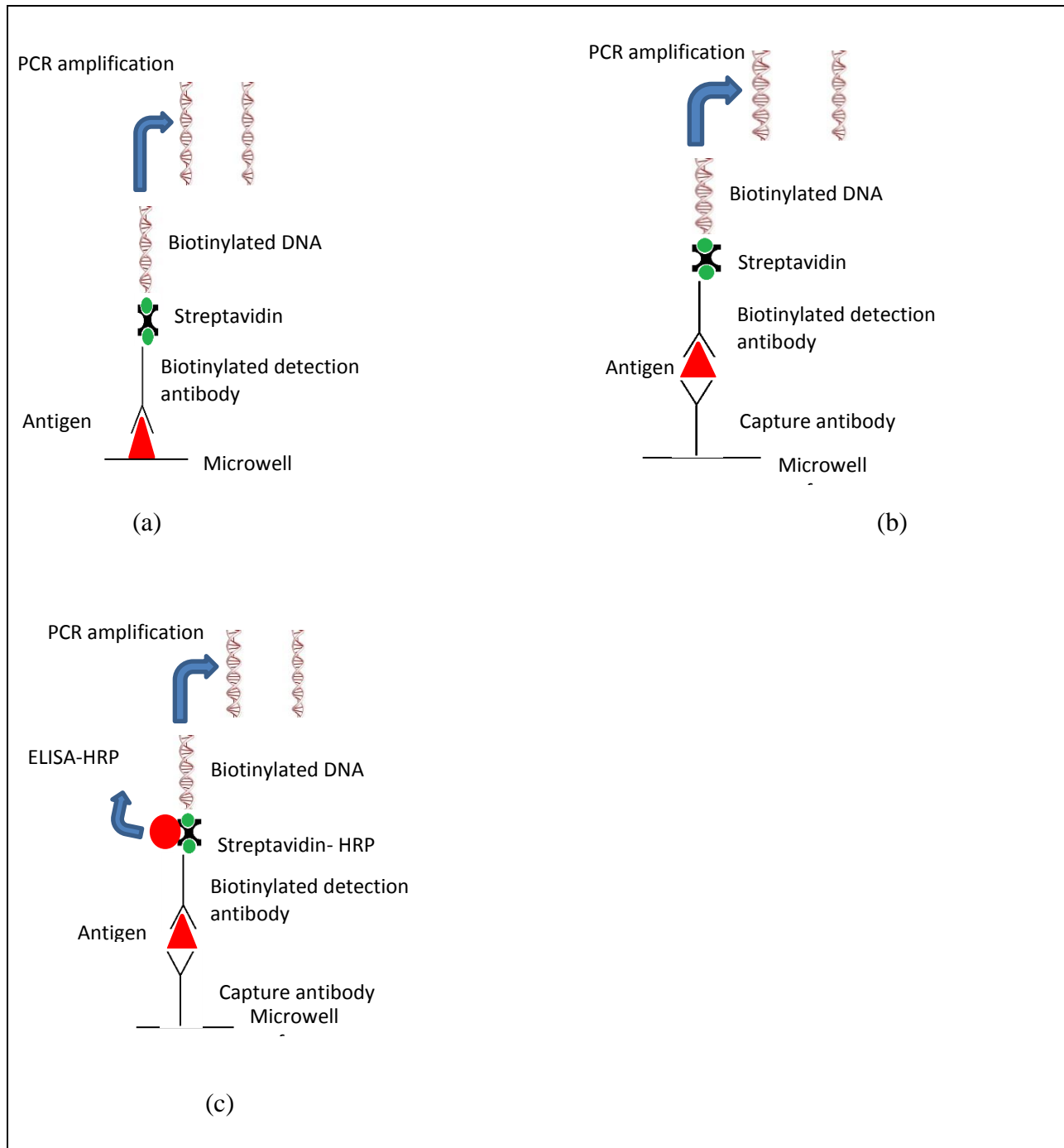


Figure 1.2. Universal iPCR formats with streptavidin linker, biotinylated antibody and reporter DNA. a) Universal iPCR for direct detection, b) Universal iPCR for sandwich format, and c) Universal iPCR combined with ELISA, employing streptavidin HRP conjugate

iPCR method has certain advantages and disadvantages, and to meet the specific demands of the desired application, the appropriate assay design and technique must be chosen accordingly. Two important iPCR steps which can vary from assay to assay have been the coupling of the reporter DNA to the antibodies and the subsequent quantification of the PCR product.

1.3.1 Antibody coupling with DNA

While a number of strategies have been developed for the coupling of antibodies to DNA, two approaches will be examined in this literature review: 1) modular *in situ* coupling and 2) pre-assembled conjugates. The modular *in situ* approach includes any iPCR which employs a multistep (sequential) protocol where streptavidin is used with biotinylated DNA and biotinylated antibodies. While *in situ* approaches use commercially available reagents and permit greater flexibility in tailoring assays, drawbacks include time-consuming washes to remove unbound reagents and lengthy multi-step protocols [18]. The sequential protocol associated with *in situ* approaches can lead to increased error rate, non-specific binding inducing high background noise, and difficulty with predicting the optimal stoichiometric ratio of reagents. Recent efforts have addressed shortcomings in reproducibility through standardized, ready-to-use reagents [19]. The increased availability of commercial reagents, such as biotinylated secondary antibodies, leads to simpler assay protocols, less nonspecific binding, and more routine diagnostics [19].

Pre-assembled DNA-antibody conjugates have been shown to outperform complexes generated using the modular approach [21, 31]. Pre-assembled DNA-antibody conjugates can be synthesized through chemical coupling or supramolecular conjugation.

1.3.2 Chemical coupling

Covalent (chemical) coupling of DNA and antibodies is another strategy available. By using pre-assembled antibody-DNA conjugates, the overall iPCR protocol is simplified and incubation steps removed. One of the major advantages of using DNA-antibody conjugates is the reduction in assay length and complexity leading to reduced non-specific binding between reagents which invariably occur with each additional incubation step [17, 18].

DNA-antibody conjugates synthesized via chemical coupling can be conveniently used in direct, indirect and sandwich formats [17]. Heterobifunctional crosslinking agents contain two reactive groups which act on separate substrates. One reactive group reacts with DNA, while the other targets the amine group present on the antibody, effectively joining the two [32]. Chemical coupling methods require complex chemistry with harsh chemicals and chromatography purification. This is a concern, as the antibodies are potentially unable to endure the severe chemical conditions of chromatographic purification [18], which are necessary to remove unreacted reagents and maintain the assay's reproducibility and sensitivity. Despite these limitations, a variety of iPCRs employ chemical coupling [32-37]. Sperl *et al.* (2005) achieved a LOD of 0.8 pg mL^{-1} of soluble murine T cell receptors, corresponding to a 125-fold increase in sensitivity over the corresponding ELISA [33]. Additionally, Henterich *et al.* (2003) directly compared iPCR with pre-conjugated chemically coupled DNA-antibody complexes with universal iPCR using *in situ* modular assembly of biotinylated-streptavidin complexes. They demonstrated an improvement in performance using the chemically coupled iPCR, and achieved a gliadin detection limit of 0.16 ng mL^{-1} , a 30-fold improvement over the corresponding ELISA [37].

1.3.3 Supramolecular conjugates

The biotin-streptavidin interaction has been exploited to create supramolecular conjugates consisting of large self-assembling networks and nanocircles of DNA fragments and streptavidin for use in iPCR [21, 38, 39]. Nanostructured networks can be created through the use of bis-biotinylated double-stranded DNA and streptavidin as the linker agent. By carefully manipulating reagent concentration and assembly parameters, highly specific antibody-streptavidin networks which contain numerous terminal streptavidin molecules can be generated. These terminal STV molecules provide convenient access to biotinylated antibodies, enabling the formation of a multitude of biotinylated DNA and antibodies within a single conjugate. With the ability to bind polyvalently to the immobilized antigens, the oligomeric conjugates have been shown to outperform traditional monomeric DNA-streptavidin conjugates [38, 39].

1.4 Signal Generation and Detection

Adequate signal analysis is necessary to ensure precise analyte quantification in any diagnostic assay. Since iPCR relies on DNA amplicons for antigen quantification, it is therefore imperative to accurately quantify the DNA products following PCR amplification. Initially, a shortcoming of the iPCR methodology was the increased labour and time required for PCR amplification when compared to ELISA. Following the addition of the enzyme's substrate in ELISA, signal generation is conveniently detected using a spectrophotometer. In iPCR however, the DNA amplicons are invisible and therefore require separate techniques for detection and quantification [18, 19]. Borrowing from established nucleic acid analysis, a wide range of proven quantification strategies for PCR-generated amplicons are now readily available in most biochemical laboratories [40-43]. Gel electrophoresis and DNA-intercalation fluorescent markers

can be useful for detection, but these methods pose some disadvantages. Quantifying band intensity over several orders of magnitude is problematic unless radiolabels or time-consuming dilution steps are employed. Additionally, for large numbers of samples, gel electrophoresis becomes very time-consuming and is expensive to automate. Despite these challenges, electrophoresis has been successfully used for DNA quantification in a number of iPCR applications [20, 25, 44-46].

Other means of DNA quantification have successfully been employed in DNA-based immunodiagnosics. Labeled probes can be hybridized with DNA amplicons, permitting sequence-specific labeling detection molecules [43]. Real-time or quantitative PCR (qPCR) [43] offers fast and direct detection of PCR products in real-time and has become the method of choice for a number of iPCR-based applications [19, 32, 35, 47-51]. An intercalation fluorescent probe with specificity for double-stranded DNA such as SYBR green or a sequence-specific fluorophore probe such as TaqMan is added to the amplification reaction and emits a fluorescent signal following the replication of amplicons [43]. The elimination of post-PCR quantification reduces the possibility of contamination or signal degradation through excessive handling, making qPCR an excellent choice with iPCR [43]. Real-time iPCR (RT-iPCR) assays have demonstrated significant improvements in sensitivity over conventional assays in the detection of viral and bacterial antigens [26, 48, 52], tumor markers [53] and environmental contaminants [54, 55]. Additionally, the high sensitivity of qPCR requires only a small sample volume, which can be highly advantageous when only small amounts of sample are available [18]. qPCR sensitivity also allows wider compatibility with complex biological matrices which can be diluted in buffer prior to analysis. The dilution of complex biological or environmental matrices can significantly reduce matrix background noise, allowing for increased sensitivity [56].

An important technical consideration when employing qPCR is microplate compatibility with a real-time thermocycler [18]. Microplate materials must have a reasonable protein binding capability, which is not necessarily a desired property of PCR microplates in most applications. While it is possible to transfer the signal generating immunoreactants to the wells of a thermocycler compatible plate, these additional handling steps increase the possibility of contamination thereby decreasing sensitivity [19, 57]. Microplates in the 96-well format, specifically designed for iPCR with high protein binding capabilities are available, and are recommended for standardized iPCR applications [19]. Real-time PCR also allows for multiplex iPCR assays where several antigens can be detected simultaneously. Unique reporter DNA can be used for detection of multiple antibody-DNA conjugates within a single experiment. Hendrickson *et al.* (1995) demonstrated the feasibility of this concept with the simultaneous detection of β -galactosidase, human thyroid-stimulating hormone and human chorionic gonadotropin [34]. Collectively, these advantages make qPCR the technique of choice for DNA quantification for iPCR.

While great potential exists for multiplex iPCR, a number of challenges are inherent to conventional multiplex-based assays which necessarily translate to multiplex iPCRs. Moreover, the widely used modular *in situ* approach is not multiplex-compatible, because it is currently impossible to distinctly label certain antibodies with reporter DNA *in situ* [57].

1.5 Reporter DNA Design

Compared to other applications employing qPCR, RT-iPCR is generally less susceptible to error. By employing several washing steps prior to the addition of the reporter DNA, all contaminants and unbound immunoreactants are removed [18]. Consequently, there should only

be one type of nucleic acid present in the sample to be amplified, therefore eliminating the possibility of mispriming or competition with other partially homologous sequences. While the sequence of the reporter DNA can be freely chosen, several published reports have described standard sequences originating from plasmids such as pUC19 [20, 21, 31, 58]. Contamination of the workplace with reporter DNA can result in false positives, therefore it is recommended to select a DNA sequence not commonly used in routine laboratory work. Additionally, if the iPCR is intended for environmental applications, it is imperative that the chosen DNA sequence will not be found in the environmental matrix. Few publications have specifically addressed the effect of the length of the reporter DNA on iPCR performance but reporter DNA ranging from less than 100 base pairs [45] up to entire plasmids [59] have been used. However, most DNA markers have been in the range of 100-300 base pairs as they are most efficiently amplified by PCR [18].

1.6 Other iPCR Strategies

The past decade has seen increasing use of nanoparticles in the field of molecular diagnostics. Because of their small size (1-100 nm) and large surface-to-volume ratio, certain classes of nanoparticles have found widespread use in a variety of diagnostic applications [60]. By controlling for size, shape and composition, it is possible to chemically tailor the physical properties of nanoparticles to provide novel binding properties and structural robustness. The selection of nanoparticle properties, now possible through recent advances in synthesis and fabrication, will further expand their diagnostic potential [61]. iPCR has recently benefited from advances in nanotechnology [17, 60].

High background signal resulting from non-specific binding of the assays reagents, problematic in iPCR methodology [17, 18] can be avoided by switching from the classical

microplate format (solid phase) to a liquid format through the use of nanoparticles or proximity ligation assays [62]. Nam *et al.* (2002) first introduced a bio-barcode technique based on oligonucleotide-modified nanoparticles for the detection of multiple protein structures in a single solution [63]. Coupling nanoparticle technology with iPCR has increased assay sensitivity and specificity while simplifying the protocols. In addition to lower detection limits, a reduction in the number of incubation and washing steps through the use of magnetic beads for the detection of the HIV-1 p34 antigen was observed [64]. Other non-specific interactions are further reduced because microwells with high binding capacity are no longer required since the large surface-to-volume ratio conferred by nanoparticles allows faster and more complete interactions between the antibodies anchored to the nanoparticles and the antigens in solution [17, 57].

Magnetic-based immunoassays have been developed using magnetosome nanoparticles [65]. In order to ensure uniform size, morphology and biocompatibility, Wacker *et al.* developed a biogenic bacterial magnetosome derived from *Magnetospirillum gryphiswaldense*, a magnetotactic bacterium [65]. These magnetosomes were chemically modified with antibodies specific to hepatitis B surface antigen (HBsAg) and combined in solution with HBsAg antibody-DNA conjugates and a serum sample containing HBsAg. The signal generating complex is formed with the HBsAg antigen acting as the linker between the magnetosome-antibody conjugate and the DNA-antibody conjugate. An external magnetic field is then used to concentrate the signal complex, which can then be re-suspended in buffer following washing to remove unbound reagents. Following addition of PCR master mix, the assay read-out is the same as conventional RT-iPCR. Using the aforementioned format, Wacker *et al.* achieved a LOD of 320 pg mL^{-1} , which was a 25-fold increase in sensitivity over RT-iPCR using commercial magnetic beads [65].

Gold nanoparticles have also successfully been employed in iPCR assays [66-68]. Perez *et al.* describes the detection of viral antigen in cell lysate employing both magnetic beads and gold nanoparticles in a nanoparticle-amplified iPCR (NA-iPCR) [68]. In this strategy, antibody-DNA functionalized gold nanoparticles (AuNPs) were used in addition to antibody functionalized magnetic microparticles (MMPs) for the detection of respiratory syncytial virus (RSV) [68]. Similar to the methodology developed for the magnetosome complex, a sandwich complex is generated linking the MMPs with the AuNPs via the RSV antigen. A magnetic field is then used to isolate the signal generating complexes for extraction. Heating the DNA releases it from the gold nanoparticles which can then be quantified using qPCR. Using the NA-iPCR, a LOD of 4.1 plaque-forming units mL⁻¹, which corresponded to a 4000 fold increase in sensitivity over the ELISA using the same antibodies, and a 4-fold improvement over qPCR using extracted viral RNA [68].

The versatility and applicability of nanoparticles in novel immunoassays suggest further growth and development as the technology continues to expand. With more and more versatile applications of nanotechnology in molecular diagnostics, it seems immunoassays will continue to be modified through the use of nanoparticles.

1.7 RT-iPCR Applications

The development of an efficient and reliable iPCR assay which can be standardized and adapted for a range of routine diagnostics is still a significant undertaking with numerous challenges. Despite these challenges, iPCR has been successfully applied for the detection of a wide range of analytes. Bacterial [26, 46] and viral [29, 58, 65, 68, 69] antigens, antibodies [34],

tumor markers [24, 59], and a variety of environmental [50, 51, 54, 55] and food contaminants [27] have been successfully detected by iPCR.

An overview of iPCR applications for a variety of antigen classes has been previously published in review articles by Niemeyer *et al.* (2005) and Adler *et al.* (2008). The focus of these reviews was primarily pertaining to medical applications for pathogens, tumour markers and biological compounds. Only recently has the use of iPCR for detection of haptens, or small molecules less than 1000 Daltons, such as polycyclic aromatic hydrocarbons (PAHs) [50] and polychlorinated biphenyls (PCBs) [54] been investigated. These haptens have significant impact on ecosystem health due to their potential carcinogenic and mutagenic properties. As a result of industrial processes and human activities, there is an increased interest in the detection and quantification of these environmental contaminants [70-74]. Typically, these compounds are detected using traditional immunoassays or chromatography coupled to mass spectrometry. A summary of iPCR applications specific to hapten quantification is presented in Table 1.1.

Table 1.1 Application of iPCR* for the detection of haptens. All of these assays used biotin-
avidin linker system

Hapten	LOD (fg mL⁻¹)	Refs
Phenanthrene	5	[50]
Naphthalene	1	[75]
Fluoranthene	5	[51]
Polychlorinated biphenyls	5	[54, 55]

Due to their acute and chronic health effects in humans [76], as well as having persistent, bioaccumulative properties [77], the need for a fast, simple and sensitive detection method for PCBs made them a prime candidate for iPCR diagnostics. While banned by many industrialized countries since the late 1970's, PCBs continue to persist as environmental contaminants following years of use as additives in a variety of oil-based products ranging from plastics to pesticides [54, 55, 78]. Historically, gas chromatography coupled to mass spectrometry (GC-MS/MS) was the standard detection method of these dioxin-like compounds. However, this method requires a well-equipped analytical chemistry laboratory, and required large sample volumes [54].

ELISAs have also been employed in PCB detection although their sensitivity is insufficient for detection of the minute quantities found in environmental samples. An iPCR assay was developed for PCB77 using a biotin-avidin linker bridging a biotinylated antibody to a biotinylated reporter DNA [55]. Using this RT-iPCR assay, a detection limit of 1.5 fg mL^{-1} was achieved, making it the most sensitive PCB detection method described to date [55]. When compared to GC-MS/MS data, the iPCR standard curve had high correlation ($r=0.99$) demonstrating its usefulness in providing rapid quantitative test results [55].

Phenanthrene (PH), a polycyclic aromatic hydrocarbon with three fused benzene rings, is an environmental contaminant with mutagenic and carcinogenic properties [50]. Presently, the maximum concentration of PH in drinking water is 50 pg mL^{-1} in China, and is monitored by HPLC-MS. Using iPCR, Zhou *et al.* achieved a detection limit of 5 fg mL^{-1} . This represented an order of magnitude increase in sensitivity over that of the ELISA assay [50].

1.8 Challenges

While iPCR diagnostic technology has proven successful in ultrasensitive detection of a variety of antigens, numerous challenges remain [17, 18]. Increased complexity and cost when compared to conventional ELISA and decreased reproducibility when compared to GC or LC-MS/MS remain important considerations when choosing an appropriate diagnostic strategy. The increased complexity and cost of iPCR when compared to ELISA makes iPCR less attractive, especially if there is no particular need for ultrasensitive detection [18].

One of the most common drawbacks associated with iPCR is its lack of reproducibility owing to excess background signal in negative control samples [19]. Nonspecific background fluorescence present in iPCR samples require that the negative threshold be raised resulting in a decrease in sensitivity [19]. High background signals can be mitigated using appropriate blocking solutions, standardized reagents and thorough washing protocols [19]. The need for exotic reagents requiring complex chemistry to synthesize, such as antibody-DNA conjugates and nanostructures, can be a major obstacle for developing iPCR as a routine diagnostic strategy. However, the use of commercial reagents can minimize these issues [18, 19, 57].

Common to all immunoassays is cross-reactivity of antibodies with structurally related compounds other than the analyte of interest that can lead to false positives. Zhou *et al.* reported cross-reactivities of PH-specific antibodies to anthracene and fluranthene at 12.5% and 11.2% respectively [50]. Chen *et al.* reported cross reactivities as high as 37.5% for PCB congeners [54].

Real-time detection of PCR amplicons has greatly reduced the number of PCR-related problems by reducing the number of handling steps, thereby simplifying and shortening the iPCR

protocol. However, iPCR with real time detection requires compatible thermocyclers, which may not be readily available. At present, iPCR is limited to well-equipped laboratories with experience in both ELISA and PCR-related technologies.

1.9 RT-iPCR Applications in Agriculture

This thesis presents the development and assessment of a universal RT-iPCR assay for the quantification of manure-borne 17β -estradiol in water, followed by antibody swapping to quantify *P. brassicae* resting spores using the same assay format, to demonstrate the potential of RT-iPCR in quantifying analytes of interest in agriculture.

1.9.1 Manure-borne estrogens

The environmental presence of natural estrogens and endocrine disrupting compounds (EDCs) has received increasing attention since the 1990's, with implications for environmental research and policy [79]. Estrogens, along with other EDCs, have been shown to disrupt the endocrine system by interfering with hormonal synthesis and degradation, thereby altering normal physiological and behavioural activity in a wide range of vertebrate species [79, 80]. Recent reviews have highlighted the ecotoxicological risk of estrogens [81, 82]. Purdom *et al.* (1994) reported that estrogen concentrations in waste water treatment plant effluents as low as 10-100 ng L⁻¹ were capable of inducing increased blood vitellogenin in male rainbow trout [83]. Despite the ecological threat posed by manure-borne estrogens, their prevalence and distribution have not been thoroughly evaluated in agricultural watersheds in southern Alberta [84]. Due to the high estrogenic potency of manure-borne estrogens and their ability to disrupt the endocrine system, it is imperative to identify potential sources and to quantify manure-borne estrogens in southern Alberta waterways.

Municipal, industrial and agricultural wastes are the primary sources of estrogens in surface and groundwater [85]. Estrogens are synthesized and excreted by all vertebrates, including humans, while concentrations and amounts excreted vary between species [86]. Agriculture is often cited as a major contributor of environmental estrogens [87] with livestock effluents and runoff from manure-enriched land acting as significant sources of estrogens in waterways [85]. Chen *et al.* estimated that 90% of environmental estrogens originate from animal waste [88]. Concentrated animal feedlot operations are of particular concern, due to the large quantities of urine and manure generated [88]. Southern Alberta has the highest density of cattle feedlots in Canada, with 700 000 heads of cattle in the County of Lethbridge alone in 2003 [89]. Previous analysis of water samples in the Oldman River has shown concentrations of 5.26 ng L⁻¹ and 1.01 ng L⁻¹ of estrone and 17 β -estradiol, respectively [84]. Consequently, surface and subsurface water contamination by manure-borne estrogens could pose a potential local problem.

A sensitive, rapid and high-throughput method for the detection and quantification of estrogens in water is therefore essential [90]. Conventional chemistry techniques, such as gas chromatography (GC) and high pressure liquid chromatography coupled to mass spectrometry (HPLC-MS) have been used to quantify natural estrogens and EDCs in environmental samples [91-93]. Unfortunately, although HPLC-MS has shown adequate sensitivity (\approx pg L⁻¹), it is time-consuming and requires expensive laboratory instrumentation in addition to skilled personnel. Considering the importance of environmental EDC monitoring, the development of a simple, practical, fast, highly sensitive, highly specific and low-cost quantification method is of utmost importance.

Because of the need for a high-throughput, ultrasensitive method for the quantification of manure-borne estrogens, an iPCR assay based on the universal format was developed for the

quantification of 17β -estradiol (E2) in water. Using iPCR, we achieved a limit of detection of 0.7 pg mL⁻¹ in water, which is comparable to the most sensitive LC-MS/MS methods published so far for E2 quantification [94]. E2 was chosen as the analyte of interest among the main manure-borne estrogens excreted by cattle since it is the natural estrogen with the highest estrogenic potential [95]. An advantage of using the universal iPCR format is the ability to swap out the detection antibodies and use the same methodology for quantification of a completely different antigen. We chose the resting spores of *Plasmodiophora brassicae* as our second target antigen for use in the antibody swapping experiment.

1.9.2 Antibody swapping: *Plasmodiophora brassicae*

P. brassicae is the causal pathogen of clubroot, a soil-borne disease that infects members of the *Cruciferae* family. Clubroot is one of the most damaging soil-borne diseases of crucifers worldwide, and has recently become established in Western Canada [96]. Infected plants suffer from tumorous galls on their roots, producing the recognizable disease symptoms that consist mainly of club-shaped roots, plant yellowing, wilting and early senescence. The galls impede water and nutrient transport, ultimately stunting growth [97, 98]. The pathogen produces resting spores that remain viable in the soil for as long as 20 years [99, 100]. As of 2012, Alberta had 1064 fields with confirmed cases of *P. brassicae* infestation, which is most likely a significant underestimate, since comprehensive surveys remain to be conducted [97]. Clubroot disease can affect up to 94% of plants infected in highly infected fields which can translate to 30-50% yield losses [96, 101]. The economic impacts of clubroot in Alberta are therefore significant and of concern to the canola industry [96, 97].

A variety of chemical control measures [102-104] have been evaluated in clubroot control, demonstrating limited success. The most practical method for clubroot control is to avoid planting susceptible crops in *P. brassicae* infested soils [105]. This can be difficult due to the long-lived nature of the resting spores.

Accurate, sensitive detection of *P. brassicae* resting spores is a critical component in minimizing the spread and infection of clubroot disease. Current detection of *P. brassicae* resting spores rely on either ELISA assays [106] or PCR-based amplification of *P. brassicae* DNA [105, 107, 108]. These techniques suffer either from lack of sensitivity or complicated extraction protocols associated with isolating *P. brassicae* DNA prior to amplification. The resting spores present an ideal target for the antibody swapping. The spores represent a completely different class of antigens when compared to 17 β -estradiol and therefore are well suited to evaluate the universal applicability of our iPCR assay.

For these reasons, *P. brassicae* is a good target for which a sensitive assay could benefit Alberta's agriculture. A sensitive *P. brassicae* RT-iPCR assay was therefore developed as part of an antibody-swapping experiment from our universal 17 β -estradiol iPCR assay.

1.10 Conclusions

Since its introduction, iPCR has evolved from a proof-of-concept to an established diagnostic method. For most applications, iPCR assays are proving to be as sensitive as conventional diagnostic methods, with the advantages of high-throughput and built-in flexibility. Although the iPCR method has gained sensitivity, robustness and standardization in recent years, challenges remain for the application of iPCR to routine clinical or environmental diagnostics. By incorporating newer technologies such as nanoparticles, iPCR assays will undoubtedly gain

further sensitivity and use as routine diagnostic method. Several iPCR assays have been successfully developed for environmental contaminants (PCBs, PAHs); however none have been used in the context of agricultural research.

The overall objective of this thesis is to investigate the feasibility of using RT-iPCR assays for agricultural applications. The specific objectives are two-fold: firstly, to develop a universal format RT-iPCR for the sensitive quantification of manure-borne estrogens in water, and secondly, to demonstrate the flexibility of the assay by swapping the antibodies and applying this modified assay for the quantification of a completely different antigen, the clubroot resting spores.

2. DEVELOPMENT OF A REAL-TIME IMMUNO-PCR ASSAY FOR THE QUANTIFICATION OF 17β -ESTRADIOL IN WATER

2.1 Abstract

A competitive real-time immuno-PCR (RT-iPCR) assay was developed for the sensitive quantification of 17β -estradiol in water. Using a universal iPCR method and polyclonal antibodies, 17β -estradiol was accurately quantified at concentrations ranging from 1 pg mL^{-1} to $10 \text{ }\mu\text{g mL}^{-1}$. The RT-iPCR assay's limit of detection was 0.7 pg mL^{-1} . The RT-iPCR assay provided an 800-fold increase in sensitivity as well as an expanded working range compared to the corresponding enzyme-linked immunosorbent assay. Assay cross-reactivity to estrone and estriol, two structurally related estrogens, was below 8%. Water samples spiked with 17β -estradiol were analyzed by RT-iPCR to determine the assay's potential as a rapid screen for the monitoring of manure-borne estrogens in water. The assay showed recoveries of 82, 102 and 103% for Milli-Q, tap and irrigation water, respectively, without requiring sample extraction or concentration prior to analysis.

2.2 Introduction

17β -estradiol (E2), estrone (E1) and estriol (E3) are natural steroidal hormones produced by all vertebrates and excreted in their urine and faeces. Since these estrogens are capable of disrupting endocrine regulation in a wide range of aquatic species [81, 82], concerns have been raised regarding their presence in the environment. Manure-borne estrogens originating from livestock production can reach surface waters and groundwater if leaching or runoff occurs following manure application to agricultural land. Estrogens can be present at high levels in manure: for example, concentrations of E2 as high as 300 ng mL^{-1} have been reported in swine

lagoons from farrowing sow feedlot operations [109]. Several studies have highlighted the risks posed to the aquatic environment from manure-borne estrogens originating from surface runoff [90, 110]. In addition, estrogens have been measured in effluents from industrial and municipal treatment plants, with concentrations ranging from several nanograms to several hundred nanograms per liter [111, 112]. Behavioural and physiological changes in aquatic species can arise from exposure to even low concentrations of estrogenic compounds; for example, a 17β -estradiol concentration of 0.5 pg mL^{-1} affected sperm motility patterns in male rainbow trout (*Oncorhynchus mykiss*) [16], while concentrations of 25 pg mL^{-1} induced vitellogenesis in minnows (*Gobiocypris rarus*) [15].

Owing to the widespread presence of estrogens in the environment and their potential impacts on aquatic species, there has been an increased interest regarding estrogen quantification [92, 113-115]. Estrogens have been measured using a variety of analytical methods including gas chromatography coupled with mass spectrometry (GC-MS/MS) [91, 116] and liquid chromatography coupled with mass spectrometry (LC-MS/MS) [92, 94, 117]. Using GC-MS/MS, 17β -estradiol was detected in sludge from domestic sewage treatment plants at concentrations as low as 49 ng g^{-1} [91], and in sewage samples at 1 pg mL^{-1} [116]. To date, LC-MS/MS is the standard and most sensitive analytical method used to quantify estrogens with limits of detection (LODs) for steroidal estrogens and their conjugated forms ranging from 0.1 to 3 pg mL^{-1} [94, 117-119]. Benijts et al. (2003) [94] reported a LOD of 1 pg mL^{-1} for 17β -estradiol in water, with a linear working range between 4 and 500 pg mL^{-1} . Ferguson et al. (2001) achieved a method detection limit (MDL) using LC-MS/MS of 0.18 pg mL^{-1} of 17β -estradiol in waste water effluent [119]. While lower limits of detection can be achieved using LC-MS/MS compared to GC-MS/MS, both methods require time-consuming pre-concentration of samples, in

addition to expensive instrumentation and skilled personnel, making routine detection difficult in the absence of a dedicated analytical laboratory.

Radioimmunoassays [120] and enzyme-linked immunosorbent assays (ELISAs), both relatively simple and low cost [121], have also been developed and used for the quantification of estrogens. A number of recent publications have employed ELISAs for the detection and quantification of estrogens in water [122-126]. ELISA kits for estrogen quantification are also commercially available [126]. The limits of detection of these assays vary, with reported LODs ranging from 0.06 to 50 ng mL⁻¹ [122-124, 126]. Farré et al. (2006) reported a 17 β -estradiol ELISA LOD of 0.06–0.30 ng mL⁻¹ with a linear range between 0.05 and 1 ng mL⁻¹ using a commercial E2 kit [126]. ELISAs are useful for environmental monitoring due to their high-throughput nature, but tend to have lower sensitivity compared to LC-MS/MS. Analytical sensitivity is especially important in the case of estrogens and estrogenic compounds, which can have environmental impacts at concentrations as low as pg mL⁻¹ (part per trillion range) [90].

The sensitivity of current ELISAs can be further improved by exploiting the amplification power of the polymerase chain reaction (PCR). By combining the specificity of antibodies with the amplification power of PCR, immuno-PCR (iPCR) permits ultrasensitive detection of a variety of antigens with a broad linear dynamic range spanning several orders of magnitude [18]. While analyte quantification using ELISA relies on a colorimetric reaction resulting in absorbance changes, iPCR depends on DNA amplification for signal generation. Originally developed by Sano et al. [20], iPCR can improve the LOD up to 10,000-fold compared to conventional ELISAs [18]. Additionally, iPCR can be readily adapted to detect any antigen of interest, as long as an antibody specific to that antigen is available [18, 21]. iPCR is also amenable to multiplex detection, where several antigens can be quantified simultaneously in

a single assay [34]. To date, a variety of bacterial, viral, prion, toxin and cancer-related antigens have been quantified using iPCR [17, 18, 27, 29, 48, 49]. However, there is a scarcity of papers reporting the quantification of haptens by this method. iPCR has been used for the detection of industrial contaminants such as polychlorinated biphenyls (PCBs), and the polycyclic aromatic hydrocarbons phenanthrene and fluoranthene [50, 51, 54, 55]. LOD's of 5 fg mL⁻¹ were reported for fluoranthene and phenanthrene, while a linear working range between 10 fg mL⁻¹ and 100 pg mL⁻¹ in water, was achieved [50, 51]. For PCB37 in soil, the iPCR assay's LOD was 5 fg mL⁻¹ with a linear working range of 10 fg mL⁻¹ to 1 ng mL⁻¹ [54].

To our knowledge, the present study is the first report of manure-borne estrogen quantification by iPCR. A real-time iPCR (RT-iPCR) assay, based on a universal iPCR model previously published [25], was developed using 17 β -estradiol-ovalbumin (17 β -estradiol:OVA) as the coating antigen [124] and streptavidin as the linker molecule between biotinylated secondary antibodies and biotinylated reporter DNA (Figure 2.1). The RT-iPCR assay was compared to its corresponding ELISA and assessed for its potential in quantifying manure-borne estrogens in water.

2.3 Materials and Methods

2.3.1 Reagents and chemicals

All chemicals including ovalbumin (OVA; M_r=45,000), 17 β -estradiol, estrone, estriol, streptavidin, Tween 20, glutaraldehyde, ethylenediaminetetraacetic acid (EDTA), RNase-free water, primers and probe were from Sigma-Aldrich (St. Louis, MO). Hot Start Fluorescent Quantitect probe PCR (Taqman) and QIAquick Gel Extraction kit were from Qiagen (Mississauga, ON). Expand High Fidelity PCR System kits were from Roche (Manheim,

Germany). Biotinylated goat anti-rabbit IgG (H+L) were from Thermo Scientific (Rockford, IL). Tris (tris(hydroxymethyl)aminomethane) was from Biorad (Hercules, CA). JM109 competent cells were from Sigma-Aldrich (St. Louis, MO). The pGEM-T Easy Vector kit was from Promega (Madison, WI). Horseradish Peroxidase (HRPO) conjugated goat anti-rabbit antibody was from Cedarlane Laboratories (Hornby, ON) and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Phosphate buffered saline (PBS) was prepared in Milli-Q water and contained 0.755 g L⁻¹ of K₂HPO₄•3H₂O, 0.25 g L⁻¹ of KH₂PO₄ and 8.2 g L⁻¹ of NaCl.

Rabbit polyclonal anti-E2 antibodies (pAbs) were obtained as reported in Caron *et al.* 2010 [124]. The 17 β -estradiol:OVA coating conjugate was synthesized using the Mannich reaction as previously described in [124].

2.3.2 ELISA development

A checkerboard ELISA was used to determine optimal conjugate and serum dilutions to obtain an absorbance value of 1. Using the optimized serum and conjugate dilutions as determined by the checkerboard ELISA, a standard curve was obtained with E2 concentrations ranging from 0.01 to 10 000 ng ml⁻¹ in PBS.

Competitive inhibition-ELISA (CI-ELISA): Microtitre plate (Nunc, Roskilde, Denmark) wells were coated with 100 μ l/well of 17 β -estradiol:OVA conjugate (diluted 1:6400 in PBS) and incubated for 17 hours at 4^oC. Wells were washed three times with PBS-Tween, and blocked with 200 μ l/well of 3% blotting grade milk (Biorad) in PBS for 1 hour at 20^oC. During that incubation time, standards and samples were mixed with an equal volume of serum diluted 1:800 in PBS and the mixture incubated for 1 hour at 20^oC. Following three washes with PBS-Tween,

100 μ l of the sample/serum mixture was added to each well and incubated for 1 hour at 20⁰C. Wells were washed three times with PBS-Tween and 100 μ l/well of HRPO goat anti-rabbit antibody (diluted 1:5000 in PBS) added and incubated for 1 hour at 20⁰C before being washed three times with PBS-Tween. 100 μ l of ABTS solution was added to each well and incubated for 15 minutes at 20⁰C prior to absorbance reading at 415 nm on a Spectramax 340PC plate reader (Molecular Devices, Sunnyvale CA). Assay completion time was 3.5 hours, in addition to the 17 hours incubation of the estradiol:OVA conjugate, for a total time of 20.5 hours.

2.3.3 CI-ELISA characterization and cross-reactivity

E1 and E3 (Table 1) are steroidal hormones structurally similar to, and commonly detected along with E2 [111] in the faeces and urine of livestock [79, 127]. E2 has been shown to have the highest estrogenic potency among natural estrogens, followed by E1 and E3 [95]. The assay's cross-reactivity with E1 and E3 was therefore assessed by comparing the inhibition concentration 50 % (IC₅₀, the concentration of hapten required to inhibit the maximum absorbance by 50%) of 17 β -estradiol with the IC₅₀ of E1 and E3. The ELISA's limit of detection was calculated as the mean of 8 replicates of the 0 pg 17 β -estradiol mL⁻¹ standard minus twice the standard deviation, as reported in [124]. Serum cross-reactivity was calculated using the 17 β -estradiol IC₅₀ of our serum divided by the IC₅₀ of E1 and E3, as reported in [128].

2.3.4 Biotinylated reporter DNA

A short (211 base-pair) double stranded sequence was randomly generated to serve as reporter DNA. The sequence was examined using the NCBI database to ensure the sequence was novel and showed no homology to previously known sequences prior to being synthesized. The reporter DNA was ligated into expression vector pGEM following manufacturer's instructions

(Promega, Madison, WI). JM109 high efficiency competent cells were transformed with the ligation products according to manufacturer's protocol (Promega, Madison, WI), and grown on Lysogeny Broth (LB) medium containing 100 mg/ml of ampicillin. A successfully transformed colony was selected and following plasmid DNA extraction, the sequence of interest was biotinylated using 5' biotinylated primers. PCR was performed as follows using a Mastercycler EP Realplex (Eppendorf, Hamburg, Germany): initial holding at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, elongation at 72°C for 30 s, and then a final elongation at 72°C for 7 min. The reaction mixture (50 µL) contained PCR buffer (10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, pH 8.3), 200 µM of each dNTP, 0.3 µM of each primer, 3.75 U Taq DNA polymerase, 40 ng of template DNA and 40.25 µL of RNase-free water. Following PCR amplification, biotinylated DNA amplicons (141 bp) were separated on a 1.5% agarose gel and purified (Qiagen DNA extraction kit, Mississauga, ON). The biotinylated reporter DNA stock was suspended in PBS to reach a concentration of 250 µg ml⁻¹.

2.3.5 RT-iPCR

A schematic representation of the RT-iPCR assay is outlined in Figure 2.1. In order to improve adsorption of the coating conjugate 17β-estradiol:OVA to the polypropylene PCR wells (Axygen, Union City, CA), the latter were treated with a 0.8% glutaraldehyde solution in Milli-Q water for 2 hours at 60°C. Following incubation, plates were washed four times with 10 second agitation using 175 µL washing buffer (20 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.05% Tween, pH 7.3) using an Aquamax 2000 from Molecular Devices (Sunnyvale, CA). All subsequent washes were performed with 175 µL wash buffer and 10 s agitation, except final wash as described below. Plates were briefly centrifuged for 5 seconds at 45xg to remove any remaining wash buffer. A 30 µL of 17β-estradiol:OVA diluted 1:6400 in PBS was dispensed into

each well. The plate was covered and incubated for 17 hours at 4⁰C. The wells were washed four times and blocked with 150 μ L of 3% OVA in PBS for 1 hour at 37⁰C. A 1 mg mL⁻¹ solution of 17 β -estradiol was prepared in acetone, and a serial dilution was prepared in Milli-Q water to obtain a set of standards with concentrations ranging from 1 pg mL⁻¹ to 10 μ g mL⁻¹. The 17 β -estradiol standards were then each mixed with an equal volume of serum diluted 1:40 000 in PBS. The mixtures were incubated at 20⁰C for 1 hour. The wells were washed four times with washing buffer and 30 μ L of each mixture was added to the appropriate wells and incubated for 1 hour at 20⁰C. Following four washes with wash buffer, 30 μ L of biotinylated secondary antibody (goat-anti rabbit IgG) diluted 1:5000 in PBS was added to each well and incubated for 1 hour at 20⁰C. Streptavidin (200 ng ml⁻¹ PBS) was mixed with an equal volume of biotinylated reporter DNA diluted 1:250 000 in PBS, and the mixture incubated for 1 hour at 20⁰C. Following four washes to remove unbound reagents, 30 μ L of the streptavidin:DNA mixture was added to each well and incubated for 1 hour at 20⁰C. For the final wash, wells were washed 8 times with 175 μ L of wash buffer with 60 s agitation, and rinsed 8 times with 175 μ L of Milli-Q water with 10 s agitation in order to remove all unbound reagents. The PCR plate was briefly centrifuged to remove any residual wash buffer and Milli-Q water, and PCR master mix (30 μ L) added. RT-iPCR was performed in a Mastercycler EP Realplex (Eppendorf, Hamburg, Germany). The PCR master mix consisted of the following: 15 μ L of 2x Quantitect Probe PCR Master Mix (Quantitect Probe PCR kit, Qiagen, Mississauga, ON), 0.3 μ M of each primer, 0.2 μ M of Taqman probe and RNase-free water. The PCR cycling parameters were an initial 15 min at 95⁰C followed by 35 cycles of 15 s at 94⁰C, and 60 s at 60⁰C.

DNA amplicons size was 70 bp. Assay completion time was 5.5 hours, in addition to 17 hour incubation of the 17 β -estradiol:OVA conjugate and two hours for the glutaraldehyde incubation, for a total time of 24.5 hours.

2.3.6 RT-iPCR characterization

A standard curve was produced by plotting the average Ct values, (n=3 times four experiments for total of n=12) against known 17 β -estradiol concentrations, and used for E2 quantification in samples.

Using a dilution series of 17 β -estradiol, a standard curve was developed between the ranges of 1 pg mL⁻¹ to 10 μ g mL⁻¹ (Fig 2). Fluorescence threshold was automatically set by the Mastercycler EP realplex instrument. Fluorescence was detected by the real-time rotary analyzer producing amplification curves for the 17 β -estradiol dilution series. In addition to the 17 β -estradiol standards, a negative control (NC) containing all assay reagents except 17 β -estradiol was included, as well as a background control (BC) containing the RT-PCR master mix only without DNA template. The Ct values are proportional to 17 β -estradiol concentrations; the sample containing the highest 17 β -estradiol concentration has the highest Ct value. The limit of detection was defined as the value of the NC plus three times the average standard deviation, as reported in [19].

2.3.7 Spiked water samples

Double-distilled, tap and irrigation water samples were collected and spiked with 17 β -estradiol at concentrations of 0.5, 250 and 500 ng mL⁻¹ to assess the performance of RT-iPCR in environmental samples. Water (1L) was collected in acetone-rinsed Nalgene bottles and stored at 4^oC until used for quantification by RT-iPCR. Tap water from the City of Lethbridge and Milli-

Q water were collected directly in the laboratory. The irrigation water was collected from an irrigation canal located in the county of Lethbridge, Alberta, Canada. The water samples were all tested for the presence of 17β -estradiol using RT-iPCR prior to being spiked. Spiked samples were prepared from a 1 mg mL^{-1} solution of 17β -estradiol in acetone, and diluted in the appropriate water matrix. Standards ranging from 1 pg mL^{-1} to $10 \text{ }\mu\text{g mL}^{-1}$ were prepared as previously described in each water matrix, and used to produce standard plots from which 17β -estradiol concentrations and recovery rates were calculated.

2.4 Results and Discussion

2.4.1 ELISA characterization and cross-reactivity

The parameters of the ELISA were calculated as previously reported in [129]. The IC_{50} for E2 in buffer was 32 ng mL^{-1} with a LOD of 0.6 ng mL^{-1} , and a working range between 1 and 100 ng mL^{-1} . To evaluate the specificity of our RT-iPCR assay, the anti- 17β -estradiol pAbs' cross-reactivity to structurally related compounds was assessed (Table 2.1). The pAbs exhibited higher cross-reactivity towards E1 (8%) compared to E3 (3%). These values are similar to cross-reactivities to E1 and E3 reported elsewhere: 17β -estradiol-specific monoclonal antibodies cross-reacted to E1 and E3 at 1.3 and 0.6 % respectively [126]. The 17β -estradiol-specific RT-iPCR was performed with both E1 and E3 and yielded poor data resolution between the standards; cross-reactivity was deemed negligible.

2.4.2 RT-iPCR characterization

The standard plot in double-distilled water had a NC value of 20.86 with a standard deviation of 0.16. The Ct LOD (21.34) was calculated as the NC Ct value (20.86) plus three times the standard deviation (0.48). Using the equation of the slope, the LOD corresponded to a

concentration of 0.7 pg 17 β -estradiol mL⁻¹ (Figure 2.2). The linear working range of the RT-iPCR assay was 0.001 ng mL⁻¹ to 10 000 ng mL⁻¹, compared to 1-100 ng mL⁻¹ for the ELISA. The RT-iPCR standard plot displayed a coefficient of determination (R^2) in double-distilled water of 0.971. The interwell variability of Ct values was determined on 8 separate wells for each 17 β -estradiol standard. Interwell relative standard variation (RSD- calculated as standard deviation divided by the mean times 100) ranged from 0.4 to 3.8% over the standard curve, with a mean interwell RSD of 1.6%. The interwell RSD for the NC was 0.9%. The interassay variability of Ct values was determined on 6 separate assays for each 17 β -estradiol standard. Interwell RSD ranged from 3.6 to 5.7% over the standard curve, with a mean interassay RSD of 4.3%. The interassay RSD for the NC was 4.9%.

The RT-iPCR assay provided an 800-fold increase in sensitivity compared to the corresponding ELISA (0.7 pg mL⁻¹ versus 0.6 ng mL⁻¹, respectively. Previous use of RT-iPCR for quantification of environmental contaminants, such as PCBs [54], phenanthrenes [50] and fluoranthenes [51] have yielded extremely low levels of detection: 3,3',4,4'-tetrachlorobiphenyl (PCB 77) was quantified in soil at concentrations ranging from 10 fg mL⁻¹ to 1 ng mL⁻¹, with a LOD of 1.5 fg mL⁻¹, representing 100-fold increase in sensitivity over ELISA [55]. PH and FL were both quantified in water between 10 fg mL⁻¹ and 100 pg mL⁻¹ with a LOD of 5 fg mL⁻¹ [50, 51]. These assays did not employ the universal iPCR format [25], but an avidin linker system. The PCB77 RT-iPCR assay employed biotinylated pAbs and directly joined the biotinylated DNA via an avidin bridge. LODs of a given ELISA are generally enhanced 100-10 000 fold by iPCR, sometimes more: Saito et al. (1999) achieved a 50 000-fold increase in sensitivity using iPCR (0.001 pg mL⁻¹) over the corresponding ELISA (50 pg mL⁻¹) for the detection of tumor necrosis factor α (TNF α) in human serum [130].

Comparing the 17β -estradiol-specific RT-iPCR assay's LOD (0.7 pg mL^{-1}) to the LODs previously obtained for 17β -estradiol using GC-MS/MS (1 pg mL^{-1}) [116] and LC-MS/MS (0.1 pg mL^{-1}) [94] reveals a slightly higher sensitivity of LC-MS/MS [94]. However the ability to analyze a large number of samples simultaneously without extraction or pre-concentration makes the RT-iPCR assay an attractive technique when environmental monitoring is performed on a large scale or when a high-throughput screen is desired prior to LC-MS/MS analysis.

The RT-iPCR assay also expanded the linear working range: from 1-100 ng 17β -estradiol mL^{-1} for the ELISA to 0.001-10 000 ng 17β -estradiol mL^{-1} by RT-iPCR. This represents an increase of five orders of magnitude in the linear working range between the two assays while using the same antibodies. When compared to the previously published linear working ranges obtained by LC-MS/MS for 17β -estradiol ($4 \text{ to } 500 \text{ pg mL}^{-1}$) [94], the RT-iPCR covers a significantly wider range of 17β -estradiol concentrations. Detection of norovirus capsid proteins using RT-iPCR also demonstrated an increase in linear working range, in addition to a 1000-fold increase in sensitivity, compared to the corresponding ELISA [69].

The R^2 value (0.971) of the 17β -estradiol RT-iPCR assay is lower than that of an optimized RT-PCR amplification of a DNA template alone (0.999) [131], or that of 17β -estradiol detection by LC-MS/MS (0.996) [94]. RT-iPCRs for the quantification of other environmental contaminants have yielded similar R^2 values: 0.974 [51], 0.972 [50] and 0.98 [54] for FL, PH, and PCBs, respectively. Unlike RT-PCR, the RT-iPCR assay combines an ELISA with PCR amplification. The ELISA adds a number of steps which could lead to increased standard deviation values when compared to a standard curve for DNA template amplification by RT-PCR alone.

2.4.3 17β -estradiol recovery from spiked samples

All water samples were tested for 17β -estradiol prior to being spiked, and were found to contain E2 values corresponding to concentrations below the assay's LOD (0.7 pg mL^{-1}). A standard plot was generated in each of the water matrices (tap, Milli-Q and irrigation), and used to determine 17β -estradiol concentration in each spiked sample. 17β -estradiol recovery rates were between 78-126% with an average of 96% for all spikes and all matrices combined ($n=27$) (Table 2.2). Average recovery rates for the 0.5, 250 and 500 ng mL^{-1} spikes in all matrices combined were 85, 110 and 91%, respectively ($n=3$). The recovery rates for the Milli-Q, tap and irrigation water were 81, 102 and 103%, respectively ($n=3$). The recoveries fall within a similar range reported in other papers using RT-iPCR for quantification of environmental contaminants: PH recovery levels in spiked Milli-Q, tap and river water ranged from 90 to 108% [50]. Previously reported 17β -estradiol recoveries for LC-MS/MS following SPE extraction ranged from 80 to 110% [94]. Our methodology does not require sample extraction or pre-concentration prior to analysis, as opposed to LC-MS/MS. The relative standard deviation within a spiked triplicate never exceeded 5% and averaged 2% among all samples tested ($n=27$), demonstrating the high performance of the assay.

With a LOD of $0.7 \text{ pg } 17\beta\text{-estradiol mL}^{-1}$ water and a linear range between 0.001 to 10 000 ng mL^{-1} , the RT-iPCR assay is sufficiently sensitive for the environmental monitoring of manure-borne estrogens at concentrations previously measured in water (1 pg mL^{-1} to 3000 pg mL^{-1}), [109, 111] which could impact aquatic ecosystems. With further refinement of the methodology, the RT-iPCR assay has the potential to be used for the routine screening of environmental water samples for E2 quantification.

2.5 Conclusion

A real-time immuno-PCR assay was developed for the rapid and sensitive detection of 17β -estradiol in water. Using 17β -estradiol-specific-pAbs in a universal iPCR format has improved significantly the sensitivity and linear working range of the corresponding ELISA. The RT-iPCR linear working range for determination of 17β -estradiol was from 1 pg mL^{-1} to $10 \text{ }\mu\text{g mL}^{-1}$, with a limit of detection of 0.7 pg mL^{-1} . This limit of detection corresponds to over 800-fold increase over the competitive ELISA using the same antibodies. By extracting and concentrating the estrogens prior to analysis through solid-phase extraction, the MDL of the RT-iPCR assay could be further improved. Although the RT-iPCR assay may not be as accurate and reliable as LC-MS/MS for environmental monitoring, it can serve as a screening assay to reduce the number of samples prior to further analysis by LC-MS-MS. Additionally, the assay has the potential to be used for the detection of other environmental contaminants for which specific antibodies are available.

2.6 Figures and Tables

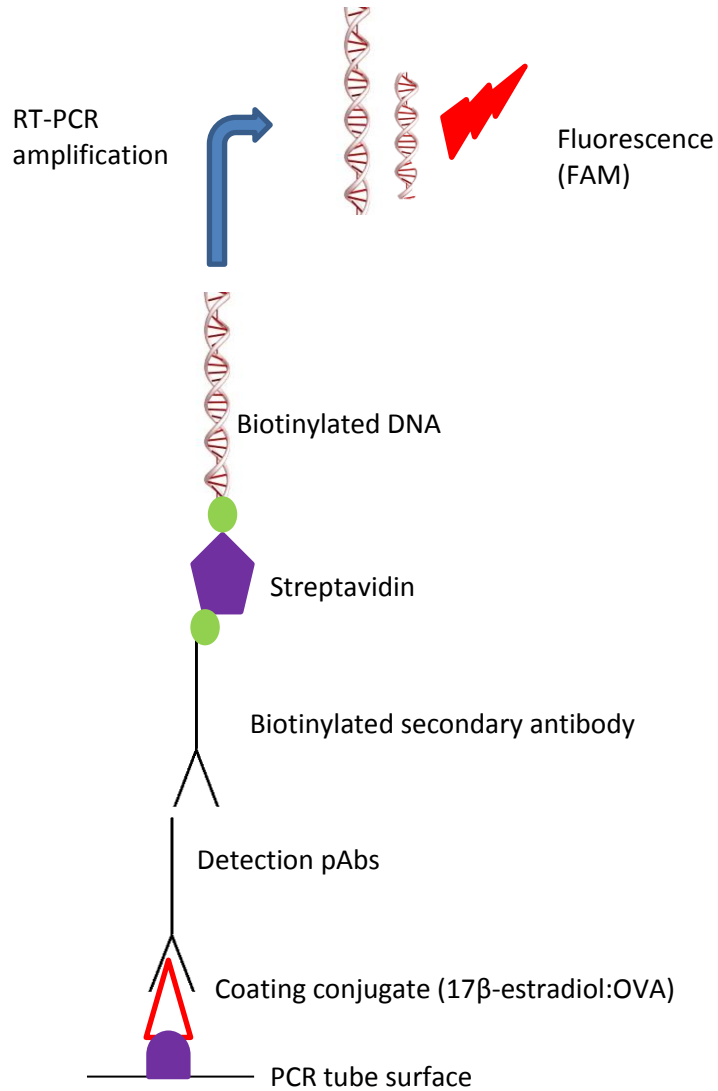


Figure 2.1. Schematic representation of real-time immuno-PCR (RT-iPCR). Coating-conjugate (OVA:17β-estradiol) was adsorbed onto the PCR tubes. 17β-estradiol thereby competes with 17β-estradiol hapten (free in solution) for binding by the anti-17β-estradiol pAbs. Biotinylated goat anti-rabbit antibodies bind to anti-17β-estradiol pAbs. Streptavidin is used as the linker

between biotinylated secondary antibodies and the 70 base pair biotinylated reporter DNA fragment. The reporter DNA fragment is amplified by PCR and detected using fluorescein amidite (FAM) for real-time analysis.

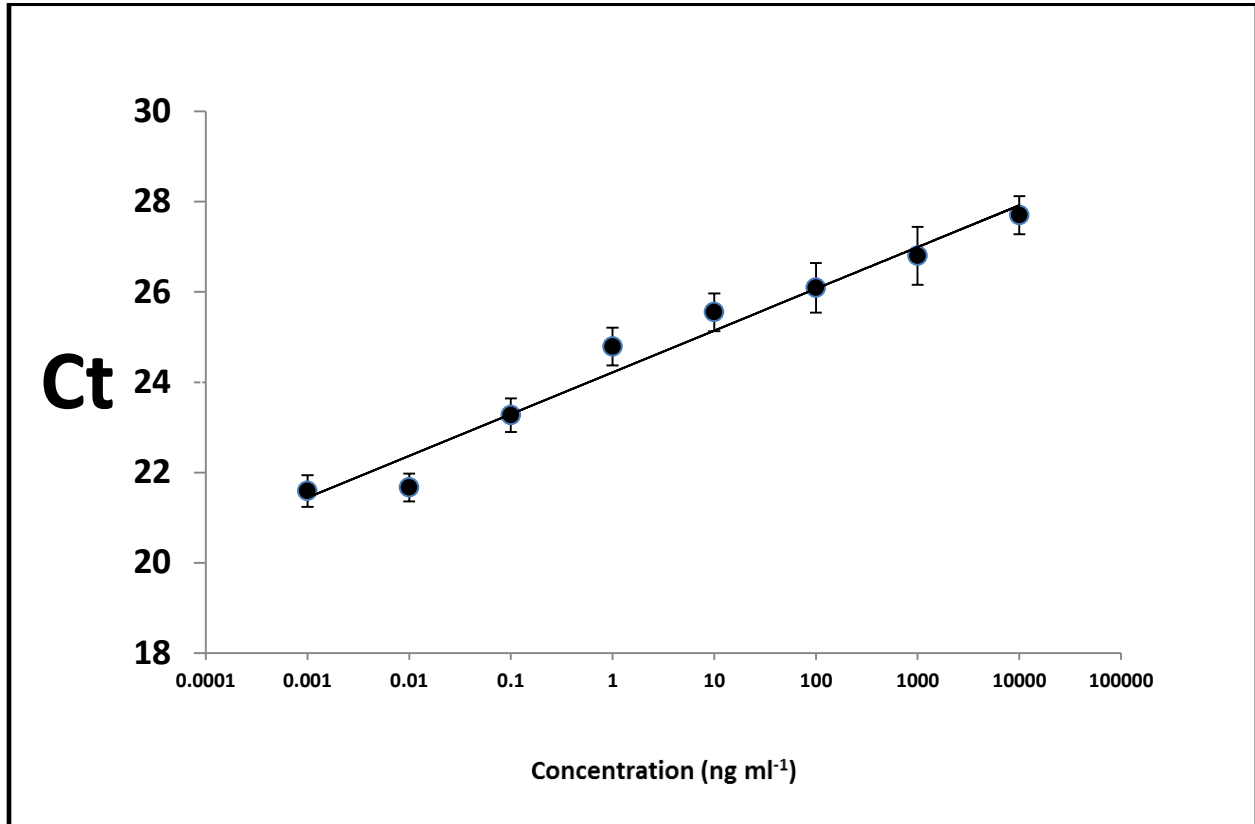


Figure 2.2 RT-iPCR standard plot: RT-iPCR was performed on a serial dilutions of known concentrations of 17β -estradiol corresponding to log-fold dilutions ranging from $10 \mu\text{g ml}^{-1}$ to 1pg ml^{-1} . Coefficient of determination: 0.9706, $n=12$. Error bars represent standard error.

Table 2.1. Cross-reactivities of Abs to structurally related compounds to 17β -estradiol, as determined by ELISA.

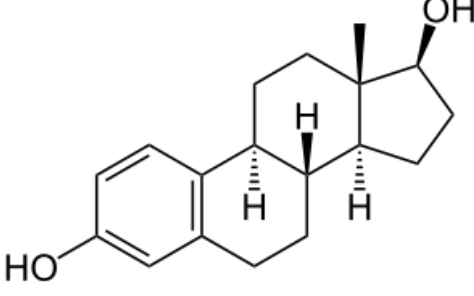
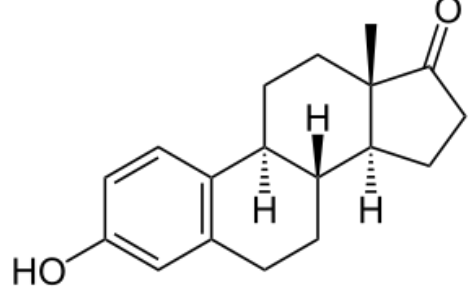
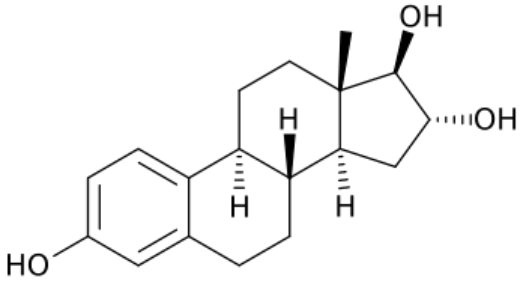
Compound	Molecular Structure	Cross-reactivity (CR%)
17β -estradiol (E2)		100
Estrone (E1)		7.9
Estriol (E3)		3.2

Table 2.2. Recovery of 17 β -estradiol from spiked water samples using RT-iPCR.

Sample	E2 levels (ng mL ⁻¹)	E2 added (ng mL ⁻¹)	Total found (ng mL ⁻¹)	Recovery (%)	RSD (n=3, %)
Double- distilled water	< LOD	500	394	78	3.2
		250	217	87	4.8
		0.5	0.42	80	0.1
Tap water	< LOD	500	493	99	1.2
		250	315	126	0.9
		0.5	0.41	82	1.2
Irrigation water	< LOD	500	483	97	0.7
		250	295	118	1
		0.5	0.47	94	1.8

3. DEVELOPMENT OF A REAL-TIME IMMUNO-PCR ASSAY FOR THE QUANTIFICATION OF *PLASMODIOPHORA BRASSICAE* RESTING SPORES

3.1 Abstract

A real-time immuno-PCR (RT-iPCR) assay was developed for the sensitive quantification of *Plasmodiophora brassicae* resting spores. Using a previously developed universal iPCR method, antibodies were swapped and *P. brassicae* resting spores were accurately quantified in the range of 50 to 10 000 spores. The RT-iPCR assay's limit of detection was 29 spores. The RT-iPCR assay provided an 8-fold increase in sensitivity as well as an expanded working range compared to the corresponding enzyme-linked immunosorbent assay. Assay cross-reactivity to *Spongospora subterranea*, a related pathogenic protist, was negligible. The assay's limit of detection is comparable to other methods for *P. brassicae*, and does not require extraction of *P. brassicae* DNA for detection.

3.2 Introduction

Clubroot, caused by the obligate parasite *Plasmodiophora brassicae* Woronin is an important soil-borne disease affecting crucifers world-wide [132, 133]. This two-phase infection cycle pathogen initially targets the root hairs, with the secondary phase affecting the cells of the cortex and stele of the root [98, 132]. The latter phase of infection involves the formation of club-shaped tumours in the roots that are induced by multinucleate plasmodia that cause improper water and nutrient transportation [98]. Infected plants develop symptoms such as stunted growth with decreased yields [98, 132]. The pathogen can persist in soil in the form of resting spores for 7-20 years [99, 100, 133], providing a long term source of inoculum. Disease development is most prevalent in moist soils with low pH [133]. Chemical control methods such

as soil sterilization [102], fungicide application [103] and lime and magnesium applications have demonstrated limited success in reducing disease severity [104]. Clubroot was recently identified in canola (oilseed rape) fields in Alberta, and as of 2012 there were 1064 fields with confirmed instances of *P. brassicae* infestation [134]. Planting susceptible crops in spore-free soils is therefore the most practical method to avoid clubroot infection, with Canadian legislation now forbidding the planting of canola crops (*Brassica. napus*) on infested land for 5 years [101].

Due to the potential economic losses arising from clubroot disease, it is imperative to detect *P. brassicae* resting spores in infested soils so that preventive strategies may be initiated. Various detection methods for *P. brassicae* have been published [105-108, 135-138]. Bioassays for clubroot, whereby cabbage was grown in soil infested with *P. brassicae* and gall formation was assessed and compared to disease indices with known spore loads have been established [135]. Microscopy has also been used to detect clubroot infection, by directly observing root hairs [136] or by staining resting spores with fluorochromes for visualization by fluorescence microscopy [137]. These techniques are labor intensive and generally impractical for routine diagnosis of *P. brassicae*. Serological detection methods for *P. brassicae* have been developed [138, 139] including an indirect enzyme-linked immunoassay (ELISA) with a detection limit of 100 spores per mL soil [106]. Such immunoassays are commonly used for detection of pathogens; however sensitivity is primarily dependant on the quality of the antisera and are typically qualitative rather than quantitative.

Techniques employing PCR or polymerase chain reaction have demonstrated great potential in the detection of pathogens including *P. brassicae* in soil samples [105, 107, 108]. Following extraction of *P. brassicae* resting spore DNA, single-tube nested PCR (STN-PCR) was performed using primers derived from the DNA sequence of the pentyltransferase gene

[108, 140]. To increase assay sensitivity, the PCR products produced from the nested reaction were then re-amplified via PCR. Using this two-step STN-PCR technique, a sensitivity of 10 resting spores g^{-1} of soil was achieved [108]. Another two-step PCR detection assay for *P. brassicae* in soil using primers specific to *P. brassicae* ribosomal DNA was able to detect 0.1 fg of pure DNA template or 1000 spores per g of plotting mix [107]. In an effort to simplify the assay and reduce the risk of contamination, a simplified and more robust single-step nested PCR diagnostic assay was developed that achieved a sensitivity corresponding to 100 fg of *P. brassicae* resting spore DNA or 1000 resting spores per g of soil [105].

Immuno-PCR, or iPCR combines PCR's amplification power with antibody's specificity and has achieved ultrasensitive detection of a wide range of antigens along with an extensive linear working range [18]. Developed in 1992 by Sano *et al.*, iPCR has increased the sensitivity of conventional ELISA by up to 10 000 fold [18]. This methodology was further refined and simplified with the development of a universal iPCR method, taking advantage of easily accessible reagents [25]. Reports of ultrasensitive detection of a range of bacterial [48], prion [49], toxin [27], viral [29] and cancer-related antigens [130] have recently been published [18]. Environmental contaminants such as PCBs and PAHs have been quantified by iPCR [50, 54, 55]. PCBs in soil were quantified by iPCR, with limits of detection (LOD) of 5 fg mL^{-1} , and a linear range of 10 fg to 1 ng mL^{-1} [54]. iPCR has also been successfully employed in the detection of antigens of the parasitic nematode *Angiostrongylus cantonensis* in serum to a sensitivity of 0.1 ng L^{-1} , that represented a 1000-fold increase in sensitivity over the corresponding ELISA [141]. iPCR offers great potential for quantification of agriculturally-important plant pathogens.

Here we report the development of real-time iPCR (RT-iPCR) assay for the detection and quantification of *P. brassicae* resting spores in soil. This RT-iPCR was adapted from a previous

universal format [25] RT-iPCR assay for the detection of 17β -estradiol in water, where antibodies were swapped to obtain a sensitive RT-iPCR assay for *P. brassicae* resting spores. A schematic representation of the iPCR methodology is outlined in Figure 3.1. Cross-reactivity was assessed against a related pathogenic protist (*Spongospora subterranea*), the causative agent of powdery scab in potatoes. The latter disease develops under conditions similar to that of clubroot and is therefore likely to be found at the same locations [142]. To our knowledge, this is the first report for the use of iPCR for the quantification of resting spores of a plant pathogen in soil.

3.3 Materials and Methods

All chemicals including ovalbumin (OVA; $M_r=45,000$), streptavidin, Tween 20, ethylenediaminetetraacetic acid (EDTA), primers and probe were from Sigma-Aldrich (St. Louis, MO). The Hot Start Fluorescent Quantitect probe PCR (Taqman) was from Qiagen (Mississauga, ON). Biotinylated goat anti-rabbit IgG (H+L) were from Thermo Scientific (Rockford, IL). Tris (tris(hydroxymethyl)aminomethane) and blotting grade non-fat dry milk was from Biorad (Hercules, CA). Horseradish peroxidase (HRPO) conjugated goat anti-rabbit antibody was from Cedarlane Laboratories (Hornby, ON) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was from Kirkegaard and Perry Laboratories (Gaithersburg, MD). RNase-free water was from Sigma. Phosphate buffered saline (PBS) was prepared in Milli-Q water and contained 0.755 g L^{-1} of $\text{K}_2\text{HPO}_4 \bullet 3\text{H}_2\text{O}$, 0.25 g L^{-1} of KH_2PO_4 and 8.2 g L^{-1} of NaCl. Mowiol was prepared according to Henkenjohann (2009) [143].

3.3.1 Spore preparation

P. brassicae resting spores were isolated according to the procedure of Castlebury *et al.* [144]. Approximately 2 g of fresh galled roots of canola infected with pathotype 3 collected at

Leduc, Alberta in 2008 were homogenized in a Waring blender in 50 ml of sterile distilled water for 2 min at high speed, and the resulting homogenate was filtered through eight layers of cheesecloth. The filtrate was centrifuged at 4°C and $4,000 \times g$ for 10 min. The supernatant was discarded and the pellet resuspended in 5 ml of 50% sucrose and centrifuged again at $2,000 \times g$ for 10 min. The resulting supernatant was transferred to a new 50-ml tube and diluted with 30 ml of sterile deionized H₂O before centrifuging at $2,000 \times g$ for 20 min. The pellet obtained was resuspended in 2 ml of sterile deionized water. The supernatant was removed and the resting spore pellet resuspended in 5 ml of sterile deionized H₂O. The resting spores were purified using a continuous gradient of LUDOX (HS-40 colloidal silica, 40% wt suspension in water; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) by centrifugation at $1,000 \times g$ for 35 min in a swinging-bucket rotor (Beckman SW 41 TI). The top two gradient bands were observed microscopically to confirm they contained purified spores and were combined, diluted with 12 ml sterile deionized water and centrifuged at $2,000 \times g$ for 10 min in a fixed angle rotor. The pellet was resuspended in sterile deionized water and recentrifuged at $2,000 \times g$ for 10 min. Pellets were resuspended in minimum volumes of sterile deionized water and diluted based on haemocytometer evaluation to give a spore concentration of 10^5 per ml. The same protocol was used to isolate and purify *S. subterranea* spores, obtained from Lacombe Research Station, Lacombe Alberta.

3.3.2 Immunization and preparation of antisera

Two 3 month old New Zealand white female rabbits were immunized. Immunization was performed with an initial intermuscular injection using a mixture of 200 μ L containing approximately 2×10^4 spores of *P. brassicae* and 200 μ L of Freund's incomplete adjuvant [145]. Three secondary interveinal boosts at biweekly intervals did not include adjuvant. Blood was

collected from the marginal ear vein and serum was prepared by adding 0.02% (w/v) sodium azide and stored at 4⁰C.

3.3.3 ELISA development

Microtiter plate (Nunc, Roskilde, Denmark) wells were coated with 100 µL/well of a serial dilution between 50 and 10 000 *P. brassicae* spores in PBS, and incubated for 16 hours at 4⁰C. Wells were washed three times with PBS-Tween, and blocked with 200 µl/well of 3% milk in PBS for 1 hour at 20⁰C. Following three washes with PBS-Tween, 100 µl of primary antibody diluted 1:5000 in PBS was added to each well and incubated for 1 hour at 20⁰C. Wells were washed three times with PBS-Tween and 100 µl/well of HRPO goat anti-rabbit antibody (diluted 1:5000 in PBS) were added to each well for 1 hour at 20⁰C and washed three times with PBS-Tween. 100 µl of ABTS solution was added to each well and incubated for 15 minutes at 20⁰C prior to absorbance reading at 415 nm on a Spectramax 340PC plate reader (Molecular Devices, Sunnyvale, CA). Assay completion time was 3.5 hours, in addition to the 16 hours incubation of the spores, for a total time of 19.5 hours.

The ELISA LOD was calculated as the mean of 8 replicates of the 0 spore standard plus twice the standard deviation, as reported in [124].

3.3.4 RT-iPCR

A schematic representation of the RT-iPCR assay is outlined in Figure 3.1. Purified *P. brassicae* spores were serially diluted in PBS to obtain various concentrations ranging from 50 to 10 000 spores/well. A negative control consisting of PBS only was included in each experiment. 30 µL of each spore dilution (50, 100, 250, 500, 1000 and 10 000 spores) were added to each well of a 96 well Axygen PCR plate (Union City, CA), sealed with a PCR plate cover, and

incubated for 16 hours at 4⁰C. The plates were then washed four times with 175µL washing buffer (20 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.05% Tween, pH 7.3) using an Aquamax 2000 from Molecular Devices (Sunnyvale, CA) with a period of 10 second agitation. Plates were briefly centrifuged for 5 seconds at 45 x g to remove remaining wash buffer. Wells were blocked with 150 µL of a 5% OVA solution in PBS for 1 hour at 37⁰C. All subsequent washes were performed with 175 µL wash buffer and 10 s agitation, except final wash as described below. *P. brassicae*-specific polyclonal rabbit antibodies were serially diluted to 1:5000 in PBS and 30 µL were added to each well for 1 hour incubation at 20⁰C with gentle rocking. The wells were washed four times with washing buffer and 30 µL of biotinylated secondary antibody (goat-anti rabbit IgG) diluted 1:5000 in PBS was added to each well and incubated for 1 hour at 20⁰C. Streptavidin (200 ng ml⁻¹ in PBS) was mixed with an equal volume of biotinylated reporter DNA diluted 1:250 000 in PBS, and the mixture incubated for 1 hour with gentle rocking at 20⁰C. Following four washes, 30 µL of the streptavidin:DNA mixture was added to each well and incubated for 1 hour at 20⁰C. For the final wash, wells were washed 8 times with 175 µL with 60 second agitation, and rinsed 4 times with 175 µL of Milli-Q water with 10 s agitation, in order to remove all unbound reagents. PCR plates were briefly centrifuged to remove any residual wash buffer and Milli-Q water, and PCR master mix (30 µL) added. RT-iPCR was performed in an Eppendorf real-time thermocycler (Hamburg, Germany). The PCR master mix consisted of the following: 15 µL of 2x Quantitect Probe PCR Master Mix (Quantitect Probe PCR kit, Qiagen, Mississauga, ON), 0.3 µM of each primer, 0.2 µM of Taqman probe and RNase-free water. The PCR cycling parameters were an initial 15 min at 95⁰C followed by 35 cycles of 15 s at 94⁰C, and 60 s at 60⁰C.

DNA amplicons size was 70 bp. Assay completion time was 4.5 hours, in addition to 16 hour overnight incubation, for a total time of 20.5 hours.

3.3.5 RT-iPCR optimization and characterization

In order to minimize non-specific binding of the iPCR reagents to the PCR wells, different blocking agents (milk and OVA) were assessed at concentrations ranging from 3-10%. To maximize PCR well coating with *P. brassicae* spores, assay performance was assessed with the spores diluted in either Milli-Q water or PBS. A standard plot was produced by plotting the average Ct values (n=6 times two experiments for a total of n=12) against known *P. brassicae* spore concentrations, and the linear range was used for spore quantification in samples.

Using a dilution series of *P. brassicae* spores, a standard plot was developed between the ranges of 50 to 10 000 spores (Fig 3.2). The Mastercycler EP realplex instrument automatically set the fluorescent threshold. Fluorescence was detected during amplification by the real-time rotary analyzer producing amplification curves for the *P. brassicae* spore dilution series. In addition to the standard dilution samples, a negative control (NC) containing all assay reagents except *P. brassicae* was included, as well as a background control (BC) containing the RT-PCR master mix only, without DNA template. The Ct values are inversely proportional to antigen concentration; the sample containing the highest spore concentration has the lowest C_t value. The LOD was defined as the value of NC minus three times the average standard deviation, as reported in [19].

Anti-*P. brassicae* pAbs cross reactivity was assessed against *S. subterranea*, the causative agent of powdery scab. Using a dilution series of *S. subterranea* spores, a standard curve was developed in PBS between the ranges of 0-100 000 spores and analyzed by RT-iPCR.

3.4 Results and Discussion

3.4.1 ELISA characterization

The performance parameters of the ELISA were calculated as previously reported in [129]. The IC_{50} for *P. brassicae* spores in PBS buffer was 4212 spores, with a LOD of 232 spores, and a linear working range between 312 and 10 000 spores.

3.4.2 RT-iPCR optimization and characterization

A 5% OVA blocking buffer solution was found to produce the highest Ct values in the negative control, therefore providing the lowest amount of non-specific binding when compared to other concentrations of OVA and milk (data not shown). The assay performed best when PBS was used as buffer for the *P. brassicae* resting spores as compared to Milli-Q water.

The standard curve in double-distilled water had a NC value of 31.53 with a standard deviation of 0.24. The Ct LOD (30.81) was calculated as the NC Ct value (31.53) minus three times the standard deviation (0.72). Using the equation of the slope, the LOD was calculated to approximately 29 *P. brassicae* spores. The linear working range of the RT-iPCR assay was 50 to 10 000 spores, compared to 312 to 10 000 spores for the corresponding ELISA. The RT-iPCR standard curve displayed a coefficient of determination (R^2) in double-distilled water of 0.979. The *P. brassicae* RT-iPCR assay provided an 8-fold increase in sensitivity compared to the corresponding ELISA (29 versus 232 spores, respectively).

The interwell variability of Ct values was determined on 8 separate wells for each *P. brassicae* standard. Interwell relative standard variation (RSD) ranged from 0.3 to 1.8% over the standard curve, with a mean interwell RSD of 0.7%. The interwell RSD for the NC was 0.3%.

The interassay variability of Ct values was determined on 3 separate assays for each *P. brassicae* standard. Interassay RSD ranged from 0.7 to 2.3% over the standard curve, with a mean interassay RSD of 1.3%. The interassay RSD for the NC was 1.9%.

The specificity of the assay was assessed against *S. subterranea*, another common pathogenic soil protist. Following analysis by RT-iPCR on serially diluted *S. subterranea* spores, Ct values were obtained and a standard curve was produced (Fig 3.3). While a trend could be observed, there was very little separation in Ct values between the negative control and 100 000 spores, suggesting little to no binding of the anti-*P. brassicae* pAbs to *S. subterranea* spores. The RT-iPCR assay was therefore specific for *P. brassicae* should field samples contain both pathogens.

Comparing the *P. brassicae*-specific RT-iPCR assay's LOD (29 spores) to the LODs of previously published detection methods employing PCR amplification reveals the sensitivity of the RT-iPCR assay: Faggian *et al.* achieved a limit of detection of 0.1 femtograms of pure *P. brassicae* DNA template, corresponding to 1000 spores per g of plotting mix [107]. By using a STN PCR method, Ito *et al.* achieved a sensitivity of 1 fg of pure *P. brassicae* DNA corresponding to 10 resting spores per g of soil [108]. Both methods required extraction of *P. brassicae* DNA from the resting spores prior to analysis. Since the RT-iPCR assay quantifies spores directly, no DNA extraction is required.

3.5 Conclusions

A real-time immuno-PCR assay was developed for the rapid and sensitive detection of *P. brassicae* resting spores. Using *P. brassicae*-specific-pAbs in a universal iPCR format has improved the sensitivity and linear working range of the corresponding ELISA. The RT-iPCR

linear working range for determination of *P. brassicae* resting spores was from 50 to 10 000 spores, with a limit of detection of approximately 29 spores. This limit of detection corresponds to an 8-fold increase in sensitivity over the ELISA using the same antibodies.

3.6 Acknowledgments

I would like to thank Dr. Larry Kawchuk for providing the *P. brassicae* and *S. subterranea* spores, as well as providing the *P. brassicae* sera, and CO-OP student Tanner Lohr for his assistance.

3.7 Figures

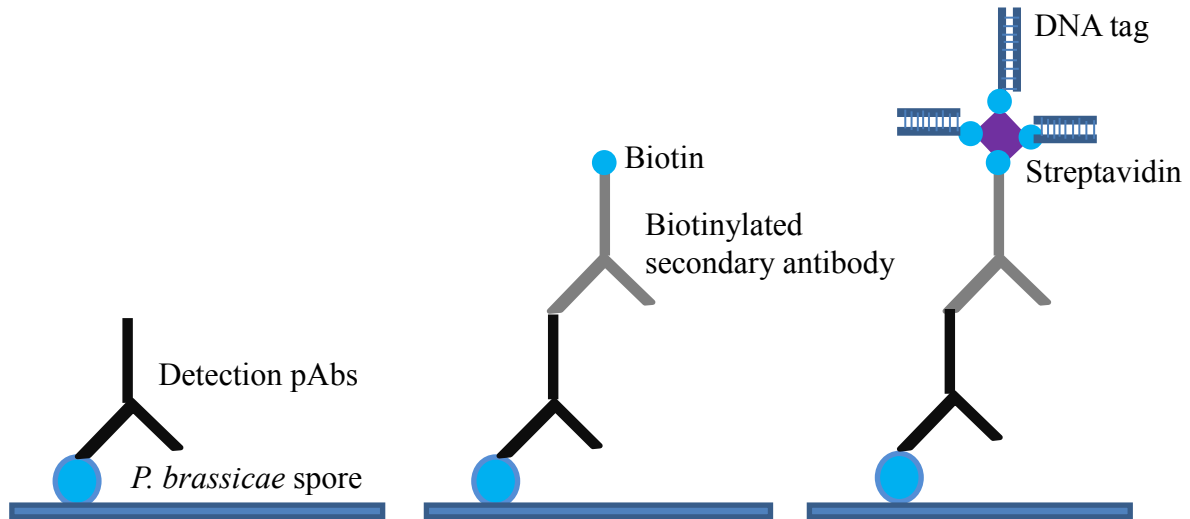


Figure 3.1. Schematic diagram of the iPCR assay. The spores were immobilized on the PCR plate wells. *P. brassicae* resting spores-specific antibodies were added, followed by biotinylated secondary antibody and the streptavidin-biotinylated DNA conjugate. The reporter DNA fragment is amplified by PCR and detected using fluorescein amidite (FAM) for real-time analysis.

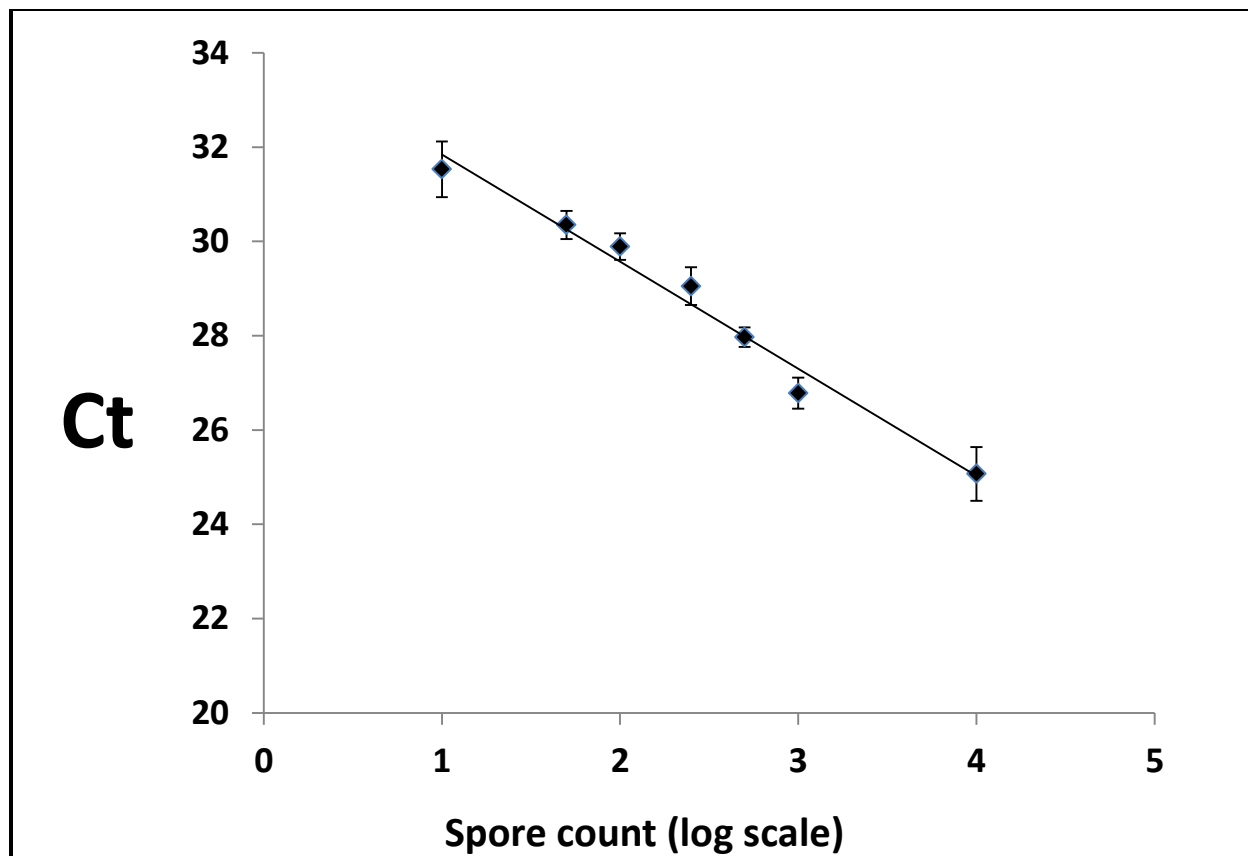


Figure 3.2. RT-iPCR standard plot: RT-iPCR was performed on a serial dilution of known concentrations of *P. brassicae* spores, corresponding to a log-fold dilution ranging from 50 to 10 000 spores. Coefficient of determination: 0.9789, $n=12$.

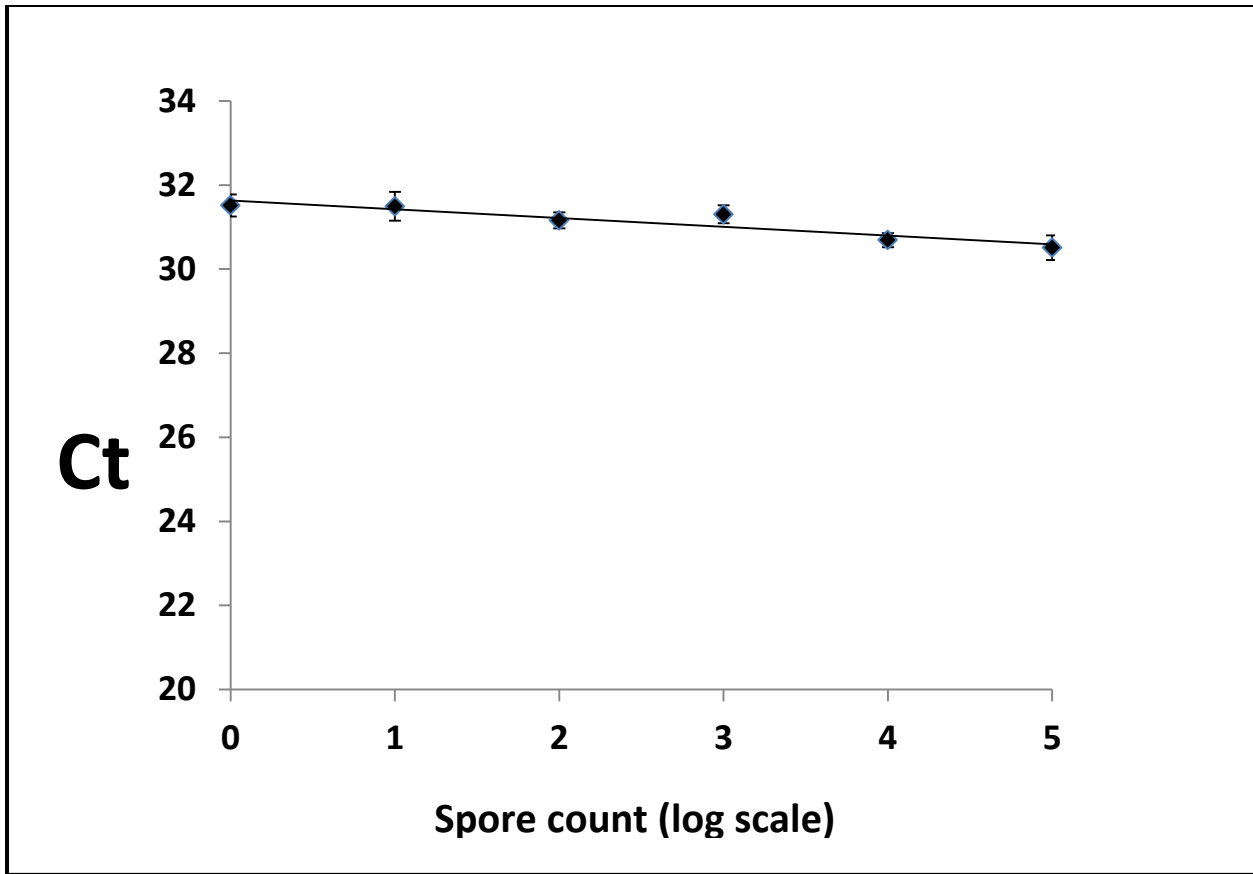


Figure 3.3. RT-iPCR standard plot: RT-iPCR was performed on a serial dilution of known concentrations of *S. subterranea* spores, corresponding to a log-fold dilution ranging from 0 to 100 000 spores. Coefficient of determination: 0.85, $n=4$.

4. DISCUSSION AND CONCLUSIONS

During the development of the RT-iPCR for 17β -estradiol, numerous challenges were encountered. Challenges included problems related to reagent preparation, equipment selection and protocol modifications. These challenges are discussed below.

Working with 17β -estradiol presented a number of difficulties. Firstly, because the molecule has a short half-life (36 hours *in vivo*), it is imperative to prepare fresh standards prior to each assay. Secondly, because of the difficulty in directly binding 17β -estradiol to the surface of an ELISA or PCR plate, the molecule must first be conjugated to a carrier protein (OVA) to facilitate binding to the microwell surface. While the coating conjugate bound well to polystyrene ELISA plates, the same was not easily achieved when switching to PCR plates. A variety of polypropylene and polycarbonate PCR plates from a variety of manufacturers were tested, with polypropylene plates from Axygen deemed most suitable. In order to improve binding, glutaraldehyde was used to modify the PCR plates surface prior to running the assay to improve coating conjugate binding. PCR plates containing various concentrations of glutaraldehyde were incubated at various temperatures and for different time periods. Plates incubated with 0.8% glutaraldehyde for 2 hours at 60°C performed best and this protocol was chosen for all subsequent experiments.

Non-specific binding was another commonly encountered challenge during RT-iPCR assay development. Blocking and washing solutions had to be optimized, along with the washing protocol, to minimize non-specific binding. A variety of different blockers (BSA, OVA, milk) were used at various concentrations (3, 5, 10%) before settling on 3% OVA solution in Milli-Q water. Additionally, the wash buffer (content) was modified to maximize its efficiency. It was found that the addition of 5 mM of EDTA greatly improved the washing and conversely reduced

non-specific binding. Modifications in the wash protocol were tested in order to maximize washing efficiency. It was found that increasing the number of washes from three to eight, and the duration of each wash cycle from 10 to 60 seconds for the final wash (prior to the addition of the PCR Master Mix) reduced the signal originating from negative control wells, suggesting a more complete removal of the unbound DNA. Since thorough washing of the wells is important for reducing non-specific binding and removing unbound reagents, we suggest the use of an automatic PCR plate washing. Manual pipetting to wash wells was found to be highly problematic and resulted in inconsistent data. With the use of an automatic plate washer however, PCR plate compatibility was problematic: not all PCR plates fit the plate washer (typically used for microtitre plates). Modifications to both the plate washer and to the plates themselves (such as the addition of weight in the form of stir bars) were necessary to ensure plate compatibility. During the development of the assay, variations in the assay protocol were assessed: it was found that pre-incubating the DNA tag and streptavidin prior to incubating in the well (as opposed to incubating each sequentially) gave much more consistent data in addition to shortening the assay by 1 hour.

Having the specifics working for the 17β -estradiol assay made the antibody swapping easier. No major changes were needed when switching to the clubroot assay.

Immuno-PCR is a rapidly evolving diagnostic technology combining the specificity of antibodies with the sensitivity of DNA amplification by PCR. The advantages conferred by iPCR are multiple: these assays are highly sensitive, high-throughput, amenable to multiplex analysis and flexible in applications and format. iPCR assays are therefore well suited for environmental analysis, whereby numerous samples are to be analyzed rapidly. Most iPCR assays have so far

been developed for biomedical applications, and have yet to be applied in agricultural research and the monitoring of agricultural contaminants.

This thesis presented the development of two universal format RT-iPCR assays for agricultural application: 1) an assay for the environmental monitoring of manure-borne estrogens; and 2) an assay for the detection of clubroot resting spores in canola fields. Both iPCRs showed significant improvements with regards to sensitivity and linear range when compared to their corresponding enzyme-linked immunosorbent assay. Both RT-iPCR have equal or improved sensitivity compared to other analytical methods published, with expanded linear working ranges and high-throughput capabilities. Additionally, the flexibility of the universal iPCR format was demonstrated by swapping out the antibodies and rapidly developing a novel assay for a completely different antigen.

In the development of any analytical method, several factors must be taken into consideration to ensure RT-iPCR performance, with significant fine-tuning of reagents concentrations, incubation times, sample preparation and assay protocol required to optimize applicability and sensitivity. Commercial reagents are helping with standardizing iPCRs, while nanotechnology and liquid-phase assays are providing novel solutions to current assay problems. These technologies require significant expertise and specialized instrumentation, but will undoubtedly result in simplified assays to be used in routine laboratory analysis in the near future.

5. REFERENCES

1. Voller, A., A. Bartlett, and D.E. Bidwell, *Enzyme immunoassays with special reference to ELISA techniques*. Journal of Clinical Pathology, 1978. **31**(6): p. 507-520.
2. Yalow, R.S. and S.A. Berson, *Assay of Plasma Insulin in Human Subjects by Immunological Methods*. Nature, 1959. **184**(4699): p. 1648-1649.
3. Voller, A., D. Bidwell, and A. Bartlett, *Enzyme immunoassays in diagnostic medicine: Theory and practice**. Bulletin of the World Health Organization, 1976. **53**(1): p. 55.
4. Engvall, E. and P. Perlmann, *Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G*. Immunochemistry, 1971. **8**(9): p. 871-874.
5. Wolters, G., et al., *Solid-phase enzyme-immunoassay for detection of hepatitis B surface antigen*. Journal of Clinical Pathology, 1976. **29**(10): p. 873-879.
6. Maskill, W.J., et al., *An evaluation of competitive and second generation ELISA screening tests for antibody to HIV*. Journal of Virological Methods, 1988. **22**(1): p. 61-73.
7. Luczynska, C.M., et al., *A two-site monoclonal antibody ELISA for the quantification of the major Dermatophagoides spp. allergens, Der p I and Der f I*. Journal of Immunological Methods, 1989. **118**(2): p. 227-235.
8. Van Emon, J.M., C.L. Gerlach, and K. Bowman, *Bioseparation and bioanalytical techniques in environmental monitoring*. Journal of Chromatography B: Biomedical Sciences and Applications, 1998. **715**(1): p. 211-228.
9. Marco, M.-P., S. Gee, and B.D. Hammock, *Immunochemical techniques for environmental analysis I. Immunosensors*. TrAC Trends in Analytical Chemistry, 1995. **14**(7): p. 341-350.
10. Abad, A., et al., *Determination of carbaryl, carbofuran and methiocarb in cucumbers and strawberries by monoclonal enzyme immunoassays and high-performance liquid chromatography with fluorescence detection: An analytical comparison*. Journal of Chromatography A, 1999. **833**(1): p. 3-12.
11. Giersch, T. and B. Hock, *Production of monoclonal antibodies for the determination of s-triazines with enzyme immunoassays*. Food and Agricultural Immunology, 1990. **2**(2): p. 85-97.
12. Stiernstedt, G.T., et al., *Diagnosis of spirochetal meningitis by enzyme-linked immunosorbent assay and indirect immunofluorescence assay in serum and cerebrospinal fluid*. Journal of Clinical Microbiology, 1985. **21**(5): p. 819-825.
13. Haber, E., et al., *Application of a Radioimmunoassay for Angiotensin I to the Physiologic Measurements of Plasma Renin Activity in Normal Human Subjects*. The Journal of Clinical Endocrinology & Metabolism, 1969. **29**(10): p. 1349-1355.

14. Skelley, D.S., L.P. Brown, and P.K. Besch, *Radioimmunoassay*. Clinical Chemistry, 1973. **19**(2): p. 146-186.
15. Liao, T., et al., *Comparative responses in rare minnow exposed to 17 β -estradiol during different life stages*. Fish physiology and biochemistry, 2009. **35**(3): p. 341-349.
16. Lahnsteiner, F., et al., *Effect of 17 β -estradiol on gamete quality and maturation in two salmonid species*. Aquatic Toxicology, 2006. **79**(2): p. 124-131.
17. Malou, N. and D. Raoult, *Immuno-PCR: a promising ultrasensitive diagnostic method to detect antigens and antibodies*. Trends in microbiology, 2011. **19**(6): p. 295-302.
18. Niemeyer, C.M., M. Adler, and R. Wacker, *Immuno-PCR: high sensitivity detection of proteins by nucleic acid amplification*. Trends in biotechnology, 2005. **23**(4): p. 208-216.
19. Niemeyer, C.M., M. Adler, and R. Wacker, *Detecting antigens by quantitative immuno-PCR*. Nat. Protocols, 2007. **2**(8): p. 1918-1930.
20. Sano, T., C.L. Smith, and C.R. Cantor, *Immuno-PCR: very sensitive antigen detection by means of specific antibody-DNA conjugates*. Science, 1992. **258**(5079): p. 120-122.
21. Niemeyer, C.M., et al., *Self-assembly of DNA-streptavidin nanostructures and their use as reagents in immuno-PCR*. Nucleic Acids Research, 1999. **27**(23): p. 4553-4561.
22. Harlow, E. and D. Lane, *Antibodies: a laboratory manual*. Vol. 559. 1988: Cold Spring Harbor Laboratory Cold Spring Harbor, NY.
23. McElhinny, A. and C. Warner, *Detection of major histocompatibility complex class I antigens on the surface of a single murine blastocyst by immuno-PCR*. BioTechniques, 1997. **23**(4): p. 660-662.
24. Ren, J.U.N., et al., *Detection of circulating CEA molecules in human sera and leukopheresis of peripheral blood stem cells with E. coli expressed bispecific CEAScFv-streptavidin fusion protein-based immuno-PCR technique*. Annals of the New York Academy of Sciences, 2001. **945**(1): p. 116-118.
25. Zhou, H., R.J. Fisher, and T.S. Papas, *Universal immuno-PCR for ultra-sensitive target protein detection*. Nucleic acids research, 1993. **21**(25): p. 6038.
26. Liang, H., et al., *A highly sensitive immuno-PCR assay for detecting Group A Streptococcus*. Journal of Immunological Methods, 2003. **279**(1-2): p. 101-110.
27. Zhang, W., et al., *New immuno-PCR assay for detection of low concentrations of Shiga toxin 2 and its variants*. Journal of clinical microbiology, 2008. **46**(4): p. 1292-1297.
28. Barletta, J.M., et al., *Detection of ultra-low levels of pathologic prion protein in scrapie infected hamster brain homogenates using real-time immuno-PCR*. Journal of Virological Methods, 2005. **127**(2): p. 154-164.

29. Maia, M., et al., *Development of a two-site immuno-PCR assay for hepatitis B surface antigen*. Journal of virological methods, 1995. **52**(3): p. 273-286.
30. Crowther, J.R., *ELISA: theory and practice*. Vol. 42. 1995: Springer.
31. Niemeyer, C.M., M. Adler, and D. Blohm, *Fluorometric Polymerase Chain Reaction (PCR) Enzyme-Linked Immunosorbent Assay for Quantification of Immuno-PCR Products in Microplates*. Analytical Biochemistry, 1997. **246**(1): p. 140-145.
32. Sims, P.W., et al., *Immunopolymerase Chain Reaction Using Real-Time Polymerase Chain Reaction for Detection*. Analytical Biochemistry, 2000. **281**(2): p. 230-232.
33. Sperl, J., et al., *Soluble T cell receptors: detection and quantitative assay in fluid phase via ELISA or immuno-PCR*. Journal of Immunological Methods, 1995. **186**(2): p. 181-194.
34. Hendrickson, E.R., et al., *High sensitivity multianalyte immunoassay using covalent DNA-labeled antibodies and polymerase chain reaction*. Nucleic Acids Research, 1995. **23**(3): p. 522-529.
35. McKie, A., et al., *A quantitative immuno-PCR assay for the detection of mumps-specific IgG*. Journal of Immunological Methods, 2002. **270**(1): p. 135-141.
36. Wu, H., et al., *Detection of Clostridium botulinum neurotoxin type A using immuno-PCR*. Letters in applied microbiology, 2001. **32**(5): p. 321-325.
37. Henterich, N., et al., *Assay of gliadin by real-time immunopolymerase chain reaction*. Food / Nahrung, 2003. **47**(5): p. 345-348.
38. Niemeyer, C.M., et al., *Nanostructured DNA-Protein Aggregates Consisting of Covalent Oligonucleotide-Streptavidin Conjugates*. Bioconjugate Chemistry, 2001. **12**(3): p. 364-371.
39. Niemeyer, C.M., *The developments of semisynthetic DNA-protein conjugates*. Trends in Biotechnology, 2002. **20**(9): p. 395-401.
40. Ferre, F., *Quantitative or semi-quantitative PCR: reality versus myth*. Genome Research, 1992. **2**(1): p. 1-9.
41. Freeman, W.M., S.J. Walker, and K.E. Vrana, *Quantitative RT-PCR: pitfalls and potential*. Biotechniques, 1999. **26**: p. 112-125.
42. Garibyan, L. and N. Avashia, *Polymerase Chain Reaction*. J Invest Dermatol, 2013. **133**(3): p. e6.
43. VanGuilder, H.D., K.E. Vrana, and W.M. Freeman, *Twenty-five years of quantitative PCR for gene expression analysis*. Biotechniques, 2008. **44**(5): p. 619.
44. Chang, T.C. and S.H. Huang, *A modified immuno-polymerase chain reaction for the detection of β -glucuronidase from Escherichia coli*. Journal of Immunological Methods, 1997. **208**(1): p. 35-42.

45. Joerger, R.D., et al., *Analyte detection with DNA-labeled antibodies and polymerase chain reaction*. *Clinical Chemistry*, 1995. **41**(9): p. 1371-7.
46. Kakizaki, E., et al., *Detection of bacterial antigens using immuno-PCR*. *Letters in applied microbiology*, 1996. **23**(2): p. 101-103.
47. Adler, M., R. Wacker, and C.M. Niemeyer, *A real-time immuno-PCR assay for routine ultrasensitive quantification of proteins*. *Biochemical and biophysical research communications*, 2003. **308**(2): p. 240-250.
48. Barletta, J., *Applications of real-time immuno-polymerase chain reaction (rt-IPCR) for the rapid diagnoses of viral antigens and pathologic proteins*. *Molecular aspects of medicine*, 2006. **27**(2): p. 224-253.
49. Barletta, J.M., et al., *Detection of ultra-low levels of pathologic prion protein in scrapie infected hamster brain homogenates using real-time immuno-PCR*. *Journal of virological methods*, 2005. **127**(2): p. 154-164.
50. Zhou, C., Q.-E. Wang, and H.-S. Zhuang, *Determination of phenanthrene by antibody-coated competitive real-time immuno-PCR assay*. *Analytical and bioanalytical chemistry*, 2008. **391**(8): p. 2857-2863.
51. Zhou, C. and H. Zhuang, *Determination of fluoranthene by antigen-coated indirect competitive real-time immuno-PCR assay*. *J. Environ. Monit.*, 2009. **11**(2): p. 400-405.
52. Barletta, J.M., D.C. Edelman, and N.T. Constantine, *Lowering the detection limits of HIV-1 viral load using real-time immuno-PCR for HIV-1 p24 antigen*. *American journal of clinical pathology*, 2004. **122**(1): p. 20-27.
53. Lind, K. and M. Kubista, *Development and evaluation of three real-time immuno-PCR assemblages for quantification of PSA*. *Journal of Immunological Methods*, 2005. **304**(1-2): p. 107-116.
54. Chen, H.-Y. and H.-S. Zhuang, *Real-time immuno-PCR assay for detecting PCBs in soil samples*. *Analytical and bioanalytical chemistry*, 2009. **394**(4): p. 1205-1211.
55. Chen, H.-Y. and H.-S. Zhuang, *A real-time immuno-PCR method for detecting 3, 3', 4, 4'-tetrachlorobiphenyl*. *Microchimica Acta*, 2011. **172**(1-2): p. 233-239.
56. Adler, M., et al., *Adaptation and performance of an immuno-PCR assay for the quantification of Aviscumine in patient plasma samples*. *Journal of Pharmaceutical and Biomedical Analysis*, 2005. **39**(5): p. 972-982.
57. Adler, M., R. Wacker, and C.M. Niemeyer, *Sensitivity by combination: immuno-PCR and related technologies*. *Analyst*, 2008. **133**(6): p. 702-718.
58. Mweene, A., et al., *Development of immuno-PCR for diagnosis of bovine herpesvirus 1 infection*. *Journal of clinical microbiology*, 1996. **34**(3): p. 748-750.

59. Ren, J., et al., *Detection of circulating gastric carcinoma-associated antigen MG7-Ag in human sera using an established single determinant immuno-polymerase chain reaction technique*. *Cancer*, 2000. **88**(2): p. 280-285.
60. Rosi, N.L. and C.A. Mirkin, *Nanostructures in biodiagnostics*. *Chemical reviews*, 2005. **105**(4): p. 1547-1562.
61. Alivisatos, P., *The use of nanocrystals in biological detection*. *Nat Biotech*, 2004. **22**(1): p. 47-52.
62. Söderberg, O., et al., *Characterizing proteins and their interactions in cells and tissues using the in situ proximity ligation assay*. *Methods*, 2008. **45**(3): p. 227-232.
63. Nam, J.-M., S.-J. Park, and C.A. Mirkin, *Bio-barcode based on oligonucleotide-modified nanoparticles*. *Journal of the American Chemical Society*, 2002. **124**(15): p. 3820-3821.
64. Barletta, J., A. Bartolome, and N.T. Constantine, *Immunomagnetic quantitative immuno-PCR for detection of less than one HIV-1 virion*. *Journal of Virological Methods*, 2009. **157**(2): p. 122-132.
65. Wacker, R., et al., *Magneto immuno-PCR: a novel immunoassay based on biogenic magnetosome nanoparticles*. *Biochemical and biophysical research communications*, 2007. **357**(2): p. 391-396.
66. Chen, L., et al., *Gold nanoparticle enhanced immuno-PCR for ultrasensitive detection of Hantaan virus nucleocapsid protein*. *Journal of Immunological Methods*, 2009. **346**(1-2): p. 64-70.
67. Potůčková, L., et al., *Rapid and sensitive detection of cytokines using functionalized gold nanoparticle-based immuno-PCR, comparison with immuno-PCR and ELISA*. *Journal of Immunological Methods*, 2011. **371**(1-2): p. 38-47.
68. Perez, J.W., et al., *Detection of respiratory syncytial virus using nanoparticle amplified immuno-polymerase chain reaction*. *Analytical Biochemistry*, 2011. **410**(1): p. 141-148.
69. Tian, P. and R. Mandrell, *Detection of norovirus capsid proteins in faecal and food samples by a real time immuno-PCR method*. *Journal of Applied Microbiology*, 2006. **100**(3): p. 564-574.
70. Sanborn, J.R., et al., *Hapten synthesis and antibody development for polychlorinated dibenzo-p-dioxin immunoassays*. *Journal of Agricultural and Food Chemistry*, 1998. **46**(6): p. 2407-2416.
71. Marco, M.P., B.D. Hammock, and M.J. Kurth, *Hapten design and development of an ELISA (enzyme-linked immunosorbent assay) for the detection of the mercapturic acid conjugates of naphthalene*. *The Journal of Organic Chemistry*, 1993. **58**(26): p. 7548-7556.
72. Li, K., et al., *Monoclonal antibody-based ELISAs for part-per-billion determination of polycyclic aromatic hydrocarbons: effects of haptens and formats on sensitivity and specificity*. *Analytical chemistry*, 1999. **71**(2): p. 302-309.

73. Karu, A.E., et al., *Synthesis of haptens and derivation of monoclonal antibodies for immunoassay of the phenylurea herbicide diuron*. Journal of Agricultural and Food Chemistry, 1994. **42**(2): p. 301-309.
74. Goodrow, M.H. and B.D. Hammock, *Hapten design for compound-selective antibodies: ELISAs for environmentally deleterious small molecules*. Analytica chimica acta, 1998. **376**(1): p. 83-91.
75. Ye, Q., H. Zhuang, and C. Zhou, *Detection of naphthalene by real-time immuno-PCR using molecular beacon*. Molecular and cellular probes, 2009. **23**(1): p. 29-34.
76. Van den Berg, M., et al., *Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife*. Environmental health perspectives, 1998. **106**(12): p. 775.
77. Campfens, J. and D. Mackay, *Fugacity-based model of PCB bioaccumulation in complex aquatic food webs*. Environmental science & technology, 1997. **31**(2): p. 577-583.
78. Safe, S., *Toxicology, structure-function relationship, and human and environmental health impacts of polychlorinated biphenyls: progress and problems*. Environmental health perspectives, 1993. **100**: p. 259.
79. Combalbert, S. and G. Hernandez-Raquet, *Occurrence, fate, and biodegradation of estrogens in sewage and manure*. Applied Microbiology and Biotechnology, 2010. **86**(6): p. 1671-1692.
80. Damstra, T., et al., *Global assessment of the state-of-the-science of endocrine disruptors*. Geneva: World Health Organization, 2002.
81. Lai, K.M., M.D. Scrimshaw, and J.N. Lester, *The effects of natural and synthetic steroid estrogens in relation to their environmental occurrence*. Critical Reviews in Toxicology, 2002. **32**(2): p. 113-132.
82. Mills, L.J. and C. Chichester, *Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations?* Science of The Total Environment, 2005. **343**(1-3): p. 1-34.
83. Purdom, C., et al., *Estrogenic effects of effluents from sewage treatment works*. Chemistry and Ecology, 1994. **8**(4): p. 275-285.
84. Jeffries, K.M., et al., *Presence of natural and anthropogenic organic contaminants and potential fish health impacts along two river gradients in Alberta, Canada*. Environmental Toxicology and Chemistry, 2010. **29**(10): p. 2379-2387.
85. Wise, A., K. O'Brien, and T. Woodruff, *Are Oral Contraceptives a Significant Contributor to the Estrogenicity of Drinking Water?*[†]. Environmental science & technology, 2010. **45**(1): p. 51-60.
86. Palme, R., et al., *Excretion of infused ¹⁴C-steroid hormones via faeces and urine in domestic livestock*. Animal Reproduction Science, 1996. **43**(1): p. 43-63.

87. Falconer, I.R., et al., *Endocrine-disrupting compounds: A review of their challenge to sustainable and safe water supply and water reuse*. Environmental toxicology, 2006. **21**(2): p. 181-191.
88. Chen, T.-S., et al., *High estrogen concentrations in receiving river discharge from a concentrated livestock feedlot*. Science of the total environment, 2010. **408**(16): p. 3223-3230.
89. Miller, J.J., et al., *Quantity and quality of runoff from a beef cattle feedlot in southern Alberta*. Journal of environmental quality, 2004. **33**(3): p. 1088-1097.
90. Hanselman, T.A., D.A. Graetz, and A.C. Wilkie, *Manure-borne estrogens as potential environmental contaminants: a review*. Environmental Science & Technology, 2003. **37**(24): p. 5471-5478.
91. Ternes, T.A., et al., *Determination of estrogens in sludge and sediments by liquid extraction and GC/MS/MS*. Analytical Chemistry, 2002. **74**(14): p. 3498-3504.
92. Baronti, C., et al., *Monitoring Natural and Synthetic Estrogens at Activated Sludge Sewage Treatment Plants and in a Receiving River Water*. Environmental Science & Technology, 2000. **34**(24): p. 5059-5066.
93. Richardson, S.D., *Environmental mass spectrometry: emerging contaminants and current issues*. Analytical chemistry, 2008. **80**(12): p. 4373-4402.
94. Benijts, T., W. Lambert, and A. De Leenheer, *Analysis of multiple endocrine disruptors in environmental waters via wide-spectrum solid-phase extraction and dual-polarity ionization LC-Ion Trap-MS/MS*. Analytical Chemistry, 2003. **76**(3): p. 704-711.
95. Metcalfe, C.D., et al., *Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays with Japanese medaka (Oryzias latipes)*. Environmental Toxicology and Chemistry, 2001. **20**(2): p. 297-308.
96. Tewari, J., et al., *Identification of clubroot of crucifers on canola (Brassica napus) in Alberta*. Canadian journal of plant pathology, 2005. **27**(1): p. 143-144.
97. Strelkov, S.E. and S.-F. Hwang, *Clubroot in the Canadian canola crop: 10 years into the outbreak*. Canadian Journal of Plant Pathology, 2014. **36**(sup1): p. 27-36.
98. Voorrips, R., *Plasmodiophora brassicae: aspects of pathogenesis and resistance in Brassica oleracea*. Euphytica, 1995. **83**(2): p. 139-146.
99. Wallenhammar, A.C., *Prevalence of Plasmodiophora brassicae in a spring oilseed rape growing area in central Sweden and factors influencing soil infestation levels*. Plant Pathology, 1996. **45**(4): p. 710-719.
100. Hwang, S.F., et al., *Effect of susceptible and resistant canola plants on Plasmodiophora brassicae resting spore populations in the soil*. Plant Pathology, 2013. **62**(2): p. 404-412.

101. Dixon, G., *The occurrence and economic impact of Plasmodiophora brassicae and clubroot disease*. Journal of Plant Growth Regulation, 2009. **28**(3): p. 194-202.
102. White, J.G. and S.T. Buczacki, *The control of clubroot by soil partial sterilization: a review*. Annals of Applied Biology, 1977. **85**(2): p. 287-300.
103. Tanaka, S., et al., *Biological Mode of Action of the Fungicide, Flusulfamide, Against Plasmodiophora brassicae (clubroot)*. European Journal of Plant Pathology, 1999. **105**(6): p. 577-584.
104. Myers, D.F. and R. Campbell, *Lime and the control of clubroot of crucifers: Effects of pH, calcium, magnesium, and their interactions*. Phytopathology, 1985. **75**(6): p. 670-673.
105. Cao, T., J. Tewari, and S.E. Strelkov, *Molecular detection of Plasmodiophora brassicae, causal agent of clubroot of crucifers, in plant and soil*. Plant disease, 2007. **91**(1): p. 80-87.
106. Wakeham, A.J. and J.G. White, *Serological detection in soil of Plasmodiophora brassicae resting spores*. Physiological and Molecular Plant Pathology, 1996. **48**(5): p. 289-303.
107. Faggian, R., et al., *Specific Polymerase Chain Reaction Primers for the Detection of Plasmodiophora brassicae in Soil and Water*. Phytopathology, 1999. **89**(5): p. 392-397.
108. Ito, S., et al., *Development of a PCR-based Assay for the Detection of Plasmodiophora brassicae in Soil*. Journal of Phytopathology, 1999. **147**(2): p. 83-88.
109. Fine, D.D., et al., *Quantitation of estrogens in ground water and swine lagoon samples using solid-phase extraction, pentafluorobenzyl/trimethylsilyl derivatizations and gas chromatography–negative ion chemical ionization tandem mass spectrometry*. Journal of Chromatography A, 2003. **1017**(1): p. 167-185.
110. Nichols, D.J., et al., *Runoff of estrogen hormone 17 β -estradiol from poultry litter applied to pasture*. J. Environ. Qual., 1997. **26**(4): p. 1002-1006.
111. Desbrow, C., et al., *Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening*. Environmental Science & Technology, 1998. **32**(11): p. 1549-1558.
112. Rodgers-Gray, T.P., et al., *Exposure of juvenile roach (Rutilus rutilus) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development*. Environmental Science & Technology, 2000. **35**(3): p. 462-470.
113. Wang, L., et al., *Assessing estrogenic activity in surface water and sediment of the Liao River system in northeast China using combined chemical and biological tools*. Environmental Pollution, 2011. **159**(1): p. 148-156.
114. Kozlík, P., et al., *Development of a solid-phase extraction with capillary liquid chromatography tandem mass spectrometry for analysis of estrogens in environmental water samples*. Journal of Chromatography A, 2011. **1218**(15): p. 2127-2132.

115. Chang, H., et al., *Occurrence of androgens and progestogens in wastewater treatment plants and receiving river waters: Comparison to estrogens*. Water Research, 2011. **45**(2): p. 732-740.
116. Ternes, T.A., et al., *Behavior and occurrence of estrogens in municipal sewage treatment plants – I. Investigations in Germany, Canada and Brazil*. Science of The Total Environment, 1999. **225**(1–2): p. 81-90.
117. Viglino, L., et al., *Analysis of natural and synthetic estrogenic endocrine disruptors in environmental waters using online preconcentration coupled with LC-APPI-MS/MS*. Talanta, 2008. **76**(5): p. 1088-1096.
118. Isobe, T., et al., *Determination of estrogens and their conjugates in water using solid-phase extraction followed by liquid chromatography–tandem mass spectrometry*. Journal of chromatography A, 2003. **984**(2): p. 195-202.
119. Ferguson, P.L., et al., *Determination of steroid estrogens in wastewater by immunoaffinity extraction coupled with HPLC–electrospray-MS*. Analytical Chemistry, 2001. **73**(16): p. 3890-3895.
120. Abraham, G.E., *Solid-phase radioimmunoassay of estradiol-17 β* . The Journal of Clinical Endocrinology & Metabolism, 1969. **29**(6): p. 866-870.
121. Hornbeck, P., *Enzyme-Linked Immunosorbent Assays*, in *Current Protocols in Immunology*. 2001, John Wiley & Sons, Inc.
122. Tanaka, T., et al., *Rapid and sensitive detection of 17 β -estradiol in environmental water using automated immunoassay system with bacterial magnetic particles*. Journal of Biotechnology, 2004. **108**(2): p. 153-159.
123. Suzuki, Y. and T. Maruyama, *Fate of natural estrogens in batch mixing experiments using municipal sewage and activated sludge*. Water Research, 2006. **40**(5): p. 1061-1069.
124. Caron, E., C. Sheedy, and A. Farenhorst, *Development of competitive ELISAs for 17 β -estradiol and 17 β -estradiol +estrone+estriol using rabbit polyclonal antibodies*. Journal of Environmental Science and Health, Part B, 2010. **45**(2): p. 145-151.
125. Swart, N. and E. Pool, *Rapid detection of selected steroid hormones from sewage effluents using an ELISA in the Kuils river water catchment area, South Africa*. Journal of Immunoassay and Immunochemistry, 2007. **28**(4): p. 395-408.
126. Farré, M., et al., *Evaluation of commercial immunoassays for the detection of estrogens in water by comparison with high-performance liquid chromatography tandem mass spectrometry HPLC–MS/MS (QqQ)*. Analytical and Bioanalytical Chemistry, 2006. **385**(6): p. 1001-1011.
127. Raman, D.R., et al., *Estrogen content of dairy and swine wastes*. Environmental Science & Technology, 2004. **38**(13): p. 3567-3573.

128. Mauriz, E., et al., *Optical immunosensor for fast and sensitive detection of DDT and related compounds in river water samples*. *Biosensors and Bioelectronics*, 2007. **22**(7): p. 1410-1418.
129. Hanselman, T.A., D.A. Graetz, and A.C. Wilkie, *Comparison of three enzyme immunoassays for measuring 17 β -estradiol in flushed dairy manure wastewater*. *J. Environ. Qual.*, 2004. **33**(5): p. 1919-1923.
130. Saito, K., et al., *Detection of human serum tumor necrosis factor- α in healthy donors, using a highly sensitive immuno-PCR assay*. *Clinical Chemistry*, 1999. **45**(5): p. 665-669.
131. Zimmermann, B., et al., *Optimized real-time quantitative PCR measurement of male fetal DNA in maternal plasma*. *Clinical chemistry*, 2005. **51**(9): p. 1598-1604.
132. Crute, I.R., et al., *Variation in *Plasmodiophora brassicae* and resistance to clubroot disease in brassicas and allied crops - a critical review*. *Plant Breeding Abstracts*, 1980. **50**(2): p. 91-104.
133. Karling, J., *The Plasmodiophorales Hafner*. New York, 1968.
134. Strelkov, S.E. and S.-F. Hwang, *Clubroot in the Canadian canola crop: 10 years into the outbreak*. *Canadian Journal of Plant Pathology*, 2013. **36**(sup1): p. 27-36.
135. Colhoun, J., *A technique for examining soil for the presence of *Plasmodiophora brassicae* Woronin*. *Annals of Applied Biology*, 1957. **45**(3): p. 559-565.
136. Macfarlane, I., *Factors affecting the survival of *plasmodio-phora brassicae* wor. in the soil and its assessment by a host test*. *Annals of Applied Biology*, 1952. **39**(2): p. 239-256.
137. Takahashi, K. and T. Yamaguchi, *Assessment of pathogenicity of resting spores of *Plasmodiophora brassicae* in soil by fluorescence microscopy*. *Annals of the Phytopathological Society of Japan (Japan)*, 1989.
138. Lange, L., et al., *Serological detection of *Plasmodiophora brassicae* by dot immunobinding and visualization of the serological reaction by scanning electron microscopy*. *Phytopathology*, 1989. **79**(10): p. 1066-1071.
139. Orihara, S. and T. Yamamoto, *Detection of resting spores of *Plasmodiophora brassicae* from soil and plant tissues by enzyme immunoassay*. *Nippon Shokubutsu Byori Gakkaiho*, 1998. **64**(6): p. 569-573.
140. Ito, S., et al., *Cloning of a single-copy DNA sequence unique to *Plasmodiophora brassicae**. *Physiological and Molecular Plant Pathology*, 1997. **50**(5): p. 289-300.
141. Chye, S.-M., et al., *Immuno-PCR for Detection of Antigen to *Angiostrongylus cantonensis* Circulating Fifth-Stage Worms*. *Clinical Chemistry*, 2004. **50**(1): p. 51-57.
142. Harrison, J.G., R.J. Searle, and N.A. Williams, *Powdery scab disease of potato — a review*. *Plant Pathology*, 1997. **46**(1): p. 1-25.

143. Henkenjohann, S., *Highly Sensitive Fluorescent Methods for the Detection of Enzymes and the Determination of their Activity by Means of Specific Hydrolases*. Ph.D. Thesis Bielefeld University 2009().
144. Castlebury, L.A., J.V. Maddox, and D.A. Glawe, *A Technique for the Extraction and Purification of Viable Plasmodiophora brassicae Resting Spores from Host Root Tissue*. Mycologia, 1994. **86**(3): p. 458-460.
145. Jones, S.L., J.C. Cox, and J.E. Pearson, *Increased monoclonal antibody ascites production in mice primed with Freund's incomplete adjuvant*. Journal of immunological methods, 1990. **129**(2): p. 227-231.

6. SUPPLEMENTARY INFORMATION

The 17β -estradiol iPCR assay performance was assessed with cattle urine to demonstrate its efficacy with a complex biological matrix. The protocol was carried out as described previously (Chapter 2). Urine collected from a female cow (Agriculture and Agri-Food Canada feedlot in Lethbridge) along with standards were analyzed by iPCR. Urine (15 μ L) was mixed with an equal volume of serum diluted 1:40000. A 1:10 and 1:100 dilution of urine was prepared in Milli-Q water and mixed with the serum dilution as well. Additional samples were prepared by spiking undiluted urine with 100 ng mL⁻¹ and 100 pg mL⁻¹ of 17β -estradiol. RT-iPCR results obtained for the urine, diluted urine and spiked samples are presented in Table 6.1. The standard curve (ranging from 10 μ g mL⁻¹ to 1 pg mL⁻¹) is presented in Figure 6.1.

Table 6.1. Average Ct values of 17β -estradiol iPCR assay with urine samples (n=3)

Urine treatment	Ct (mean \pm SE)
Urine undiluted	27.2 \pm 0.19
Urine diluted 1/10	23.8 \pm 0.08
Urine diluted 1/100	23.9 \pm 0.08
Undiluted urine with 100 ng mL ⁻¹ 17β -estradiol	28.2 \pm 0.5
Undiluted urine with 100 pg mL ⁻¹ 17β -estradiol	27.1 \pm 0.18

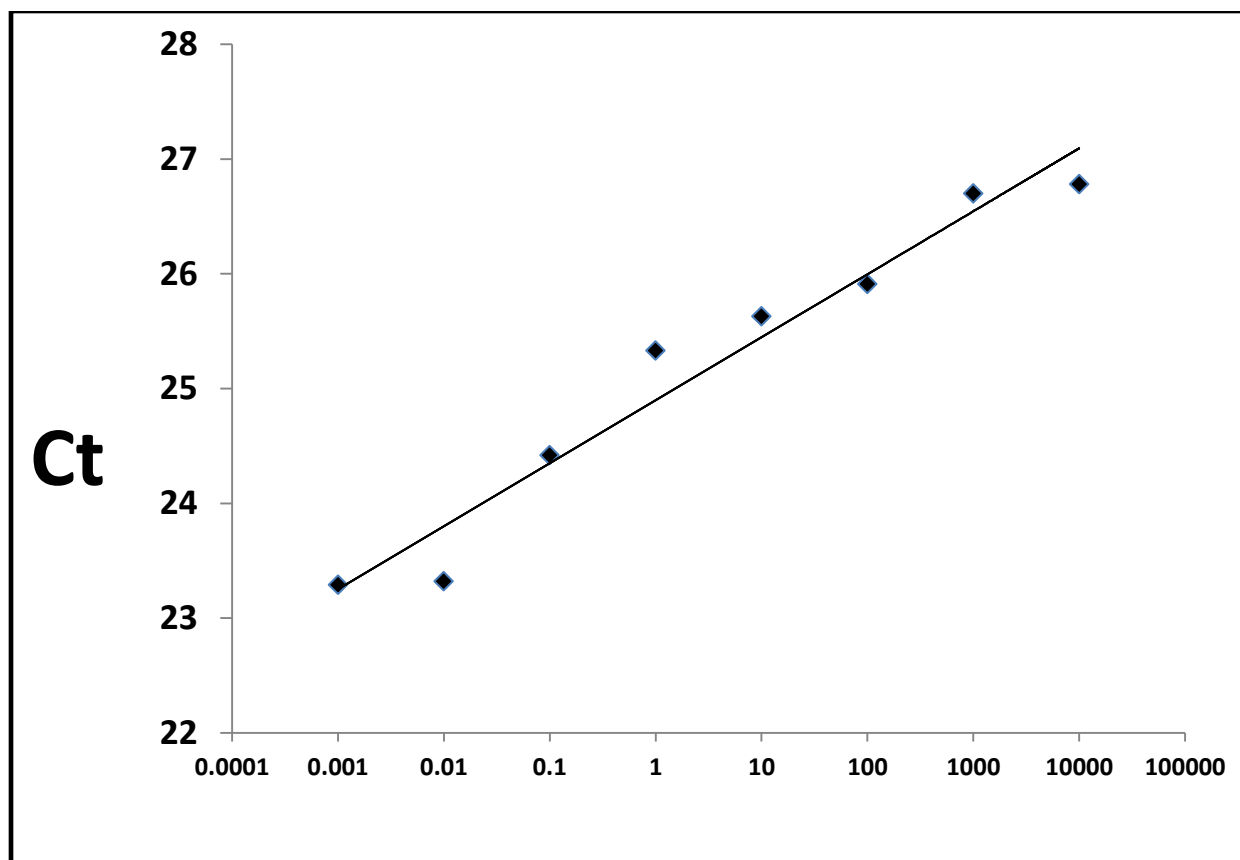


Figure 6.1. RT-iPCR standard curve: RT-iPCR was performed on a serial dilutions of known concentrations of 17β -estradiol corresponding to log-fold dilutions ranging from $10 \mu\text{g ml}^{-1}$ to 1pg ml^{-1} . Coefficient of determination: 0.955, $n=3$