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Inhibitory Effects of Food Matrices on Inhibition Real-Time Reverse Transcription Polymerase Chain Reaction Detection of Foodborne Viruses

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INHIBITORY EFFECTS OF FOOD MATRICES ON REAL-TIME
REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION DETECTION OF
FOODBORNE VIRUSES

by

KEVIN PATRICK MCMULLEN

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Public Health
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Inhibitory Effects of Food Matrices on Real-Time Reverse Transcription Polymerase Chain Reaction Detection of Foodborne Viruses

Kevin Patrick McMullen

ABSTRACT

The Centers for Disease Control and Prevention estimated 23,000,000 cases of viral gastroenteritis caused by Norovirus in 2000, 40% of which were transmitted by food including: a variety of fresh produce, cake, deli meats, fruit salad, cheeses and ice. (CDC, 2003). An estimated 83,391 cases of Hepatitis A virus was reported in 2000, of which 5% was attributed to foodborne transmission (CDC, 2003). These figures underscore an urgent need for a method that can isolate virus from a variety of food matrices.

The aim of this study was to develop an overall assessment of the inhibitory effects of a variety of food matrices on Real Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Additionally, to compare a sequence specific hybridization probe amplification format to a non sequence specific SYBR Green format using the Roche LightCycler. The secondary aim was to evaluate the effectiveness of a food virus concentration and isolation protocol under development at the Florida Department of Health Bureau of Laboratories, Tampa.

Three food specimens consisting of prepackaged smoked ham, fresh cilantro, and Thompson's green grapes were seeded with three dilutions of poliovirus 3 (Sabin strain). A viral concentration procedure under development at the Florida Department of Health

Bureau of Laboratories, Tampa was used to isolate the virus. Real Time RT-PCR was carried out on the Roche LightCycler in SYBR Green and Hybridization probe formats.

Spiking the virus-negative samples of each matrix with a dilution series of poliovirus 3 created post-flocculation spikes. This post-flocculation dilution series amplification allowed a standard curve to be created unique to each food matrix. The flocculation and concentrations specimens were then amplified and the standard curves from the post-flocculation seed were used to calculate the loss associated with the concentration procedure.

This study reports significant differences ($p < 0.05$) in recovery detected between the various matrices, and Real Time RT-PCR formats. The concentration protocol under development at the Florida Department of Health Bureau of Laboratories, Tampa, demonstrates a 12-78% recovery of seeded virus in a simulated “real world” virus contamination event among the various matrices.

Introduction

Noroviruses

Noroviruses (NV) previously known as Norwalk or Norwalk-like viruses (NLV's), and initially referred to as small round structured viruses (SRSV's) are members of the family Caliciviridae. NV's are approximately 27nm in diameter, nonenveloped, and icosahedral. The NV's capsid surrounds a linear, single-stranded, positive-sense RNA genomes that range from 7.3 to 8.3 kilo-bases containing three major open reading frames (ORF). ORF1 and ORF3 are in the same reading frame and encode for nonstructural proteins. ORF2 encodes for structural proteins and lies in a second reading frame (Green, 2001).

NV's are named after Norwalk, Ohio, where in 1968 the Centers for Disease Control (CDC) investigated an outbreak of acute gastroenteritis in an elementary school. Classical microbiological methods failed to yield an etiologic agent, until a bacterial-free stool filtrate was fed to volunteers. Those volunteers subsequently developed acute gastroenteritis. Bacterial-free stool filtrates were serially passaged to other volunteers who also developed gastroenteritis, each group experiencing a 50% attack rate (Dolin, 1972). In 1972, Kapikian et al. identified 27-32 nm viral particles from the experimentally passaged stool. The visualized particles were incubated with prechallenge and convalescent phase serum to confirm an association to the acute gastroenteritis (Kapikian, 1972). A thick layer of antibodies was observed surrounding the viral particles

incubated with the convalescent serum, indicating a specific serologic response, which the prechallenged specimen lacked.

NV's enter the body predominately by the oral route, although some evidence suggests virus may be transmitted via aerosols generated during violent vomiting that is often a symptom of illness (Caul, 1994). The primary replication site of NV in the gastrointestinal tract has not yet been experimentally determined, but is expected to be in the upper intestinal tract. Jejunum biopsies of volunteers that developed gastrointestinal illness after administration of Norwalk virus exhibited histopathic lesions characterized by blunting and broadening of the small intestine (Agus, 1973; Schreiber, 1973). Meeroff et al. observed a marked delay in gastric emptying in infected volunteers who became ill and had jejunal mucosal lesions; they proposed abnormal gastric motor function is responsible for the nausea and vomiting that is associated with the illness (Meeroff, 1980).

NV's are highly infectious and only a small inoculum of 10 to 100 virions is required; attack rates range from 50% to 90% (Sheih, 2000). NV's symptoms include vomiting, diarrhea, nausea, and abdominal cramps typically lasting less than 72 hours. The incubation time generally ranges from 12-48 hours, and is communicable during the first stage and at least up to 48 hours after diarrhea subsides. RT-PCR has detected shedding at least up to seven days after the symptomatic illness ends. No long-term immunity has been demonstrated with these viruses, so an individual is susceptible throughout their life (Graham, 1994).

NV's are acid stable and can remain infective in stool filtrate at pH 2.7 for three hours at room temperature, and at 60°C for 30 minutes (Dolin, 1972). They are also

resistant to ether and 0.5 to 1mg/L free residual chlorine, which allows them to survive in treated chlorinated water (Schaub, 2000).

NV outbreaks are the most common cause of gastroenteritis in the United States. The CDC estimates 23 millions cases of acute gastroenteritis annually are cause by Norovirus. Typically peaking in cooler months, outbreaks occur worldwide in all age groups throughout the year (Mounts, 2000). Foodborne outbreaks have been attributed to the consumption of raw oysters, salads, deli meat, and cake frosting (Kuritsky, 1984). Outbreaks have also been associated with drinking fecally contaminated water (Schaub, 2000; Wilson 1982), raw salads, cake frosting (Kuritsky, 1984), consumption of raw or undercooked contaminated oysters (Lees 1995; Le Guyader 1996), and contaminated delicatessen foods (Schwab, 2000), in addition to person-to-person contact.

These outbreaks have proved difficult to control. Since the illness is self-limiting and the symptoms subside in two or three days patients rarely visit their physician, therefore many illnesses go unreported (Mounts, 2000). Extended periods of asymptomatic shedding of Norovirus has a profound impact on the management of outbreaks involving infected food handlers. Food handlers returning to work after symptoms subside may still be shedding virus for several days. General universal sanitary measures such as effective hand washing and proper disposal and/or disinfection of contaminated material may decrease transmission. In the case of oysters, care must be taken not to contaminate oyster beds contaminated with feces or sewer effluent (Shieh, 2000).

Hepatitis A Virus

Hepatitis A virus (HAV) is a Hepatovirus belonging to the family *Picornaviridae*. HAV is nonenveloped and approximately 27-32 nm in diameter was first described by Feinstone et al.(Feinstone, 1973). The genome consists of approximately 7.5 kilobases of single stranded RNA, containing a single (ORF) that encodes capsid and nonstructural proteins. The ORF is flanked by a highly conserved 5' nontranslated region (NTR) and a 3' poly (A) tail consisting of 40 to 80 nucleotides (Bradley, 1984). HAV was initially classified as an enterovirus due to its biophysical and biochemical features. Later studies demonstrated nucleotide and amino acid sequences, and protein sizes differ from that of enteroviruses. Unlike enteroviruses, HAV only has one known serotype (Emerson, 1996). Also, HAV replicates very slowly in tissue culture often with no cytopathic effects, and is resistant to pH and temperatures that inactivate enteroviruses (Hollinger, 2001).

In the 17th to 19th centuries outbreaks of jaundice among diverse populations were recorded. The disease, called campaign jaundice, was common among the military. Studies using human volunteers conducted during and after World War II confirmed the viral etiology of the disease named hepatitis A, and differentiated it from serum jaundice later known as hepatitis B which has a longer incubation period (Havens, 1946; Boggs, 1970).

HAV's host cell receptor has not yet been identified, but some studies suggest it may be a class I glycoprotein (Kaplan, 1996; Ashida 1997). Incubation period is from 10-50 days, with greater doses reducing the incubation period (Havens, 1946). HAV is shed in feces up to 10 days before clinical symptoms appear. Transmission is the greatest concern at this phase. Infectious HAV has been found in patients as early as two weeks

before and 8 days after jaundice occurs (Havens, 1946; Krugman 1959). Rosenblum et al. reported detecting HAV RNA in infant stools up to 5 months after they were diagnosed as infected (Rosenblum, 1991).

Infection with HAV usually follows ingestion of the virus. The infected may experience anorexia, fever, fatigue, nausea, malaise, diarrhea (in children), and vomiting. The icteric phase is characterized by golden-brown urine and yellowing of the mucosal membranes, conjunctivae, and skin. Liver functions are compromised and serum bilirubin remains elevated above 10mg/dL for more than 12 weeks (Hollinger, 2001). Although there is slow resolution of the disease, patient recovery is usually complete.

HAV is relatively resistant to heat. At neutral pH it is only partially inactivated after 10 to 12 hours at 60°C. With a relative humidity of 42% at 25°C, HAV infectivity can be maintained for 1 month after drying (Sobsey, 1988). HAV has been found to persist days and even months longer than poliovirus in contaminated freshwater, seawater, live oysters, and even crème filled cookies (Sobsey, 1988). HAV is inactivated by UV radiation (1.1W for 1 minute), formalin (8% at 25°C for 1 min.), iodine (3mg/L), and by free chlorine, 2.5ml/L for 15 minutes (Siegl, 1984).

Enteroviruses

Poliovirus is a 30 nm, spherical, nonenveloped enterovirus belonging to the family *Picornaviridae*. Enteroviruses contain a single strand positive sense RNA genome that ranges from 7,209 to 8,450 bases in length that contains a single long ORF (FV). The ORF is divided into three regions: P1 which encodes the capsid proteins, and the P2 and P3 regions that encode for protein processing and genomic replication (Forss, 1982).

Poliomyelitis was first clinically described in the 1800s when physicians reported cases of paralysis with fever (Ranciello, 2001). During the 1900s there were great advancements in the understanding of the infectious nature the poliovirus. Subsequent work has had a significant impact on the field of molecular biology; poliovirus was the first animal virus cloned and sequenced, and the first human virus to have its three-dimensional structure verified by x-ray crystallography (Pallansch, 2001).

Polioviruses enter the cell via receptor CD155, an integral membrane protein and member of the immunoglobulin superfamily of proteins. CD155 is thought to be the only receptor required for poliovirus binding and entry into the cell (Mertens, 1983). Expression of this receptor on cell lines from several animal species leads to susceptibility to poliovirus infection (Langford, 1988).

Most enterovirus infections, including poliovirus, are asymptomatic; but infection can lead to serious illness in infants and immunocompromised individuals. Enteroviruses are the most common cause of aseptic meningitis (Ranceillo, 2001).

Polioviruses are thermostable, but less so than HAV (Pallansch, 2001). They are stable and remain infective at pH values of 3.0 and lower (Ackerman, 1970). Polioviruses are somewhat thermostable, most are inactivated at 42°C. UV light, strong acid, formaldehyde, gluteraldehyde, and sodium hypochlorite readily inactivates these viruses when free in solution (Ackerman, 1970). The degree of viral loss depends on the presence of organic material (Abad, 1994).

Poliovirus 3 Sabin strain was chosen as a model for NV's and HAV and enteroviruses due to its similar environmental tolerances. Its ability to be readily grown in tissue culture allows the quantitative analysis that could not be easily replicated with

NV's or HAV. In addition, the poliovirus's proclivity to bind very tightly to substrates makes it a good model for examination of the flocculation and elution protocols. The intrinsic safety of manipulating this attenuated, vaccine strain virus was also taken into consideration.

Traditional RT-PCR

Reverse-Transcription Polymerase Chain Reaction (RT-PCR) is a molecular biological technique capable of amplifying one copy of a RNA target approximately 500,000,000 times in 35 cycles. Viral genomes, viable and non-viable particles, copies present in quantities below the detection limit of classical virological techniques are amplified to detectable quantities, usually within 1.5 to 3 hours. The amplified products are separated and identified by length in an ethidium bromide poly-acrylamide gel. When available, specific internal probes via Southern Hybridization identify the products. This entire process may take from 8 to 24 hours.

Light Cycler Real™ Time RT-PCR

The Roche Light Cycler™ (LC) is a rapid thermal cycler that utilizes alternated heated and ambient air as a medium for rapid temperature transfer (LC manual). The RT-PCR reaction and detection takes place in a closed tube format in approximately 60 to 75 minutes. The LC can operate in a sequence dependent (hybridization probe) or sequence independent (SYBR Green) format. The fluorescence is detected in each cycle, and amplification curves can be monitored as the reaction is proceeding on a computer monitor.

Advantages and Disadvantages

A major advantage of traditional RT-PCR is that it is currently being conducted, or can be conducted in almost any laboratory with minimal start up costs. A Southern hybridization probe assay can be conducted to confirm target when probes are available. A major disadvantage is that it is only an endpoint detection assay. Only the plateau phase of the amplification reaction is visualized in the poly-acrylamide gel. The reaction tubes have to be opened to load the polyacrylamide gels, which can lead to cross contamination of samples. Also, amplification, gel visualization, and southern hybridization may take up to 24 hours.

A major advantage of Real-Time RT-PCR is its ability to quantify samples, amplify, and detect target in a closed tube format, reducing potential cross-contamination, within 60 to 75 minutes. Real-Time RT-PCR's primary disadvantage is the high initial capital expenditure for the machine. Also, hybridization probes are not as readily available as in traditional RT-PCR and Southern hybridization.

Literature Review

Food Viral Detection

Detection of human enteric viruses in food is hindered by a myriad of problems. The most formidable is that the viruses do not replicate in the food, unlike many bacterial pathogens (Richards, 1999). Until recently, the study and the detection of these viruses has been a slow evolution primarily because most of these viruses have been difficult, if not impossible to propagate in mammalian cell culture lines, and therefore can not be studied by traditional virological methods (Richards, 1999).

During the last decade, advances in molecular biology has made it possible to detect viral presence in clinical, food, and water samples without classical tissue culture techniques. Polymerase Chain Reaction (PCR) and more specifically RT-PCR have allowed the amplification of RNA genomes including poliovirus, hepatitis A, and Norwalk viruses.

There are limitations to using present molecular biological techniques with food specimens. First, is the labor-intensive virus isolation that requires multiple steps, and skilled personal. This isolation process must extract the virus, release, purify, and concentrate the nucleic acids (Richards, 1999). Added to this is the need to remove inhibitors inherent in the matrix that may otherwise interfere with downstream PCR applications. These processes involve the use of toxic compounds such as, trichlorotrifluoroethane, guanidinium isothiocyanate, phenol, and chloroform, and must

be completed in the least amount of steps to minimize viral loss. This compounded with already minute quantity of virus present in the food sample makes this a challenging task.

In addition, sample size must also be taken into consideration. A large sample size is usually required to detect low viral concentrations virus in naturally contaminated environmental specimens (Leggitt, 2000).

There is an urgent need for a reliable, repeatable, cost-effective protocol to detect non-culturable viruses in food samples. To date, the most extensive work has been performed on shellfish (Leggitt, 2000). As filter feeders, shellfish are natural concentrators of viruses, bacteria, and toxins. Culinary practices of eating these mollusks raw or undercooked poses a substantial health risk (Shieh, 2000). Since the cloning and characterization of the NLV genome in the 1990's, they have been found as the primary etiological agents among reported cases of infectious diseases associated with shellfish consumption. In 1999 Shieh et al. proposed a method to detect low levels of enteric NVs in shellfish. The method involves an acid-adsorption and elution step, followed by a PEG-precipitation, solvent extraction, a 2nd PEG precipitation, RNA extraction, and assayed by traditional gel based RT-PCR and Southern hybridization (Sheih, 1999). Cromeans proposed a detection protocol for hepatitis A virus that also included homogenization of oyster meat in sterile water, centrifuged and resuspended in a glycine buffer (pH 9.5) to elute the virus, treat with trichlorotrifluoroethane, and the extracts combined and tested by immunocapture PCR (Cromeans, 1997). The sensitivity of the RT-PCR assay was determined by serial dilutions of cell culture derived HAV. The immunocapture RT-PCR reported detection consistently at 0.5 PFU (Cromeans, 1997).

The detection of NV's and HAV in other food poses a greater problem since these foods do not concentrate the virus like the bivalve mollusks. Furthermore, each food matrix may contain unique compounds inhibitory to RT-PCR detection. Ill food handlers most commonly contaminate these foods (Schwab, 2000). Schwab et. al was able to isolate and amplify Norovirus G2 in a sample of ham taken from a Texas university cafeteria that was the suspected vehicle of transmission (Schwab, 2000). Sequencing of the PCR amplicons revealed the ham had 283 base pairs in common with the Norovirus isolated from the outbreak stool specimens. This sequence was identified Norovirus Genogroup 2, Lordsdale cluster.

Outbreak-associated NV's and HAV's are a major health concern worldwide. Research to date has had only limited success in directly linking food associated with NV or HAV to the strain identified in the stools. The 1999, the CDC's estimated that 96% of the non-bacterial gastroenteritis in the United States are caused by NV's (Schaub, 2000). It is of the utmost importance that an efficient, reliable, and sensitive protocol be developed to detect these viruses.

This thesis presents an assessment of a method under development at the Florida Department of Health Bureau of Laboratories, Tampa, for virus concentration, isolation, and Real-Time RT-PCR based detection protocol from four distinct food matrices. It examines each matrix's effect on real-time RT-PCR detection in sequence and non-sequence specific formats to create a unique inhibition profile for each matrix.

Materials and Methods

Seed Virus Propagation

Poliovirus 3 (Sabin strain) was propagated in a monolayer of BGM cells grown in Corning 25cm² cell culture bottles. The poliovirus 3 cell culture was frozen and thawed, an aliquot diluted 1:10 in Earles Balanced Salt Solution (BBSS, Sigma#E6132), and 1 ml was then inoculated onto BGM cells five corning 25cm² flasks. An additional flask was inoculated with 1 ml of EBSS. The flasks were placed on a rocker at 37°C, and the virus was allowed to adsorb for one hour.

Nine milliliters of Eagles Minimal Essential Medium. Earle's Salts (EMEM, Sigma#M0643)/ 5% fetal calf serum (FCS) was then added to the flasks and they were monitored by microscope every 12 to 18 hours. After 48 hours the cells were over 90% lysed; the bottles were frozen at -75°C.

A poliovirus 3 stock flask was thawed in a 37°C water bath, frozen at -70°C and thawed again. The contents of the bottle was transferred to a sterile 15ml Falcon tube and centrifuged at 5,000 RCF for 10 minutes in a Beckman J6B centrifuge with a Beckman 5.2 swinging bucket rotor. One hundred and twenty five microliters of the supernatant was pipetted into each of 96 sterile, tapered, 500µl micro centrifuge tubes and frozen at -75°C.

Seed Virus Quantification

Three separate 125µl seed stocks were thawed at 37°C. Each was serially diluted in log increments to a 10⁻⁸ dilution in EBSS. A 100µl aliquot of each dilution from 10⁻⁵ to 10⁻⁸ from each aliquot was inoculated into 5 wells in a six well tissue culture plate leaving the sixth inoculated with EBSS as a cell control. The virus was allowed to adsorb to the cells for one hour at 37°C/ 5% CO₂. The cells were then fed with 4 ml of EMEM/ 5% FCS and placed back in the incubator. Examination for cytopathic effect (CPE) was at four and five days post inoculation. The virus titer per 100µl was determined to be 10^{7.49} TCID₅₀ (30,902,954 TCID₅₀) for two of the seed aliquots and 10^{7.5} (31,622,776 TCID₅₀) for the third aliquot. The results were averaged and rounded to 31,000,000 TCID₅₀ per 100µl virus stock.

Matrix Preparation

Matrices consisted of pre-sliced and prepackaged smoked deli ham (3 slices), fresh whole leaf and stem cilantro (17-19 stalks), Thompson's green seedless grapes (11-15 grapes), and strawberries (6 berries) all purchased the day before seeding. Each food was first weighed on an analytical balance and divided into four 600ml polypropylene beakers per food type, then stored, covered aluminum foil with at 4°C until seeding (Table 1).

Table 1 Food Matrix Weights for Flocculation Specimens

Sample I.D.	BBE†	Sample I.D.	Ham (g)	Sample I.D.	Cilantro (g)	Sample I.D.	Grapes (g)	Sample I.D.	Strawberries (g)
1	n/a	5	56.40	9	17.55	13	80.50	17	142.50
2	n/a	6	56.85	10	17.45	14	76.30	18	139.20
3	n/a	7	56.35	11	17.65	15	76.40	19	133.45
4	n/a	8	55.65	12	17.90	16	79.85	20	139.55

†Specimens 1-4 that are matrix negative specimens, so no matrix weight is applicable.
Viral Seed Preparation

A 125µl aliquot of Poliovirus 3 was thawed in a 37°C water bath. Seven 2.0ml Eppendorf tubes were labeled, and appropriate quantities of RNase free water (table 4) was pipetted into each. The poliovirus 3 aliquot was vortexed quickly on highest setting and a 64.6µl portion was removed with a Ranin 100µl micropipette and diluted in 935.4 µl of RNase free water to yield 2,000,000 TCID₅₀ /100µl. This poliovirus 3 seed stock was then diluted in log₁₀ increments to 20 pfu/100µl (Table 2).

Table 2 Poliovirus 3 Dilution Series

Log	10 ⁻¹ (µl)	10 ⁻² (µl)	10 ⁻³ (µl)	10 ⁻⁴ (µl)	10 ⁻⁵ (µl)	10 ⁻⁶ (µl)
Poliovirus 3	64.5	100	100	100	100	100
Water	935.5	900	900	900	900	900
TCID ₅₀ / 100µl	2,000,000	200,000	20,000	2,000	200	20
TCID ₅₀ / 10µl	200,000	20,000	2,000	200	20	2

†The 100µl volume will be used to seed the flocculation matrix samples.

‡The 10µl volume will be used to spike the post flocculation spike samples.

Seed Virus Verification

An additional 10µl of the afore mentioned seed stock was diluted into 990µl water of EBSS to yield a 10⁻² dilution to prepare a back titration. This was serially diluted to a 10⁻⁸ dilution of stock. Each dilution from 10⁻⁵ to 10⁻⁸ was inoculated into 6 wells

plates. They were examined by microscope at four and five days post inoculation. The titer was confirmed to be $10^{7.49}$ TCID₅₀ per 100µl of stock virus.

Viral Seeding

The food specimens were removed from the refrigerator and the test specimens (Table 3) were seeded with a 100µl of poliovirus 3 containing 200,000; 20,000; or 2,000 TCID₅₀ in a 100µl total volume. The control specimens were seeded with 100µl RNase free water. Seed was applied dropwise from a Ranin 100µl pipettor on an exposed food surface. Seeded specimens were covered and allowed to dry for 2½ hours in a Bio Safety cabinet (type A/B3) at ambient temperature, then placed at 4°C until the next morning. The viral seed dilutions were subjected to the same temperature conditions. Total drying time was approximately 24 hours. The viral seed drying process was designed to mimic a real world contamination event by food handler preparing food in advance for a buffet or holiday style gathering. In this scenario, food would be left out for the initial event then placed in the refrigerator, and possibly consumed the next day as “leftovers”.

Table 3 Viral Seeding (TCID₅₀)

I.D.	†BBE	I.D.	Ham	I.D.	Cilantro	I.D.	Grapes	I.D.	Strawberries
1	(-)‡	5	(-)‡	9	(-)‡	13	(-)‡	17	(-)‡
2	200,000	6	200,000	10	200,000	14	200,000	18	200,000
3	20,000	7	20,000	11	20,000	15	20,000	19	20,000
4	2,000	8	2,000	12	2,000	16	2,000	20	2,000

†Buffered Beef Extract eluate only controls

‡ Virus negative specimens seeded with 100µl water

Flocculent Preparation

Bacto™ Beef Extract (BBE, Difco catalogue #0115-17) 30g/L and glycine (Sigma #G8770) 4.5g/L was dissolved in reagent grade water, and autoclaved (15 min., 121°C). Tween 20 (20ml/L) was added and the pH adjusted to 7.5 with 5N HCl.

Viral Flocculation and Concentration

The flocculation specimens (Table 1, #'s 1-20) were removed from the 4°C refrigerator and 200ml 3% BBE flocculent was added to each of the beakers. The beakers were vortexed on a Glas-Col Multi-Pulse Vortexer, at a motor speed of 40 for 15 minutes, and were then transferred to a Lab-Line Instruments Inc., L.E.D. Orbital Shaker at 125 rpm for 15 minutes. The eluent was decanted into a 250ml polypropylene centrifuge bottle containing a 2.5 cm teflon coated magnetic stir bar, leaving the food matrix in the beaker to be discarded. Any eluate that adhered to the matrix was also discarded. The eluent pH is adjusted to 3.5 (\pm 0.1) by the addition of 5N HCl, and stirred slowly for 30 minutes to allow the BBE proteins to form a large floc that co-precipitates the virus. The pH is checked periodically to be sure it is still at 3.5 (\pm 0.1). The stir bar is removed and the bottles centrifuged at 4,200 g for 20 minutes (Beckman J6B). The supernatant was discarded, the precipitate resuspended in 9 ml of 4M guanidine isothiocyanate (GITC) lysis buffer (Organon Technica #284407), and transferred to a 15ml sterile polypropylene Falcon conical tube. At this point the virus capsid is lysed and the RNA is in solution; it is protected from degradation by RNases by GITC.

Samples were frozen in a -70°C freezer, quick thawed in a 37°C water bath, and centrifuged for 15 minutes at 4,200 g (Beckman J6B). The supernatant was decanted into

another 15 ml sterile polypropylene Falcon conical tube, the volume recorded, and the precipitate discarded. This freeze thaw facilitates the breaking up of remaining food matrix that may bind and precipitate viral RNA in the centrifugation step.

Post-Flocculation Matrix Spikes

One –tenth of the total resuspended volume from each negative flocculation matrix specimen was transferred to seven 1.5 ml RNase free micro centrifuge tubes. Ten microliters of each of the viral seed dilutions (200,000; 20,000; 2,000; 200; 20; 2 TCID₅₀) was spiked into each matrix. Ten microliters of water pipetted into the remaining tube to serve as the negative control. The spiked samples were incubated at ambient temperature for 10 minutes and frozen at –70°C. These samples will serve as post-flocculation spikes to determine the viral loss due to the concentration procedure.

Viral RNA Isolation

Approximately 900µl of lysate from the post flocculation spiked specimens, and 10% of each positive flocculation specimen (~900 µl, Table 1, #1-20) was processed for RNA isolation with Qiagen RNeasy RNA isolation kits (Qiagen #74104) (Appendix B). RNA was eluted in 100µl RNase free water.

QIAshredder

The QIAshredder (Qiagen #79654) is a homogenizing system utilizing a biopolymer filter in spin-column format. Qiagen claims the biopolymer filters out insoluble debris and reduced sample viscosity. A Qiagen technical representative

suggested the Qias shredder might remove some inhibitory compounds from our food viral lysates

One-tenth of each lysed 20,000 TCID₅₀ flocculation specimen (Table 3, #'s 3, 7, 11, 15, and 19) from BBE, Ham, Cilantro, and green grapes was aliquoted into two RNase-free micro-centrifuge tubes. One tube from each matrix was processed with a Qias shredder step immediately before RNA isolation using the Qiagen RNeasy kit. The RNA from the other set of tubes was isolated using the Qiagen RNeasy isolation kit without pretreatment (Appendix B). The RNA in both sets was eluted in 100µl RNase free water.

Five microliters of each specimen was amplified in triplicate using the hybridization probe format in the Roche LightCycler.

Detection

Pan enterovirus primers, dubbed ENT 3 and ENT 4, were used to amplify the poliovirus 3 RNA. Ent 3 and 4 primers amplify a 196-nucleotide segment from the 5' non translated region of the poliovirus genome (Sheih, 1997). The pan enterovirus 1 and 2 hybridization probes, developed at the Florida Department of Health Bureau of Laboratories, Tampa, bind near the 3' end of the amplified segment.

Pan Enterovirus Primer Antisense

Ent 3 5'-CCT-CCG-GCC-CCT-GAA-TG-3'

Panenterovirus Primer Sense

Ent 4 5'-ACC-GGA-TGG-CCA-ATC-CAA-3'

Pan Enterovirus Hybridization Probe 1

5'-CTG-TCG-TAA-CGC-GCA-AGT-CYG-TGG-C X

Pan Enterovirus Hybridization Probe 2

5'-LCRed640-ACC-GAC-TAC-TTT-GGG-TGT-CCG-TGT-TTC-A-p

Real-Time PCR Optimization

The Roche LightCycler was used for the Real-Time RT-PCR detection of viral RNA. The MgCl₂ concentration was optimized for the poliovirus 3 template amplified with Ent 3 and Ent 4 primers, and Ent 1 and Ent 2 probes using the Qiagen Quantitech™ RT-PCR Probe (Qiagen #204443) and SYBR green kits (Qiagen #204243). Poliovirus 3 RNA at 50 TCID₅₀/5µl and 5 TCID₅₀/µl that was previously isolated with the Qiagen RNeasy Kit RNA, was used as the template.

One master mix each for the hybridization probe kit and the SYBR green kit was prepared. Each kit was run simultaneously in two LightCyclers. Three replicates were run for each concentration of virus from 2.5mM to 4.0mM in 0.5mM increments.

Real-Time RT-PCR

Five microliters (1/20th) of the 100µl RNA eluate was amplified. The final RNA input values ranged from 1,000 to 10 TCID₅₀ in log increments (Figure 1). The post flocculation spike dilutions final RNA input that range from 10,000 to 0.1 TCID₅₀ in log increments (Figure 1).

Figure 1

Final Virus TCID₅₀ Input in the Amplification Assay Flow Chart

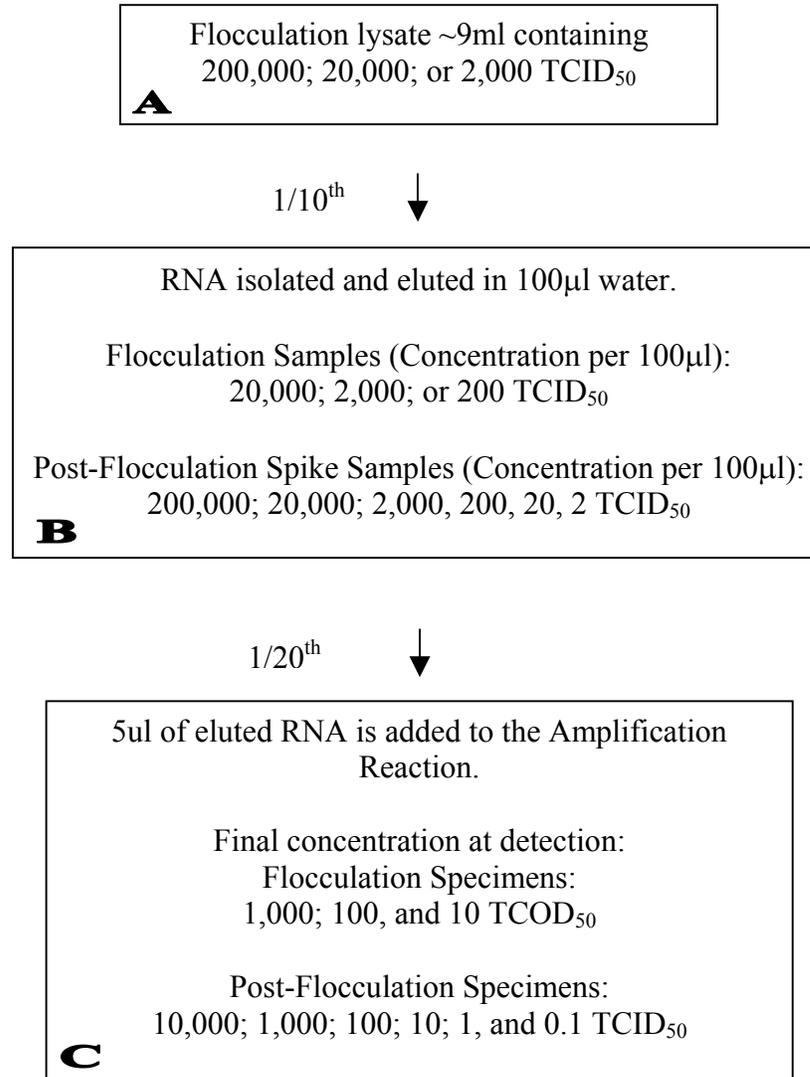


Figure 1. Viral TCID₅₀ calculations at amplification. One-tenth of the flocculation lysate (~1ml) (A) RNA is isolated and eluted in 100µ water (B). The matrix virus negative samples are then spiked with 10µl of 200,000 to 2 TCID₅₀ into 1/10 (~1ml) of negative sample flocculation lysate (B). 1/20 of the RNA eluate is amplified yielding 1,000 to 10 TCID₅₀ final viral concentrations for the flocculation samples, and 10,000 to 0.1 TCID₅₀ final viral concentrations for the post flocculation spike samples.

Post-Flocculation Spike Amplification

The flocculation and concentration matrix-negative, and extraction-negative specimens were assayed first to verify no cross-contamination occurred between the negative and positive samples. Post-flocculation spike amplifications were assayed in each format (SYBR Green and hybridization probe) one for each food and a virus control (total 6). These assays provided a post-flocculation standard curve. Three replicates of the 10,000- 100 TCID₅₀ dilutions and six replicates of the 10 to 0.1 dilutions were amplified.

Data was then analyzed for each food and standard curves created to determine the level of inhibition before proceeding to the amplification of the specimens. At this time, the strawberries were determined unsuitable for further processing, due to the lack of amplification in the post-flocculation spikes below 10,000 TCID₅₀.

The flocculation-seeded food specimens (Table 1, #'s 1-20) were then amplified in each format. Three replicates of the 1,000 TCID₅₀, four replicates of 100 TCID₅₀, and five replicates of the 10 TCID₅₀ in buffered beef extract, ham, cilantro, and green grapes matrices were assayed unless otherwise indicated. In addition 10,000, 1,000, and 100 TCID₅₀ post-flocculation spike specimens were run with each food matrix to monitor the inter-run variation of the standard curve created by the post-flocculation spike amplifications.

Statistical Data Analysis

Student t-tests and one-way ANOVA's were calculated in Microsoft's Excel (Microsoft Corporation, 2000). Tukey's HSD (honestly significant difference) test was calculated manually (Kuzma, 1998).

Results

Real-Time RT-PCR Optimization

A paired t-test demonstrated no significant difference ($p>0.05$) in crossing points or channel 1 fluorescence (F1) values observed in the 50 TCID₅₀ template concentration. At the 5 TCID₅₀ template only the 2.5mM MgCl₂ concentration amplified all three replicates in the hybridization probe format (Table 4). The 2.5mM MgCl₂ concentration in the SYBR green format produced the highest average F1 values (Table 4). Therefore, the 2.5mM MgCl₂ concentration was chosen for the experiments.

Table 4 MgCl₂ Optimization

Hybridization Probe Crossing Points					SYBR Green Crossing Points				
MgCl ₂ (mM)	Poliovirus 3 TCID ₅₀	Average C.P.	Std. Dev.	C.V.	MgCl ₂ (mM)	Poliovirus 3 TCID ₅₀	Average F1†	Std. Dev.	C.V.
2.5	50	33.84	0.34	0.99	2.5	50	0.92	0.05	5.43
3.0	50	33.28	0.14	0.42	3.0	50	0.86	0.11	12.79
3.5	50	33.32	0.21	0.64	3.5	50	0.83	0.04	4.82
4.0	50	33.38	0.10	0.30	4.0	50	0.75	0.02	2.67
2.5	5	34.68	0.32	0.91	2.5	5	0.91	0.06	6.59
3.0	5	34.36‡			3.0	5	0.88	0.01	1.13
3.5	5	34.53‡			3.5	5	0.56	0.11	1.96
4.0	5				4.0	5	0.36‡		

† The F1 value measures total fluorescence of the product at the target DNA melting temperature. This value is obtained at the end of the PCR run and is similar to fluorescence reading of a target in a traditional polyacrylamide gel.

‡ In these concentrations only one replicate amplified, therefore the standard deviations and C.V.'s are not available.

QIAshredder

A t-test detected a significant difference ($p < 0.05$) was observed in crossing points in the BBE and ham matrices. Crossing points were lower (i.e. larger copy input or less inhibition observed) for these matrices without the use of the QIAshredder. No significant difference ($p > 0.05$) was observed with the cilantro or green grape matrix. Therefore, the RNA isolations were conducted without the QIAshredder.

Standard Curves

Standard curves for each matrix and control were created with the LightCycler Fit Point analysis software (Roche, 2000). This analysis tool allows the user to adjust the “noise band” to a level where the samples log phase growth curves are parallel. Parallel amplification curves in the log growth phase allow a comparison and quantification of samples (Figure 3).

Poliovirus 3 post-flocculation matrix spikes in water, buffered beef extract, ham, cilantro, and green grapes standard curves, were calculated from the LightCycler crossing points for the 10,000; 1,000; 100; and 10 TCID₅₀ dilutions. Standard curves for some matrices could be calculated to include the 1 TCID₅₀ dilution, however, to maintain uniformity standard curves were only calculated to 10 TCID₅₀. Inter assay variation was monitored in each matrix flocculation amplification assay by inclusion of a 10,000; 1,000; and 100 TCID₅₀ sample from the post flocculation spike, creating an internal standard curve. The slope from this internal standard curve was compared to the post- flocculation spike standard curve. All internal standard curves slopes that were

amplified in the flocculation assay were equivalent to the standard curve created from the post-flocculation spike specimens within ± 0.05 (Table 5, figure 2 and 3). The slope of the standard curve, y-intercept, and sample crossing point was used to calculate the TCID₅₀ of virus recovered in each sample. The coefficient of correlation was -1.00 in all standard curves indicating a near perfect linearity of data points.

Table 5 Standard Curve Analysis
Virus in Matrix Hybridization Probe

Virus in Matrix Hybridization Probe				Virus in Matrix SYBR Green			
Matrix	slope	intercept	r	Matrix	slope	intercept	r
water	-3.62	34.83	-1.00	water	-3.39	28.26	-1.00
BBE	-3.42	36.64	-1.00	BBE	-3.35	29.63	-1.00
ham	-2.97	33.71	-1.00	Ham	-2.99	38.07	-1.00
cilantro	-3.24	37.89	-1.00	cilantro	-3.25	32.13	-1.00
green grapes	-3.47	37.36	-1.00	green grapes	-3.46	33.91	-1.00

Note. r represents the coefficient of correlation.

Figure 2: Hybridization Probe Food Matrix Standard Curves

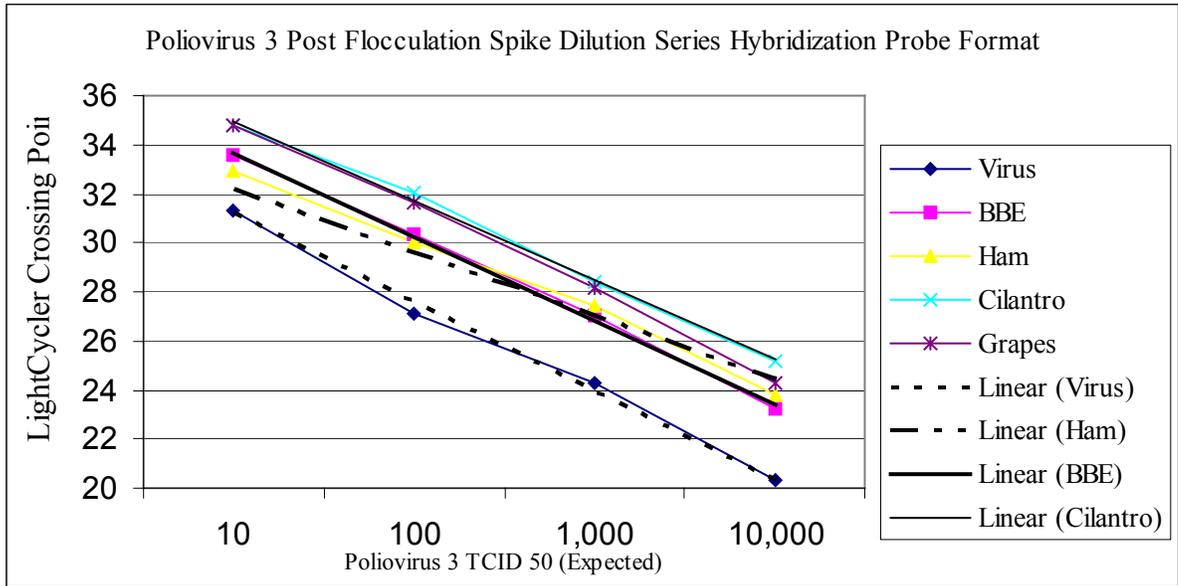


Figure 2. This figure depicts standard curves created by the hybridization probe post flocculation spike amplification reactions. The actual crossing point values are represented by colored symbols. The slope and y-intercept of the linear regression (black lines), and crossing points for the flocculation specimens (not shown) were used to determine the recovered TCID₅₀.

Figure 3: SYBR Green Food Matrix Standard Curves

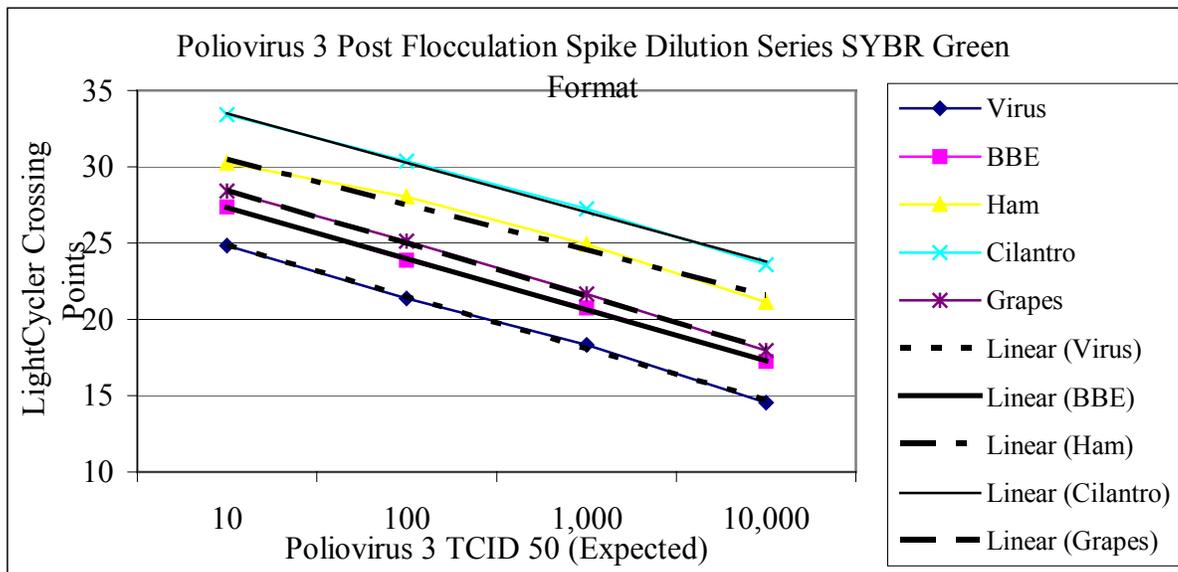


Figure 3. This figure depicts standard curves created by the SYBR Green post flocculation spike amplification reactions. The actual crossing point values are represented by colored symbols. The slope and y-intercept of the linear regression (black lines), and crossing points for the flocculation specimens (not shown) were used to determine the recovered TCID₅₀.

Figure 4: LightCycler Real-Time Amplification

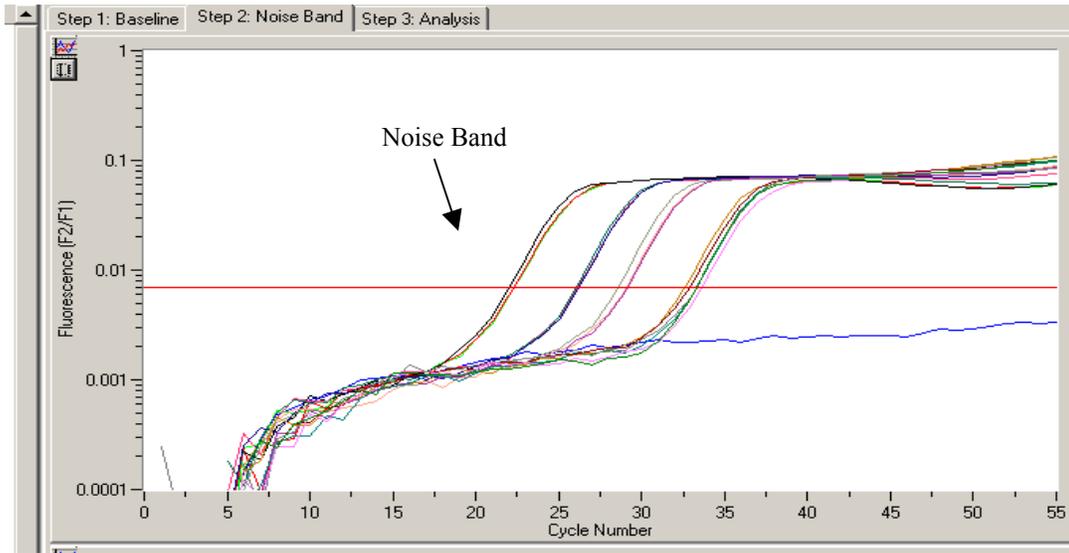


Figure 4. Above is a LightCycler noise band adjustment screen for the LightCycler amplification for the cilantro standard curve assay. The noise band (shown in red) is adjusted so it bisects each set of dilution amplification curves at a point where every sample is parallel to every other sample. This allows a comparative analysis of the samples.

Virus Recovery

Viral flocculation recoveries were calculated using post-flocculation spike $TCID_{50}$ and observed $TCID_{50}$ values obtained by applying the matrix regression equation to the corresponding crossing point for the seeded samples. The average values were calculated and the observed $TCID_{50}$ value was divided by the seeded $TCID_{50}$ value to obtain a percent recovery for the 1000 $TCID_{50}$ and 100 $TCID_{50}$ flocculation samples (Tables 6). The 10 $TCID_{50}$ samples amplification curves did not parallel the 1000 $TCID_{50}$ and 100 $TCID_{50}$ curves, so they could not be the quantitative recovery calculations. The cilantro 100 $TCID_{50}$ (Table 6) and green grapes 1000 $TCID_{50}$ (Table 6) results were suspected to be compromised by pipetting errors and excluded from the recovery calculations.

Virus recoveries ranged from 78.6% in the 100 TCID₅₀ cilantro matrix SYBR Green format to 12.2% in the 100 TCID₅₀ ham matrix. Grapes and ham recoveries were the lowest in both amplification formats. SYBR Green recoveries were generally lower than the hybridization probe recoveries (Table 6).

Analysis of flocculated samples using a one-way ANOVA indicated no significant differences ($p>0.05$) in the 1000 or 100 TCID₅₀ samples in the hybridization probe format.

In the SYBR Green format Tukey's HSD test detected a significant difference ($p<0.05$) between the BBE and ham, and ham and cilantro in the 1000 TCID₅₀ samples. A significant difference was also detected between BBE and ham, ham and grape, and ham and cilantro matrices in the 100 TCID₅₀ samples (Table 6).

Table 6 Post-Flocculation Spike Recoveries

Hybridization Probe Format				SYBR Green Format			
Matrix	Seeded TCID ₅₀	Rec. TCID ₅₀	% Rec.	Matrix	Seeded TCID ₅₀	Rec. TCID ₅₀	% Rec.
Water	832.2	n/a	n/a	water	868.2	n/a	n/a
	135.2	n/a	n/a		107.1	n/a	n/a
BBE	904.9	600.8	66.4	BBE	940.9	372.6	39.6
	92.4	69.3	75.9		112.9	57.7	51.1
Grapes	857.8	191.9‡	22.4‡	Grapes	917.9	207.1‡	22.6‡
	88.3	52.9	59.9		93.3	46.1	49.4
Cilantro	1092.5	743.6	68.1	Cilantro	843.8	498.2	59.1
	78.4	77.1†	98.3†		91.7	72.0	78.6
Ham	732.8	435.2	59.4	Ham	781.7	188.4	23.1
	99.6	60.3	60.6		67.9	8.3	12.2

† A pipetting error may have caused this unusually high recovery result.

‡ Low recovery most likely due to a pipetting error in the seeding procedure.

Hybridization Probe vs. SYBR Green Format Inhibition Comparison

No significant difference ($p>0.05$) in detection was observed between the results for the hybridization probe and SYBR Green formats in any matrix in the post-flocculation spike samples. However, a one-way ANOVA detected a significant difference ($p<0.05$) between the formats in the ham matrix at 1000 TCID₅₀ and 100 TCID₅₀ samples flocculation samples (Table 3, #'s 5). The hybridization probe yielded better recovery in both concentrations.

Sensitivity of Viral Isolation Processes

Sensitivity was defined as the most dilute concentration of virus that was detected in the post-flocculation spiked specimens. The Roche's Second Derivative Maximum LightCycler analysis software was used to determine sensitivity. This analysis allows each sample to be analyzed independently from the other specimens without manually adjusting a uniform threshold for all samples. This sensitivity analysis will only identify the absence or presence of amplification, it does not take into account crossing points. Primer dimers in the SYBR Green format contribute to the crossing point fluorescence that results in decreased crossing points (Figure 5), falsely increasing concentration calculations in low-available template copy samples.

The hybridization probe and the SYBR Green formats sensitivities were equal from the 10,000 TCID₅₀ to the 10 TCID₅₀ concentrations in all food matrices. The SYBR Green format was more sensitive in water detecting 5 of 6 replicates at the 0.1 TCID₅₀ while the hybridization probe detected only 3 of 6 replicates. The hybridization probes

were more sensitive in the BBE, cilantro, and green grape matrices than the SYBR Green. Both formats performed with equal sensitivity in the ham matrix (Table 5).

Figure 5

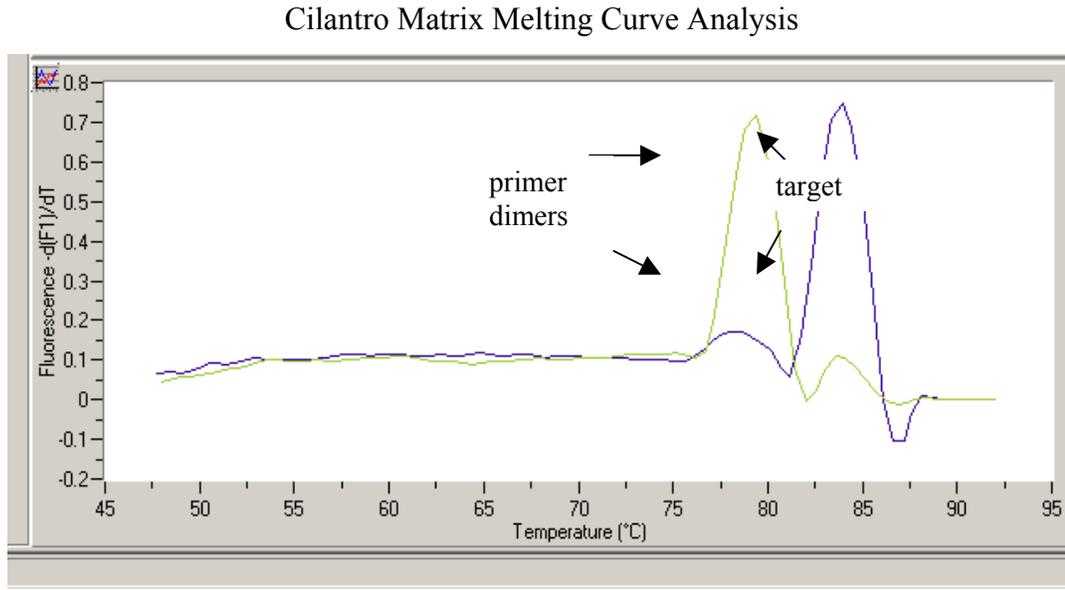


Figure 5. Cilantro Melting Curve Analysis. The tall green peak to the left is the primer dimer of a 10 TCID₅₀ flocculation sample replicate. The smaller green peak to the right is the poliovirus 3 196 nt amplicon. The tall blue peak to the right is the target poliovirus 3 196 nt amplicon from a 1,000 TCID₅₀ flocculation sample replicate. The small blue peak to the left is the primer dimer. Although the difference is clear in this melting curve analysis the amplification curve crossing points are very similar (data not shown).

Detection Sensitivity of Flocculation and Concentration Samples

Viral RNA was detected in the buffered beef extract, ham, cilantro, and green grapes seeded flocculation lysate for all replicates in all three concentrations (1,000; 100; and 10 TCID₅₀). The crossing points in both formats and the F1 fluorescence data demonstrate buffered beef extract negative matrix seeded concentration control has the least inhibition in our Real-Time RT-PCR assay. The green grapes, cilantro, and ham followed, in order from least to most inhibitory in terms of total sensitivity (Table 7).

Table 7 Matrix Effect on Virus Detection in Post Flocculation Spike Samples

Matrix	Hybridization Probe						SYBR Green					
	10,000	1,000	100	10	1	0.1	10,000	1,000	100	10	1	0.1
water	3/3	3/3	3/3	6/6	5/5	3/6	3/3	3/3	3/3	6/6	5/6	5/6
BBE	3/3	3/3	3/3	6/6	6/6	3/6	3/3	3/3	3/3	6/6	6/6	1/6
ham	3/3	3/3	3/3	6/6	0/6	0/6	3/3	3/3	3/3	6/6	0/6	0/6
cilantro	3/3	3/3	3/3	6/6	3/6	1/6	3/3	3/3	3/3	6/6	0/6	0/6
grapes	3/3	3/3	3/3	6/6	3/6	0/6	3/3	3/3	3/3	6/6	0/6	0/6

Note. The numbers above depict the number of positive replicates/ total number of replicates in each matrix in the hybridization probe and SYBR Green format.

Discussion

In Gastroenteritis outbreaks, foodborne or otherwise, health officials often have to take action before an etiologic agent can be identified. An overlap in clinical symptoms can make distinguishing between a bacterial and viral illness difficult (Mead, 1999). Increasing numbers of outbreaks in child daycare centers, nursing homes, and cruise ships are being attributed to NV (CDC, 2003). In the United States, an estimated 23 million cases of NV illness occur yearly, of which 9.2 million (66.6%) are transmitted by food. There are an estimated 20,000 hospitalizations from these suspected foodborne cases that result 124 deaths annually. An estimated 4,170 cases of foodborne HAV infection occur each year resulting in 125 hospitalizations and 4 deaths. Foodborne infections of Rotavirus and Astrovirus combined account for an estimated 78,000 illnesses yearly, 725 requiring hospitalizations (Mead, 1999). These figures underscore an urgent need for an assay that can rapidly concentrate and detect virus from a variety of food products. An assay, such as the one presented, which can isolate a viral agent from a suspected vehicle in less than 8 hours may have a real time impact on outbreak management, potentially effecting clinical treatment of the affected persons in the primary outbreak, and possibly preventing a secondary outbreak.

To date, very few studies have reported methodologies to detect virus contamination in food other than shellfish (Leggitt, 2000). Schwab detected NV contamination in deli meat using a guanidinium-based wash procedure from a NV

outbreak in a university cafeteria (Schwab, 2000). It should be mentioned that this study was one of the rare instances NV was isolated from a suspected food vehicle. The virus from the food was sequenced and matched to the virus sequenced from the outbreak victims' stool specimens. Gouvea et al. developed methods to concentrate and detect Rotavirus and NV in orange juice, lettuce, and milk. This method involved homogenization of the matrix and subsequent elution and precipitation of virus. They reported 1,000 particle detection limits using a nested RT-PCR procedure (Gouvea, 1994). Although generally considered sensitive, a major concern with nested or semi-nested RT-PCR procedures is the potential for cross-contamination of amplified PCR products during the second amplification reaction. To reduce the chance of cross-contamination, a laboratory area separate from the specimen processing, RNA isolation, and PCR preparation area is required. Many laboratories may not have the space required for this detection procedure.

This thesis presented a procedure to concentrate virus from three distinct food matrices. The virus seeding portion of the experiment was designed to replicate a natural contamination event by allowing the virus was to adsorb to the matrix for 2 ½ hours at ambient temperature and approximately 21 hours at 4°C. Replication of a “real world” contamination event was essential in providing an accurate analysis of the concentration assay.

Seeding was followed by a rapid viral isolation procedure that consisted of washing the matrix with 3% BBE/ 2% Tween 20 to elute the virus. Unlike shellfish, for these matrices, virus would only be present on the food surface, thus washing may have avoided the excessive inhibitory effects of plant-derived carbohydrates on PCR based

detection method described by other investigators, especially when sample homogenization is employed (Leggitt, 2000). The virus was concentrated by flocculation of the BBE protein by acid precipitation. Concentrated viral RNA was isolated with a Qiagen RNeasy RNA isolation kit followed by Real Time RT-PCR detection, allowing the entire assay to be completed in less than 8 hours. The closed tube Real Time RT-PCR procedure eliminates potential for cross-contamination of amplified product that can be a problem in traditional PCR, nested, and semi-nested PCR procedures.

The natural seeding event and a subsequent virus concentration, isolation, and detection procedure from three distinct food matrices identified differences in detection. Significant differences ($p < 0.05$) in detection were observed between food matrices, recovery was most efficient for cilantro, then BBE, then green grapes, and finally ham in the SYBR Green format. Cilantro yielded slightly better recovery than BBE. This may have been due to the poliovirus 3 binding more tightly to the bottom of the polypropylene beaker of the matrix-negative BBE sample than to the cilantro leaves. The level of inhibition observed in the cilantro matrix may apply to other herbs such as parsley and mint that have with similar surface characteristics.

Recovery was most effective for BBE, then cilantro, then ham, and finally green grapes in the hybridization format. The waxy surface of the green grapes may have washed off and eluted with the virus, potentially imparting more inhibitors to the RT-PCR assay than the other matrices. Or, virus may bind more tightly to the surface of the green grapes reducing the efficiency of the elution process. This effect may apply to other produce such as blueberries that have a similar waxy surface. Additionally, the surface of

the ham may have not allowed the virus to dry as on the green grapes, thus allowing a more efficient elution.

The hybridization probe yielded better recovery, even though it was not expected to perform as well as the SYBR Green in these complex food matrices. It was hypothesized that the hybridization probe format would be more susceptible to inhibitors, due to the more complex binding kinetics required for probe hybridization, and subsequent fluorescence resonance energy transfer. The hybridization probe amplification assay demonstrated ruggedness in all matrices, and even superiority in the smoked ham matrix where significant differences ($p < 0.05$) in detection were observed between the formats.

Although not significantly different ($p > 0.05$), the greatest inhibition in the post-flocculation spike samples was demonstrated by cilantro followed by ham, green grapes, BBE and finally virus in water, which demonstrated the least inhibition in both RT-PCR formats. Since these samples are spiked after the flocculation and concentration procedure, the lack of significant differences between the food matrices may indicate a rugged RNA isolation and/or detection procedure, regardless of matrix.

The strawberry matrix samples failed to amplify viral nucleic acid in the flocculation seed. Nor, was signal was detected below 10,000 TCID₅₀ in the post-flocculation spike samples. Absence of amplification in the post-flocculation samples below 10,000 TCID₅₀ may indicate substantial PCR inhibitors in the matrix, rather than a viral elution or flocculation and concentration procedure problems. Also, the strawberry matrix may contain compounds that prevent efficient RNA isolation.

The 10 TCID₅₀ flocculation samples, although tested positive in all matrices, cannot be used in the recovery calculations because their amplification curves were not parallel to the 1,000 TCID₅₀ and 100 TCID₅₀ specimens. Amplification curves that are not parallel indicate non-equal reaction efficiencies. Crossing points derived from samples with non-equal reaction efficiencies cannot be used to accurately determine virus concentration using the standard curve. The LightCycler can detect, but the LightCycler statistical software (version 3.53) cannot accurately quantify samples below 10 copies. The copies available for amplification in the 10TCID₅₀ flocculation samples may have been below the 10 copies required.

The hybridization probes major advantage lies in its RT-PCR and sequence-specific detection performed in a closed tube format, and completed in less than 75 minutes. Also, two or three probes could be designed for a multiplex reaction that allows the user to detect several variants of the target in one reaction tube. This could not be accomplished with the SYBR Green format or traditional RT-PCR. The most significant disadvantage is the probes for each primer set have to be available, or the user must design their own. As more probes become available in the literature, this will become less of an obstacle.

The major advantage of the SYBR Green format is that sequence-specific probes do not have to be developed; however, the main disadvantage is that it is not a sequence-specific assay. Therefore, confirmation of the target via Southern hybridization, sequencing, or other means is necessary.

The knowledge of level of inhibition present in the suspected foods can impact identification of a viral agent. For example, foods demonstrating substantial inhibition may require examination of large samples in order to have a reasonable chance of detecting virus. Food classes known to exhibit greater degrees of inhibition may require modifications of the isolation process (i.e. freon treatment of a matrix with high lipid content may be required). Foreknowledge of the effects of food inhibitors before sample processing will save precious sample and laboratory processing time.

The CDC estimates a total of 13.8 million cases of food related acute viral gastroenteritis per year, 9.2 million are caused by NV (Goodgame, 2001). This assay may have the most immediate impact on the detection of viruses on contaminated fresh produce. Particularly, with the growing popularity of “pre-washed” and “ready to eat” produce, which seem to imply the product is free from chemical and biological contaminants. Regular samples of fresh produce taken from the field or packing plants and processed by this procedure may be able to detect viral contamination event caused by a food handler shedding virus before symptoms appear. Or it may indicate a large-scale contamination if many samples, from different parts of the plant, test positive for virus due to a contaminated reclaimed water supply used for irrigation (Abad, 1994). Since this assay can be completed in less than 8 hours it may be able to prevent potentially contaminated produce from leaving the plant, or allow a recall to be implemented before a contaminated lot is delivered to its final destination. Further development of a sensitive, rapid food virus isolation method, similar to the one presented, could have a substantial impact on food virus detection in various food

matrices, potentially impacting current policies concerning monitoring and recalls of virus contaminated food products.

References

Abad FX, Pinto RM, Bosch A. Survival of enteric viruses on environmental fomites. *Appl Environ Microbiol* 1994;60:3704-3710.

Ackerman WW, Fujioka RS, Kurtz HB. Cationic modulation of the inactivation of poliovirus by heat. *Arch Environ Health* 1970;21:377-81.

Agus SG, Dolin R, Wyatt RG, et al. Acute infectious nonbacterial gastroenteritis: Intestinal histopathology. Histologic and enzymatic alterations during illness produced by the Norwalk agent in man. *Ann Intern Med* 1973;79:18-25.

Ashida M, Hamada C. Molecular cloning of the hepatitis A virus receptor from a simian cell line. *J Gen Virol* 1997;78:1565-1569.

Boggs JD, Melnick JL, Conrad ME, et al. Viral hepatitis: Clinical and tissue culture studies. *JAMA* 1970;214:1041-1046.

Bradley DW, Schable CA, McCaustland KA, et al. Hepatitis A virus: Growth characteristics of in vivo and in vitro propagated wild and attenuated strains. *J Med Virol* 1984;14:373-386.

Caul EO. Small round structured viruses: Airborne transmission and hospital control. *Lancet* 1994;343:1240-1242.

CDC. Norovirus activity-United States 2002. *MMWR* 2003; 52 (03) 41-45

Cromeans TL, Nainan OV, Margolis HS. Detection of hepatitis A virus RNA in oyster meat. *J Infect Dis* 1997;63(6):2460-63.

Dolin R, Blacklow NR, DuPont H, et al. Biological properties of Norwalk agent of acute infectious nonbacterial gastroenteritis. *Proc Soc Exp Biol Med* 1972;140:578-583.

Emerson SU, Tsarev SA, Govindarajan S, et al. A simian strain of hepatitis A virus, AGM-27, functions as an attenuated vaccine for chimpanzees. *J Infect Dis* 1996;173:592-597.

Endres JF, Weller TH, Robbins FC. Cultivation of the Lansing strain of poliomyelitis in cultures of various human embryonic tissues. *Science* 1949; 109:185-230.

Feinstone SM, Kapikian AZ, Purcell RH. Hepatitis A: Detection by immune electron microscopy of a viruslike antigen associated with acute illness. *Science* 1973;182:1026-28.

Forss S, Schaller H. A tandem repeat gene in picornaviruses. *Nuclear Acid Res* 1982;10:6441-50.

Glass RI, Noel J, Ando T, Fankhauser R, Belliot G, Mounts A, Parashar UD, Bresee JS,

Monroe SS. The epidemiology of enteric caliciviruses from humans: a reassessment using new diagnostics. *J. Infect. Dis.* 2000 181(S2): S254-61.

Goodgame R. Viral causes of diarrhea. *Infect. Diarrhea* 2001 30(3): 779-95.

Goueva V, Santos N, Carmo-Timenetsky, Estes MK. Identification of Norwalk virus in artificially seeded shellfish and selected foods. *J Virol Meth.* 1994;48:177-87.

Graham DY, Jiang X, Tanaka T, et al. Norwalk virus infection of volunteers: New insights based on improved assays. *J Infect Dis* 1994;170:34-43.

Green KY, Chanock RM, Kapikian AZ. Human caliciviruses. In: Knipe DM, Howley PM, editors. *Fields Virology* 4th Ed. Volume 1. Philadelphia: Lippincott Williams and Wilkens; 2001. p 842-866.

Havens WP Jr. Periods of infectivity of patients with experimentally induced infectious hepatitis. *J Exp Med* 1946;83:251-258.

Hollinger FB, Emerson SU: Hepatitis A virus: In: Knipe DM, Howley PM, editors. *Fields Virology* 4th Ed. Volume 1. Philadelphia: Lippincott Williams and Wilkens; 2001. p 799-829.

Kapikian AZ, Wyatt RG, Dolin R, et al. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. *J Virol* 1972;10:1075-1081.

Kaplan G, Totsuka A, Thompson P, et al. Identification of a surface glycoprotein on African green monkey kidney cells as a receptor for hepatitis A virus. *EMBO J* 1996;15:4282-4296.

Krugman S, Ward R, Giles SP. Infectious hepatitis: detection of virus during the incubation period and in a clinically inapparent infection. *N Engl j Med* 1959;261:729-34.

Kuritsky JN, Osterholm MT, Greenberg HB, et al. Norwalk gastroenteritis: A community outbreak associated with bakery product consumption. *Ann Intern Med* 1984;100:519-521.

Kuzma, JW. Basic statistics for the health sciences. California: Mayfield Publishing Company; 1998. 344 p.

Langford MP, Kadi RM, Ganley JP, Yin-Murphy M. Inhibition of epidemic isolates of coxsackievirus type A24 by recombinant and natural interferon alpha and interferon beta. *Intervirol* 1988;320-327.

Le Guyader F, Neill FH, Estes MK, Monroe SS, Ando T, Atmar RL. Detection and analysis of a small round –structured virus strain in oysters implicated in an outbreak of acute gastroenteritis. *Appl Environ Microbiol* 1996;62(11):4268-72.

Lees DN, Henshilwood K, Green J, Gallimore CI, Brown DW. Detection of small round –structured viruses by reverse transcription-PCR. *Appl Environ Microbiology* 1995;61:4418-24.

Leggit PR, Jankus LA. Detection methods for human enteric viruses in representative foods. *J Food Prot* 2000 53 (12): 1738-44.

Meroff JC, Schreiber DS, Trier JS, Blacklow NR. Abnormal gastric motor function in viral gastroenteritis. *Ann Intern Med* 1980;92:370-373.

Mounts AW, Ando T, Koopmans M, Bresee JS, Noel J, Glass RI. Cold weather seasonality of gastroenteritis associated with norwalk-like viruses. *J Infect Dis* 2000 181: S284-7.

Mounts AW, Ando T, Koopmans M, et al. Cold weather seasonality of gastroenteritis associated with Norwalk-like viruses. *J Infect Dis* 2000;181(suppl 2):S284-287.

Pallansch MA, Roos RP. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In: Knipe DM, Howley PM, editors. *Fields Virology* 4th Ed. Volume 1. Philadelphia: Lippincott Williams and Wilkins; 2001. p 723-767.

Ranciello VR. Picornaviridae: the viruses and their replication. In: Knipe DM, Howley PM, editors. *Fields Virology* 4th Ed. Volume 1. Philadelphia: Lippincott Williams and Wilkins; 2001. p 685-715.

Richards GP. Limitations of molecular biological techniques for assaying the virological safety of foods. *J Food Prot* 1999;62(6):691-97.

Roerink F, Hashimoto M, Tohya Y, Mochizuki M. Organization of the canine calicivirus genome from the RNA Polymerase gene to the poly(A) tail. *J Gen Virol* 1999;80:929-935.

Rosenblum LS, Villarino ME, Nainan OV, Hepatitis A outbreak in a neonatal intensive care unit: Risk factors for transmission and evidence of prolonged viral excretion among pre-term infants. *J Infect Dis* 1991;164:476-82.

Schaub SA, Oshiro RK. Public health concerns about caliciviruses as waterborne contaminants. *J Infect Dis* 2000;181(2):s374-80.

Schreiber DS, Blacklow NR, Trier JS. The mucosal lesion of the proximal small intestine in acute infectious nonbacterial gastroenteritis. *N Engl J Med* 1973;288:1318-1323.

Schwab KJ, Neil FH, Fankhauser RL, Daniels NA, Monroe SS, Bergmire-Sweat DA, Estes MK, Atmar RL. Development of methods to detect "norwalk-like viruses" (NLVs) and hepatitis A virus in delicatessen foods: application to a food-borne NLV outbreak. *Appl Environ Microbiol* 2000: 213-18.

Schwab KJ, Neill FH, Fankhauser RL, Monroe SS, Bergmire-Sweat DA, Estes MK, Atmar RL. Development of methods to detect Norwalk-like viruses (NLV's) and hepatitis A virus in delicatessen foods: application to a foodborne NLV outbreak. *Appl Environ Microbiol* 2000;66(1):213-18.

Sheih C, Baric RS, Sobsey MD. Detection of low levels of enteric viruses in metropolitan and airplane sewage. *Appl Environ Microbiol* 1997;63:4401-07.

Shieh C, Monroe S, Frankhauser RL, Daniels NA, Lagolis GW, Burkhard W, Bank RS. J Detection of Norwalk-virus in shellfish implicated illness. *Infect Dis* 2000;181(2):s360-66).

Siegl G, Weitz M, Kronaaur G. Stability of hepatitis A virus. *Intervirology* 1984;22:218-26.

Sobsey MD, Shields PA, Hauchman FS, et al. Survival and persistence of hepatitis A virus in environmental samples. In: Zuckerman AJ, ed. *Viral hepatitis and liver disease*. New York: Alan R. Liss, 1988: 121-124.

Wilson R, Anderson LJ, Holman RC, Gary GW, Greenberg HB. Waterborne gastroenteritis due to the Norwalk agent: a clinical epidemiological investigation. *Amer J of Public Health* 1982;72(3):72-74.

APPENDICES

Appendix A

Nucleic Acid Isolation with Qiagen's RNeasy™ Spin Columns

The following was adapted from the Qiagen RNeasy RNA Isolation Kit Protocol for animal cell RNA isolation (Qiagen, 2001).

1. The specimens were removed from -70°C storage and placed in a 37°C water bath for 20 minutes.
2. The samples were vortexed on high for 30 seconds and placed in the Beckman J6-B and centrifuged at 4,200g for 10 minutes.
3. The specimens were removed, with care to avoid disturbing the precipitate, from the centrifuge and decanted into a 15ml sterile Falcon blue top tube. The precipitate was discarded.
4. The volume of the specimens was measured in a 10ml disposable pipette, and recorded.
5. One-tenth of the specimen(range 8.6-9.5 ml) was transferred to a 2.0ml sterile screw top micro centrifuge tube and 500 μl of 100% absolute ethanol added.
6. The specimen was vortexed and quick spun. Then 700 μl was loaded in a Qiagen RNeasy spin column.
7. The column was centrifuged at 10,000g for 30 seconds. The rest of the sample was then loaded and again centrifuged for 30 seconds at 10,000g.
8. The flow through was discarded. Then 700 μl of buffer RW1 was pipetted into the spin column and centrifuged at 12,000g for 30 seconds.
9. The flow through was discarded. Then 500 μl of buffer RPE was pipetted into the spin column and centrifuged at 12,000g for 30 seconds.
10. Step 9 was repeated
11. The flow through was discarded and the column centrifuged at 20,000g for 2 minutes.
12. The column was then placed in a 1.5ml DNase/ RNase free micro centrifuge tube.

Appendix A (Continued)

13. 50 μ l of RNase free water was placed in the column, directly in the center of the silica filter and allowed to stand at RT for 1 minute.
14. Step 13 was repeated to get a 100 μ l final volume of RNA.
15. The sample was then centrifuged at 20,000g for one minute.
16. At this point all of the post flocculation RNA eluate samples are divided into two aliquots of 70 μ l and 30 μ l. This will minimize RNA degradation due to freeze thawing during subsequent amplifications.

Appendix B

LightCycler Amplification Protocol

1. Master mix preparation

Master Mix Formulation

Reagent	Final Conc.	Hyb. Probe (μ l)	SYBR Green (μ l)
Water	N/A	4.1	4.5
2X Enzyme Mix	1X	10	10
MgCl ₂ (25mM)*	2.5mM	Incl.	Incl.
Ent 3 (100mM)	0.5 mM	0.15	0.15
Ent 4 (100mM)	0.5 mM	0.15	0.15
Polio FL (10mM)	0.2 mM	0.2	0.2
EVL640 (10mM)	0.2 mM	0.2	0.2
Template	variable	5	5

*Note: MgCl₂ is included in the 2X enzyme mix at a final concentration of 2.5mM.

1. Fifteen micro-liters of either Probe or SYBR green master mix is placed in each capillary tube.
2. Water, 5 μ l, is then placed in the amp (-) tube.
3. The lowest concentration of template is pipetted into the capillaries, and then they are capped.
4. The next lowest concentration is then pipetted and capped. This continues until all the samples are in the capillaries.
5. The amplification negative is then capped
6. The carousel was centrifuged in the LightCycler centrifuge for 15 seconds, and then placed in the LightCycler instrument.

Appendix B (Continued)

Hybridization Probe Amplification Thermal Cycling Protocol			
Reverse Transcription			
Time (min.)	°C		
20	50		
Taq Activation			
Time (min.)	°C		
15	95		
Polymerase Chain Reaction (55 cycles)			
Time (sec.)	°C	Cycle Description	
0	95	Denaturation	
30	52	Annealing	
30	72	Extension	

SYBR Green Amplification Thermal Cycling Protocol			
Reverse Transcription			
Time (min.)	°C		
20	50		
Taq Activation			
Time (min.)	°C		
15	95		
Polymerase Chain Reaction (55 cycles)			
Time (sec.)	°C	Description	
15	95	Denaturation	
15	52	Annealing	
30	72	Extension	

Appendix C

Simple Food Processing Protocol Bench Sheet

Experiment: _____ Date: _____ Tech: _____

	Procedure	Time	Comment
1.	Weigh food sample food on top loader balance.		
2.	Place the sample food in a 0.6 liter disposable plastic beaker. Add as small a quantity of 3% buffered beef extract (BBE) sufficient to cover sample.		
3.	Place beaker with sample on vortexer and vortex at 50rpm for 15min.		
4.	Adjust the pH to 7.0 ± 0.1 . Place on L.E.D. Orbital Shaker (speed ~130) for 15min. Maintain pH 7.0.		
5.	Insert a pH electrode into the eluate. Add 5M HCl slowly to the solution until the pH of the beef extract reaches 3.5 ± 0.1 . Do not allow the pH to drop below 3.4.		5 M: ___ ml
6.	Stir slowly for 30 min, monitoring the pH. Adjust if necessary. Remove the electrode from the beaker and disinfect it.		
7.	Pour the eluate into a sterile 250ml-centrifuge bottle.		
8.	Centrifuge at 5000 rpm for 20 min in the J6B at 4°C (4100 X g).		
9.	Decant and discard the supernatant . Save the solids.		
10.	Place a stir bar in the centrifuge bottle containing the precipitate. Add 9 ml GITC lysis buffer (NASBA) to dissolve the precipitate.		
11.	Stir until the precipitate is dissolved completely.		
12.	Place in an appropriate tube and store at -70°C until processing.		

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Appendix D

The following information contained in Appendix D was provided by the LightCycler™ instruction manual for version 3.5 software (Roche, 2000).

Background. The Roche LightCycler™ is a rapid thermal cycler that utilizes alternated heated and ambient air as a medium for temperature transfer. This technique uses glass capillaries that have a large surface area to volume ratio that transfers heat efficiently, thus allowing rapid cycling conditions. Real-Time RT-PCR combines the amplification and detection, with or without sequence specific detection, in a closed tube format in 60 to 90 minutes.

SYBR Green. The LightCycler (LC) can operate in a sequence specific hybridization probe format, or a non-sequence specific SYBR Green format. SYBR Green is a ds DNA binding dye. It fluoresces only upon binding to the minor groove of the DNA double helix. SYBR Green does not intercalate, thus is much less toxic than ethidiumbromide. In the denaturation phase at 95 °C, only melted ss DNA is present (template and primers), and SYBR Green which does not fluoresce as there is no ds DNA present. As the primers bind to DNA in the annealing phase creating ds DNA. SYBR Green binds to the ds DNA and emits fluorescent light upon excitation with the light of the blue LED. During the elongation phase the Taq polymerase is creating a DNA strand complementary to the template. As more ds DNA is formed, more SYBR Green binds. At

the end of the elongation cycle all the template DNA is double stranded and the peak SYBR Green fluorescence is reached. The LC acquires the signal at this point in the cycle.

Hybridization Probe. The hybridization probe format that use uses two sequence specific oligonucleotide probes that are complementary to an internal sequence of the amplified fragment, along with the normal PCR primers. The 5' probe is labeled at the 3' end with

Appendix D (Continued)

fluorescein. The 3' probe is labeled at the 5' end with LC Red 640 fluorophore, and a phosphorated 3' end that blocks elongation by Taq polymerase. During the denaturation phase only ss DNA is present. In the annealing phase the primers and the probes bind to their target sequences. Upon binding, the probes get into close proximity. The light of the blue LED excites the fluorescein donor. The fluorescein then transfers its energy on to LC Red640 acceptor fluorophore. The LC Red begins emitting fluorescent light. The intensity of this signal is proportional to the target copies present in the reaction. This is a photon-free process based on dipole-dipole interactions called Fluorescence Resonance Energy Transfer (FRET). During the elongation phase the probes are displaced by the Taq polymerase causing the fluorescence to drop until the end of the cycle when all probes have been displaced. Since Hybridization probes create a fluorescent signal only when bound to a specific target sequence, primer dimers and other amplification by-products do not contribute to the fluorescent signal.