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# Propagation of Homalodisca Coagulata Virus-01 via Homalodisca vitripennis Cell Culture

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# PROPAGATION OF *HOMALODISCA COAGULATA VIRUS -01* VIA *HOMALODISCA VITRIPENNIS* CELL CULTURE

by

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Abstract

PROPAGATION OF *HOMALODISCA COAGULATA VIRUS -01* VIA *HOMALODISCA VITRIPENNIS* CELL CULTURE

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The glassy-winged sharpshooter (*Homalodisca vitripennis*) is a highly vagile and polyphagous insect, which feeds on more than 100 plant species throughout the southwestern United States. Sharpshooters are the predominant vector of *Xylella fastidiosa* (Xf), a xylem-limited bacterium that is the causal agent of Pierce's disease (PD) of grapevine. Infected *H. vitripennis* transmit the bacterium while feeding. The rise of PD has been economically damaging for agriculture and *H. vitripennis* have become a target for disease control. A dicistrovirus identified as *Homalodisca coagulata virus-01* (HoCV-01) has been associated with an increase in mortality rates within infected *H. vitripennis* populations. A host is required for HoCV-01 replication and cell culture provides the logistically and economically valuable means for producing a virus biopesticide. In this study, we developed a system for large-scale propagation of *H. vitripennis* cells via tissue culture, providing viral replication machinery. Cells were inoculated with low levels of HoCV-1, medium was removed every 24H for 168H, RNA extracted using TRIzol and analyzed with qRT-PCR. Cells were also trypan blue stained

and counted to determine cell survivability. Whole virus particles were extracted within 72-96H after infection before total cell culture collapse occurred. This study shows that *H. vitripennis* cells are capable of being cultured and used for virus mass production, suitable to produce a biopesticide.

# Introduction

Successful agricultural production often depends on pest and pathogen management, in many systems invasive species management is imperative. The glassy-winged sharpshooter (GWSS, *Homalodisca vitripennis* Germar 1821) has been identified as the predominant vector of *Xylella fastidiosa*, the causal agent of Pierce's disease of grapevine (PD) in North America (Takiya et al. 2006). Insect population management has quickly become the focus of research to combat this devastating problem to the viticulture industry in California and across the southern United States. A positive-sense, single-stranded, RNA virus belonging to the Family: Dicistroviridae, *Homalodisca coagulata virus-01* (HoCV-01*),* has been identified in wild *H. vitripennis* populations and shown to increase mortality, (Hunter et al. 2006; Hunnicutt et al. 2006, 2008), while lowering the insect's resistance to insecticides.

Development of methods to effectually rear infected GWSS to adulthood in a laboratory setting have been difficult because *H. vitripennis* have different stage-specific nutritional needs that require a variety of host plants presenting a barrier in live insect rearing (Setamou 2005; Turner and Pollard 1959; Brodbeck et al. 1996, 1999). Specific facilities are also required to cultivate live *H. vitripennis* colonies in the United States, making cell culture techniques a more economical and viable alternative, as well as increasingly vital for HoCV-01 detection and replication (Kamita et al. 2005; Hunter 2006). Utilizing cell culture techniques versus live insect colonies can potentially circumvent these issues.

While basic methods for establishing cell cultures of *H. vitripennis* are described, these methods have not yet been utilized for commercial production of biological control agents, such as viruses (Hunter 2006). Viral replication requires a living cell, which is why successfully cultivating and optimizing *H. vitripennis* cultures is vital to the progress of producing a high concentration of virus suitable for utilization as a biological control agent.

Chapter One

Literature Review

## Section I

### *Xylella fastidiosa*

*Xylella fastidiosa* (Wells) is a gram negative, γ-proteobacterium that is xylem-limited and belongs to the Xanthomodaceae family (Hopkins 1973; Wells et al. 1997). The bacterium disperses through insect vectors like *Homalodisca vitripennis* that are xylophagous. In spite of being limited to feeding on nutrient poor environments, *X. fastidiosa* can be found in a broad range of plant hosts including both wine and table grapes, citrus plants, coffee plants, almonds, alfalfa, oleander, mulberry, oak, elm, sycamore, plum, peach, as well as other reservoir hosts of less economic importance (Barnard 1998; Costa et al. 2004; Henneberger et al. 2004; Hernandez-Martinez et al. 2006, 2007; Hill and Purcell 1995; Hopkins 1989; Hopkins and Purcell 2002; Li et al. 2001; McGaha et al. 2007; Paradela-Filho et al. 1997; Purcell et al. 1999; Winstrom and Purcell 2005). *Xylella fastidiosa* is the causal agent in an assortment of scorch-like diseases found in plants of both agricultural and ornamental importance including citrus, grapes, and almonds (Hoddle 2004). Two major *X. fastidiosa*-associated diseases that have increased in importance in the past decade are citrus variegated chlorosis (caused by *X. fastidiosa pauca*) and Pierce's disease (PD) of grapevines (caused by *X. fastidiosa pierci*) (Redak et al. 2004).

The development of disease from *X. fastidiosa* is dependent on a systemic infection (Chatterjee et al. 2008; Hopkins 1989). For a systemic infection to occur, *X. fastidiosa* must move between xylem vessels through pit membranes, a process that is dependent on polygalacturonase-mediated degradation of the pit membranes, allowing *X. fastidiosa* to travel through xylem vessels, attaching to them and forming a biofilm (Baccari and Lindow 2011; Chatterjee et al. 2008; Newman et al. 2004; Roper et al. 2007; Varela et al. 2001). Cell-cell signaling regulates biofilm production within the xylem vessels, but is also dependent on quorum sensing within *X. fastidiosa* (Newman et al. 2004). The occlusion of xylem vessels often leads to impaired hydraulic conduction and reduced leaf water potential, as well as green epidermal patches on the stem, marginal leaf necrosis and the presence of petioles (where leaves attach to the stem) remaining on the stem after leaves have fallen off of the vine (Goheen and Hopkins 1988; Goodwin et al. 1988; Purcell 1986; Stevenson et al. 2005). Death will only occur when infections are systemic, as having high numbers of localized bacteria does not necessarily correlate to severity of symptoms in a plant (Gambetta et al. 2007; Newman et al. 2003). Asymptomatic plants can have high numbers of bacteria in localized areas, indicating a failure of *X. fastidiosa* to move across the pit membranes and become systemic (Alves et al. 2004; Baccari and Lindow 2011; Fry and Milholland 1990; Gambetta et al. 2007; Hopkins 1989; Krivanek and Walker 2005; Newman et al. 2003; Newman et al. 2004).

There is no known direct causal mechanism of pathogenicity for *X. fastidiosa*, but the progression of disease induces an infected plant to mimic the effects of a drought leading to the conclusion that bacterial obstruction in the xylem causes water stress in infected plants leading to leaf and shoot dieback and eventual plant death (Chatterjee et

al. 2008; Gambetta et al. 2007; Newman et al. 2003; Setamou 2005). The occlusion of xylem vessels is exacerbated by water stress, which occurs commonly before harvest in wine grapes (McElrone et al. 2001).

Thriving in more moderate climates, *X. fastidiosa piercei* is distributed mostly throughout North America. While some native plants that are considered wild have expressed tolerance to bacterial colonization, the bacteria have been affecting plants considered exotic to the continent, including many of the agricultural crops (Redak et al. 2004). Motility of *X. fastidiosa* is limited because it is completely xylem-limited and it is aflagellate; thus, in order for it to move through xylem vessels it relies on twitching motility via type I and IV pili (Baccari and Lindow 2011; Hopkins 1989; Hopkins and Purcell 2002; Meng et al. 2005; Newman et al. 2003). Having limited motility necessitates a direct transmission into host plant xylem, consequently requiring insect vectors such as leafhoppers. In California, *H. vitripennis* have potentially altered the ecology and movement of *X. fastidiosa* by exposing native plants that lack evolutionary resistance to this pathogen (Hoddle 2004).

While a great deal of research has been conducted on *X. fastidiosa*, there is still no known cure for the diseases caused in various plant hosts. Controls of major agricultural diseases caused by *X. fastidiosa* have moved towards vector management approaches as a means to combat emerging diseases.

**Xylem Feeding Insects**

Xylem fluid-feeding insects belong to the order Hemiptera and appear to have a single evolutionary origin (Sorensen 1995). The three families of xylem fluid-feeding insects are Cercopoidea, Cicadoidea and Cicadellidae; however, Cicadellidae was

organized into two tribes: Proconiini and Cicadellini, with Cicadellini being the more diverse tribe (Young 1977). Cicadellini are found in all zoogeographical regions, whereas Proconiini are found strictly in the New World (Young 1977). Cicadellinae leafhoppers possess unique physical features designed for feeding on a difficult food source. Insects belonging to this group have an inflated clypeus encasing the muscalture that connects to the cibarium, thus permitting them to feed on xylem fluid in high negative tension situations (Redak et al. 2004) (Fig. 1.1).

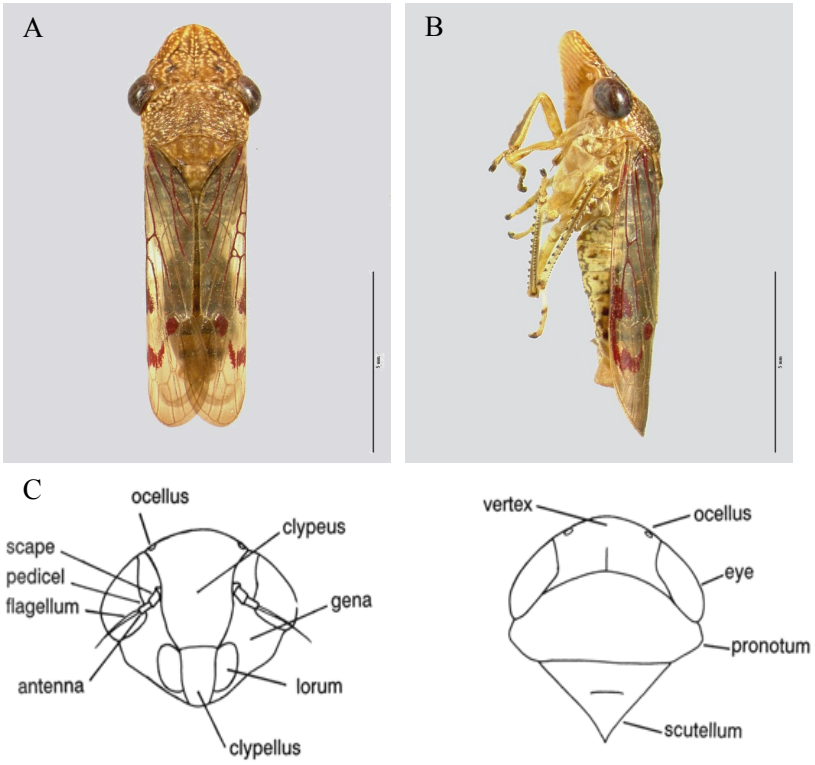


**Figure 1.1**. Leafhopper morphology. (A) Top view of an *H. vitripennis* specimen collected in Texas. (B) Side view of an *H. vitripennis* specimen collected in Texas. (C) Generalized head morphology of leafhoppers. In *H. vitripennis* the clypeus is inflated, allowing them to feed on xylem fluid that has high negative tension. Image from Wilson et al. 2009.

They are also linked to an extensive and devastating group of plant diseases because of their ability to transmit disease causing xylem-limited bacteria, particularly

*Xylella fastidiosa*, which has been linked to non-curable diseases in grape vines, citrus, almonds, alfalfa, stone fruits, and several other types of plants (Hopkins 1989; Hopkins 2002; Purcell 1979; Purcell 1989). The main vectors of *X. fastidiosa* common in North America are *Xyphon (Carneocephala) fulgida* (Nottingham), *Draeculacephala minerva* (Ball), *Graphocephla atropunctata* (Signoret), *Oncometopia* spp. and *Homalodisca vitripennis (*formerly *Homalodisca coagulata*) (Say) (Nielson 1968; Purcell and Frazier 1985; Turner and Pollard 1959; Takiya et al. 2006). Many other species could potentially be vectors of pathogens, but limited information is available in the literature.

*Glassy-winged sharp shooter*

*Homalodisca vitripennis* commonly referred to as the glassy-winged sharpshooter, have become a species of great interest, as it is highly invasive. It is indigenous to the southern United States and northeastern Mexico, with a natural geographic distribution among the southeastern Gulf States and Texas (Hunnicutt et al. 2007; Triapitsyn 2000). *Homalodisca vitripennis* have successfully invaded new territory, including California, the Hawaiian island of Oahu, Central America and French Polynesia in the past century (Hunnicutt et al. 2007; Goheen et al. 1979; Plant Protection Service 2002). Potential ranges of *H. vitripennis* based on climate includes major wine grape growing regions in New Zealand and Australia, as well as Italy, Chile and the Western Cape Province in South Africa (Hoddle 2004) (Fig. 1.2). Smaller leafhopper species indigenous to the newly acquired *H. vitripennis* territories, *Draeculacephala minerva* Ball, *Graphocephala atropunctata* (Signoret), and *Xyphon fulgida* Nottingham (Cicadellini), have been linked to outbreaks of other agricultural disease, but none have been as destructive as *H. vitripennis* (Winkler 1949; Young 1977; Purcell 1980, 1981;

Hopkins and Purcell 2002). Agricultural productivity has been threatened by *H. vitripennis* since its accidental introduction to southern California, likely as eggs on nursery stock, from the southeastern states during the late 1990's (Phillips 1999; Sorensen and Gill 1996).

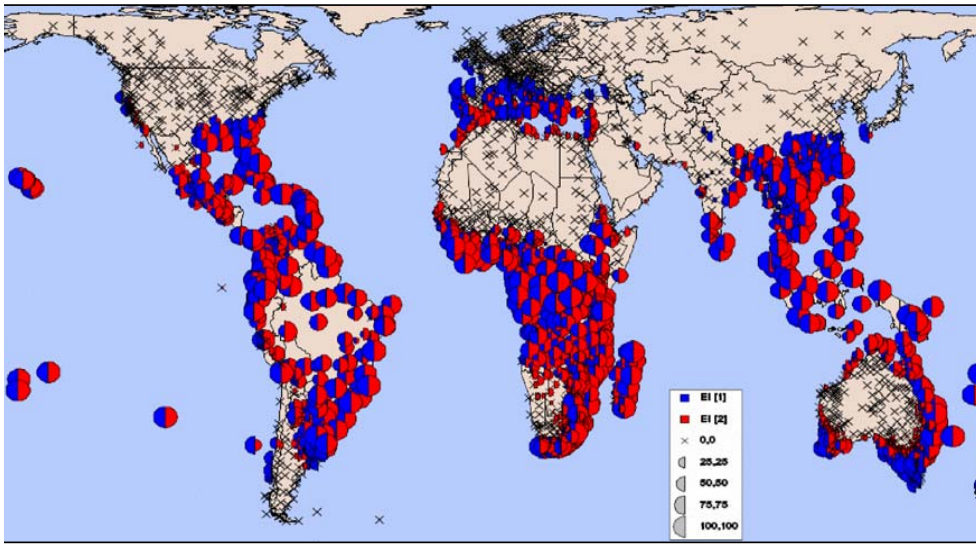


**Figure 1.2**. Map showing the potential global distribution model of *H. vitripennis* (blue) and the PD causing strain of *X. fastidiosa* (red) generated by CLIMEX (from Hoddle 2004).

As a highly vagile and polyphagous insect, *H. vitripennis* adults and late-instar nymphs disperse long distances to find more than 100 plant hosts belonging to 35 families including woody dicot, herb, and grass families on which to develop and feed (Mizell and French 1987; Andersen et al. 2003; Hunnicutt et al. 2006). Cicadellidae fecundity and development are influenced by host plants species, implying that the survival of *H. vitripennis* varies with host plant species available, genotypes of host plant species, and with plant growing conditions (Van Rensburg 1982; Brodbeck et al. 1999, 2004).

The nutritional ecology of *H. vitripennis* is distinctive because they are voracious xylem feeders, enabling them to survive off of a fluid low in nutritional value, and they can rapidly spread plant pathogens as they access a host plant's xylem repeatedly during feeding (Raven 1983). There have been two to three generations of *H. viterpennis* per year documented in California populations, and as a paurometabolous insect that undergoes five ecdyses during development, *H. vitripennis* have different stage-specific nutritional requirements, making insect rearing for study difficult (Blua 1999; Setamou 2005; Turner and Pollard 1959; Brodbeck et al. 1996, 1999). The number of generations in Texas has yet to be confirmed in the literature. Successful nymph development occurs on host plants with a balanced amino acid profile in its xylem fluid versus adults that thrive on xylem fluid containing higher concentrations of amides, requiring nymphs to disperse to better host plants in order to complete their development (Brodbeck et al. 1995; Tipping et al. 2004). With a diverse agricultural setting present in California, *H. vitripennis* have flourished by moving from one preferred host to another year-round (Lauziere 2008). The pathogen transmission ability of *H. vitripennis* is ascribed to these biological characteristics and it has been identified as one of the principle vectors of a devastating disease of grapevines (Redak et al. 2004).

*Pathogen transmission efficiency*

Insect vectors must acquire *X. fastidiosa* by feeding from infected plants, or transovarially (transmission of pathogens through pathogen invasion of ovary tissue and subsequently the eggs within the host) in some species. In *H. Vitripennis,* no evidence of transovarial transmission has been observed, meaning that they become infective by feeding on infected plants (Freitag 1951). Evidence from transmission studies confirmed

that vectors would cease transmitting the bacterium after molting, but would resume transmission once they fed on an infected plant (Purcell and Finlay 1979). Not only was this indicative of how *H. vitripennis* become infective, but also that the bacteria are harbored and transmitted from the external surface of the insect's foregut, which is lost during molting (Redak et al. 2004). Forgut-borne pathogens like *X. fastidiosa* are retained in the cuticular surface of the anterior forgut, which contains the precibarium and the cibarium (Almeida and Purcell 2006; Brlanksy et al. 1983; Purcell et al. 1979; Nault 1997). *Xylella fastidiosa* is the only known forgut-borne bacteria to be semipersistently transmitted, indicating that the bacterium infects plants from retention sites in the precibarium or cibarium (Backus 2011). Since *H. vitripennis* lose the bacteria during each molt, this presents a potential area of vector management because infections are not permanent until adulthood (Almeida and Purcell 2003; Backus and Morgan 2011).

Rates of vector transmission depend greatly upon the species and host plant of interest (Redak et al. 2004). Transmission rates of *X. fastidiosa* tend to be higher in grape plant varieties than peach or almonds (Redak et al. 2004). Infected adults are capable of transmitting persistently for several months and during that time, when they feed on a host plant; their stylets repeatedly pierce the plant tissue and transfer the bacterium with each insertion (Almeida and Purcell 2003; Severin 1949). Physical damage to host plants can often be observed owing to these multiple, aggressive insertions of their stylets during feeding (Hunnicutt et al. 2006).

**Pierce's Disease of Grapevine**

Pierce's disease (PD) is a bacterial disease of increasing importance in grapevines. The disease is caused by *X. fastidiosa*, which causes 'scorch-like' symptoms, reduced yields, and vine death. The main insect vector responsible for rapid pathogen spread is *H. vitripennis*. The most notable symptoms are the appearance of water stress, marginal leaf burn, and uneven cane lignification (Ruel and Walker 2006). While the disease has been found in multiple grape production states, California is the largest grape producer, followed closely by Texas, and both have become a focal area for study of the disease. The PD causing strain of *X. fastidiosa* has only been found in areas with mild climates, as the bacterium is not cold tolerant and is consequently unable to survive freezing temperatures (Purcell 1997; Feil and Purcell 2001; Hopkins and Purcell 2002). The climates of California and Texas align with the needs of the PD *X. fastidiosa* strain and have permitted sharpshooter populations to flourish. The blue-green sharpshooter, *G. atropuctata,* is often seen in coastal California infections because of a high occurrence of riparian areas near vineyards; two grass feeding sharpshooters have also been identified as vectors in central California as vineyards are often adjacent to pastures, hayfields and canals; the most recent outbreaks in southern California have been attributed to *H. vitripennis* and present a real danger because of the association of these outbreaks with urban areas and citrus groves (Blua et al. 1999; Goodwin and Purcell 1992; Hewitt et al. 1949; Purcell and Frazier 1985). The increase in PD infections can also be attributed to the knowledge that *H. vitripennis* will often feed on stems of vines rather than leaves and petioles, which may lead to the high overwinter survival of bacteria as infected areas are

not trimmed off during winter pruning and the infection can become systemic (Purcell and Saunders 1999).

The spread of *X. fastidiosa* and resulting PD epidemiology has been a source of great economic loss in many agricultural settings throughout North America, largely in grape vineyards across California and Texas. Introduction of *H. vitripennis* into new vineyards has been linked directly with an increase in PD (Perring et al. 2001). As a top producing state in agriculture, California's production was valued at $38.4 billion in 2009, of that approximately $3.27 billion originates from grapes and $2.3 billion originates from almonds, which are both primary hosts of *H. vitripennis* (Izumi 2010). Additional economic costs other than crop loss include spread containment measures (CDFA 2003). Grape production in Texas in 2009 was valued at $1.17 million and the state is ranked fifth in grape production with a value exceeding $200 million (NASS 2010; Dodd et al. 2006). The full economic impact of the wine and wine grape industry in California and Texas is summarized in Table 1.1 (Wine Institute 2009; Texas Wine Marketing Research Group 2011).

Pierce's disease of grapevines presented itself in Texas after the introduction of *Vitis vinifera* and central Texas had an increase in PD incidence in the 1990's (Lauziere 2008). Production of grapes and value-added wine products are key players in the economies of both of these states as well as other PD effected areas. With the import and export of wine and fruit, the risks increase for global distribution of infected *H. vitripennis* that can potentially transfer inside crates.

**Table 1.1**: Summary values of the economic impact of wine and wine grape industry in California and Texas. Values illustrate the potential detrimental impact of Pierce's Disease.

| Economic Impact of Wine and Grape Industry | | |
|---|---|---|
| | California | Texas |
| Full-time Equivalent Jobs | 330,000 | 10,756 |
| Wages Paid | $12.3 billion | $379 million |
| Wine Produced (cases) | 196.3 million | 1.2 million |
| Retail Value of State Wine Sold | $18.5 billion | $117.5 million |
| Number of Wineries | 2,843 | 188 |
| Number of Grape Growers | 4,600 | 315 |
| Grape-Bearing Acres | 482,000 | 3,300 |
| Wine-Related Tourism Expenditures | $2.1 billion | $379.5 million |
| Number of Wine-Related Tourists | 20.7 million | 1,363,000 |
| Taxes Paid: Federal/State and Local | $3.9 billion/ $3.3 billion | $78.9 million/ $63.3 million |

## Section II

### Cell Culture

Insect rearing is a costly and ineffectual approach to investigate *H. vitripennis* control methods. The different stage-specific nutritional requirements and lack of an artificial diet make mass rearing of *H. vitripennis* difficult (Kamita et al. 2005). Tissue culture initially began with a small portion of tissue from a vertebrate being removed and grown on a sealed slide with fresh body fluids from the same region for several weeks (Harrison 1906). The first insect cell cultures were attempted using Harrison's method on sperm cells of *Samia cercropia* L. in haemolymph and were kept alive for up to three weeks (Goldschmidt 1915). Day and Grace (1959) detailed the history of cell culturing and divided it into three distinct phases. The first phase focused on gametogenesis and growth in haemolymph or simple saline solutions, but growth did not persist beyond several weeks (Glasser 1917). The second phase moved towards a focus on development and refinement of culture medium and cells survived an average of three months, allowing progress towards virus propagation techniques in cultured cells (Trager 1953)

(Fig. 1.3). The third, and most dynamic phase, involved the development of culture medium based on insect tissue chemistry and led to establishing cell lines from pupal ovarian tissues (Grace 1962).

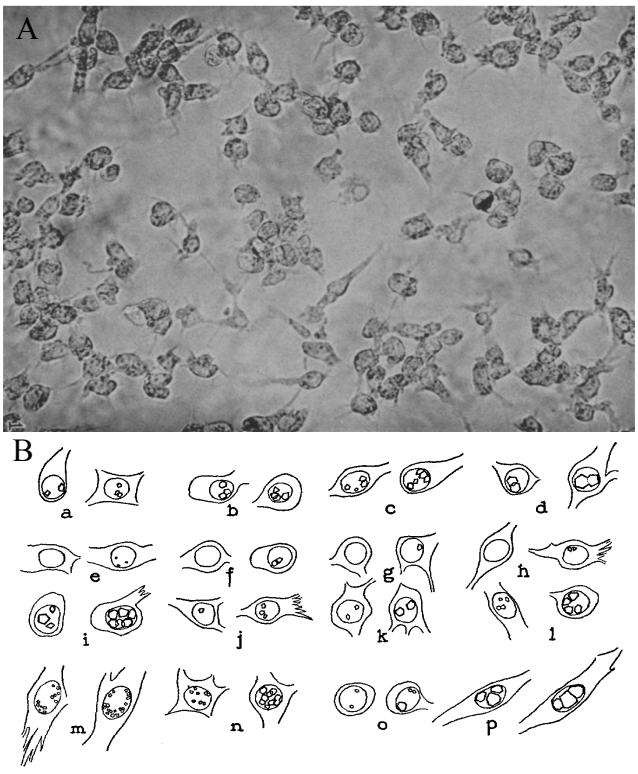


**Figure 1.3**. Early cell culture. (A) Photograph of six day old culture of silk-worm tissue. (B) Stages of polyhedra formation in silk-worm tissue culture. Figures from Trager 1952.

Since the 1950's, the use of insect cell culture for research has continued to increase, especially in the field of viral propagation. The numbers of established insect cell lines and the types of tissues that lines can begin from have continued to rise (Smagghe et al. 2009). Cell lines are now being utilized for a wide variety of things such as protein production, production of bioinsecticidal viruses and studying insect cell functions (Elias et al. 2007; Smagghe et al. 2009). In so far as viruses are concerned, cell lines are vital in understanding virus-cell interactions (Smagghe et al. 2009). While the

field has progressed, there is still much to be learned about current established cell lines, as well as continuing to increase the diversity of lines available.

**Section III**

**Methods of Control**

Control of vector species is crucial to controlling and preventing the further spread of PD as removal of symptomatic vines has been shown to not be an effective mechanism to staving off vineyard infections (Hewitt et al. 1949). Use of traditional vector management techniques, such as the application of pyrethroid and neonicotinoid insecticides has been employed in infected areas with limited success. The most commonly used neonicotinoid is imidacloprid, which is an insecticide approved for use both indoors and outdoors. Application of non-toxic insecticidal soaps and oils have been employed, but they have been found to be less effective and only target soft-bodied nymphs (Varela 2001). Problems arising with these methods in commercial vineyards are that such insecticides are non-specific and lead to problems including insecticide resistance of pest population, non-target organism impacts and residue contamination (Hunnicutt et al. 2006).

A shift towards utilizing naturally occurring parasites of *H. vitripennis* occurred once it was discovered that *H. vitripennis* were able to become established in southern California because of a lack of naturally occurring parasitic wasps and entomopathogenic fungi to act as natural enemies (CDFA 2003). Two species of entomopathogenic fungus were identified as potentially virulent towards *H. vitripennis*, *Psuedogibellula formicarum* (Mains) Samson and Evans (1973) and *Metarhizium anisopliae* (Metschinkoff) 5630 (Ecoscience, New Bruinswick, NJ) (Kanga et al. 2004).

Mymarid wasps (Hymenoptera: Mymaridae) have been identified as being the most recognized, naturally occurring egg parasitoid of leafhoppers (Huber 1986; Döbel and Denno 1993). In 1995, a mymarid wasp, *Gonatocerus ashmeadi* Girault, was identified as being an egg parasitoid of *H. vitripennis* (Triapitsyn et al. 1998). Studies of *G. ashmeadi,* a solitary endoparasitoid, have focused on parasitism, overwintering biology, and field release investigations, and have shown to account for 80-95% of observed egg parasitism in *H. vitripennis* in California (Chen et al. 2006; Huber 1988; Irvin and Hoddle 2005; Lopez et al. 2004; Phillips 2000). Two other species have been identified as successful parasitoids of *H. vitripennis* eggs: *Gonatocerus triguttatus* Girault (Hymenoptera: Mymaridae), a solitary endoparasitoid, and *Gonatocerus fasciatus* Girault (Hymenoptera: Mymaridae), a gregarious endoparasitoid (Triapitsyn et al. 2003). Different species have been observed to parasitize different egg age categories. Eggs 1-2 days of age were parasitized by *G. ashmeadi* and *G. fasciatus*, while eggs 3-4 days of age were parasitzed by *G. ashmeadi and G. triguttatus* (Irvin and Hoddle 2005). Age specific attacks would require multiple species to be employed for an effective control system; however, interspecies competition is a risk and could hinder population growth of the wasps and not impact *H. vitripennis* populations to as great of a degree.

The practice of utilizing naturally occurring entomopathogenic fungi and mymarid wasps as self-sustaining biocontrol agents, have been an incomplete methodology for combating this pest (Kanga et al. 2004; Irvin and Hoddle 2005). An insect pathogen that is presently found in nature can reduce pest populations in the wild and would be a much more effective biocontrol method by presenting a targeted approach for pest management (Hunter-Fujita et al. 1998; Hunnicutt et al. 2006).

**Dicistroviridae**

There are seven classes of eukaryotic viruses; the largest group is the positive RNA ((+) RNA) viruses. They are obligate, intracellular parasites that require host cells for replication. Dicistroviridae are a recently described family of single-stranded (ss), positive sense (+) RNA viruses with a genome in a dicistronic arrangement. They are also found strictly in invertebrate hosts (Christian 1998). The International Committee on the Taxonomy of Viruses (ICTV) has characterized them as belonging to the order *Picornavirales*; however, they are distinct from other members within the order because of the structural proteins located at the 3′ end of the genome and by having two open reading frames (ORF) (Bonning 2009). There are currently 2 genera within Dicistroviridae, *Aparavirus* and *Cripavirus,* and a total of 15 species (six in the former genus, nine in the latter). The distinction between the two genera is based on the type of internal entry site (IRES) on the intergenic region (IGR) of the genome. Species belonging to *Apavirus* have an additional stem loop found on the 3′ region of the IGR IRES that is not present in members of *Cripavirus*, as well as there is a conserved bulge sequence found in members of *Cripavirus* (Bonning 2009).

*Virion Structure*

Though there are similarities in the three dimensional structure of Dicistroviridae viruses to other picornaviruses, they have shown to be stable at both highly alkaline and acidic conditions (Tate 1999). The versatility in these conditions allows dicistroviruses to survive in different environments within a host. The virion is a non-enveloped icosahedral approximately 25-30nm in diameter (Tate 1999). There are 60 protomers that comprise the virion, and each of those consists of one molecule of capsid proteins

(CP) 1, 2 and 3 (Bonning 2009). Capsid protein 4 is found in some Dicistroviruses, but is much smaller and found under the surface of CP1, providing a link between the RNA genome and the capsid protein (Bonning 2009).

*Genome Structure*

A (+) RNA virus has a single-stranded RNA genome that can be directly translated by host cell machinery once it uncoats after entering the cell, because the genome functions like mRNA, making it highly infectious (Bonning 2009). The genomic RNA also drives viral replication in an involved process that forms membrane-associated replication complexes (RC) (Bonning 2009). Once the viral polyprotein(s) are processed (translated), viral RNA-dependent RNA polymerase (RdRp) combines with the viral RNA, viral non-structural proteins and host factors to form the RC for viral particle synthesis (Wang and Lui 2012). The genome is linear and has a viral genome-linked protein at the 5' end, and a polyA tract on the 3′ end (Bonning 2009). The first ORF is responsible for coding for nonstructural proteins such as helicase, protease, RDRP; the second ORF encodes for structural proteins. Replication of the genome can begin at ORF 2 without the complex initiation process and resulting strands can either be translated and continue the replication cycle, or be packaged into virions and released to infect more cells (Bonning 2009; Shüler 2006; Wang and Lui 2012) (Fig. 1.4). The virus does not encounter a host immune response because it lacks a start codon for translation initiation (Bonning 2009).

The small size of the genome limits the amount of proteins it can code for, requiring the virus to greatly rely on host cell intracellular machinery to complete replication. Dicistroviridae have been shown to enter host cells through

clathrin-mediated endocytosis and negative-sense complementary ssRNA are synthesized, which in turn synthesize new genomic RNA (Cherry 2004). As obligate, intracellular parasites, (+) RNA viruses hijack host cells and exploit them for proteins, as well as: membranes, lipids and microRNAs (Wang and Lui 2012). All aspects of the (+) RNA virus life cycle have been linked to needed participation from the host machinery, including virion release. The replication process drastically alters intracellular membranes in the host cell to facilitate replication and potentially shield RC components from cellular degradation; the levels of needed cellular components are also increased during this process (Wang and Lui 2012).

Transfer of virions between infected cells is unclear across the whole family. Some appear to be lytic, thus sacrificing their host cell to infect others, like Cricket Paralysis Virus (CrPV); others appear to be non-lytic, spreading from cell-to-cell without causing clear cytopathology (Bonning 2009). Transmission of Dicistroviruses from one host has been shown to occur: horizonitally *per os* from females to males and vertically by transovum and transovarial transmission (Bonning 2009; D'Arcy 1981; Gomirez-Zilber 1993; Reinganum 1970). In other words, viruses have been shown to transmit between species that are not in a parent-child relationship and from mother to offspring through infected reproductive tissue and eggs. With multiple modes of transmission, these viruses have great potential for spread within insect populations.

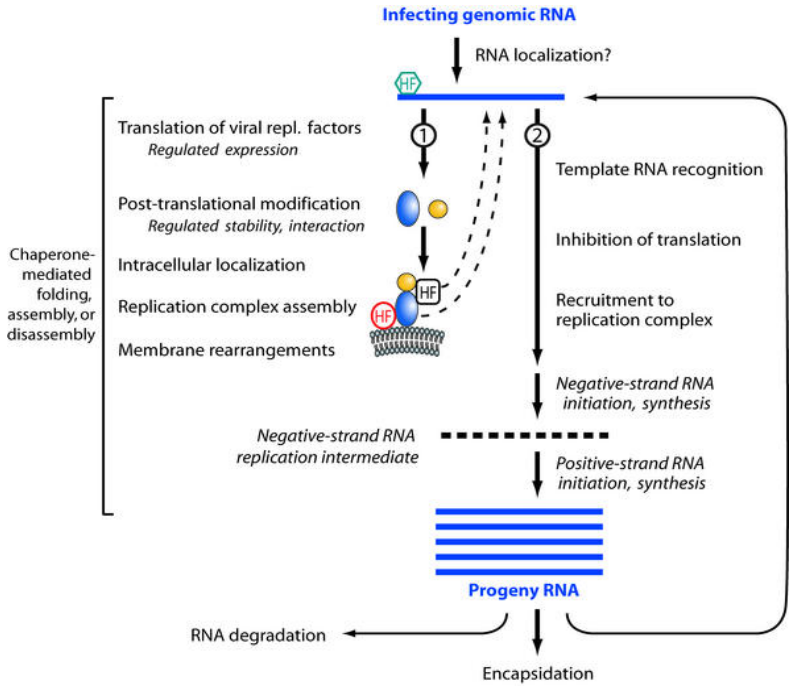


**Figure 1.4**. Simplified diagram of (+) RNA virus replication pathway. (Wang and Lui 2012)

*Study Virus*

A novel virus, *Homalodisca coagulata virus-01* (HoCV-01), has been identified in field collected *H. vitripennis* populations and classified as belonging to the genus *Cripavirus* in the family Dicistroviridae, based on capsid protein analysis and other molecular traits (Hunnicutt et al. 2006, 2008; Hunter 2006). *Homalodisca coagulata virus-01* and related virus species have been shown to increase mortality rates and reduce fecundity in insect populations (Hunnicutt et al. 2008). Production of viable biopesticides is becoming critical in battling invasive pests and in regards to *H. vitripennis*, HoCV-01 could be used to target low-density populations that occur when preferred host plants are unavailable in late winter, thus reducing number of offspring in first generations found in late spring (Blua et al. 2001).

**Purpose**

With the capability to exploit a broad array of host plants and the ability to cover large ranges, *H. vitripennis* present a great risk to agriculture within the United States as well as internationally if this invasive species is not managed. The following research questions will be addressed in this study:

(1) Can *H. vitripennis* cell lines be optimized for increased growth and development of a lab stock?

(2) What are the optimal concentrations and replication times of HoCV-01 in vitro?

(3) What levels of HoCV-01 are detectable using qRT-PCR and other quantification methods?

Resulting data will provide insight into an economically damaging invasive pest while presenting an alternative integrated pest management technique, biological control, to manage the main vector of the pathogen.

Chapter Two

Methods

**Cell Culture**

*Homalodisca vitripennis* cell lines established by the Hunter lab at the USDA Agricultural Research Service were used to establish a lab stock composed of mixed cell stages including initial fibroblast growth and monolayers. Cells were cultivated in H2G+ Leafhopper medium, a modified WH2 honeybee media (Hunter 2010) (Table 2.1). Medium was mixed and passed through a sterile 0.22 μm filter. Fetal bovine serum was added after filtration and 5 mL aliquots of medium were placed in a light-proof cabinet for three days at room temperature to test for bacterial contamination. Fungin (Cat. No. ant-fn-2, 200mg) was added to culture medium to inhibit mold growth. Cultures were maintained in Corning 25cm$^2$ and 75 cm$^2$ tissue culture flasks treated with CellBIND to promote cell attachment (Corning®, Lowell, MA) (Hunter 2010).

Culture flasks were kept in an incubator at 24°C with 53% humidity and examined using an inverted microscope (Olympus DP30BW, IX2-SP, IX71) at 100X magnification. Complete medium change was done every 10 days without disturbing the culture surface and cultures were passed when approximately 80% confluent. A 0.25% Trypsin EDTA solution (Invitrogen™, Carlsbad, CA) was used to dissociate cells. Trypsin is a proteolytic enzyme that breaks down the proteins allowing cells to adhere to the surface of the flask but can also damage sensitive cells. Cultures were exposed to minimal amounts of trypsin for short periods of time (5-10 min) to achieve complete

dissociation. For stubborn cells, gentle pipetting across the culture surface was used to detach cells. Once cells were completely dissociated, they were centrifuged at 350 RPM, in an Eppendorf 5804R Centrifuge centrifuge at 4°C for six minutes. Cultures were passed at a 1:2 ratio for 25 $cm^2$ flasks and a 1:1 for 75 $cm^2$ flasks. The supernatant was drawn off and the cell pellet gently suspended in 4 mL of fresh medium per 25 $cm^2$ flask being seeded and 9 mL per 75 $cm^2$ flask being seeded. Freshly passed cultures were left untouched for 48 hours to allow cells in suspension to attach securely to the surface of the flask.

**Table 2.1:** H2G+ Leafhopper medium components

| | |
|---|---|
| Grace's Insect medium (supplemented, 1X) | 210 mL |
| 0.06M L-histidine monohydrate solution (pH = 6.5) | 290 mL |
| Medium 199 (10X) | 10 mL |
| Medium 1066 (1X) | 17 mL |
| Hank's Balanced Salts (1X) | 33 mL |
| L-Glutamine (100X) | 1.5 mL |
| MEM, amino acid mix (50X) | 1.5 mL |
| 1 M MgCl solution | 6 mL |
| Pen-Strep (w/ Glutamine) | 2.5 mL/500 mL |
| Nystatin | 1.0 mL/500 mL |
| Gentamycin | 1.5 mL/500 mL |
| Dextrose | 1.8 g |
| Fetal Bovine Serum | 10% of final volume |

**Total volume of medium ~600mL; pH adjusted to 6.4-6.5 with 1M NaOH or HCl

Cells were also cultivated in to 48-well sterile tissue culture plates with a growth surface of 1 $cm^2$, that were surface treated to promote cell attachment (GREINER CELLSTAR®, Monroe, NC), for experimental purposes. Plates were seeded with 250 µL of cells in medium and the plates were sealed with parafilm to prevent contamination. Medium was replaced every 10 days without disturbing the culture surface and cultures were utilized for experimental procedures when approximately 80% confluent.

*Light microscopy*

All cultures were examined daily under an inverted microscope (Olympus DP30BW, IX2-SP, IX71) at 100X magnification, beginning 48 hours post-seeding. Images were captured at full light exposure with high contrast, taking care to image the correct field of vision. Five fields were imaged per culture to compare cross-flask cell growth.

*Viral replication*

Virus positive whole body *H. vitripennis* were homogenized and virus extracted previously in the Bextine lab (Bextine et al. 2009). The resulting HoCV-01viral pellet from that work was subjected to a 10-fold dilution series up to 1:100,000. Utilizing cell culture plates, all rows were grown until 80% confluent (approximately 72 hours post-pass). When ready, plates were inoculated with 10 µL of varying viral dilutions except for the top row. The top row was used as a control and 10 µL of ddH20 was added to each well for volume control (Fig. 2.1). After viral inoculation, culture plates were examined every 24 hours for any color change in the medium indicating a pH change and for cell morphology changes. At each time point, one column of the test plate was imaged using an inverted microscope at 100X magnification. All medium was removed from the same column and stored at -20°C for RNA extraction and viral quantification. After medium was removed, cells were dissociated from the culture plate surface. Cell counts were completed using a hemocytometer and trypan blue stain after cell dissociation for each 24 hour period over one week. Prior to viral inoculation, the first well of each row was removed to establish a baseline starting cell concentration.

**RNA**

*RNA extraction*

Whole RNA was extracted from medium samples collected during each one week virus trial using TRIzol® LS ( Invitrogen™, Carlsbad, CA) per the manufacturers protocol, with no modifications, and stored at -80°C. In short, liquid medium samples were homogenized in TRIzol LS by pipetting and chloroform was used to induce a phase separation allowing for removal of the RNA captured in the aqueous layer. Sample RNA was precipitated out using isopropanol and then washed in 75% ethanol. The final RNA pellet was resuspended in RNase free water and quantified using spectrophotometry.

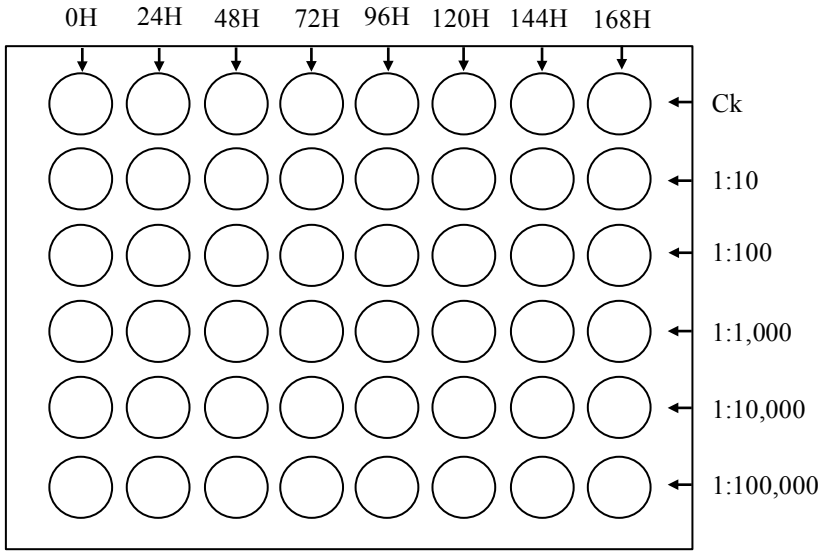


**Figure 2.1.** Diagram of experimental plates indicating control and treatment rows. Column labels represent the time point that the sample was removed.

*Whole virus extraction*

Infected *H. vitripennis* cells were removed from culture flasks, pelleted and homogenized by vortexing in 100mL of phosphate buffer containing 0.02mg DETCA. The following virus extraction is a slightly modified version used to collect virus from whole body *H. vitripennis* (Bextine et al. 2009). Modifications were made to

centrifugation speeds and times to account for the difference for extracting virus from cells grown in vitro versus whole body insects. The homogenate was then transferred to 50mL centrifuge tubes and centrifuged at 300 rpm for 20 minutes in an Eppendorf 5804R Centrifuge (Eppendorf, Hamburg, Germany). The resulting supernatant was split into two ultra-centrifuge tubes, vortexed, and ultra-centrifuged at 22,000rpm for 16 hours in a Sorvall® RC-5B Refrigerated Superspeed Centrifuge (DuPont Instruments, Wilmington, DE). Following ultra-centrifugation, the supernatant was discarded, and the resulting pellet was dissolved with 5mL phosphate buffer containing 4% Brij 52 and 0.4% Na-deoxycholic acid. The resulting solution was centrifuged at 300 rpm for 15 minutes and passed through a 0.45μm filter into large Eppindorf collection tubes. The impure HoCV-01 solution was transferred to a dialysis membrane and placed in a large beaker in a refrigerator at 4°C containing a stir-bar and ddH20. The ddH20 was changed every hour for a period of five hours until a white precipitate was observed in the dialysis membrane. The purified HoCV-01solution was stored at -80°C.

**RT-PCR**

*Virus standards*

To establish viral standards for RT-PCR, traditional PCR was run using the primer pair HoCV RT-PCR primer 1 (forward 5′-GCTCCCCGGCTTTGCTGGTT-3′, reverse 5′-ACGACGGATCTGCGTGCCAA-3′) using virus isolate from whole body *H. vitripennis*. Samples were electrophoresed for 60 minutes at 120 volts in a 2% agarose gel containing 0.1% ethidium bromide. Bands were excised from the gel and purified using the QIAquick® (Qiagen, Carlsbad, CA) gel extraction kit. Purified samples were quantified using spectrophotometry, combined together and subjected to an ethanol

precipitation to increase the overall sample concentration. Pooled samples were re-quantified using spectrophotometry.

A ten-fold serial dilution was performed on the purified sample ranging from 57ng/μL to 57ag/μL ($10^{-18}$). To determine detection limits on the dilution series, qRT-PCR was done. It was determined that viral concentrations lower than $5x10^{-3}$ copies were not detectable.

*Experimental samples*

RNA was extracted from experimental samples as described previously and quantified using spectrophotometry. All samples were normalized to 5ng/μL using nuclease free water. All samples were subjected to qRT-PCR in duplicate as 25μL reactions using the QuantiFast® RT-PCR kit (Qiagen, Carlsbad, CA) as follows: 50°C hold for 10 minutes; 95°C hold for 5 minutes; 30 cycles of 95°C for 10 seconds, 60°C for 30 seconds; melt from 50-99°C for 5 seconds on each step. Each reaction mixture contained 12.5 μL of master mix, 1.0 μL of forward primer, 1.0 μL of reverse primer, 0.25 μL of reverse transcriptase and variable amounts of template based on standardization values. Total reaction volume was brought to 25 μL with RNase free water. Five standard concentrations were included in each PCR run with the following copy numbers: $5x10^{-10}$, $5x10^{-8}$, $5x10^{-6}$, $5x10^{-4}$, and $5x10^{-2}$ copies. The threshold for each run was set to just below a fluorescence of $10x^{-2.5}$ to reduce noise during early acquisition at the beginning of each run.

**Confocal Microscopy**

*Homalodisca vitripennis* cells were grown in a twelve well plate containing glass coverslips measuring 18mm in diameter in each well. Once a monolayer was achieved,

one column on the plate was inoculated every 24-hours for a period of four days. Each column contained a control well, a low viral dilution (1:10) well and a high viral dilution (1:100,000) well. At the end of the four-day period, the resulting cells had four different time points of viral infection (24, 48, 72 and 96 hours).

On the fifth day, media was removed and the cells washed twice with 1X PBS (pH 7.4) and prepared for confocal microscopy. Cells were fixed with cold 4% paraformaldehyde at 4°C for 30 minutes. After fixing, cells were washed three times with 500µL of 1X PBS for 10 minutes at room temperature on a rocker at low speed. Cells were permeabilized using 500µL of 0.1% Triton X-100 for 10 minutes at room temperature. Cells were washed again with 500µL of 1X PBS, three times for 10 minutes at room temperature on a rocker at low speed. A 5% bovine serum albumin (BSA) solution was used to block cells at room temperature for two hours and then removed from the cells.

To stain for F-actin, Rhodamine red-conjugated phalloidin (RCP) was used. Stock RCP was diluted in 5% BSA and 250µL of the dilution was added to each well. The plate was covered in aluminum foil to prevent the dye from bleaching. Cells were incubated at 4°C overnight. The next day, the RCP was removed and replaced with 250µL of DAPI diluted in 5% BSA, to stain the nuclei of the cells. The DAPI was incubated at room temperature for one hour. The cells were then washed three times with 1X PBS as previously described, the coverslips were gently removed from the wells, mounted to microscope slides using mounting media with an anti-fade reagent and were allowed to dry in light proof boxes until viewed under the confocal microscope.

The stained cells were imaged using an LSM510 Meta Confocal System (Carl Zeiss, Germany) equipped with an Axio Observer Z1 microscope (Zeiss) using a 63X (oil) plan-apochromate lens. The laser setting wavelengths were 543 ± 10 nm excitation and 575 ± 10 nm emission for Rhoadmine red-conjucated phalloidin, and 369 ± 10 nm excitation and 450 ± 30 nm emission for DAPI. All images were obtained using identical gain and off-set settings for the detector. The images were processed using LSM Zen 2007 (Zeiss) software and imported to Pixelmator (v. 2.1.4) for compilation of figures.

**Statistical Analysis**

Cell count and qRT-PCR data were tested for normality and analyzed using a two-way ANOVA with Bonferroni post-hoc comparisons to look at differences between treatment groups at each time point. Group means were plotted with standard deviation values and also subjected to Kaplan-Meier survival analysis and Cox proportional hazards analysis. A threshold value for survival was set at $25x10^4$ cells/mL to determine whether an event (cell decline) occurred or not for each experiment group at each time point. All data was analyzed using Prism (v. 5.0b) and R (v. 2.15.1) for Mac.

# Chapter Three

# Results

## Cell Culture

### *Lab stock*

Cell attachment and growth was seen within 48-hours of passage in both small and large culture flasks, from primary cultures and continued passages. Fibroblast growth and development was also observed within this time frame. When newly seeded flasks were disturbed before 48 hours, there was a visible decline in cell attachment, leading to slower growing cultures and sometimes no attachment or growth at all. Cells were approximately 80% confluent within one week of passing and formed a monolayer in 10-14 days (Fig. 3.1).

Bacterial contamination in the medium wiped out the newly established lab stock seven months after initial formation. The source of the contamination was determined to be an improperly filtered and stored buffer and caused the medium in culture flasks to turn milky and cells to detach completely from the culture surface. New primary cultures received were cultivated and have survived 20+ cell passages without any morphological deterioration or overall cell viability decline (Appendix A).

### *Experimental plates*

Cell attachment and growth was seen within 48-hours of passage from flasks to plates. Monolayer formation was achieved in a shorter time period, approximately 5-6 days, as it is a smaller growth surface.

**Viral Effects on Cell Culture**

*Light microscopy*

Infected cultures photographed at 100X under light microscopy showed signs of morphological changes and cell deterioration approximately 72-96 hours after being infected with non-diluted HoCV-01 (Fig. 3.2).

*Viral treatments*

Mean live cell counts for control and experimental samples were calculated (Table 3.1) and plotted to show differences in abundance of live cells between viral loads over time (Fig. 3.3). The counts show a consistent increase in live cells for the control group, indicating healthy cells. Comparatively, all treatment groups show a marked decline in the number of live cells present over time. The higher viral treatment groups indicate a much more marked decline in culture health with a major drop in live cells between 48-72H, while the lower viral groups slowly decline until dropping off around 144H.

A two-way ANOVA with Bonferroni post-hoc analysis was used to test the differences in cell culture kill rates by HoCV-01 based on the live cell counts in each treatment group compared to each time point in the study, as well as between groups. The two-way analysis of variance showed a significant main effect of the time factor, $F(7, 432) = 82.5$, $p < 0.0001$, suggesting that the lengths of time cultures were exposed to treatment affected culture longevity. The effect of the type of treatment cultures received was significant as well, $F(5, 432) = 170.6$, $p < 0.0001$, indicating that the amount of viral load a culture initially receives affects culture survival. The results also indicate a significant effect in the interaction between the time factor and treatment factor,

$F$ (35, 432) = 17.63, $p < 0.0001$, underlining that the higher the viral load received the shorter amount of time needed to reduce culture fitness and conversely the lower the viral load, the longer period of time required for the same effect. Bonferroni post-hoc tests are summarized in Table 3.2, and illustrate a significant difference between treatment and control groups, indicating a notable dose response.

Kaplan-Meier curves showing survival probability also indicate a lower survival rate with higher viral treatments over time (Fig. 3.4), correlating to the conclusions drawn from the mean live cell count analyses. The survival curves indicate a 100% survival rate for control or non-infected cells. Cells exposed to the high viral treatments had a marked decline in survival probability over time, while lower viral treatments have a greater probability of survival until the 144H, then a decline in survival probability is present (Appendix B). Cox proportional hazards model analysis was not significant, treatment coef = 0.8812 (95% CI [0.76, 1.02]), $p > 0.05$. While not significant, the data suggests that cells exposed to virus are 88% more likely to exhibit lower survival rates over time.

**Viral RT-PCR**

Resulting curves from qRT-PCR runs illustrates that higher viral standards ramp up earlier during the run than lower viral standards and experimental samples (Fig. 3.5). From each run, replicate Ct values were analyzed using a two-way ANOVA to test for differences in the abundance of HoCV-01 RNA present in experimental samples and compare values to multiple control values. The two-way analysis of variance showed no significant main effect of the time factor, $F$ (7, 289) = 0.38, $p > 0.05$, or in the interaction between time and treatment groups, $F$ (63, 289) = 0.14, $p > 0.05$. There was a significant main effect between treatment groups, $F$ (9, 289) = 135.7, $p < 0.0001$, indicating that

amount of virus initially introduced to cell culture affects the amount of viral RNA detected. Bonferroni post-hoc tests were run and show significant interactions between treatment groups and control measures, but no significant interactions between treatment groups alone, indicating no measureable dose response (Appendix C).

**Confocal Microscopy**

Differences in cell morphology of healthy and HoCV-01 infected *H. vitripennis* cells at 24 and 72 hours can be seen under fluorescence. The decline of number of nuclei present as well as the misshapen appearance of F-actin in the cells exposed to HoCV-01 as compared with controls indicate that the virus has a major impact on culture health. Cells exposed to the higher 1:10 viral load show greater distress than the cells exposed to the lower viral treatment. Control cells appear more abundant and to have normal morphology between the two time points. (Fig. 3.6)

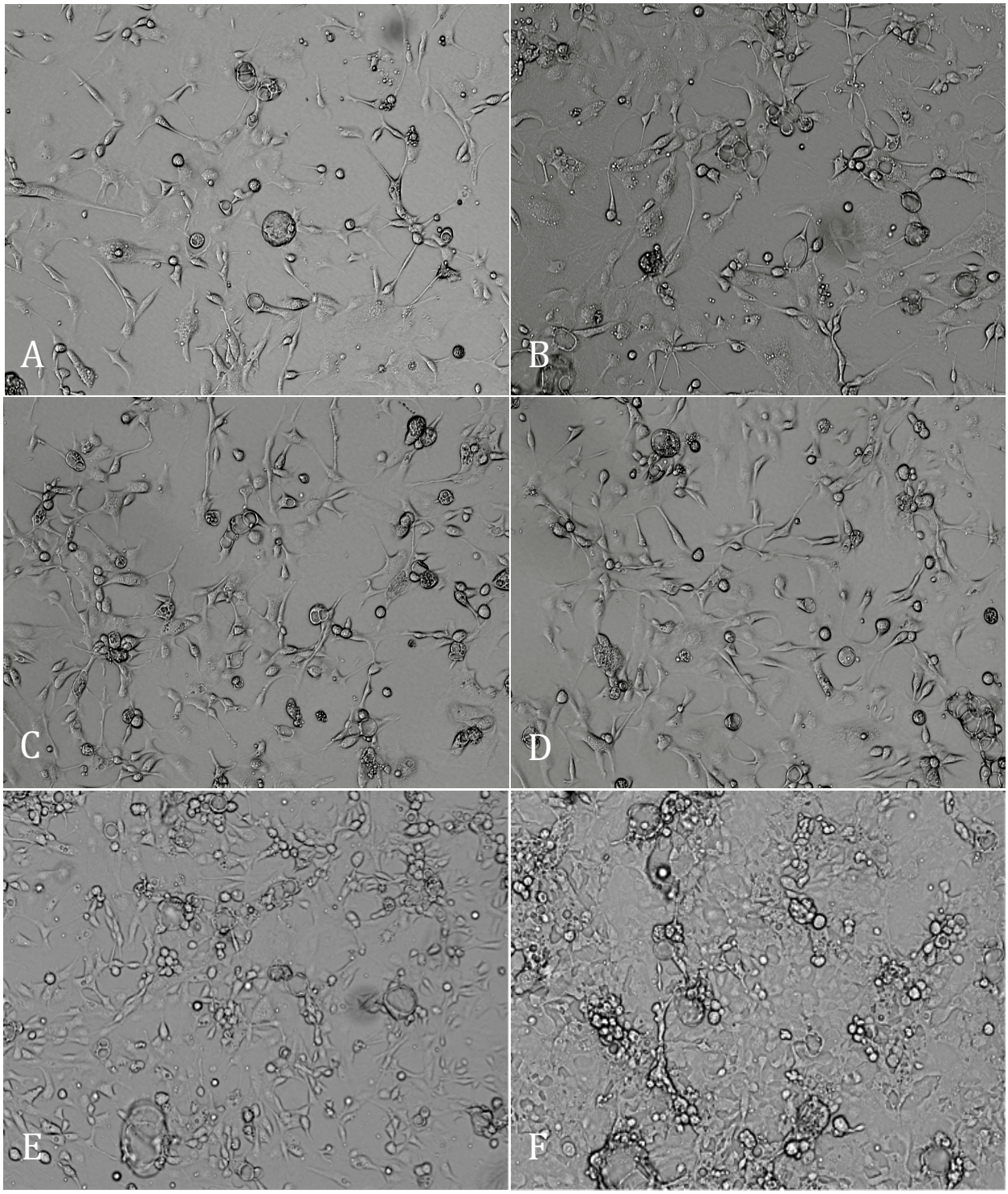


**Figure 3.1**. Images of *Homalodisca vitripennis* cell growth in vitro captured at 100X. (A) Cells two days (48H) post-passage exhibiting attachment and fibroblast development. (B-E) Cells four, six, eight and ten days post-passage continuing to grow across culture surface. (F) Monolayer formation occurring ~10-14 days post-passage.

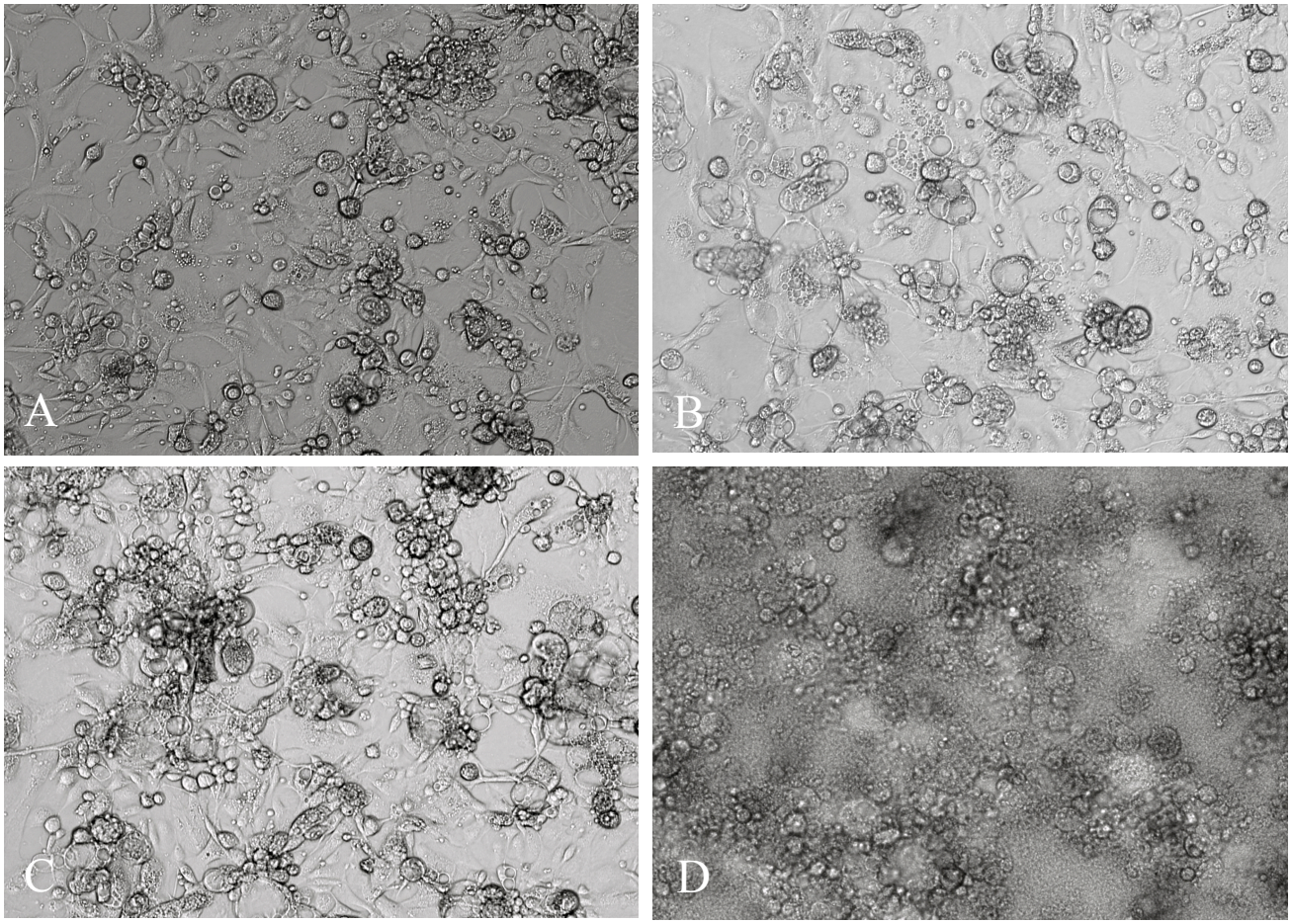


**Figure 3.2**. Infected *Homalodisca vitripennis* cells imaged at 100X magnification to capture morphological changes. (A) Fibroblast growth prior to inoculation. (B) Cells 24H post-infection. (C) Cells 48H post infection. (D) 96H post infection cells have mostly detached from the culture surface and medium has become cloudy.

**Table 3.1**. Mean live cell count data. Live cell counts were recorded for each treatment group at 24H intervals. Mean live cell counts and standard deviation were calculated for each treatment group per time point.

| Time point (in hours) | Cell counts ($10^4$/mL) | | | | | |
|---|---|---|---|---|---|---|
| | Ck | 1:10 | 1:100 | 1:1,000 | 1:10,000 | 1:100,000 |
| 0 | 38.22 ± 5.51 | 42.93 ± 6.56 | 44.71 ± 4.87 | 44.21 ± 2.36 | 39.87 ± 11.78 | 43.05 ± 5.94 |
| 24 | 40.69 ± 4.37 | 39.91 ± 7.61 | 40.50 ± 4.58 | 39.91 ± 6.87 | 42.88 ± 2.49 | 42.13 ± 7.14 |
| 48 | 42.01 ± 6.16 | 24.87 ± 2.28 | 38.54 ± 4.62 | 39.59 ± 4.71 | 41.53 ± 2.28 | 38.48 ± 9.19 |
| 72 | 46.00 ± 4.44 | 21.76 ± 0.71 | 37.90 ± 3.55 | 37.87 ± 5.33 | 38.33 ± 3.63 | 38.54 ± 6.59 |
| 96 | 45.90 ± 5.06 | 19.04 ± 2.22 | 35.28 ± 5.14 | 36.44 ± 4.59 | 36.54 ± 3.50 | 37.38 ± 4.59 |
| 120 | 50.74 ± 7.41 | 16.71 ± 2.26 | 32.56 ± 5.72 | 34.57 ± 4.37 | 34.97 ± 3.49 | 35.65 ± 4.45 |
| 144 | 52.48 ± 5.14 | 15.24 ± 2.26 | 22.89 ± 2.03 | 24.95 ± 2.67 | 25.48 ± 1.63 | 27.05 ± 1.10 |
| 168 | 55.17 ± 5.05 | 13.24 ± 2.06 | 19.16 ± 1.31 | 22.19 ± 2.80 | 22.93 ± 1.38 | 24.37 ± 0.82 |

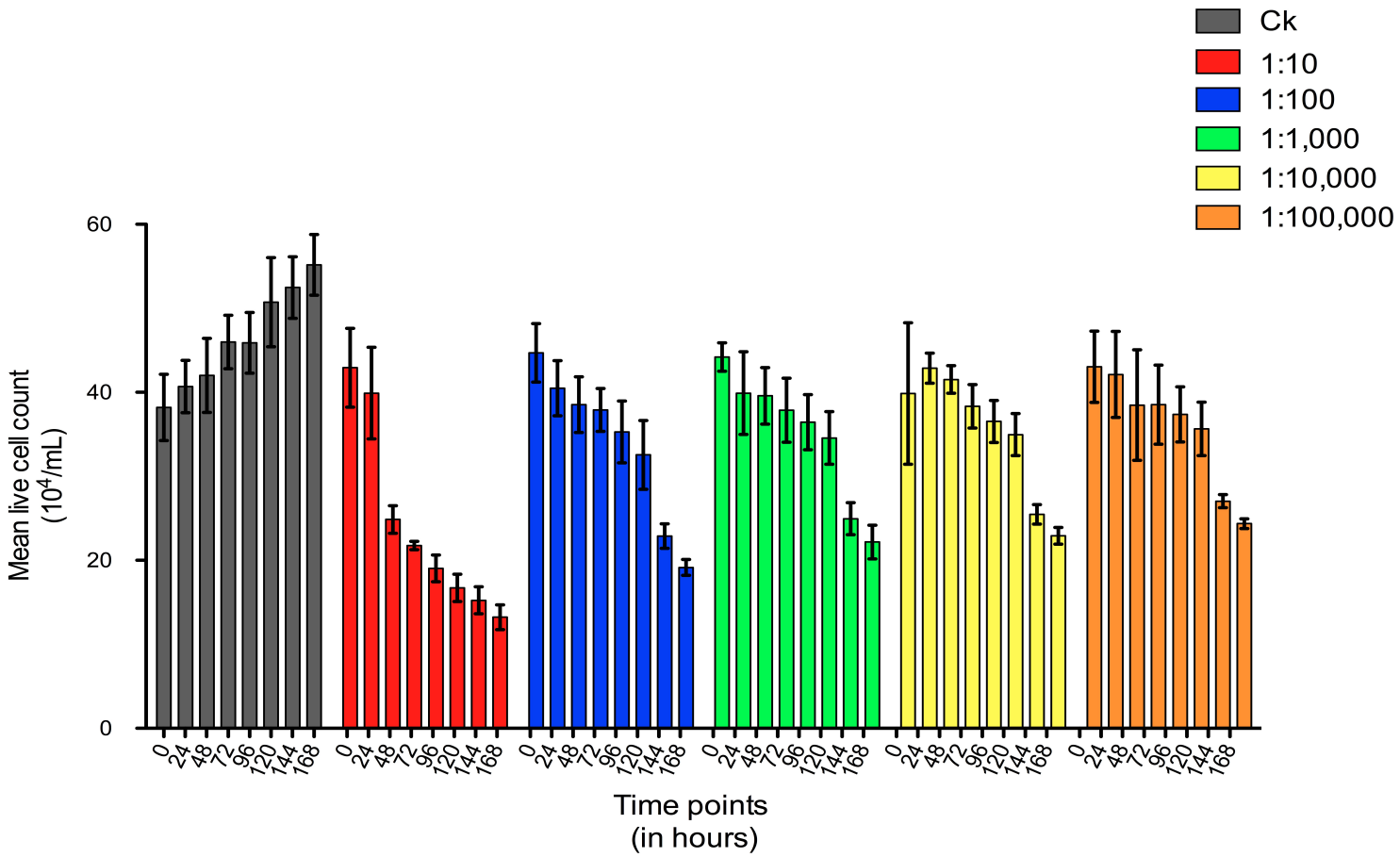


**Figure 3.3**. Bar chart showing mean live cell counts for experimental samples. Mean live cell counts were calculated for experimental samples by viral load received for each day during the experimental period and are shown here with standard deviation bars. Mean cell counts show a significant decrease in live cells ~72H post-infection with high viral loads and significant decreases in live cell counts at ~144H post-infection with lower viral loads.

**Table 3.2**. Summary of Bonferroni post-hoc test results for cell count data. Tests were between treatment groups compared to the control group showing significance within treatment groups at different time points

| | | | Sources of variation (N=10) | | | | |
|---|---|---|---|---|---|---|---|
| Groups | Difference | p-value | Significant | Group | Difference | p-value | Significant |
| Ck vs 1:10 | | | | Ck vs 1:10,000 | | | |
| 0H | 4.72 | > 0.05 | No | 0H | 1.65 | > 0.05 | No |
| 24H | -0.78 | > 0.05 | No | 24H | 2.191 | > 0.05 | No |
| 48H | -17.14 | < 0.001 | Yes | 48H | -0.48 | > 0.05 | No |
| 72H | -24.24 | < 0.001 | Yes | 72H | -7.67 | < 0.01 | Yes |
| 96H | -26.86 | < 0.001 | Yes | 96H | -9.36 | < 0.001 | Yes |
| 120H | -34.03 | < 0.001 | Yes | 120H | -15.78 | < 0.001 | Yes |
| 144H | -37.23 | < 0.001 | Yes | 144H | -27.00 | < 0.001 | Yes |
| 168H | -41.93 | < 0.001 | Yes | 168H | -32.34 | < 0.001 | Yes |
| Ck vs 1:100 | | | | Ck vs 1:100,000 | | | |
| 0H | 6.50 | < 0.05 | Yes | 0H | 4.83 | > 0.05 | No |
| 24H | -0.19 | > 0.05 | No | 24H | 1.44 | > 0.05 | No |
| 48H | -3.47 | > 0.05 | No | 48H | -3.53 | > 0.05 | No |
| 72H | -8.10 | < 0.01 | Yes | 72H | -7.46 | < 0.01 | Yes |
| 96H | -10.62 | < 0.001 | Yes | 96H | -8.52 | < 0.001 | Yes |
| 120H | -18.19 | < 0.001 | Yes | 120H | -15.10 | < 0.001 | Yes |
| 144H | -29.59 | < 0.001 | Yes | 144H | -25.43 | < 0.001 | Yes |
| 168H | -36.01 | < 0.001 | Yes | 168H | -30.80 | < 0.001 | Yes |
| Ck vs 1:1,000 | | | | | | | |
| 0H | 5.99 | < 0.05 | Yes | | | | |
| 24H | -0.78 | > 0.05 | No | | | | |
| 48H | -2.42 | > 0.05 | No | | | | |
| 72H | -8.13 | < 0.01 | Yes | | | | |
| 96H | -9.46 | < 0.001 | Yes | | | | |
| 120H | -16.18 | < 0.001 | Yes | | | | |
| 144H | -27.53 | < 0.001 | Yes | | | | |
| 168H | -32.98 | < 0.001 | Yes | | | | |

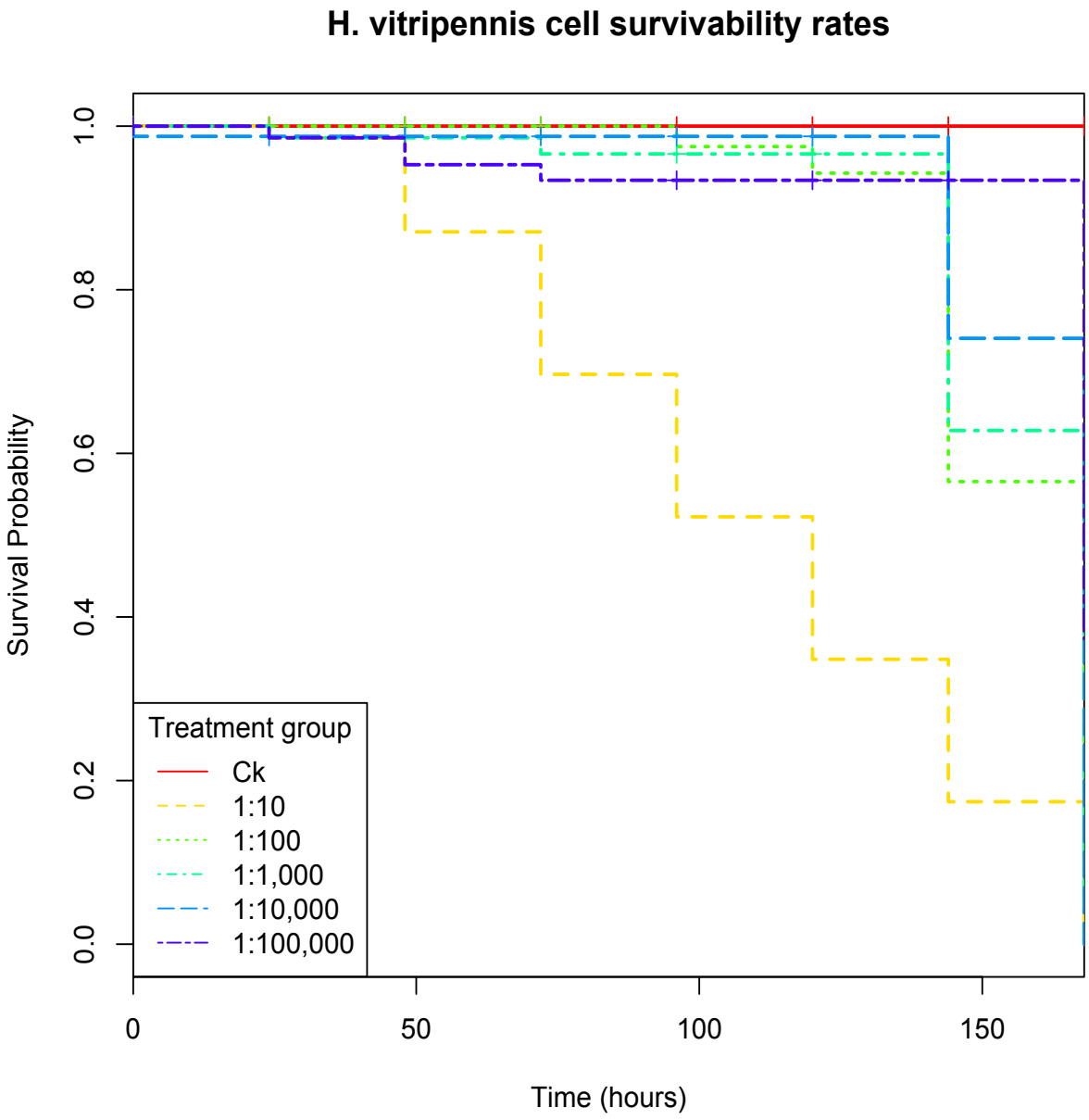


**Figure 3.4**. Kaplan-Meier survival analysis of five different HoCV-01 treatments compared to a non-infected control in *H. vitripennis* cell cultures. Control cultures maintained a 100% survival rate compared to the five treatment groups. The lowest survival probability was seen in the high treatment group and all treatment groups head towards zero survival probability at 168H when n=10.

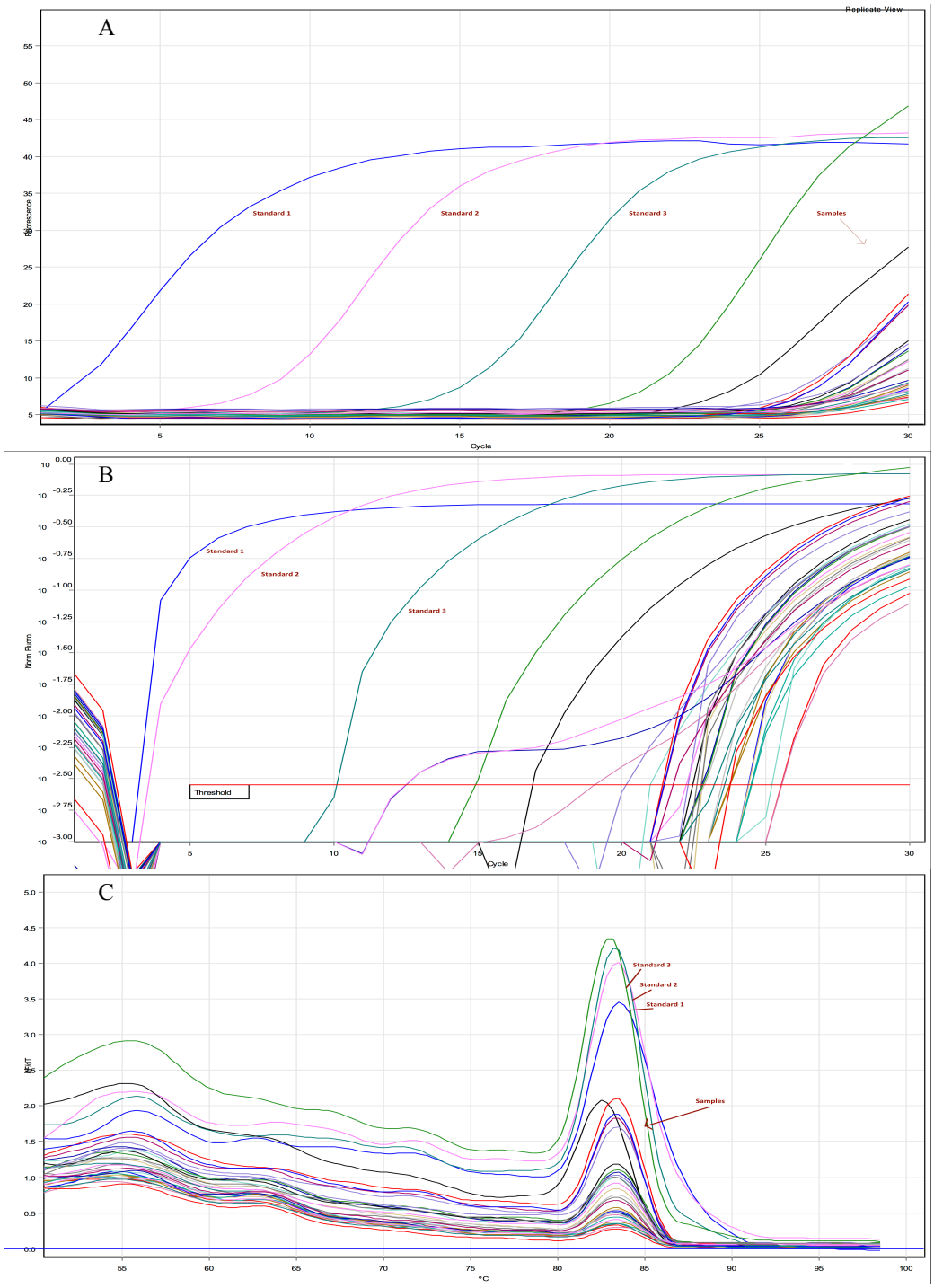


**Figure 3.5**. Curves from a qRT-PCR run showing viral standards and experimental samples from different time points. (A) Quantitation curves showing viral standards ramping up 10-15 cycles prior to experimental samples. (B) Cycling curves correlating to quantitation curves showing higher concentrations of viral standards ramping up before lower concentration standards and low concentrations from experimental samples. (C) Melt curves of all samples run showing the same average melt temp across samples in the run illustrating that the same piece of RNA was copied.

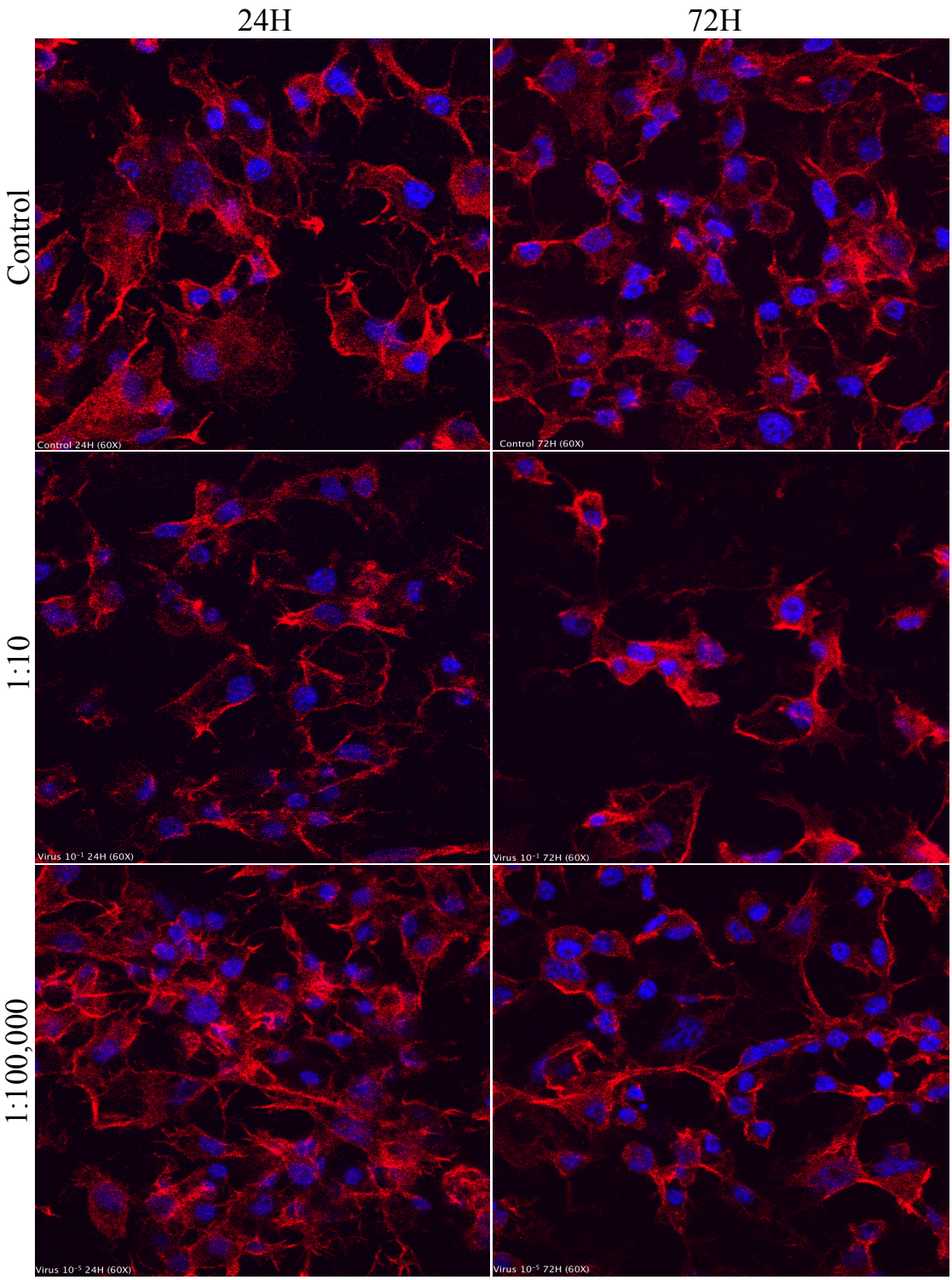


**Figure 3.6.** Confocal images of control and infected *H.vitripennis* cells. *Homalodisca vitripennis* cells were infected with serial diluted HoCV-01 in 1:10 and 1:100,000 concentrations at 24H intervals. Cells were treated with rhodamine phalloidin and DAPI stains to visualize F-actin and nuclei within the cultures. Confocal images were captured at 60X and show a break down in cell morphology at 72H at both low and high viral dilutions.

# Chapter Four

## Discussion

Rising concern regarding the influx of invasive species in agriculture has lead to an increased demand for new methodologies to defend against emerging diseases, as well as to combat established epidemics. Managing pathogen vectors has become a focus for disease prevention and control, and was the primary target of this study. Economics play a vital role in the decision to produce this type of biopesticide to manage pathogen vectors in agriculture because the practical application needs to be large quantities over large areas but at a low cost (Rhodes 1996). The practice of utilizing cell culture for research and development has become increasingly common and as such, the impacts of this study are significant. Identifying economically feasible integrated pest management (IPM) strategies is key to continued successful agricultural production worldwide and the findings in this study contribute to progress in improving IPM strategies and reducing the occurrence of PD of grapevine.

In this study, the first question addressed was the capability of extended periods of increased *H. vitripennis* cell culture propagation. Primary *H. vitripennis* cell cultures were propagated and maintained for over a year without any visible morphological deterioration. Passage numbers can drastically affect the results of in vitro studies in mammalian cells by changing cell metabolism and growth characteristics (Briske-Anderson et al. 1997). As cells replicate in vivo and in vitro, telomeres shorten with each round of cell replication and eventually reach a critical limit where telomeres become too

short and induce cellular senescence (Chin et al. 1999). When severe telomere shortening occurs, it leads to genetic instability and finally crisis, or massive cell death (Counter et al. 1992). Because of this phenomenon, it was previously determined that cultures rarely survive beyond 50 subcultivations or one year, deemed the Hayflick limit, based on the following criteria: retention of sex chromatin, histotypical differentiation, inadaptability to suspend culture, non-malignant characteristics in vivo, finite limit of cultivation, similar cell morphology to primary tissue, increased acid production compared to cell lines, retention of Coxsackie A9 receptor substance and ease with which strains could be developed (Hayflick and Moorhead 1961). The potential complications of passage numbers were not observed during the duration of this study indicating that this method of cell line propagation is capable of continual production over extended periods of time. Because utilization of cell culture over live insect rearing or other expensive and complicated production methods is rapidly becoming commonplace across many disciplines, the longevity of this type of cell line has many practical applications. If maintained properly, a single primary cell line could be used for multiple rounds of virus production.

The two major factors in successful long-term maintenance of these cells were disturbance time for freshly seeded cultures and proper medium preparation. Cultures that remained untouched for the first 48H after passage showed a marked increase in cross-flask growth compared to those that were moved within that initial window. When left undisturbed with cell passage ratios of 1:2 and 1:3, the rapid replication of cells achieved monolayers in as little as ten days post-passage in culture flasks. Medium preparation was as vital during the study as disturbance time as far as general culture

health was concerned. Even with antibiotics present in the medium, bacterial contamination was still an important factor to consider when preparing medium. By allowing aliquots of medium to remain at room temperature for several days before use in cultures, the likelihood of a devastating series of culture collapses because of bacterial contamination is reduced to a nearly non-existent factor. Antibiotics are commonly used in cell culture medium to combat bacteria found within the cells and any outside contamination, especially gentamicin and streptomycin (pen-strep), which are both present in the medium used in this study. Both of these antibiotics have been linked to a depression of cell growth in mammalian cultures and to a decrease in the use of aseptic techniques and concern for increasing the likelihood of developing antibiotic resistant strains of bacteria (Goetz et al. 1979; Coriell 1973). While antibiotic use should not be excessive, it is a necessary tool for combating contamination problems within cell culture.

The implications of these factors are such that up scaling production of cells is a viable option for quick mass production of biopesticide materials with minor steps to ensure quality of cell cultures. Bioreactors emerged in the 1950's and 1960's, and have since evolved to provide efficient means of producing billions of cells in an exceptionally short amount of time (Hambor 2012). There are many types of bioreactors that could be utilized to dramatically increase the number of *H. vitripennis* cells produced at one time and the process of developing this type of production system would require the development of a method to treat cells to prevent shearing from growth surfaces in bioreactors.

Successful continued growth of cell lines is crucial to HoCV-01 replication and once achieved, can be used to address the second objective of how much virus is needed for quantifiable in vitro replication and how long should the virus be allowed to remain within cultures. Two potential techniques for viral replication within *H. vitripennis* cell cultures were determined during the study. A clear correlation was found between amount of initial viral load received by cells and the duration of time virus particles were permitted to incubate within cells. The higher the viral load received, the lower the time requirement for cell death, indicating rapid viral replication. However, across all treatments, cell numbers declined to below the threshold value of $25x10^4$ cells/mL at approximately 144H post-infection, demonstrating an overarching cell culture survivability threshold. The results illustrate that large amounts of virus can be produced quickly if larger amounts of virus are readily available for initial infection, or that increased amounts of virus can be produced in a longer period of time with lower initial dosage. Variability in the relationship between concentration and time factors allows some flexibility in production options for larger-scale studies with an optimal viral extraction time of 72-96H post-infection.

Using cell cultures for viral studies is dependent on the ability to detect the target virus and quantify the results of the study. A reliable method for this is to use PCR to check for the presence of viral RNA sequences within experimental samples. The analysis of Ct values from PCR data in this study does not give a clear answer to what the optimal extraction time of virus would be, however lack of a definitive extraction time is not indicative of an inability to replicate HoCV-01 in vitro, but of the sensitive nature of viral studies be from cell cultures across treatment groups. The trypan cell counts do lend

to a clearer view of optimal extraction times but are by no means a definitive answer. Cell death data illustrates that high viral loads lead to highly decreased cell survivability after 72H, indicating that viral extraction between 48H and 72H post-infection may be ideal to reduce cellular breakdown of viral particles as the cells in the culture begin to die exponentially. Extraction times at lower initial viral loads are more ambiguous, but with dramatic decreases in cell survivability after 144H, it can be speculated that optimal extraction time would be 24-48H prior to that time point. Determination of optimal viral extraction time is vital to effectual production of biopesticides for use against *H. vitripennis* infestations and this study has taken an important step towards determination of those times.

Microscopy is a key tool for cell culture analysis and this study is the first one to use confocal microscopy with *H. vitripennis* cells. Imaging protein attachment increase or decline and abundance of cell nuclei is the first step towards more detailed studies into the intracellular activity of HoCV-01 in vitro. Throughout this study, cell cultures were maintained with no visible morphological deteriorations. However, when infected cultures were imaged with confocal microscopy, cell morphology deterioration was observed, especially at the 72H time point, with both high and low initial viral loads. The implications from this first use of higher resolution microscopy correlate to the results seen in the cell survivability analysis and give rise to other possible uses for increased viral studies. Advanced microscopy techniques could be utilized to its maximum capabilities if antibody development for HoCV-01 was conducted. Antibodies for the virus would not only allow visualization of intercellular workings of the viral particles but could also help determine proliferation rates and even more precise extractions times.

The process of scaling up the production methods developed in this study to produce an effective biological control agent to a point where large biomasses of cells are harvested for virus and then applied to fields is mostly a matter of cost. Initial costs of building up large scale systems is high and many factors have to be considered, such as: profitability, cell and virus productivity, cell culture medium costs, application rate, production scale and batch production costs (Rhodes 1996). Despite initial cost, the payoffs have the potential to outweigh the cost.

*Future Work*

With cell culture already being utilized for production of proteins, biopesticides and other pharmaceuticals, the economic value for this area of research is increasing. For large-scale production in agriculture, it would be beneficial to try a similar trial with larger cell growth systems. Bioreactors and the new methodology of 3D matrix cell growing systems allow for larger volume production of cells and, in the same respect, larger volumes of viral production (Abbot and Cyranoski 2003). While the initial cost of building up large-scale systems is high, the payoff in the amount of product able to be produced has the potential to be even greater.

Other areas of study would be to use extracted whole virus from cell culture in trials for infecting live insects and testing for survival and transmission rates. Antibody design has been used increasingly in viral studies for diseases like HIV and Hepatitis C, and while it is a time consuming and detailed process, for HoCV-01 it would allow for visualization of viral activity in vitro with confocal microscopy and could lead to other areas of investigation.

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Appendix A: Images of cell growth from primary culture over time

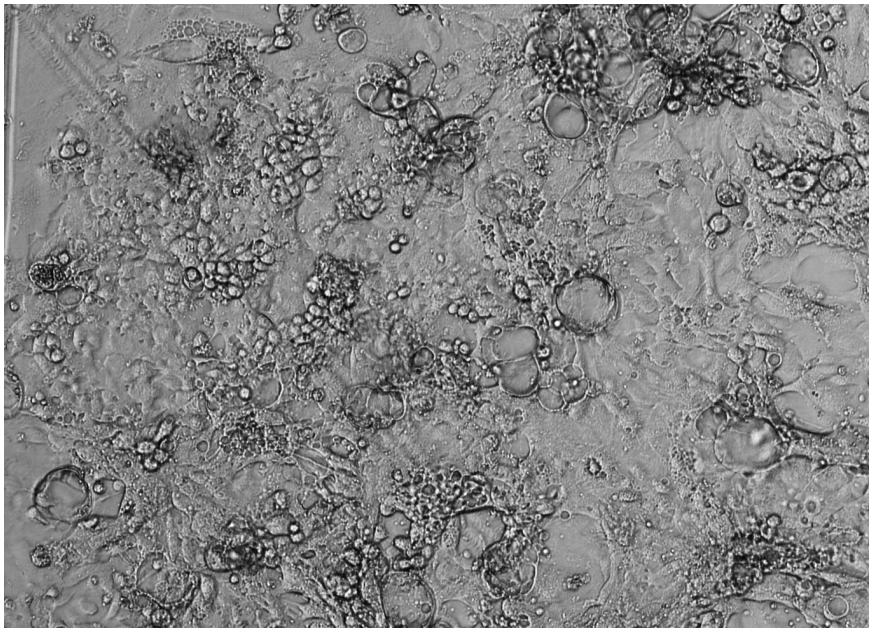

**Figure A-1**. Primary culture received from USDA-ARS 23-02-12.

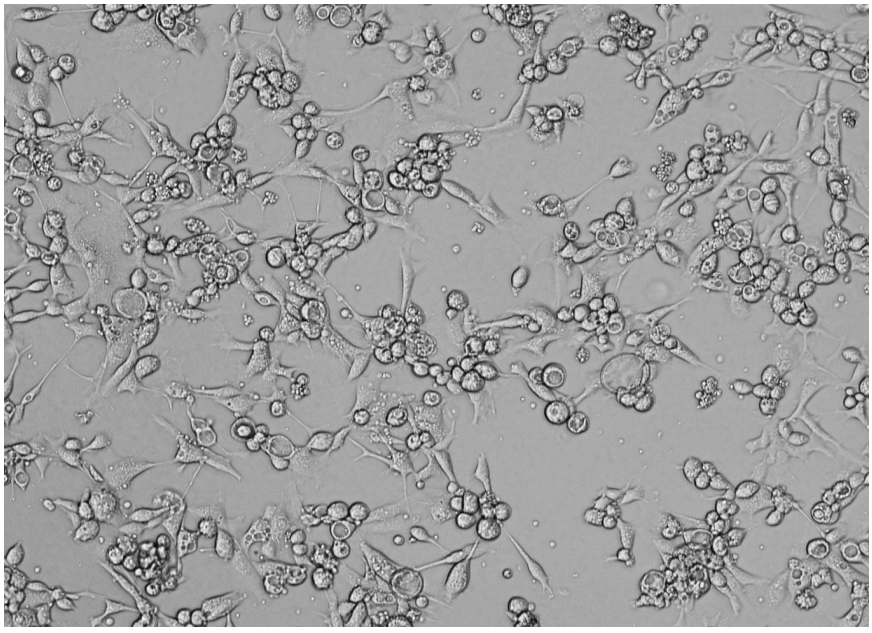

**Figure A-2**. Cells from primary culture received from USDA-ARS at 100X after 2 passes (March 2012).

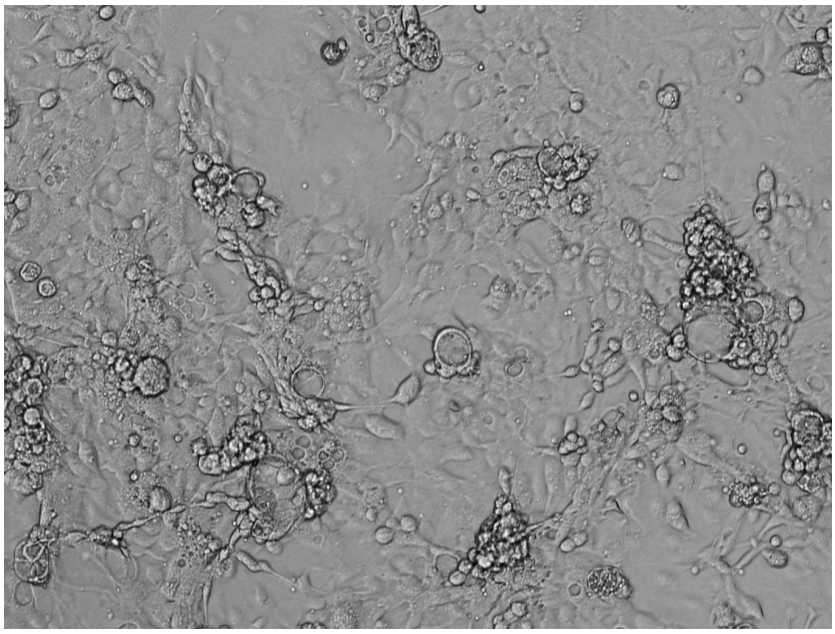

**Figure A-3**. Cells from primary culture received from USDA-ARS at 100X after 4 passes (April 2012).

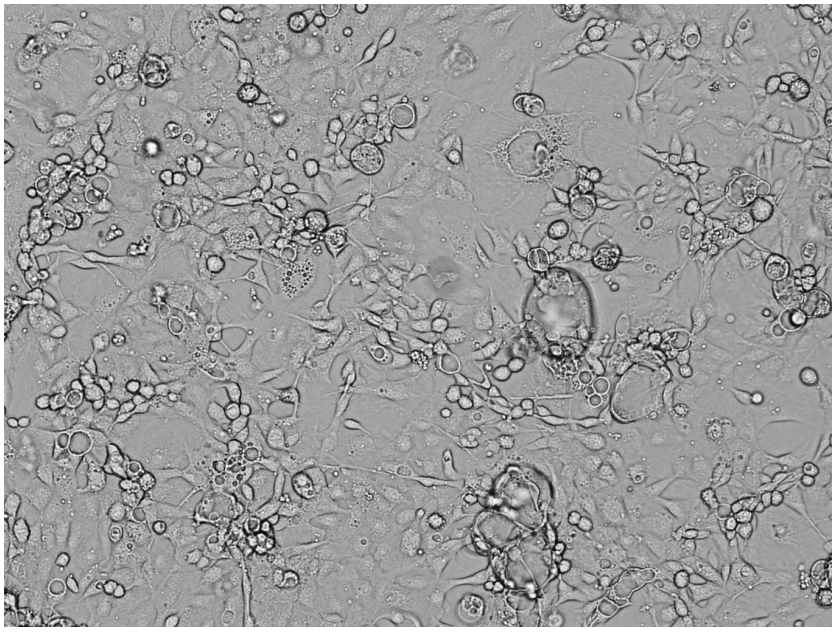

**Figure A-4**. Cells from primary culture received from USDA-ARS at 100X after 7 passes (May 2012).

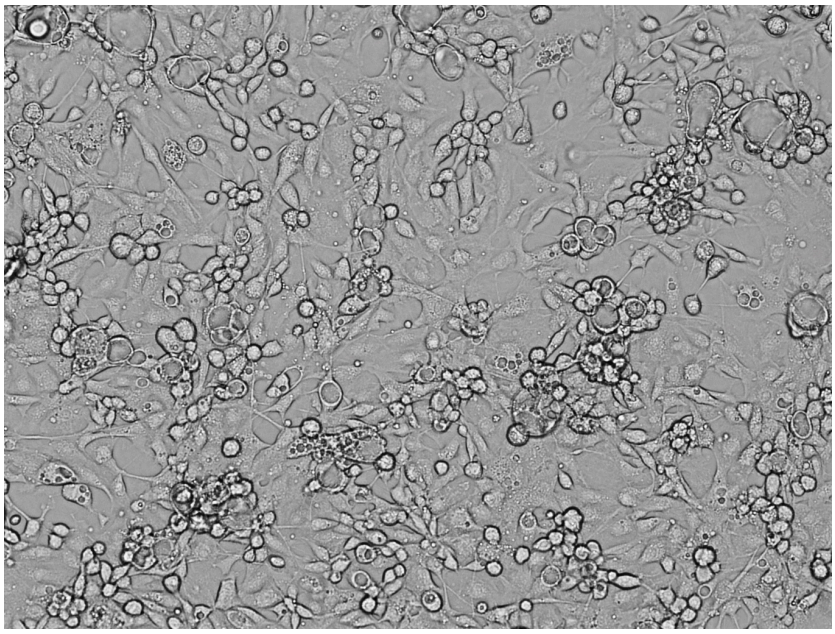

**Figure A-5**. Cells from primary culture received from USDA-ARS at 100X after 9 passes (June 2012).

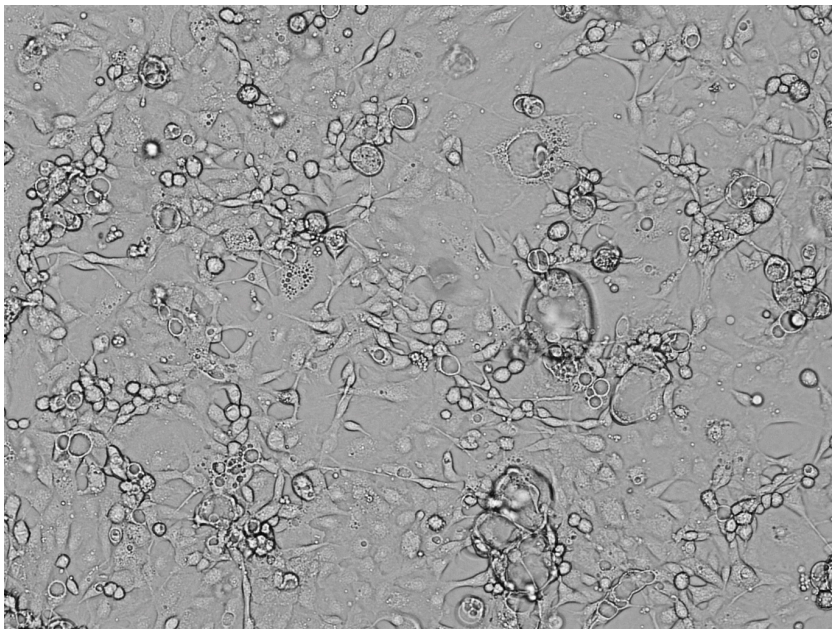

**Figure A-6**. Cells from primary culture received from USDA-ARS at 100X after 11 passes (July 2012).

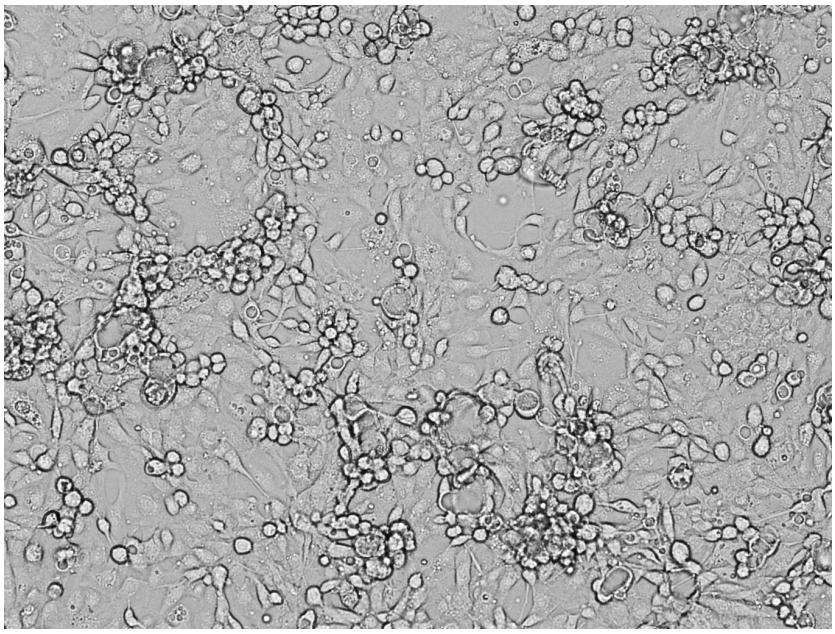

**Figure A-7**. Cells from primary culture received from USDA-ARS at 100X after 13 passes (August 2012).

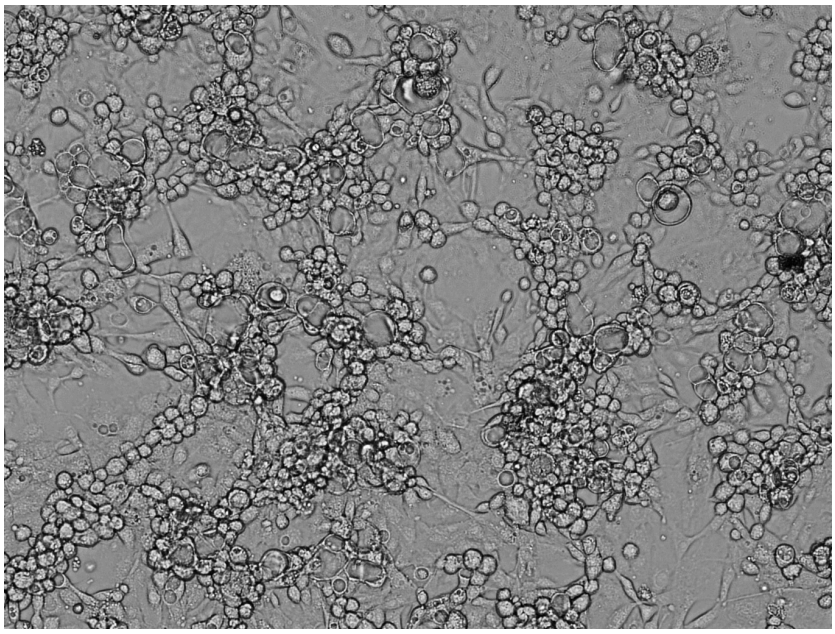

**Figure A-8**. Cells from primary culture received from USDA-ARS at 100X after 16 passes (September 2012).

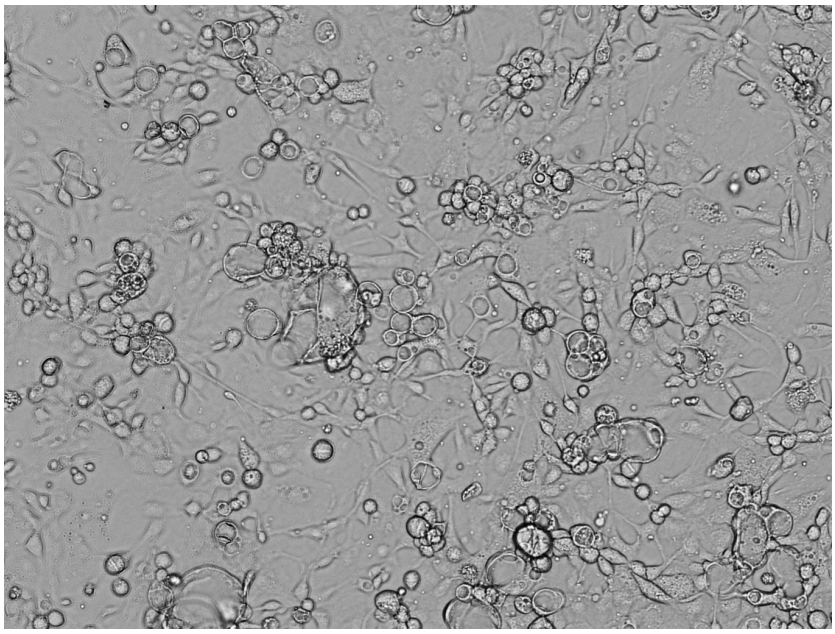

**Figure A-9**. Cells from primary culture received from USDA-ARS at 100X after 18 passes (October 2012).

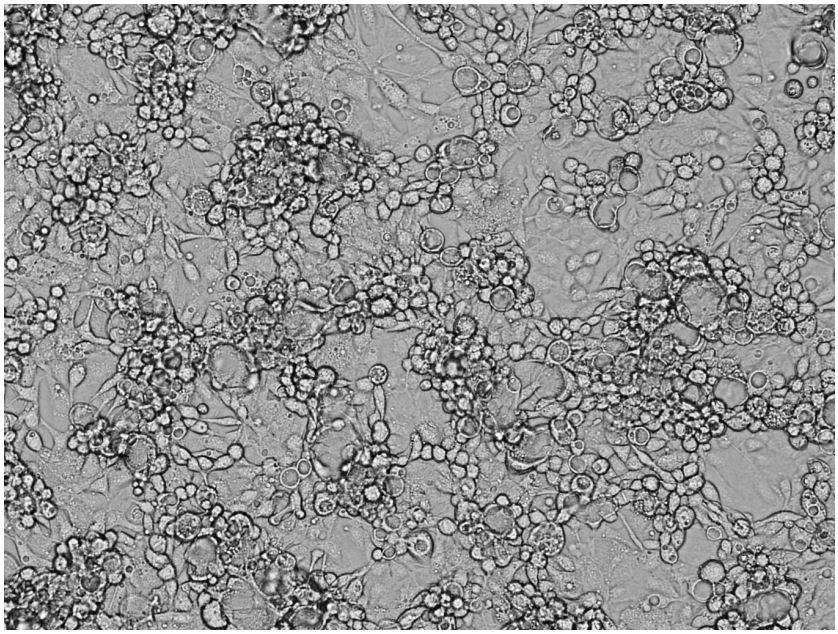

**Figure A-10**. Cells from primary culture received from USDA-ARS at 100X after 20 passes (November 2012).

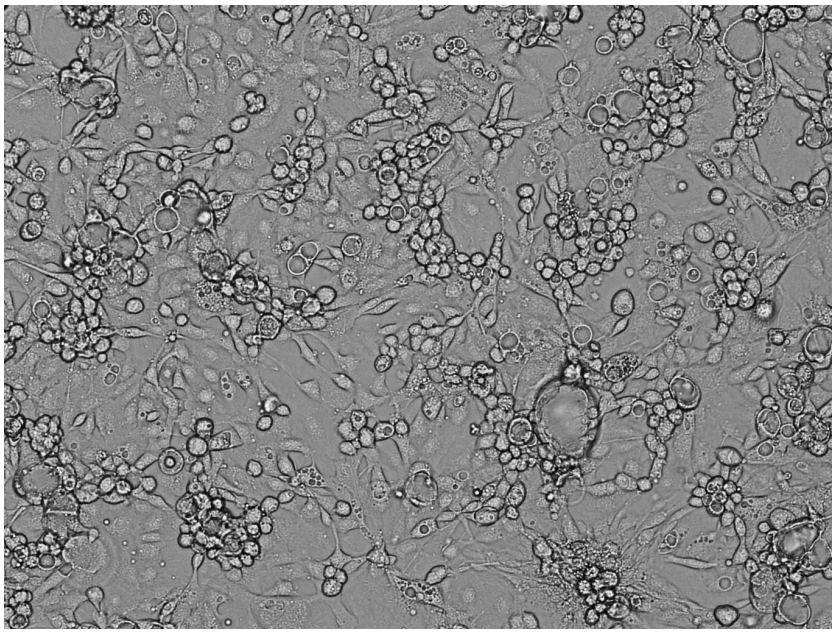

**Figure A-11**. Cells from primary culture received from USDA-ARS at 100X after 22 passes (December 2012).

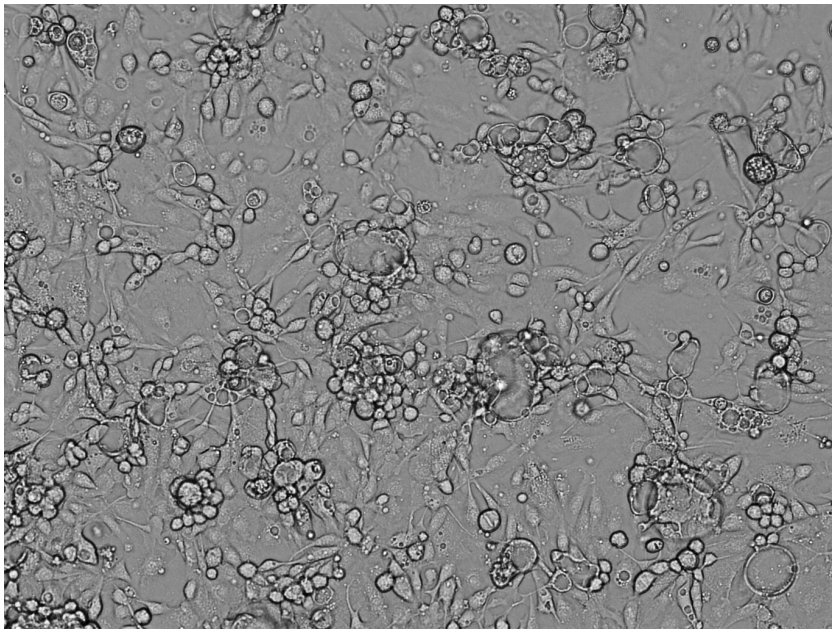

**Figure A-12**. Cells from primary culture received from USDA-ARS at 100X after 25 passes (January 2013).

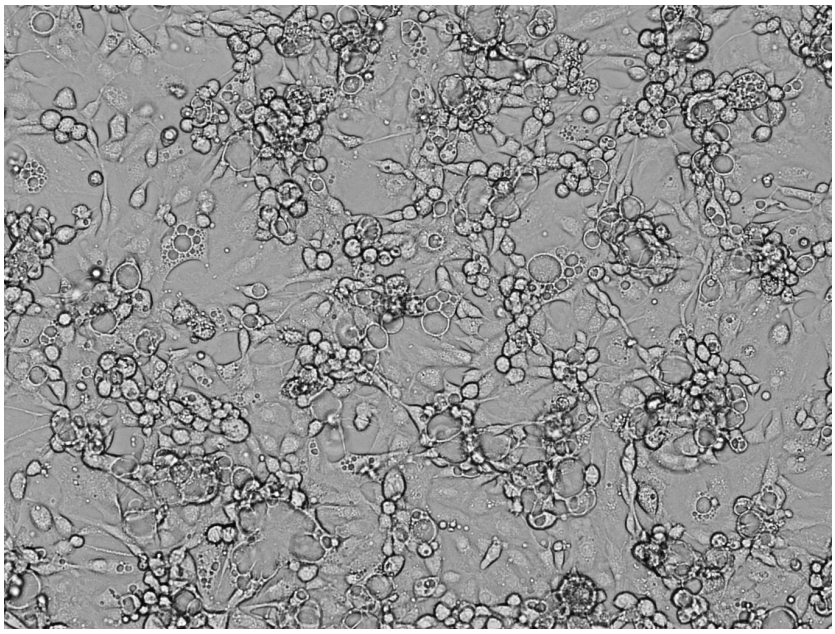

**Figure A-13**. Cells from primary culture received from USDA-ARS at 100X after 27 passes (February 2013).

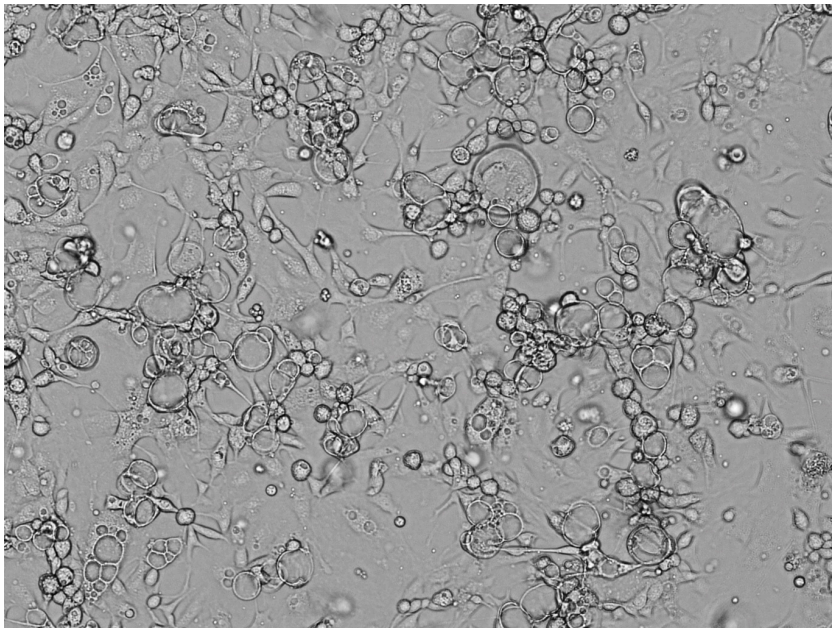

**Figure A-14**. Cells from primary culture received from USDA-ARS at 100X after 29 passes (March 2013).

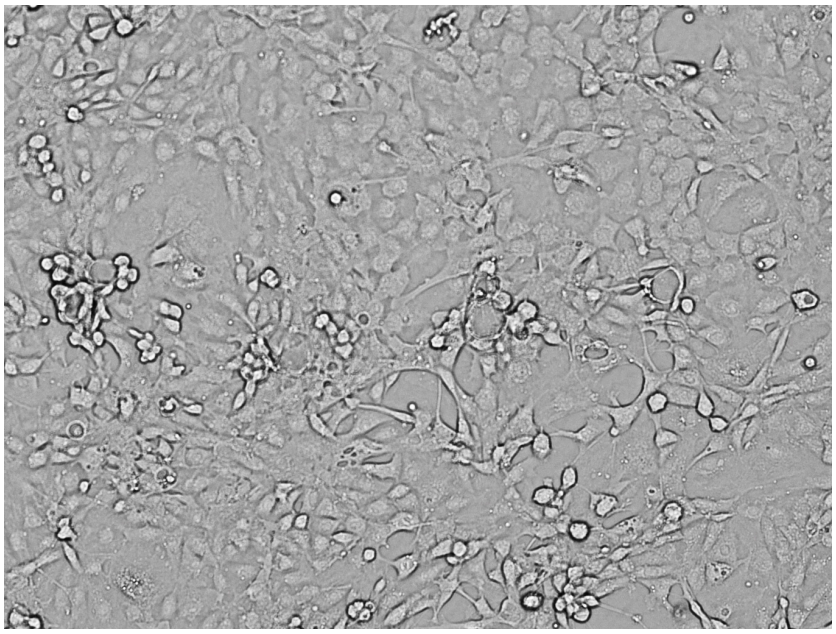

**Figure A-15**. Cells from primary culture received from USDA-ARS at 100X after 31 passes (April 2013).

# Appendix B: Full summary of Kaplan-Meier survival analysis

**Table B-1**. Mean values of Kaplan-Meier survival analysis of cell count numbers. Survival rate indicates the likelihood of all groups surviving at each time point.

| KM Survival Analysis<br>Mean values | | | | | | |
|---|---|---|---|---|---|---|
| Time (hours) | Number at risk | Number of events | Survival rate | SE | Lower 95% CI | Upper 95% CI |
| 0 | 480 | 1 | 0.998 | 0.002 | 0.994 | 1.000 |
| 24 | 420 | 3 | 0.991 | 0.005 | 0.982 | 1.000 |
| 48 | 260 | 9 | 0.966 | 0.009 | 0.948 | 0.984 |
| 72 | 300 | 12 | 0.927 | 0.014 | 0.900 | 0.955 |
| 96 | 240 | 11 | 0.885 | 0.018 | 0.850 | 0.922 |
| 120 | 180 | 11 | 0.831 | 0.023 | 0.786 | 0.878 |
| 144 | 120 | 30 | 0.623 | 0.037 | 0.554 | 0.701 |
| 168 | 60 | 45 | 0.156 | 0.036 | 0.099 | 0.245 |

**Table B-2**. Kaplan-Meier survival analysis of cell count numbers for treatment group 1. Survival rate indicates the likelihood of the 1:10 viral treatment group surviving at each time point.

| KM Survival Analysis<br>1:10 Treatment | | | | | | |
|---|---|---|---|---|---|---|
| Time (hours) | Number at risk | Number of events | Survival rate | SE | Lower 95% CI | Upper 95% CI |
| 24 | 70 | 1 | 0.986 | 0.014 | 0.958 | 1.000 |
| 48 | 60 | 7 | 0.871 | 0.042 | 0.791 | 0.959 |
| 72 | 50 | 10 | 0.697 | 0.060 | 0.588 | 0.825 |
| 96 | 40 | 10 | 0.522 | 0.066 | 0.409 | 0.668 |
| 120 | 30 | 10 | 0.348 | 0.063 | 0.245 | 0.496 |
| 144 | 20 | 10 | 0.174 | 0.050 | 0.099 | 0.306 |
| 168 | 10 | 10 | 0.000 | NA | NA | NA |

**Table B-3.** Kaplan-Meier survival analysis of cell count numbers for treatment group 2. Survival rate indicates the likelihood of the 1:100 viral treatment group surviving at each time point.

| KM Survival Analysis 1:100 Treatment | | | | | | |
|---|---|---|---|---|---|---|
| Time (hours) | Number at risk | Number of events | Survival rate | SE | Lower 95% CI | Upper 95% CI |
| 96 | 40 | 1 | 0.975 | 0.025 | 0.928 | 1.000 |
| 120 | 30 | 1 | 0.943 | 0.039 | 0.867 | 1.000 |
| 144 | 20 | 8 | 0.566 | 0.106 | 0.392 | 0.817 |
| 168 | 10 | 10 | 0.000 | NA | NA | NA |

**Table B-4**. Kaplan-Meier survival analysis of cell count numbers for treatment group 3. Survival rate indicates the likelihood of the 1:1,000 viral treatment group surviving at each time point.

| KM Survival Analysis 1:1,000 Treatment | | | | | | |
|---|---|---|---|---|---|---|
| Time (hours) | Number at risk | Number of events | Survival rate | SE | Lower 95% CI | Upper 95% CI |
| 24 | 70 | 1 | 0.986 | 0.014 | 0.958 | 1.000 |
| 72 | 50 | 1 | 0.966 | 0.024 | 0.920 | 1.000 |
| 144 | 20 | 7 | 0.628 | 0.104 | 0.454 | 0.869 |
| 168 | 10 | 9 | 0.063 | 0.061 | 0.009 | 0.415 |

**Table B-5**. Kaplan-Meier survival analysis of cell count numbers for treatment group 4. Survival rate indicates the likelihood of the 1:10,000 viral treatment group surviving at each time point.

| KM Survival Analysis 1:10,000 Treatment | | | | | | |
|---|---|---|---|---|---|---|
| Time (hours) | Number at risk | Number of events | Survival rate | SE | Lower 95% CI | Upper 95% CI |
| 0 | 80 | 1 | 0.988 | 0.012 | 0.963 | 1.000 |
| 144 | 20 | 5 | 0.741 | 0.096 | 0.574 | 0.955 |
| 168 | 10 | 10 | 0.000 | NA | NA | NA |

**Table B-6**. Kaplan-Meier survival analysis of cell count numbers for treatment group 5. Survival rate indicates the likelihood of the 1:100,000 viral treatment group surviving at each time point.

| KM Survival Analysis 1:100,000 Treatment | | | | | | |
|---|---|---|---|---|---|---|
| Time (hours) | Number at risk | Number of events | Survival rate | SE | Lower 95% CI | Upper 95% CI |
| 24 | 70 | 1 | 0.986 | 0.014 | 0.958 | 1.000 |
| 48 | 60 | 2 | 0.953 | 0.027 | 0.902 | 1.000 |
| 72 | 50 | 1 | 0.934 | 0.032 | 0.873 | 0.999 |
| 168 | 10 | 6 | 0.374 | 0.145 | 0.174 | 0.800 |

**Table C-1**. Summary of Bonferroni post-hoc test results of Ct values between all sample groups.

| Sources of variation (N=5) | | | | | | | |
|---|---|---|---|---|---|---|---|
| Groups | Difference | p-value | Significant | Group | Difference | p-value | Significant |
| Standard1 vs Standard2 | | | | Standard1 vs Ck | | | |
| 0H | 0.90 | > 0.05 | No | 0H | 25.28 | < 0.001 | Yes |
| 24H | 0.70 | > 0.05 | No | 24H | 22.16 | < 0.001 | Yes |
| 48H | 1.15 | > 0.05 | No | 48H | 23.54 | < 0.001 | Yes |
| 72H | -0.58 | > 0.05 | No | 72H | 22.45 | < 0.001 | Yes |
| 96H | -0.66 | > 0.05 | No | 96H | 24.07 | < 0.001 | Yes |
| 120H | -0.73 | > 0.05 | No | 120H | 24.11 | < 0.001 | Yes |
| 144H | -0.66 | > 0.05 | No | 144H | 23.59 | < 0.001 | Yes |
| 168H | -0.84 | > 0.05 | No | 168H | 23.52 | < 0.001 | Yes |
| Standard1 vs Standard3 | | | | Standard1 vs 1:10 | | | |
| 0H | 2.86 | > 0.05 | No | 0H | 20.99 | < 0.001 | Yes |
| 24H | -2.10 | > 0.05 | No | 24H | 22.85 | < 0.001 | Yes |
| 48H | 2.86 | > 0.05 | No | 48H | 24.41 | < 0.001 | Yes |
| 72H | -1.67 | > 0.05 | No | 72H | 22.26 | < 0.001 | Yes |
| 96H | -1.70 | > 0.05 | No | 96H | 23.13 | < 0.001 | Yes |
| 120H | -1.67 | > 0.05 | No | 120H | 23.32 | < 0.001 | Yes |
| 144H | -1.81 | > 0.05 | No | 144H | 22.56 | < 0.001 | Yes |
| 168H | -1.93 | > 0.05 | No | 168H | 22.37 | < 0.001 | Yes |
| Standard1 vs NTC | | | | Standard1 vs 1:100 | | | |
| 0H | 3.18 | > 0.05 | No | 0H | 24.59 | < 0.001 | Yes |
| 24H | 2.35 | > 0.05 | No | 24H | 22.71 | < 0.001 | Yes |
| 48H | 1.54 | > 0.05 | No | 48H | 21.81 | < 0.001 | Yes |
| 72H | -4.17 | > 0.05 | No | 72H | 22.48 | < 0.001 | Yes |
| 96H | 1.33 | > 0.05 | No | 96H | 23.01 | < 0.001 | Yes |
| 120H | 1.50 | > 0.05 | No | 120H | 23.74 | < 0.001 | Yes |
| 144H | 1.45 | > 0.05 | No | 144H | 23.23 | < 0.001 | Yes |
| 168H | 1.28 | > 0.05 | No | 168H | 27.59 | < 0.001 | Yes |

**Table C-1** cont.

| Groups | Difference | p-value | Significant | Group | Difference | p-value | Significant |
|---|---|---|---|---|---|---|---|
| Standard1 vs 1:1,000 | | | | Standard2 vs Standard3 | | | |
| 0H | 24.61 | < 0.001 | Yes | 0H | 1.95 | > 0.05 | No |
| 24H | 23.45 | < 0.001 | Yes | 24H | -2.80 | > 0.05 | No |
| 48H | 23.37 | < 0.001 | Yes | 48H | 1.71 | > 0.05 | No |
| 72H | 22.62 | < 0.001 | Yes | 72H | -1.09 | > 0.05 | No |
| 96H | 22.40 | < 0.001 | Yes | 96H | -1.04 | > 0.05 | No |
| 120H | 23.23 | < 0.001 | Yes | 120H | -0.94 | > 0.05 | No |
| 144H | 22.71 | < 0.001 | Yes | 144H | -1.15 | > 0.05 | No |
| 168H | 22.82 | < 0.001 | Yes | 168H | -1.10 | > 0.05 | No |
| Standard1 vs 1:10,000 | | | | Standard2 vs NTC | | | |
| 0H | 24.46 | < 0.001 | Yes | 0H | 2.28 | > 0.05 | No |
| 24H | 23.32 | < 0.001 | Yes | 24H | 1.65 | > 0.05 | No |
| 48H | 22.68 | < 0.001 | Yes | 48H | 0.38 | > 0.05 | No |
| 72H | 21.52 | < 0.001 | Yes | 72H | -3.59 | > 0.05 | No |
| 96H | 23.35 | < 0.001 | Yes | 96H | 1.99 | > 0.05 | No |
| 120H | 23.54 | < 0.001 | Yes | 120H | 2.23 | > 0.05 | No |
| 144H | 23.37 | < 0.001 | Yes | 144H | 2.11 | > 0.05 | No |
| 168H | 23.56 | < 0.001 | Yes | 168H | 2.12 | > 0.05 | No |
| Standard1 vs 1:100,000 | | | | Standard2 vs Ck | | | |
| 0H | 24.37 | < 0.001 | Yes | 0H | 22.37 | < 0.001 | Yes |
| 24H | 24.08 | < 0.001 | Yes | 24H | 21.45 | < 0.001 | Yes |
| 48H | 22.18 | < 0.001 | Yes | 48H | 22.39 | < 0.001 | Yes |
| 72H | 23.01 | < 0.001 | Yes | 72H | 23.03 | < 0.001 | Yes |
| 96H | 23.91 | < 0.001 | Yes | 96H | 24.73 | < 0.001 | Yes |
| 120H | 24.12 | < 0.001 | Yes | 120H | 24.83 | < 0.001 | Yes |
| 144H | 23.68 | < 0.001 | Yes | 144H | 24.25 | < 0.001 | Yes |
| 168H | 21.96 | < 0.001 | Yes | 168H | 24.35 | < 0.001 | Yes |

**Table C-1** cont.

| Groups | Difference | p-value | Significant | Group | Difference | p-value | Significant |
|---|---|---|---|---|---|---|---|
| Standard2 vs 1:10 | | | | Standard2 vs 1:10,000 | | | |
| 0H | 20.09 | < 0.001 | Yes | 0H | 23.56 | < 0.001 | Yes |
| 24H | 22.15 | < 0.001 | Yes | 24H | 22.62 | < 0.001 | Yes |
| 48H | 23.26 | < 0.001 | Yes | 48H | 21.53 | < 0.001 | Yes |
| 72H | 22.84 | < 0.001 | Yes | 72H | 22.11 | < 0.001 | Yes |
| 96H | 23.79 | < 0.001 | Yes | 96H | 24.01 | < 0.001 | Yes |
| 120H | 24.05 | < 0.001 | Yes | 120H | 24.26 | < 0.001 | Yes |
| 144H | 23.22 | < 0.001 | Yes | 144H | 24.03 | < 0.001 | Yes |
| 168H | 23.21 | < 0.001 | Yes | 168H | 24.39 | < 0.001 | Yes |
| Standard2 vs 1:100 | | | | Standard2 vs 1:100,000 | | | |
| 0H | 23.69 | < 0.001 | Yes | 0H | 23.46 | < 0.001 | Yes |
| 24H | 22.00 | < 0.001 | Yes | 24H | 23.38 | < 0.001 | Yes |
| 48H | 20.66 | < 0.001 | Yes | 48H | 21.02 | < 0.001 | Yes |
| 72H | 23.06 | < 0.001 | Yes | 72H | 23.59 | < 0.001 | Yes |
| 96H | 23.67 | < 0.001 | Yes | 96H | 24.56 | < 0.001 | Yes |
| 120H | 24.46 | < 0.001 | Yes | 120H | 24.85 | < 0.001 | Yes |
| 144H | 23.89 | < 0.001 | Yes | 144H | 24.34 | < 0.001 | Yes |
| 168H | 24.10 | < 0.001 | Yes | 168H | 22.79 | < 0.001 | Yes |
| Standard2 vs 1:1,000 | | | | Standard3 vs NTC | | | |
| 0H | 23.71 | < 0.001 | Yes | 0H | 0.32 | > 0.05 | No |
| 24H | 22.75 | < 0.001 | Yes | 24H | 4.45 | > 0.05 | No |
| 48H | 22.21 | < 0.001 | Yes | 48H | -1.32 | > 0.05 | No |
| 72H | 23.21 | < 0.001 | Yes | 72H | -2.50 | > 0.05 | No |
| 96H | 23.06 | < 0.001 | Yes | 96H | 3.03 | > 0.05 | No |
| 120H | 23.96 | < 0.001 | Yes | 120H | 3.17 | > 0.05 | No |
| 144H | 23.37 | < 0.001 | Yes | 144H | 3.26 | > 0.05 | No |
| 168H | 23.65 | < 0.001 | Yes | 168H | 3.22 | > 0.05 | No |

**Table C-1** cont.

| Groups | Difference | p-value | Significant | Group | Difference | p-value | Significant |
|---|---|---|---|---|---|---|---|
| Standard3 vs Ck | | | | Standard3 vs 1:1,000 | | | |
| 0H | 20.42 | < 0.001 | Yes | 0H | 21.75 | < 0.001 | Yes |
| 24H | 24.26 | < 0.001 | Yes | 24H | 25.55 | < 0.001 | Yes |
| 48H | 20.68 | < 0.001 | Yes | 48H | 20.51 | < 0.001 | Yes |
| 72H | 24.12 | < 0.001 | Yes | 72H | 24.30 | < 0.001 | Yes |
| 96H | 25.77 | < 0.001 | Yes | 96H | 24.10 | < 0.001 | Yes |
| 120H | 25.77 | < 0.001 | Yes | 120H | 24.90 | < 0.001 | Yes |
| 144H | 25.40 | < 0.001 | Yes | 144H | 24.53 | < 0.001 | Yes |
| 168H | 25.45 | < 0.001 | Yes | 168H | 24.75 | < 0.001 | Yes |
| Standard3 vs 1:10 | | | | Standard3 vs 1:10,000 | | | |
| 0H | 18.14 | < 0.001 | Yes | 0H | 21.61 | < 0.001 | Yes |
| 24H | 24.95 | < 0.001 | Yes | 24H | 25.42 | < 0.001 | Yes |
| 48H | 21.55 | < 0.001 | Yes | 48H | 19.82 | < 0.001 | Yes |
| 72H | 23.93 | < 0.001 | Yes | 72H | 23.20 | < 0.001 | Yes |
| 96H | 24.83 | < 0.001 | Yes | 96H | 25.05 | < 0.001 | Yes |
| 120H | 24.99 | < 0.001 | Yes | 120H | 25.20 | < 0.001 | Yes |
| 144H | 24.37 | < 0.001 | Yes | 144H | 25.18 | < 0.001 | Yes |
| 168H | 24.30 | < 0.001 | Yes | 168H | 25.49 | < 0.001 | Yes |
| Standard3 vs 1:100 | | | | Standard3 vs 1:100,000 | | | |
| 0H | 21.74 | < 0.001 | Yes | 0H | 21.51 | < 0.001 | Yes |
| 24H | 24.81 | < 0.001 | Yes | 24H | 26.18 | < 0.001 | Yes |
| 48H | 18.96 | < 0.001 | Yes | 48H | 19.32 | < 0.001 | Yes |
| 72H | 24.15 | < 0.001 | Yes | 72H | 24.68 | < 0.001 | Yes |
| 96H | 24.71 | < 0.001 | Yes | 96H | 25.61 | < 0.001 | Yes |
| 120H | 25.40 | < 0.001 | Yes | 120H | 25.79 | < 0.001 | Yes |
| 144H | 25.05 | < 0.001 | Yes | 144H | 25.49 | < 0.001 | Yes |
| 168H | 25.19 | < 0.001 | Yes | 168H | 23.89 | < 0.001 | Yes |

**Table C-1** cont.

| Groups | Difference | p-value | Significant | Group | Difference | p-value | Significant |
|---|---|---|---|---|---|---|---|
| NTC vs Ck | | | | NTC vs 1:1,000 | | | |
| 0H | 20.10 | < 0.001 | Yes | 0H | 21.43 | < 0.001 | Yes |
| 24H | 19.81 | < 0.001 | Yes | 24H | 21.10 | < 0.001 | Yes |
| 48H | 22.00 | < 0.001 | Yes | 48H | 21.83 | < 0.001 | Yes |
| 72H | 26.62 | < 0.001 | Yes | 72H | 26.79 | < 0.001 | Yes |
| 96H | 22.74 | < 0.001 | Yes | 96H | 21.07 | < 0.001 | Yes |
| 120H | 22.61 | < 0.001 | Yes | 120H | 21.73 | < 0.001 | Yes |
| 144H | 22.14 | < 0.001 | Yes | 144H | 21.26 | < 0.001 | Yes |
| 168H | 22.23 | < 0.001 | Yes | 168H | 21.53 | < 0.001 | Yes |
| NTC vs 1:10 | | | | NTC vs 1:10,000 | | | |
| 0H | 17.81 | < 0.001 | Yes | 0H | 21.28 | < 0.001 | Yes |
| 24H | 20.50 | < 0.001 | Yes | 24H | 20.97 | < 0.001 | Yes |
| 48H | 22.87 | < 0.001 | Yes | 48H | 21.14 | < 0.001 | Yes |
| 72H | 26.43 | < 0.001 | Yes | 72H | 25.69 | < 0.001 | Yes |
| 96H | 21.80 | < 0.001 | Yes | 96H | 22.02 | < 0.001 | Yes |
| 120H | 21.82 | < 0.001 | Yes | 120H | 22.04 | < 0.001 | Yes |
| 144H | 21.11 | < 0.001 | Yes | 144H | 21.92 | < 0.001 | Yes |
| 168H | 21.08 | < 0.001 | Yes | 168H | 22.27 | < 0.001 | Yes |
| NTC vs 1:100 | | | | NTC vs 1:100,000 | | | |
| 0H | 21.41 | < 0.001 | Yes | 0H | 21.19 | < 0.001 | Yes |
| 24H | 20.36 | < 0.001 | Yes | 24H | 21.73 | < 0.001 | Yes |
| 48H | 20.28 | < 0.001 | Yes | 48H | 20.64 | < 0.001 | Yes |
| 72H | 26.65 | < 0.001 | Yes | 72H | 27.18 | < 0.001 | Yes |
| 96H | 21.68 | < 0.001 | Yes | 96H | 22.58 | < 0.001 | Yes |
| 120H | 22.24 | < 0.001 | Yes | 120H | 22.62 | < 0.001 | Yes |
| 144H | 21.78 | < 0.001 | Yes | 144H | 22.23 | < 0.001 | Yes |
| 168H | 21.97 | < 0.001 | Yes | 168H | 20.67 | < 0.001 | Yes |

**Table C-1** cont.

| Groups | Difference | p-value | Significant | Group | Difference | p-value | Significant |
|---|---|---|---|---|---|---|---|
| Ck vs 1:10 | | | | Ck vs 1:10,000 | | | |
| 0H | -2.28 | > 0.05 | No | 0H | 1.189 | > 0.05 | No |
| 24H | 0.70 | > 0.05 | No | 24H | 1.16 | > 0.05 | No |
| 48H | 0.87 | > 0.05 | No | 48H | -0.86 | > 0.05 | No |
| 72H | -0.19 | > 0.05 | No | 72H | -0.93 | > 0.05 | No |
| 96H | -0.94 | > 0.05 | No | 96H | -0.72 | > 0.05 | No |
| 120H | -0.78 | > 0.05 | No | 120H | 0.14 | > 0.05 | No |
| 144H | -1.03 | > 0.05 | No | 144H | 0.05 | > 0.05 | No |
| 168H | -1.15 | > 0.05 | No | 168H | 0.01 | > 0.05 | No |
| Ck vs 1:100 | | | | Ck vs 1:100,000 | | | |
| 0H | 1.32 | > 0.05 | No | 0H | 1.09 | > 0.05 | No |
| 24H | 0.55 | > 0.05 | No | 24H | 1.93 | > 0.05 | No |
| 48H | -1.73 | > 0.05 | No | 48H | -1.37 | > 0.05 | No |
| 72H | 0.03 | > 0.05 | No | 72H | 0.56 | > 0.05 | No |
| 96H | -1.06 | > 0.05 | No | 96H | -0.17 | > 0.05 | No |
| 120H | -0.37 | > 0.05 | No | 120H | 0.02 | > 0.05 | No |
| 144H | -0.36 | > 0.05 | No | 144H | 0.09 | > 0.05 | No |
| 168H | -0.26 | > 0.05 | No | 168H | -1.56 | > 0.05 | No |
| Ck vs 1:1,000 | | | | 1:10 vs 1:100 | | | |
| 0H | 1.33 | > 0.05 | No | 0H | 3.60 | > 0.05 | No |
| 24H | 1.29 | > 0.05 | No | 24H | -0.15 | > 0.05 | No |
| 48H | -0.17 | > 0.05 | No | 48H | -2.60 | > 0.05 | No |
| 72H | 0.17 | > 0.05 | No | 72H | 0.22 | > 0.05 | No |
| 96H | -1.67 | > 0.05 | No | 96H | -0.12 | > 0.05 | No |
| 120H | -0.88 | > 0.05 | No | 120H | 0.41 | > 0.05 | No |
| 144H | -0.88 | > 0.05 | No | 144H | 0.68 | > 0.05 | No |
| 168H | -0.70 | > 0.05 | No | 168H | 0.89 | > 0.05 | No |

**Table C-1** cont.

| Groups | Difference | p-value | Significant | Group | Difference | p-value | Significant |
|---|---|---|---|---|---|---|---|
| 1:10 vs 1:1,000 | | | | 1:100 vs 1:1,000 | | | |
| 0H | 3.62 | > 0.05 | No | 0H | 0.02 | > 0.05 | No |
| 24H | 0.56 | > 0.05 | No | 24H | 0.74 | > 0.05 | No |
| 48H | -1.04 | > 0.05 | No | 48H | 1.56 | > 0.05 | No |
| 72H | 0.36 | > 0.05 | No | 72H | 0.144 | > 0.05 | No |
| 96H | -0.73 | > 0.05 | No | 96H | -0.61 | > 0.05 | No |
| 120H | -0.09 | > 0.05 | No | 120H | -0.51 | > 0.05 | No |
| 144H | 0.16 | > 0.05 | No | 144H | -0.52 | > 0.05 | No |
| 168H | 0.45 | > 0.05 | No | 168H | -0.44 | > 0.05 | No |
| 1:10 vs 1:10,000 | | | | 1:100 vs 1:10,000 | | | |
| 0H | 3.47 | > 0.05 | No | 0H | -0.13 | > 0.05 | No |
| 24H | 0.47 | > 0.05 | No | 24H | 0.62 | > 0.05 | No |
| 48H | -1.73 | > 0.05 | No | 48H | 0.87 | > 0.05 | No |
| 72H | -0.74 | > 0.05 | No | 72H | -0.96 | > 0.05 | No |
| 96H | 0.22 | > 0.05 | No | 96H | 0.34 | > 0.05 | No |
| 120H | 0.21 | > 0.05 | No | 120H | -0.20 | > 0.05 | No |
| 144H | 0.81 | > 0.05 | No | 144H | 0.14 | > 0.05 | No |
| 168H | 1.19 | > 0.05 | No | 168H | 0.30 | > 0.05 | No |
| 1:10 vs 1:100,000 | | | | 1:100 vs 1:100,000 | | | |
| 0H | 3.38 | > 0.05 | No | 0H | -0.23 | > 0.05 | No |
| 24H | 1.23 | > 0.05 | No | 24H | 1.38 | > 0.05 | No |
| 48H | -2.24 | > 0.05 | No | 48H | 0.36 | > 0.05 | No |
| 72H | 0.75 | > 0.05 | No | 72H | 0.53 | > 0.05 | No |
| 96H | 0.77 | > 0.05 | No | 96H | 0.89 | > 0.05 | No |
| 120H | 0.80 | > 0.05 | No | 120H | 0.39 | > 0.05 | No |
| 144H | 1.12 | > 0.05 | No | 144H | 0.45 | > 0.05 | No |
| 168H | -0.41 | > 0.05 | No | 168H | -1.30 | > 0.05 | No |

**Table C-1** cont.

| Groups | Difference | p-value | Significant | Group | Difference | p-value | Significant |
|---|---|---|---|---|---|---|---|
| 1:1,000 vs 1:10,000 | | | | 1:10,000 vs 1:100,000 | | | |
| 0H | -0.15 | > 0.05 | No | 0H | -0.10 | > 0.05 | No |
| 24H | -0.13 | > 0.05 | No | 24H | 0.76 | > 0.05 | No |
| 48H | -0.69 | > 0.05 | No | 48H | -0.51 | > 0.05 | No |
| 72H | -1.10 | > 0.05 | No | 72H | 1.48 | > 0.05 | No |
| 96H | 0.95 | > 0.05 | No | 96H | 0.55 | > 0.05 | No |
| 120H | 0.31 | > 0.05 | No | 120H | 0.59 | > 0.05 | No |
| 144H | 0.65 | > 0.05 | No | 144H | 0.31 | > 0.05 | No |
| 168H | 0.74 | > 0.05 | No | 168H | -1.60 | > 0.05 | No |
| 1:1,000 vs 1:100,000 | | | | | | | |
| 0H | -0.24 | > 0.05 | No | | | | |
| 24H | 0.63 | > 0.05 | No | | | | |
| 48H | -1.19 | > 0.05 | No | | | | |
| 72H | 0.38 | > 0.05 | No | | | | |
| 96H | 1.51 | > 0.05 | No | | | | |
| 120H | 0.89 | > 0.05 | No | | | | |
| 144H | 0.96 | > 0.05 | No | | | | |
| 168H | -0.86 | > 0.05 | No | | | | |