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Large Scale Amplification and Purification of Replication Competent Vaccinia Virus

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My final project was carried out for Cancer Gene Therapy Group, University of Helsinki, Finland; to enable further research projects with vvD-GFP.The main aim of my work was to carry out and describe a large scale vaccinia virus (VV) preparation and to amplify the titer of vvD-GFP. Due to the problematic nature of fetal bovine serum (FBS) in cell cultures, one aim was to investigate whether culturing in serum-free medium affected the cell viability of A549 cells and to find out whether it was possible to amplify vvD-GFP in serum-free culture. In order to get reliable virus titer results, the aim was to compare titers between two titering methods; plaque and $TCID_{50}$ assay.

I carried out two large scale VV preparations, one in culture with 2% FBS and one in serum-free culture. The existence of transgene, GFP, was confirmed with a fluorescent microscope. In addition, I incubated A549 cells in serum-free medium and in medium with 10% FBS and measured the cell viability with tetrazolium (MTS) based assay on three consecutive days. Then, I compared the results and demonstrated them in diagram. Furthermore, I quantified the virus titer from both virus preparations using two different titering methods, compared the titers with both assays and evaluated the reliability of these results.

It was possible to amplify vvD-GFP in serum-free culture without remarkable loss of virus titer. The virus titer in serum-free culture was 12% lower than in culture with 2% FBS. The cell culture of A549 cells in serum-free medium was possible at least for a short time but it was less efficient. Difference in virus titers between the two different titering methods occurred.

Keywords	vaccinia virus, large scale virus preparation, serum-free cell culture, $TCID_{50}$, plaque-assay

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Tein opinnäytetyöni Helsingin Yliopiston Cancer Gene Therapy Group-tutkimusryhmälle. Työni päätavoitteena oli tuottaa tiettyä vaccinia-virusta (vvD-GFP) tulevia tutkimuksia varten sekä kuvata tuotantoprosessin eri työvaiheet. Soluviljelyssä käytettävän seerumin (FBS) ongelmallisuuden vuoksi, tavoitteenani oli myös selvittää vaikuttaako seerumiton kasvatusliuos A549-solujen elinkykyyn sekä selvittää, onko vaccinia-virusta mahdollista tuottaa ilman seerumin käyttöä. Luotettavan virustitterin saamiseksi, tavoitteenani oli lisäksi tehdä virustitterin määritys kahdella eri menetelmällä, plakki- ja $TCID_{50}$-menetelmällä, sekä verrata niistä saatuja tuloksia keskenään.

Tein vaccinia-viruksesta kaksi virusvalmistetta. Toisen valmisteen tuotin seerumittomassa soluviljelmässä ja toisen 2 % seerumia sisältävässä soluviljelmässä. Viruksen GFP-transgeenin osoitin fluoresenssimikroskoopin avulla. Kasvatin A549-soluja sekä seerumittomassa että 10 % seerumia sisältävässä kasvatusliuoksessa ja mittasin solujen elinkykyä kolmena peräkkäisenä päivänä MTS-analyysillä. Vertasin saamiani tuloksia keskenään ja havainnollistin tuloksia taulukon avulla. Määritin virustitterit molemmista valmisteista kahdella eri titteröinti menetelmällä ja vertasin tuloksia keskenään sekä arvioin menetelmien luotettavuutta.

Tulosten mukaan vaccinia-virusta (vvD-GFP) oli mahdollista tuottaa A549-soluissa seerumittomassa viljelmässä ilman merkittävää virustitterin laskua. Seerumittomassa soluviljelmässä tuotetun virusvalmisteen titteri oli noin 12 % pienempi kuin tavallisessa, seerumia sisältävässä, viljelmässä. A549-solujen viljely seerumittomassa kasvatusliuoksessa oli mahdollista ainakin lyhyen aikaa, mutta solut eivät kasvaneet siinä yhtä tehokkaasti. Virustittereissä kahden eri menetelmän välillä esiintyi eroja.

Avainsanat	vaccinia virus, viruksen valmistus, seerumiton soluviljely, plakki-määritys, $TCID_{50}$

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1   Introduction

Cancer Gene Therapy Group (CGTG) is a research group of University of Helsinki, Finland, led by research professor Akseli Hemminki (MD, PhD). The mission of this group is to improve cancer treatments by exploring and developing novel, more efficient cancer treatments. The approaches to this development are the use of gene therapy and oncolytic viruses. Under special interest are such malignant cancers which currently are lacking effective treatments.

Approximately, every second man and every third woman is affected by cancer at some point in their life. Conventional cancer therapies, like surgery, chemo- and radiotherapy, are not always effective enough alone to defeat cancer, and often they have severe side effects. New therapy options are desperately needed for treating cancer, and hence, the design of efficient cancer treatments is one of the major challenges of medical science. A combination of traditional and novel cancer treatment may improve the patient's prognosis, or even cure the patient if the cancer is detected early enough. Gene therapy and oncolytic viruses hold great promise for the development of novel cancer therapies. Oncolytic vaccinia virus (VV) has emerged as a promising candidate for gene therapy agent. Different generated recombinant vaccinia viruses have shown convincing preclinical results; however, this new type of therapy also raises some safety concerns. Hence, more research work is needed and the antitumor efficacy and safety need to be demonstrated by randomized clinical trials. (Cross – Burmester 2006: 218-219; Guse – Cerullo – Hemminki 2011: 603; Rajecki – Joensuu – Hemminki 2008: 3085, 3091.)

This final project was carried out in co-operation with Cancer Gene Therapy Group. The practical execution of this project was carried out during a six weeks practical training period, under the guidance of supervisor Kilian Guse, laboratory technician Eerika Karli and the assistance of other members of the group. The subject of this final project came from supervisor Kilian Guse. The subject developed according to the needs of the research group at that time and obtained the final form during the execution period.

The aim of the project was to carry out a large scale vaccinia virus preparation to produce large amounts of a recombinant VV, named as vvD-GFP, which would then be used by the research group in experiments. To be able to reduce the use of fetal bo-

vine serum (FBS), I carried out another VV preparation in serum-free cell culture beside the actual preparation and compared the results with each other. In addition, I tried to find out how serum-free culture affects the cell growth and viability of the VV producer cell line A549. I performed a cell viability assay using a tetrazolium (MTS) based CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay. The final aim was to quantify the virus titers from both VV preparations using two tittering methods; plaque and TCID$_{50}$ assay, and compare the results. Finally, I tried to evaluate the reliability of the methods.

2   Background

2.1   Gene Therapy in General

Gene therapy can be defined as a transfer of genetic material into patient´s somatic cells to treat or prevent disease. Genes can be used to treat both acquired and inherited diseases, by replacing the defective gene with the functioning version or by transferring genes, which encode therapeutic proteins. Gene transfers can be carried out using *ex vivo* or *in vivo* methods. In *ex vivo* approaches, the cells, isolated from the patient, are modified outside of the patient's body and then returned to the patient by transplantation. In *in vivo* methods, the therapeutic gene is introduced directly to the desired location in the body. Gene therapy is an experimental treatment and that is why it can be used only in very serious diseases that lack efficacious treatment; such as cancers, cystic fibrosis, duchenne muscular dystrophy and lysosomal storage diseases. Other disease areas which gene therapy is being studied are immunodeficiency (ADA, SCID-XI), Parkinson´s disease, Alzheimer´s disease, muscle ischemia, arthritis and even AIDS. The gene therapy applications in preventing and treating diseases may prove to be more effective and more common in the future than traditional therapy applications. Furthermore, gene therapy may complete and improve conventional therapies. Successful gene therapy includes clear understanding in pathogenesis of diseases and characteristics of target tissues, effective therapeutic gene or genes, and appropriate animal models for preclinical testing. (Robbins – Ghivizzani 1998: 35-36, 43-44; Roemer – Friedmann 1992: 211-212; Ylä-Herttuala 2009: 1729, 1733, 1735.)

Nucleic acids degrade quickly in the body by the influence of nucleases, and naked DNA enters cells poorly. Therefore, gene transfer requires the use of gene carriers, also known as vectors. Virus is an organism which is specialized to transport its genes to host cells. Because of this character, viruses can be modified into efficient vectors for gene transfers. Nevertheless, both viral and nonviral vectors have been generated for this purpose. The efficiency of DNA transfer depends on the target-cell type and the state of cellular replication at the time of delivery, and even the most developed virus vector systems has its limitation. Hence, it is unrealistic to imagine that only one virus vector is suitable for all applications in gene therapy. So, there are virus vectors from many different virus families. Viruses have evolved as highly effective tools for gene delivery to specific cell types. However, viruses are pathogens and more or less immu-nogenic. Nonviral vectors are mainly nonpathogenic, but are at the same time less effi-cient in the gene transfer. Vector systems require further modification and development to make the vector more suitable and specific for gene therapy applications. (Hukkanen – Hemminki – Ylä-Herttuala 2010: 706-707; Robbins – Ghivizzani 1998: 36, 45; Roe-mer – Friedmann 1992: 212.)

Depending on the disease, the treatment requires either long-term g or short-term gene expression. Inherited diseases require long-term gene expression which is possible with virus vectors that integrate their gene into host cell genome, such as retroviruses, adeno-associated viruses (AAV) and lentiviruses. The ability of these viruses to insert their genome into the cellular DNA allows the expression of a therapeutic gene for the life of the infected cell. For the treatment of acquired diseases, like cancer, a therapeu-tic gene is transferred into the body. For this purpose virus vectors with short-term gene expression can be used. (Robbins – Ghivizzani 1998: 36-40; Ylä-Herttuala – Äyräpää 2009: 1730-1731, 1733.) It also possible to develop a chimeric virus system in which features of one viral system are combined with features of another viral sys-tem. This type of approach can make the gene expression more effective. Genes can be delivered into cell also with nonviral vectors, such as liposomes, gene gun, DNA conjugates, nonviral hybrids and transposons. These nonviral vectors are nonpatho-genic and relatively cheap and easy to produce, but the efficiency compared to viral vector is poorer. (Robbins – Ghivizzani 1998:41-42; Ylä-Herttuala 2009: 1733.)

2.2 Gene Therapy for Cancer

The term gene therapy includes a wide range of treatments that all use genetic material to modify cells (Cross – Burmester 2006: 218). In the review article of Deanna Cross and James Burmester, the field of cancer gene therapy is broken into three categories: gene transfer, immunotherapy and oncolytic virotherapy.

In gene transfer or insertion, the foreign gene (therapeutic gene) is introduced into the cancer cell or surrounding tissue. A number of different genes have been proposed as therapeutic agents, including suicide genes, antiangiogenesis genes and cellular stasis genes. For delivering these genes a number of viral and nonviral vectors have been used in clinical trials. Most commonly used and currently most efficient viral vector has been replication incompetent adenovirus. The advantages of adenovirus based vectors are the ability of infecting dividing and nondividing cells and the ability of achieving a high level transient gene expression. Herpes simplex virus (HSV) is also being developed for gene therapy applications. The ability to cause a latent infection in neuronal cells makes HSV an attractive vector especially for treating neurological disorders. The choice of delivery vector depends on the specificity and desired duration of the gene transfer therapy. With integration capable viruses extra precautions must be taken to ensure the therapeutic gene does not integrate into unwanted cell type or to unwanted area of the genome. (Cross – Burmester 2006: 223; Robbins – Ghivizzani 1998: 37-38.)

One goal of cancer treatment has been to harness the immune system and to invoke long-term immune response to target and destroy cancer cells without autoimmunity. Immunotherapy aims to eradicate the tumor cells by increasing the patient's immune responses against tumor antigens. For this purpose recombinant cancer vaccines has been created. Live cancer vaccines are not meant to prevent diseases, but stimulate the immune system to recognize the cancer cells. (Cross – Burmester 2006: 219.) The development of an effective cancer vaccine requires an efficient antigen delivery system. For this purpose various bacterial and viral vectors have attracted a great attention. The advantage of live vectors is their ability to produce the desired antigen in its native form, which is important in production of neutralizing antibodies. Attenuated bacteria vectors, like *Salmonella*, *Listeria monocytogenes* and *Lactococcus latis*, have many advantages that make them suitable for cancer treatment. They are quite inexpensive to prepare, powerful immune stimulators, safe and well tolerated and treatable

with antibiotics. (Bolhassani – Zahedifard 2012: 1733-1734.) Viral vectors are capable for inducing antibody responses and eliciting T lymphocytes, which are important for control of intracellular pathogens and cancer. Many viruses have been evaluated as recombinant vectors for vaccines, such as alphaviruses, adeno-associated viruses, poliovirus and hepatitis B virus. The most widely used evaluated vectors are human adenovirus and some types of poxviruses. (Bolhassani – Zahedifard 2012: 1738).

Oncolytic viruses hold a great potential as novel therapeutic agents for treating malignancies like cancer. Oncolytic viruses are genetically engineered to specifically infect and destroy cancer cells, while being not harmful for the rest of the body. Oncolytic viruses destroy cancer cells by spreading of the virus, expression of cytotoxic proteins and lysing the cell through virus replication. Many different viruses have been used as oncolytic vectors, including vaccinia virus, adeno virus, herpes simplex virus type 1, reovirus and Newcastle disease virus. Many of viruses chosen for this purpose have a natural tropism to cancer cells. The results from oncolytic virus therapies have been promising. However, the therapy has some challenging obstacles. One of these is the existing antibodies in humans which may clear the viral agent before it has time to infect cells. The clinical trials are also more expensive and complicated because of safety issues in use of replication competent viruses. (Cross – Burmester 2006: 221-222.)

2.3   Vaccinia Virus

Vaccinia Virus (VV) has the longest and most extensive history of use in humans and has been studied extensively in the laboratory. VV is the first animal virus seen microscopically, grown in tissue culture, accurately titered, physically purified, and chemically analyzed. VV is known for its role in smallpox eradication as an effective smallpox vaccine. There are many strains of VV with different characteristics, pathogenicity and host range. Western Reserve (WR) is one of the most commonly used strains in laboratory and preclinical studies. (Moss 2001:2849-2850; Shen – Nemunatis 2005: 180-181.)

Vaccinia virus is a member of a Poxviridae family and belongs to Orthopoxvirus genus. VV has a wide host range and the ability to replicate in almost all vertebrate or invertebrate cell types. The replication of the virus occurs in the cytoplasm of the infected cell. The origin and the natural host of VV are unknown. The VV virion is a large, physically complex, brick- or oval-shaped particle with highly organized subvirion structure (figure 1). VV is one of the largest known viruses, with virion dimensions of 300 x 240 x 120

nm (Moss 2001: 2849-2851; Shen – Nemunatis 2005:181).The genome composes of a single linear double-stranded (ds) DNA molecule of approximate 195 kilobase pairs with hairpin loop at each end (Laliberte - Moss 2010: 973; Moss 2001:2850).

VV produces three forms of infectious particles (Shen – Nemunatis 2005: 181). The majority of the VV particles are of the mature virion (MV) form (alternate name intracellular mature virion: IMV). MV form is the simplest infectious form of poxvirus particles with only a single lipid bilayer envelope. Mature virions are released upon cell lysis and may be important for animal-to-animal transmission. In addition, there is less abundant enveloped virion (EV, including cell-associated enveloped virion [CEV] and extracellular enveloped virion [EEV]), which is essentially an MV with additional lipoprotein membrane. EVs are released from the host cell by exocytosis, without lysing the cell. EVs are important for efficient virus spread inside the infected host. The additional lipid membrane protects EVs against host immune defenses. The cell-associated EV is largely responsible for cell-to-cell spread. MV and EV forms are antigenically different from each other. (Laliberte – Moss 2010: 973, 979.)
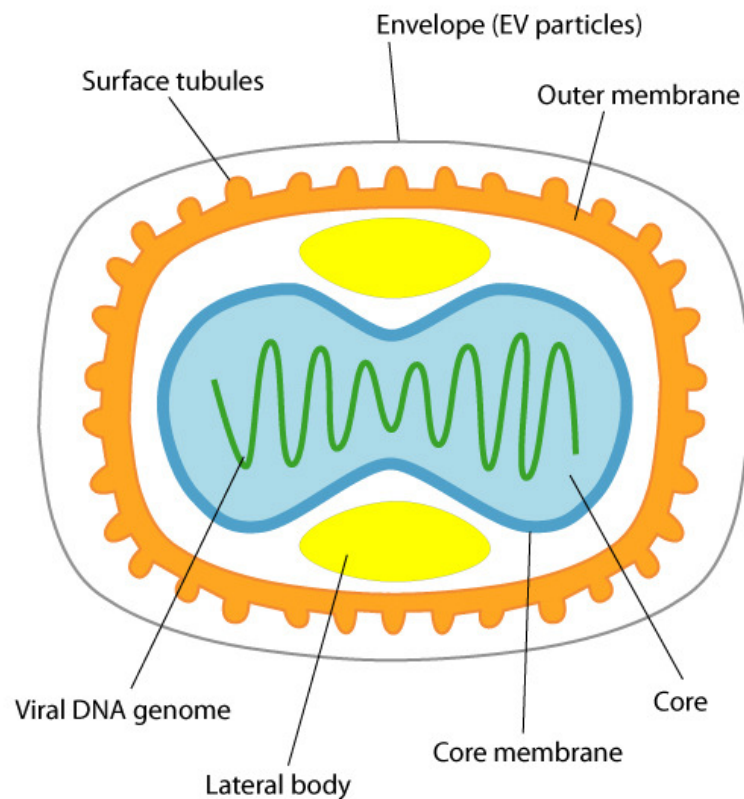

Figure 1. Structure of vaccinia virion (Eposito – Fenner 2001: 2886.)

Due to the multiple forms of infectious virions, the process of cell entry by VV is not well understood and the receptor for infection has not been identified (Shen – Nemunatis 2004: 181). MV particles can enter, and release the viral core, to the host cell cytosol by both direct fusion with the plasma membrane and fusion with endosomal vesicles. These two actions can occur even in the same cell. Entry of EV particle requires rupture of the outer membrane "wrapper" to expose the MV membrane. Thus, EV particles shed their extra membrane prior to direct fusion of the MV membrane with the cell plasma membrane. Entry through endosomal route is also possible for EV. It has been observed that the EV outer, "wrapper", membrane is not fusogenic with cell plasma membrane. (Laliberte – Moss 2010: 974, 976.)

The entire life cycle of VV occurs in the cytoplasm of host cells. The double-stranded DNA genome, packaged in the viral core, encodes approximately 200 gene products. These gene products include, for example, RNA polymerase, early transcription factors, mRNA capping enzymes and protein kinases, which enables viral protein synthesis and virion assembly. (Laliberte – Moss 2010: 973; Shen – Nemunatis 2005:181; Wagner – Hewlett 2004: 338.) VV expresses a temporally-regulated gene expression program. That is, viral replication can be separated into three specific temporal phases in the infected cells. VV replication is initiated by entry of the viral core into the cytoplasm where the following steps of the cycle take place. The replication occurs at special sites in cytoplasm termed viral factories. During early events the virion is partially uncoated to enable the synthesis and expression of early viral proteins required for DNA replication. The replication of viral DNA is followed by expression of intermediate proteins during the intermediate stages of replication. Late mRNA encodes late gene products which consist mainly of structural proteins needed in virion assembly and other proteins involved with virus maturation. In summary, expression of early genes triggers the expression of intermediate genes which encodes late gene specific transcription factors. (Laliberte – Moss 2010: 973; Shen – Nemunatis 2005: 182; Wagner – Hewlett 2004: 338-340.) The entire life cycle of VV takes approximately 24 hours, whereupon as many as 10,000 IMV particles are released upon cell lysis. CEV and EEV particles may be released from cells as soon as 6 hours after post-infection and allow rapid long-term spread. (Shen – Nemunatis 2005:187-188.)

2.4   Vaccinia Virus in Gene Therapy

Vaccinia virus (VV) has been studied for its use in cancer gene therapy mainly in three ways: as a gene therapy vector of therapeutic genes, as a tumor selective replicating oncolytic virus and as a cancer vaccine expressing tumor antigens and/ or immunostimulatory molecules. A number of unique features make VV attractive for research and in the development of biotherapeutics. VV is highly immunogenic virus and is able to elicit both strong T-cell mediated and antibody responses. This character makes VV an efficient vaccine. VV can be studied in many different animal models, due to its wide host range. The entire replication cycle of VV occurs in the cytoplasm of the host cell, so the genome of virus never enters to the cell nucleus. Hence, there is no possibility of DNA integration. In addition, in the unlikely case of uncontrolled replication, there are several antiviral agents available. Furthermore, VV can be produced to relatively high titers, the particles are stable and can be stored both in frozen solution and dry powder without significant loss of infectivity. (Guse et al. 2011: 596.)

VV can be used as a vector for therapeutic genes. VV has the capacity to hold up to 25 kb of foreign DNA in its genome, gene expression occurs extremely efficiently and it is capable to infect many different tissues. Although VV is highly immunogenic, which may lead to rapid clearance of the vector, efficient infection is possible despite the presence of neutralizing antibodies. The VV strains most widely used are highly attenuated, such as Modified Vaccinia Ankara (MVA) and New York Vaccinia virus (NYVAC). (Guse et al. 2011: 596-597.)

As previously mentioned, VV is highly immunogenic and it is capable of inducing strong humoral and cell mediated immune responses. In addition, VV infection provides "danger signals" that help to shape T cell responses effectively. Thus, VV represent an attractive candidate for cancer immunotherapy. A number of VV vector based cancer vaccines have shown promising results in several preclinical and clinical trials. For instance, VV has been engineered to deliver tumor-associated antigens (TAAs) to elicit antigen-specific immune responses, or immune modulating genes, like cytokines and costimulatory molecules, to change the tumor's microenvironment. (Shen – Nemunatis 2005: 185.)

VV is also very attractive vector for developing oncolytic viruses, due to its efficient replication, cell lysis, spread, broad host range, natural tropism to tumor tissues and

remarkable safety record in human use (Shen – Nemunatis 2005:187). The virus has been genetically modified to create an oncolytic vector that specifically infect and replicate in tumor cells. Western Reverse (WR) strain has been used for tumor-selective oncolytic VV because it is more efficient in killing cancer cells than other strains. (Shen – Nemunatis 2005: 188.)

The strategies used to create tumor-selective oncolytic viruses can be grouped in to three categories. The first strategy is to delete such gene functions that are crucial for efficient viral replication in normal cells but unnecessary in tumor cells. The second strategy is to limit the expression of critical viral gene to tumor tissues through the use of certain promoters. The third strategy is to modify viral tropism through modification of surface proteins. An example of generating tumor specificity of VV using the gene deletion are the deletions of the thyminide kinase (TK) and vaccinia growth factor (VGF) genes. TK is necessary for replication in normal cells, but is not necessary in cancer cells because of high concentrations of intracellular nucleotides in these cells. VGF involves in proliferation in normal cells, but is dispensable in tumor cells because they are naturally proliferating. Thus, virus with the VGF gene deleted replicates primarily in tumor cells. Double deletion (dd) of TK and VGF enhances the tumor specificity further. (Shen – Nemunatis 2005: 188-189.)

3   Final Project Aims

Virus preparation process requires special expertise. To be able to carry out a high-quality large scale vaccinia virus preparation, the methods and techniques need to be properly practiced. The preparation is always carried out by humans and hence the qualification of the worker correlates directly with the results, in this case with the virus titer. High quality and reliable high titer of virus preparation require competent workers. Hence, the main aim of my project was to carry out a large scale vaccinia virus preparation with assistance of experts, and learn and practice the methods and techniques to be able to ensure high quality. In addition, another goal was to describe the different stages of the process.

Routine cell culture generally demands the use of fetal bovine serum (FBS) as constituent of complete growth medium to ensure proper cell growth. Nevertheless, serious ethical concerns are raised about the harvest and collection of FBS, focused mainly on

the painful methods of collecting the serum from animals. In addition to ethical concerns, serum has a number of other disadvantages, such as physiological variability, qualitative variation in the composition, cost, and it may contain adverse factors or it can be a potential source of microbial contaminants, like bacteria, viruses and prions. Hence, the use of animal proteins in cell cultures needs to be decreased for ethical and practical reasons as well as for involved safety issues. (Freshney 2005: 129, 132-134; Gstraunthaler 2003: 275-276.) Due to these facts, one aim of my work was to investigate how serum-free culture affects to cell viability of A549 cells and virus production.

Virus titers have inherently large deviation, due to biological variation and variations in serial limiting dilutions. These sources of error are only partly controlled. In addition, the practical problems in titer assays are that they are time consuming and require cell culture reagents and skilled personnel. Accurate titer determination is vital for the reliability of any preclinical experiment and clinical studies. (David – LaBarre – Lowy 2001: 107-108.) Therefore, it is important, that the defined virus titers are reliable. Hence, one objective of my project was to compare the common plaque titering assay with $TCID_{50}$ assay, and to compare the result with each other.

The purpose of my final project is summarized in the specific aims below:

- To learn how to make a large scale preparation of VV with high quality and high virus yield and describe the preparation process.

- To evaluate the effect of serum-free medium on A549 cell cultures.

- To evaluate whether vaccinia virus can be produced without using the serum in viral culture medium.

- To evaluate whether $TCID_{50}$ assay can be used as reliable titering method in VV preparations.

4   Materials and Methods in Vaccinia Virus Preparation Process

4.1   Cell lines

When choosing the cell line, specific functional requirements and a number of general parameters need to be considered. It is essential to consider whether the cell line needs to be finite or continuous, normal or transformed, and the importance of the species. One important issue is the growth characteristics of the cells, such as doubling time, yield, plating efficiency, growth fraction and the ability to grow in suspension. Some other considerations are in addition availability of the cell line, how well the cell line is characterized, expression of phenotype, is a control cell line required and how stable is the cell line. (Freshney 2005: 204-205.) In this process there were two cell lines in use in CGTG, Vero and A549 cell line.

In this project, the A549 cell line was used for viral culture and to test the effect of serum-free growth medium on cell viability. A549 cell line was obtained from American Type Culture Collection (ATCC), The Global Bioresource Center. The A549 cells are from lung carcinomatous tissue from a 58-year-old Caucasian male. The cell line was initiated in 1972 by D.J. Giard, et al. through explant culture. The cells are hypotriploid with modal chromosome number of 66. The chromosomes are abnormal with unusual amount of chromosome copies in cells and other chromosome mutations. Biosafety level with this cell line is 1, so appropriate safety procedures are always needed. Cell line is intended only for research purposes. (Product Information Sheet for ATCC CCL-185™: 1-2.) The detailed instructions for handling and subculturing are found from product information sheet.

In this project, the Vero cell line was used in both virus titration methods. Vero cells were a kind gift of John Bell, Ottawa Health Research Institute, Canada. This Vero cell line has been in use in GGTG for many years. Vero cells are from the kidney of a normal adult African green monkey. The cell line was initiated in1962, by Y. Yasumura and Y. Kawakita in Chiba, Japan. Vero cell line has hypodiploid chromosome count, with modal chromosome number of 58. Biosafety level is 1, so appropriate safety procedures are always needed with this cell line as well. Also this cell line is intended only for research purposes. (Product Information Sheet for ATCC CCL-81™: 1-2.) Elaborated

instructions for handling and subculturing are found in the specific product information sheet.

4.2   Recombinant Vaccinia Virus vvD-GFP

This recombinant vaccinia virus was kindly provided by Andrea McCart, Toronto General Research Institute, Canada. This virus has also been used in CGTG's previous studies. The strain of this recombinant vaccinia virus is Western Reserve (WR). This vvD-GFP has a deletion in its thyminide kinase (TK) gene and a green fluorescent protein (GFP) gene as transgene in its genome. Specifically, the TK gene has been deactivated by adding a sequence of foreign DNA, the GFP gene, in the middle of the functional TK gene. The virus can be used as oncolytic virus vector.

4.3   Aseptic Technique

Tissue culture can be contaminated easily by microorganisms, such as bacteria, mycoplasma, yeast and fungal spores. Contamination can be confined only to one or two cultures, or it can spread and infect the whole experiment. In the worst case, the contamination can ruin even the laboratory's whole stock. The source of contamination can be the operator itself, the atmosphere, work surfaces, solutions or some other factor. With proper aseptic technique it is possible to minimize the risk and even eliminate such contaminants. A strict code of practice and commitment to it is essential. (Freshney 2005: 73.)

To minimize the risk of contamination the cultures need to be checked by eye and on a microscope every time they are handled, reagents and bottles of medium need to be checked for sterility and not shared with other people or used for different cell lines and the standard of sterile technique is always kept high. Aseptic technique provides a barrier between non sterile environment and uncontaminated culture within its flask or other dish. Hence, all material and equipment which will have a direct contact with culture need to be absolutely sterile. (Freshney 2005: 73.)

Aseptic environment has some certain elements. The area or room for sterile work should be selected so that it is free from air currents, like doors and windows, and have no through traffic. A use of laminar flow cabinet, the hood, is highly recommended. The

area should be kept clean and free from dust, and contain only equipment used in tissue culture. Activity in the area should be limited to tissue culture and all nonsterile activities should carry out elsewhere. Keeping the work surface clean and tidy is crucial. The work should always start and finish with a completely clean work surface. Generally, the surfaces are wiped before and during the work with 70% alcohol. Personal hygiene of the operator is also essential, including proper hand hygiene, use of gloves or other protective device when needed, long hair tied back and keeping talking to a minimum. Medium and reagents should be wiped with 70% alcohol always when they come from refrigerator or water bath, and kept open only in laminar flow cabinet. Cultures with different cell line, or virus infected cells, should be handled separately. (Freshney 2005: 74-76.)

Sterile handling includes wiping with 70% alcohol, use of clean caps, most preferably deep screw caps, and occasionally even flaming. In addition, 1.5% Barrydin and UV light can be used in culture rooms to destroy viruses more effectively. When handling bottles and culture flasks, a hand should never pass over an open vessel. Therefore, the bottles and flasks should left open only as short time as needed. The pipettes should be disposable plastic pipettes or in some cases standard glass. The pipettes packed individually should not be opened until in the hood, and unused pipettes should be stored in dust-free container. (Freshney 2005: 76-78.)

The working environment can be protected from dust and other possible contaminations by a constant, stable flow of filtered air passing over the work surface. That is the major advantage of working in a laminar flow hood. There are two main types of flow: horizontal and vertical flow. In horizontal flow the airflow blows from the side facing you, parallel to the working surface and is not recirculated. In vertical flow the air blows down from the top of the hood onto the work surface and it is drawn through it and then recirculated or vented. Horizontal flow hood gives the best sterile protection to the culture while vertical flow hood gives more protection to the operator. (Freshney 2005: 78-79.)

4.4   Culture of Mammalian Cells

Tissue culture was invented already at the beginning of the twentieth century as a method for studying the behavior of animal cells. The technique started from growing of solid fragments of tissue and developed into disaggregation of explanted cells and into

re-plating of these dispersed cells. HeLa cell line was the first continuous human cell line established. Tissue culture is used for many applications, such as investigation of intracellular activity (DNA transcription, protein synthesis etc.) and intracellular flux, investigation of environmental interactions (infection, cytotoxity etc.), cell-to-cell interactions, and investigation of genetics and cell products and secretion. Cancer research and virology are highly dependent on tissue culture techniques. (Freshney 2005: 1, 4.)

Cell culture is one type of tissue culture *in vitro*. Cell culture implies that the tissue from the original tissue (primary culture or cell line) is dispersed mechanically, enzymatically or chemically into a cell suspension and is then cultured as an adherent monolayer on a solid artificial substrate or free floating in suspension (Freshney 2005: 3, 8.) For successful growth and maintenance of mammalian cells in vitro, appropriate culture conditions are required. That means that, growth environment should provide favorable growth conditions to the cells, including physiological, physiochemical and nutrient conditions. (Gstraunthaler 2003: 276.)

Physiological conditions are defined as the use of appropriate culture dishes which allow the cells to attach and spread. Most normal cells grow as adherent monolayer; unless they have transformed and become anchorage independent. The cells need to attach and spread out on the substrate before they will start to proliferate. (Freshney 2005: 31.) The substrate must allow adhesion of attachment factors, which will allow the cell adhesion and spreading. The extracellular matrix adheres to the substrate, and the cells bind to the matrix via specific receptors. For cell adhesion and spreading the substrate needs to have slightly negative charge. Glass was the original substrate because of its optical properties and surface charge, but it has been replaced by irradiation, chemically or electric ion treated plastic, commonly polystyrene. (Freshney 2005: 31, 105.)

Physiochemical conditions comprise of incubation temperature, constitution of gas phase (oxygen requirement), suitable pH (CO_2) and osmolality (water status). The temperature recommended for most human and warm-blooded animal cell lines is normal body heat, +37°C. For energy production cultured cells are often relying on glycolysis which may be anaerobic. Providing the correct oxygen tension is always a compromise between fulfilling the respiratory requirement and avoiding toxicity. Dissolved oxygen can be toxic to the cells due to the increase of the free radicals. Most cell lines grow well at pH 7.4, but the pH varies among different cell strains. CO_2 acts

as a pH buffer and stabilize the pH in cell cultures. The buffer can be incorporated into the growth medium, but exogenous CO_2 is generally still required. The optimum osmolality has been assumed to be at the same level where it would be in origin's plasma. (Freshney 2005: 115-118.) For controlling the humid, temperature and CO_2 tension in culture atmosphere, a humid CO_2 incubator is optimal. It will circulate the air around and keeps the temperature and CO_2 level consistent. (Freshney 2005: 63.)

The culture medium is one of the most important single factors in cell culture. The growth medium should contain all essential nutrients for cell metabolism, growth and proliferation, such as amino acids, vitamins, salts, glucose, hormones and grow factories. (Freshney 2005: 115-123; Gstraunthaler 2003: 276.) Serum is generally added to culture medium as a supplement. The role of serum in culture medium is to provide hormonal factors for cell growth and proliferation; transport proteins carrying hormones, minerals, trace elements and lipids; attachment and spreading factors and stabilizing and detoxifying factors. (Gstraunthaler 2003: 276-277.) Complete medium signifies a medium that has all of needed ingredients added and is ready to use. Some constituents may be added just before use, such as serum and glutamine. Dulbecco's modification of Eagle's MEM (DMEM) is a complex medium with high nutrient concentration, and is optimized for viral propagation. (Freshney 2005:120.)

The cell lines used in this project were grown in T-175 tissue culture flasks (Tissu Culture Flask, 550 ml, 175 cm^2, with filter, Greiner bio-one) and maintained in a humified incubator at 37°C with 5% CO_2. Both cell lines were propagated every 3-4 days and split 1:2 or 1:3. First, the old medium was aspirated from the T-175 flasks and the cells were washed with 5-6 ml of PBS (Phosphate Buffered Saline: 0,0067 M PO_4, without Ca and Mg, Lonza BioWhittaker). Then, the PBS was aspirated from the flasks and 5 ml of 1x trypsin (with Versene [EDTA], Lonza BioWhittaker) was added to each flask. Flasks were kept in the incubator for 5-10 minutes until the cells were detached. 5ml of appropriate medium was added to the flasks to inactivate the trypsin. Finally, the cells were suspended by pipetting, added to new flasks with fresh medium containing a total volume of 25 ml, and transferred to incubator.

A549 cell line was used for viral culture and to test cytotoxic effects of serum-free growth medium. A549 cells were grown in DMEM (Dulbecco´s Modified Eagle´s Medium) with 1 g/l glucose, without L-glutamine (Lonza BioWhittaker). To make complete growth medium 1% L-glutamine (200 mM in 0,85% NaCl Solution, Lonza

BioWhittaker), 1% PEN-STREP (10,000 U Penicillin/ml and 10,000 U Streptomycin/ml, Lonza BioWhittaker) and 10% FBS (Lonza BioWhittaker) were added.

Vero cell line was used for virus titration with plaque assay and $TCID_{50}$ assay. Vero cells were grown in DMEM (Dulbecco´s Modified Eagle´s Medium) with high 4.5 g/l glucose, without L-glutamine (Lonza BioWhittaker). To make complete growth medium 1% L-glutamine (200 mM in 0,85% NaCl Solution, Lonza BioWhittaker), 1% PEN-STREP (10,000 U Penicillin/ml and 10,000 U Streptomycin/ml, Lonza BioWhittaker) and 10% FBS (Lonza BioWhittaker) were added.

4.5   Infection with Vaccinia Virus

As previously mentioned, the replication of VV occurs in the cytoplasm of the host cell. Therefore, the cells need to be infected by the virus for the virus replication and amplification to take place.

For large scale VV preparation, ten 100% confluent T-175 flasks of A549 cell were prepared. Because two virus preparations were made, the amount of flasks was double. The growth medium was aspirated from the flasks and the cells were infected with vvD-GFP with a multiplicity of infection (MOI) of 0.1 PFU/cell. The virus stock was maintained in Tris base solution (1 mM Tris, pH 9.0) at -80°C until use. For the first VV preparation the calculated volume of virus was added to 5 ml of growth medium with 2% FBS per flask. The other VV preparation was carried out the same way but without use of FBS. 5 ml of infecting solution was added to each flask and incubated one hour in the incubator at 37°C with 5% CO_2. After infection, 25 ml of proper growth medium, with 2% FBS or serum-free, was added into the flasks and the flasks were placed in incubator at 37°C with 5% CO_2 for three days until extensive CPE (cytopathic effect) was visible. The cells were monitored daily with microscope for morphological changes (CPE).

4.6   Vaccinia Virus Purification

Virus purification means the physical separation of virus from the host cell in which it has replicated. Ultracentrifugation is the standard technique for the purification of small particles of defined size, like virions, from the contaminating material. Ultracentrifuge is

a powerful centrifuge whith maximum speed in excess of 30,000 rpm up to 600,000 g with sophisticated refrigeration and vacuum system (Jones – Reed – Weyers 2007: 362).The particle's rate of sedimentation depends on the size, density and morphology of the particle, but also on the characteristics of the medium in which the particles are suspended. With differential centrifugation (pelleting) it is possible to successfully separate particles which have large differences between their mass and densities. More accurate virus purification can be achieved by the technique of density gradient centrifugation, where separation of the particles is achieved by sedimentation through a density gradient. Sucrose is an ideal medium for making of sharp and easily isolated bands of pure virus (Killington – Stokes – Hierholzer 1996: 71-72, 75.)
.

When CPE was visible, the cells with virus in them were collected from the flasks. Cells were released by scraping the bottom of the flask with scrapers. The cell suspension, loose cells and medium, was collected in 50 ml Falcon tubes. The tubes were spun down in a centrifuge with 1000 rpm for 10 minutes at 4°C and the supernatant was then discarded. The cell pellets were resuspended altogether with 30 ml of Tris base solution (1 mM Tris, pH 9.0) and put in freezer at -80°C.

The viruses were released from A549 cells by cell lysing with freeze-thaw cycles. The cell suspension was first thawed in water bath, mixed vigorously by using Vortex and then frozen at -80°C. Freezing took approximately two hours. This procedure was repeated three times. After the last thaw the suspension was spun down in centrifuge with 3000 rpm for 10 minutes at 4°C. The supernatant containing the virus was collected and put on ice. The remaining cell debris pellet was resuspended with 10 ml of Tris base solution (1 mM Tris, pH 9.0), mixed by using Vortex and spun down. The supernatant was also collected from the latter and combined with earlier collected supernatant. This was performed to ensure that all virus particles are used in the further purification.

The actual purification was performed by differential centrifugation with a sucrose gradient. 10 ml of 36% sucrose was added to two ultracentrifuge tubes (Ultra-Clear™ Centrifuge Tubes, 25 x 89 mm; Beckman). The viral sample (supernatant) was then layered carefully on top of the sucrose cushion. Before ultracentrifugation, the tubes were accurately balanced using a scale. Then, the tubes were centrifuged for 90 minutes at 20,000 g at 4°C using Optima™ XL-80K Ultracentrifuge, Beckman Coulter. During ultracentrifugation the virus pellet travelled through the sucrose cushion to the bottom of

the tube. After centrifugation, the supernatant was instantly discarded from the tube, carefully without disturbing the pellet. Viral pellets from two tubes were resuspended with 1 ml of Tris base solution (1 mM Tris, pH 9.0) and combined. Finally, the virus suspension was divided to aliquots in Eppendorf tubes and stored in freezer at -80 °C. Eppendorf tubes and tags for storing the virus aliquots were prepared while tubes were centrifuged.

4.7   Quantification of Vaccinia Virus and Titer Calculations

The concentration of the infectious virus particles is essential for most virology experiments. Animal viruses are quantified by infectivity assay (e.g. $TCID_{50}$ and plaque assay), by some other biological or chemical assay (e.g. haemagglutination) or by direct total virus particle counting using the electron microscope. Plaque assay has been considered to be the easiest, most accurate and sensitive form of assaying virus infectivity. However, plaque assay has some disadvantages and hence other methods are needed. (Hierholzer – Killington 1996: 35.)

The replication of virus in the host cell causes several biochemical and morphological changes within the cell and generally results in cell death. These morphological changes are called as cytopathic effect (CPE) which may take several forms, such as cell rounding, cell fusion or total cell lysis. CPE can be visible by naked eye, but it is generally observed with light microscope. Infectivity assays are designed to enable the calculation of virus titer (the number of infectious units per unit volume). A virus particle able to instigate an infection, in other words an infectious unit, is called a plaque forming unit (PFU) (Wagner – Hewlett 2004: 136). Determination of virus titer by infectivity assays requires making accurate serial dilutions of virus suspension. 10-fold dilutions are commonly carried out for routine use. (Hierholzer – Killington 1996: 35-36.)

4.7.1   Plaque Assay

The plaque assay quantifies the number of infectious units in a given virus suspension. Plaques are discrete foci of infection originated from a single infectious virion. A plaque denotes a spot of cell lysis or CPE, in other words a hole, within the cell monolayer. In monolayer plaque assay the virus dilution is added to the previously seeded confluent cell monolayer for virus infiltration. Before incubation an overlay medium, composed

from agarose, is added to the cell monolayer. This overlay medium prevent the development of secondary plaques by forcing the released virus particles from the infected cell to infect only neighbouring cells. The assay dishes are then incubated at appropriate temperature until the plaques are visible. Finally, the plates are fixed, stained with crystal violet and observed either with the naked eye or with microscope. For statistical reasons, to minimize the error, 20-100 plaques per monolayer (e.g. well) are ideal to count. The infectivity titer is expressed as the number of the plaque forming units per ml (PFU/ml). (Hierholzer – Killington 1996: 38.) The formula below can be used for calculation of the titer of a virus stock:

$$V_f = V_0 \, / \, D$$

V_f is the final concentration (PFU/ml), V_0 is the original concentration in ml (number of viruses i.e. plaques in the volume of diluted virus solution per well) and D is the dilution factor. (Wagner – Hewlett 2004: 138.) In other words, the calculation can be carried out as following:

Number of plaques/ (dilution factor x volume of diluted virus solution)

E.g. If the average number of plaques is 50 in the 10^{-5} dilution well, and if the volume of added diluted virus solution is 1 ml, the titer is then 50/ (0.0001 x 1) = 5 x 10^5 PFU/ml.

In this project the plaque assays were performed according to the instructions in appendix 3. Virus quantification with plaque assay was carried out with two 6 well plates per virus preparation in parallel. Vero cells were seeded at 1 x 10^6 cells per well in 2 ml growth medium with 2% FBS per well. Cells were incubated in the incubator at 37 °C with 5% CO_2 for 24 hours, and infected with five ten-fold virus dilutions (10^{-5}-10^{-9}). 1 ml of each of five virus dilution was added to one well on the plate and incubated for one hour in the incubator. One well was used as negative control (mock) which received growth medium with 2% FBS without any virus. After infection, virus dilutions were aspirated from the wells, and the cells were overlaid with 1.3% agarose (Bactoagar) mixed with 2xDMEM (1:1). After three days, the occurrence of plaques was checked with a microscope. The cells were then fixed with methanol-acetic acid fixative and stained with Coomassie® Brilliant Blue staining solution (Bio-Rad). Then, plaques of the two plates were counted manually, and the titer was determined as the average of PFU/ml titers of the single plate. In figure 2 is an example of a 6-well plaque assay

plate. The cell monolayer is stained blue, and the plaques can be seen as clear punctures.
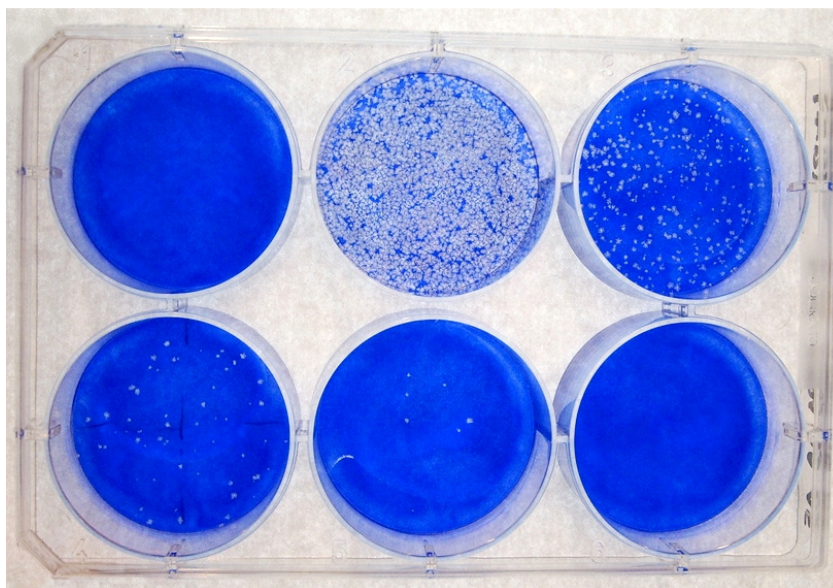

Figure 2. An example of plaque assay plate, and plaques in different virus dilutions.

4.7.2 TCID$_{50}$ Assay

TCID$_{50}$ is an abbreviation for median tissue culture infectious dose, which denotes the amount of a virus required to infect 50% of inoculated cell cultures. TCID$_{50}$ is a dilution endpoint method and the assay relies on presence and detection of virus particles capable to produce CPE. The virus dilutions are added to host cells which are grown in confluent monolayers in 96-well tissue culture plates. The number of wells per dilution affects to the accuracy of the method. During the incubation the virus replicates and the released progeny virions infect the healthy cells in the monolayer. The CPE is allowed to develop over period of days and during that time the monolayers are observed microscopically. The time required depends on virus and host cell type. After incubation the wells are scored for the presence of CPE and the TCID$_{50}$ is calculated either by the Reed-Muench or by the Spearman-Kärber method. The results do not directly indicate how many infectious units are present, but reflect the dilution of virus which produces CPE in 50% of the inoculated cells. TCID$_{50}$ relates to the average number of PFUs in the aliquot, and thus it is possible to transform the results in PFU/ml. (Hierholzer – Killington 1996: 36; Wagner – Hewlett 2004: 141-142.)

In this project, two 96-well plates were prepared for parallel quantification of each virus preparation. $TCID_{50}$ was performed by seeding the Vero cells at 1×10^4 cells per well in 96 well plate with 100 µl growth medium with 2% FBS per well. After incubation for 24 hours in the incubator, the cells were infected with 100 µl per well of virus sample from both virus preparations. Each 96 well plate was infected with eight ten-fold virus dilution series (10^{-5}-10^{-12}), so that each plate had all eight dilutions and two negative control columns. $TCID_{50}$ was carried out according the instructions in appendix 4. After 10 days, the plates were read manually with a microscope to determine CPE of each well, and the titers were calculated using the Spearman-Kärber statistical method and then transformed in PFU/ml. The formula below was used to calculate the titers:

$$T = 10^{1+d(S-0.5)}$$

T is the titer for 100 µl aliquot of virus, d is the log 10 of the dilution (1 for ten-fold dilution) and S is the sum of ratios. After this calculation, the titers in $TCID_{50}$/µl were transformed into $TCID_{50}$/ml and futher to PFU/ml. First, the titer in $TCID_{50}$/µl was multiply by 10 to get the titer in $TCID_{50}$/ml (e.g. 10^8 $TCID_{50}$/µl x 10 = 10^9 $TCID_{50}$/ml). Then, the titer in $TCID_{50}$/ml was transformed in PFU/ml by subtracting 0.7 from the log of the titer (e.g. $10^{9-0.7} = 10^{8.3}$ PFU/ml). (AdEasy™ - Vector System: 39.)

4.8   Detection of GFP transgene with Fluorescent Microscope

Fluorophores (also known as fluorochromes) are certain chemical compounds that absorb ultraviolet radiation and release some of the radiation energy in the longer, visible wavelengths. This phenomenon is called fluorescence. The presence of fluorophores in cells can be observed using a fluorescence microscope. In a fluorescence microscope the light source produces a beam of ultraviolet light that travels through a filter. The filter blocks all the other wavelengths except that which is capable to excite the fluorophore. When the fluorophore has become excited by the beam it starts to emit light of visible wavelength. The objects with fluorophore can be seen brightly against a black background. (Karp 2005: 737.)

Jellyfish *Aequorea victoria* produces bioluminescent green light. The light produces when calcium binds to its photoprotein called aequorin. Aequorin itself produces blue light when activated. However, the jellyfish produces green light. This green light is

caused by another protein in *A. victoria*, the green fluorescent protein (GFP). (Chalfie et al. 1994: 802.) Green fluorescent proteins are found in many organisms. However, the gene of A. victoria´s GFP was the first which was cloned and expressed. This GFP is also used in most of the tracer studies. (Zimmer 2001: 759.) GFP is a protein of 238 amino acids. The crystal structure of wild-type GFP is dimer and it resembles a shape of a cylinder. A cromophore, the "pigment" of GFP fluorophore molecule, is situated in the center of the cylinder, and in this way it is well protected in the "can". GFP absorbs blue light (398 nm - 475 nm) and emits green light (509 nm - 540 nm). (Chalfie et al.1994: 802; Zimmer 2001: 760-761.) Nowadays, the GFP is commonly used as a biological marker in molecular biology, medicine and cell biology. GFP has many good qualities which make it good research tool in living systems. (Zimmer 2001: 759.)

The ability of vvD-GFP to express GFP protein in infected cells was checked with the fluorescence microscope (Zeizz Axiovert 200; Carl Zeizz Inc.) Vero cells from one plaque assay plate were examined and photographs were taken.

4.9 Effects of Serum-free Medium on A549 Cell Growth *in vitro*

According to the ATCC product information sheet for A549 cell line, the complete growth medium needs to contain 10% of FBS. Hence, the interest was to investigate how serum-free medium affects to the cell viability of A549 cells compared to the complete medium. A549 cells were incubated 1-3 days in both serum-free growth medium and in complete growth medium with 10% FBS. Cell viability was measured using tetrazolium (MTS) based colorimetric CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation assay on day one, two and three.

4.9.1 Execution of Cell Viability Measurement for A549 Cells

A549 cells were seeded in 16 wells (two columns) in three 96-well plates. A549 cells were seeded at 1×10^4 cells per well in 100 µl growth medium with 10% FBS and incubated for 24 hours. On next day the medium was changed. Old medium was aspirated carefully from each well, then 100 µl of medium with 10% FBS was added to wells in first column and 100 µl of serum-free medium was added to the wells in second column (Figure 3). The procedure was repeated to ensure absence of serum in wells with serum-free medium. The cell viability of A549 cells in different serum concentrations was

measured using CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay. The measurement was carried out for one, two and three days after the medium change. In addition, the cells were checked daily with light microscope.

A	10%	0%										
B	10%	0%										
C	10%	0%										
D	10%	0%										
E	10%	0%										
F	10%	0%										
G	10%	0%										
H	10%	0%										

Figure 3. Example of media with different serum concentrations on 96-well plate in cell viability assay.

4.9.2   Measuring the Cell Viability with CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay

CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay (Promega) is a colorimetric method for determining the number of viable cells in proliferation or cytotoxity assays. The reagent contains a novel tetrazolium compound (MTS) and electron coupling reagent (PES). Metabolically active cells bioreduce the MTS tetrazolium compound into a colored formazan product that is soluble in tissue culture medium. The assay is performed by adding 20 µl of the reagent directly to culture wells and incubating 1 – 4 hours. After incubation the absorbance is recorded at 490 nm with 96-well reader. The amount of 490 nm absorbance is directly proportional to the number of living cells in culture. (CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay. Technical bulletin No. 245.)

Before adding the reagent, fresh medium with 10% FBS was changed to each culture well. Medium had to be the same in all wells because of the color. In addition, four wells with no cells (blank) were filled with the same medium as background control. After medium change, 20 µl of CellTiter 96® AQ$_{ueous}$ One Solution- reagent was added to each well and incubated one hour. After incubation, the absorbance from the wells was recorded at 490 nm with spectrophotometric plate reader (Multiskan Ascent, Thermo Labsystems) and results were analyzed with MS Excel.

5   Results

5.1   Large Scale Preparation of Vaccinia Virus Using Standard Techniques

Different stages of the preparation process progressed in logical order, according to the plan. The work instructions of VV preparation process can be found on appendices 1 and 2. The titer of this virus preparation according to the plaque assay results was 7.6 x 10^8 PFU/ml, which is higher than the titer of previous vvD-GFP preparation, which had been made by a different operator (5.5 x 10^8 PFU/ml). The stages of the large scale VV preparation process are shown in the workflow diagram below (Figure 4).

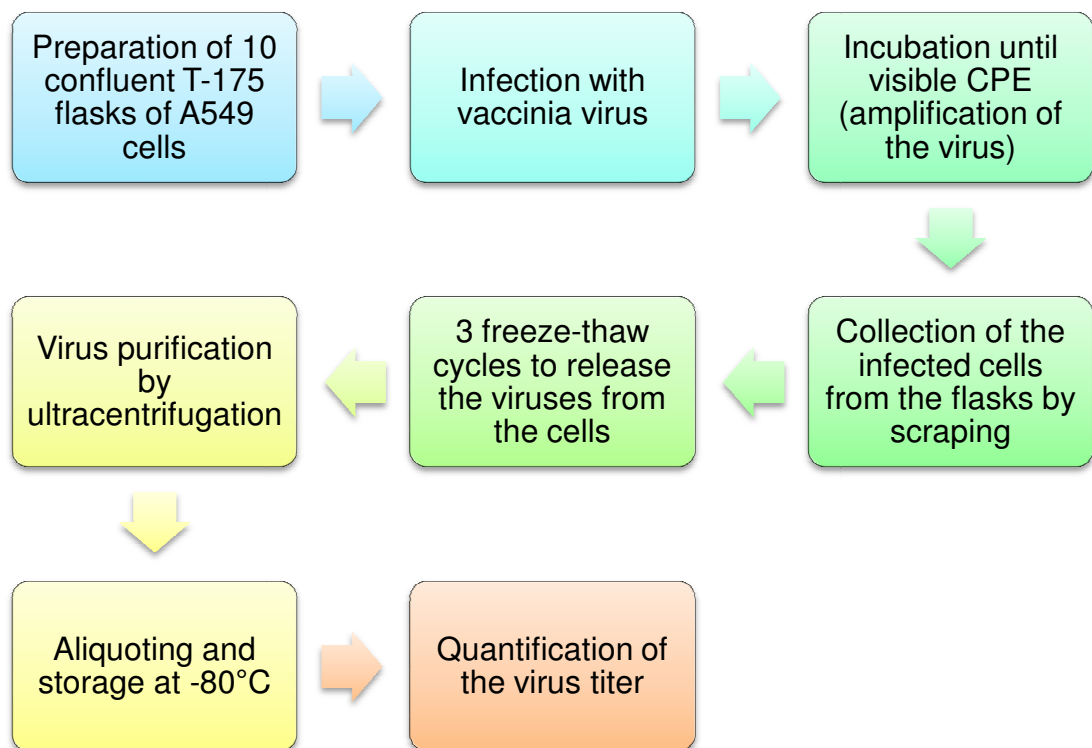Figure 4. Work flow diagram of vaccinia virus preparation process.

The presence of GFP transgene in vaccinia virus genome was indicated with fluorescence microscopy. Vero cell monolayers infected with virus of the vvD-GFP preparation developed plaque (Figure 5 D), which was positive for GFP (Figure 5 E and F). In addition, PCR for the viruses' TK region was carried out. The PCR was carried out with two

primer combinations, namely TK_F (forward) and P7.5 R (reverse) and P7.5 F (forward) and TK_R (reverse). The PCR was partly successful, having a positive band with primer combination of P7.5 F and TK_R and negative water controls (Figure 6). However, due to the absence of positive control the PCR results cannot be considered completely reliable.
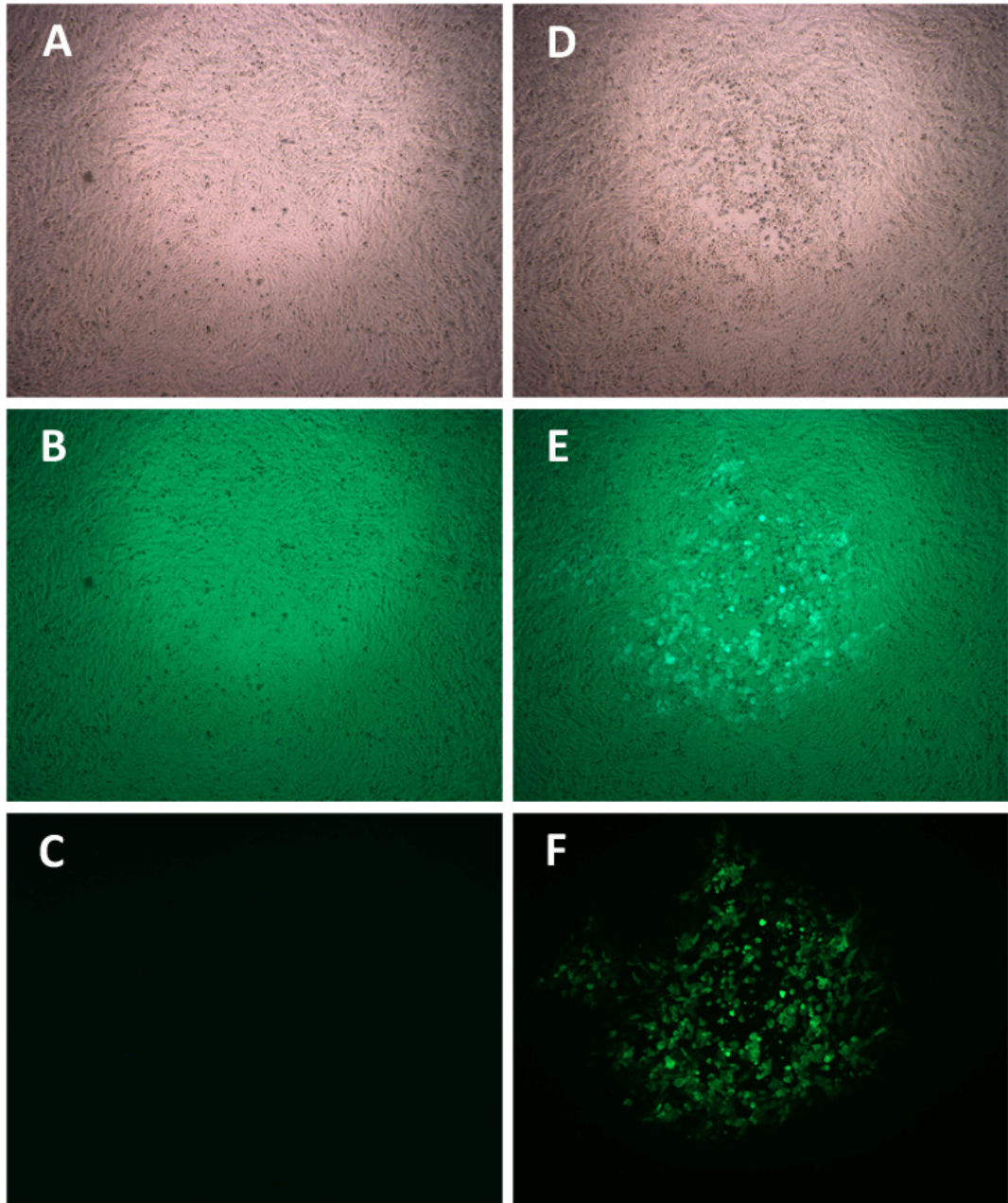

Figure 5. Fluorescence microscope picture of viral GFP in Vero cell culture. Normal Vero cells in negative control well (A), a brightfield picture of negative control (B), a GFP picture of negative control (C). A plaque in Vero cell monolayer developed by vvD-GFP preparation (D), a brightfield picture of GFP positive plaque (E), a GFP picture of GFP positive plaque (F).
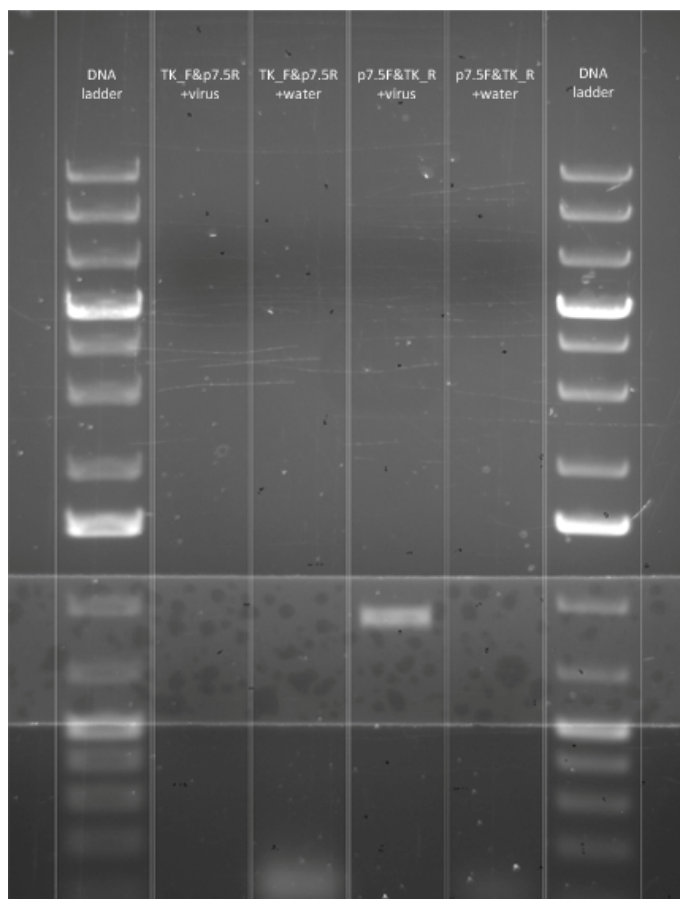
Figure 6. A positive band in gel electrophoresis with primer combination of P7.5 F and TK_R after amplification of viral DNA via PCR. Water controls are negative. Positive control is lacking.

5.2   Effect of Serum-free Growth Medium on A549 Cell Cultures

The effect of serum-free growth medium on A549 cell growth and viability was examined with MTS assay (CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay). The cell growth and viability were monitored for three days and the cell viability assays were carried out on three consecutive days; one, two and three days after the medium was changed. The background was taken away from the absorbance and the average of absorbance was determined from ten wells (n=10). The results from the assays are shown in figure 7.

After incubating for one day the number of live cells in serum-free medium was 76% compared to the number of cells in medium with 10% FBS. On second day the number of live cell in serum-free medium was 71% and on third day it was 67% compared to cultures with 10% FBS. It can only be noted that during the incubation the cells man-

aged better in medium with serum than in serum-free medium. The standard deviation (SD) of absorbance was more uniform in cultures with medium containing serum. The standard deviation of absorbance in cultures with serum-free medium varied between 0.07-0.21, where in cultures with serum it varied between 0.10-0.18.
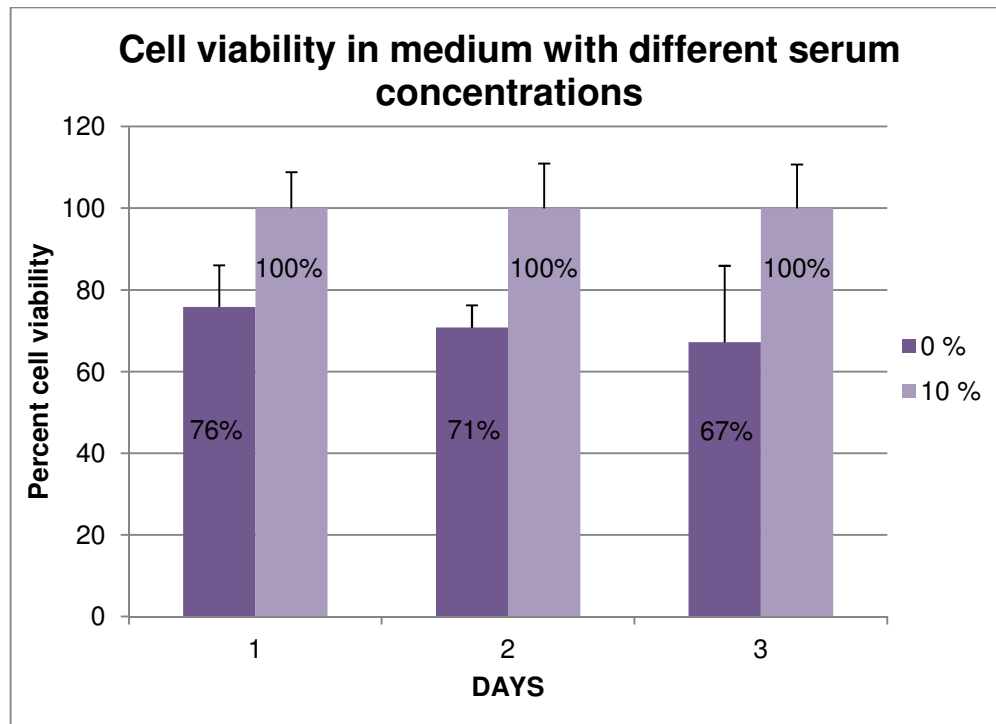

Figure 7. Diagram of ratios of live cells in cultures with 0% and 10% serum (FBS).

5.3   Effect of Serum-free Growth Medium on Vaccinia Virus Yield

The serum-free growth medium had an influence on the cell growth of A549 cells. According to the results, the cell growht was approximately 29% lower in serum-free growth medium than in medium with serum. Question remained whether the reduced cell growth would affect virus yield to the same extent.

The plaque assay is routinely used in vaccinia virus titration around the world and that is why the results from the plaque assay are used for the comparison of the virus titers. Figure 8 shows the difference between the titers in serum-free medium and medium with 2% serum. The virus titer in normal preparation was $7,6 \times 10^8$ PFU/ml and in proportion in serum-free medium the virus titer was $6,7 \times 10^8$ PFU/ml. Consequently, the virus titer in serum-free medium was approximately 12% lower than the virus titer in

normal circumstances. This result does not correlate well with the reduced cell growth. Alternatively, in TCID$_{50}$ assay the difference between the titers was bigger (27%) which correlates better with the difference in cell growth (29%).
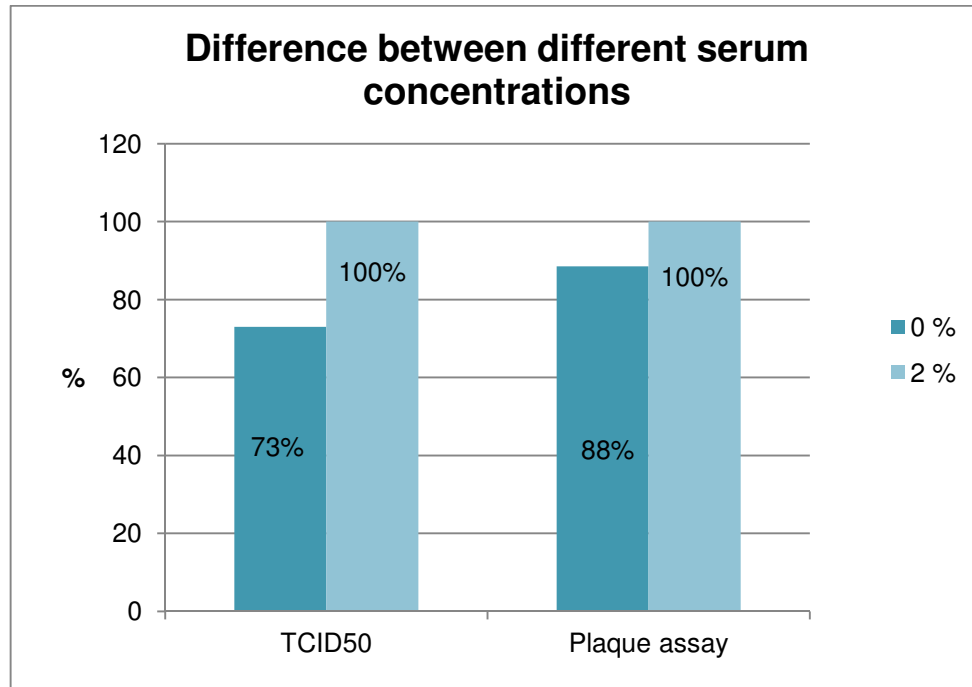

Figure 8. Comparison in virus yields between different serum concentrations by using both titering methods.

5.4   Comparison of Titering Methods for Vaccinia Virus Preparation

In this final project, one goal was to evaluate the comparability of two different titering methods. In addition to the plaque assay results, the virus titers were quantified also by TCID$_{50}$ assay. The difference in virus titers between these titering methods is shown in figure 9. The titer generated by plaque assay was an average of two plaque assay plates (n=2), while the titer generated by TCID$_{50}$ assay was an average of four 96-well plates (n=4). The virus titers generated by TCID$_{50}$ assay were higher than the titers of the plaque assay. The virus titer from normal virus preparation by plaque assay was 7.60 x 10^8 PFU/ml (CV 8.4%), where the titer generated by TCID$_{50}$ was 1.89 x 10^9 PFU/ml (CV 10.9%). In serum-free virus preparation the virus titer by plaque assay was 6.73 x 10^8 PFU/ml (CV 4.7%) and by TCID$_{50}$ it was 1.38 x 10^9 PFU/ml (CV 30.9%). Variation in titers was higher in TCID$_{50}$ assay. The difference between the virus titers from virus preparations cultured in different serum concentrations was clearer in TCID$_{50}$ than in plaque assay.
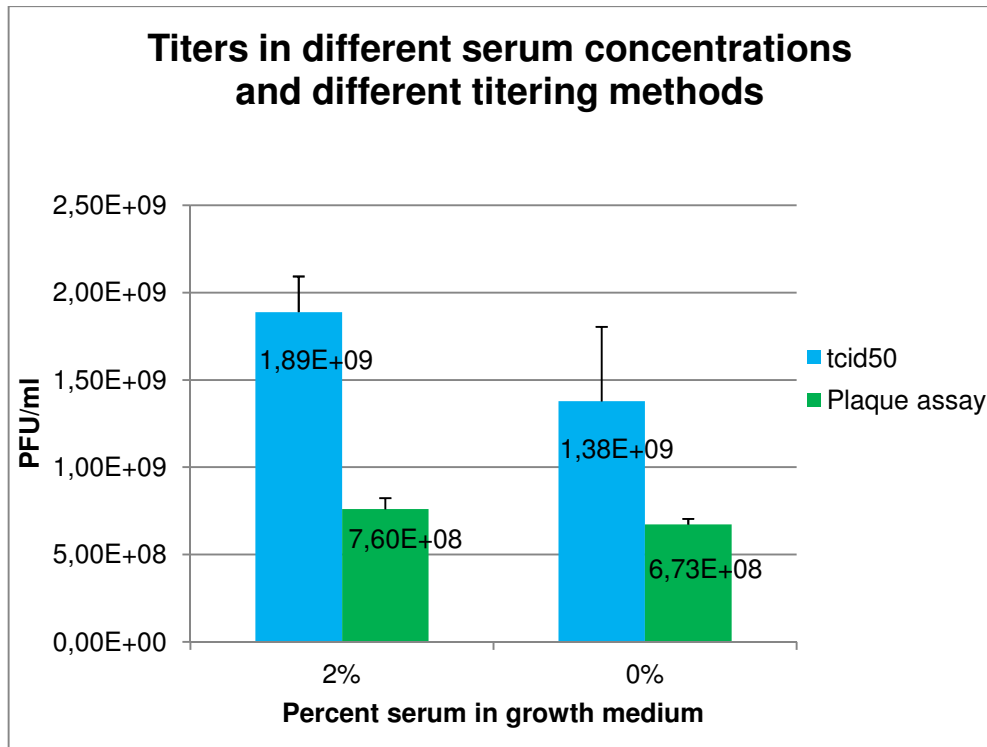
Figure 9. Comparison of virus titers in different serum concentrations and titering methods.

It was found in the studies that plaque assays yield approximately 55% smaller titers than TCID$_{50}$ assays (Figure 10).
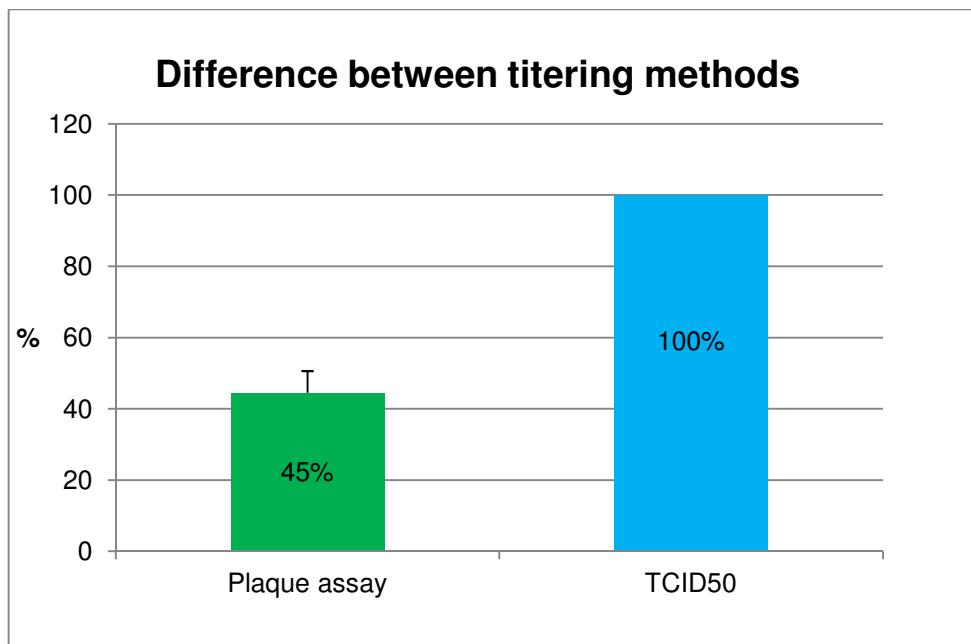

Figure 10.   Comparison of virus titers between plaque and TCID$_{50}$ assay.

5.5 Reliability of Results

Notable is that this large scale vaccinia virus preparation was the operator's first one which affected the working speed and may have a slight effect on the virus titer. Nevertheless, the virus preparation process was successful. High quality virus preparation requires experience. The virus was positive for GFP which was indicated reliably with fluorescence microscope. The virus preparation process and all assays were carried out according to instructions and with high accuracy. In addition, both tittering assays were carried out twice in parallel to ensure the validity of the virus titers. Therefore, the results can be considered reliable. However, the first plaque assay plates were impossible to read, so data from plaque assays was smaller. Because of the limited material, the results are only suggestive. More data and material are needed for reliable statistical analysis, for evaluating the significance of the results and for reliable conclusions.

6 Discussion

6.1 Ethical Speculation

Medicine has developed from people´s urge to understand the mechanisms of diseases and to be able to avoid and cure them (Karttunen – Soini – Vuopala 2005: 12). An international group of Hastings Centers project has defined four goals of medicine: "The prevention of disease and injury and the promotion and maintenance of health; The relief of pain and suffering caused by maladies; The care and cure of those with a malady, and the care on those who cannot be cured; The avoidance of premature death and the pursuit of a peaceful death" (The Goals of Medicine: Setting New Priorities 1996: 9-14). Some diseases, like malignant cancers, cannot usually be cured by conventional therapies. Therefore, novel therapies are sorely needed. To be able to response to these goals of medicine, it is essential to discover and constantly develop novel and more efficient treatments and approaches to care and cure patients. (Rajecki et al. 2008: 3085, 3091.)

Research work, especially cancer research, requires use of animal models. Animal testing is sensitive issue which divides views universally and strikes a chord with a lot of people. The issue is nevertheless two-sided; novel therapies are desperately needed, but animal models are often difficult to replace. Recently, a concept of 3R (re-

placement, reduction, refinement) has fortunately become well-known. These three Rs refer to the basics of a humane laboratory animal research: replacement of animal models and reduction of number and pain of test animals. Possibly, by changing the culture in science, it is possible to increase the use of alternative methods. (Hirsijärvi – Mäkiniemi – Saunaoja – Siitonen 2005: 103, 106, 113-114.)

The use of test animals for experimental purposes is defined in the Finnish act on the use of animals for experimental purposes (Laki koe-eläintoiminnasta 62/2006). The use of animals for experimental purposes is allowed only with the permission of the State Provincial Office (experimental animal establishment authorization). Animal experiments are authorized in Finland by the national Animal Experiment Board. The objective of the Act is to ensure that the animals are used and kept for experimental purposes only for necessary and important reasons. The object is also to ensure that a minimum number of animals are used and that the use of animals causes the least possible amount of pain, suffering, distress or lasting harm to the animals. The requirements of experimental animal establishments and requirements for the personnel performing the experiments are also specified in the act. These statutes may still not be sufficient enough to ensure that alternative methods are always used when possible (Hirsijärvi et al. 2005: 112).

Serum is commonly used in cell culture medium as one constituent. In most cases, routine cell culture demands the use of animal-derived products, mainly fetal bovine serum (FBS). The estimated amount of FBS produced for the world market is approximately 500,000 liter per year, which makes at least 1 million bovine fetuses to be harvested. This number is expected to increase annually. The use and the current methods of collecting FBS raise ethical and moral concerns. In addition, scientific and technical problems are related to use of FBS in cell cultures. (Gstraunthaler 2003: 275; Jochems – van der Valk – Stafleu - Baumans 2002: 219.) The fetal blood is commonly harvested by cardiac puncture from at least 3 months old cow fetuses during the slaughter of the mother cow. The cardiac puncture is performed without anesthesia and hence the fetuses may possibly suffer. (Jochems et al. 2002: 222-223.) In addition to these ethical reasons, the use of animal serum in cell cultures has a number of disadvantages; serum is an ill-defined medium supplement, there exist quantitative and qualitative variation and it can contain adverse factors and microbial contaminants (Gstraunthaler 2003: 277). Especially, when virus is produced for use in humans, the major concerns are the contaminants. Special standard in the production of FBS, called

Good Manufacturing Practice (GMP), needs then to be fulfilled, which increases the price significantly. In addition, prions can not to be tested for and therefore the FBS must come from prion-free countries. (Guse 2012.) For all of these reasons above, FBS should be replaced or reduce the use of it in cell culture.

Issues around the use of test animals are complicated and there is not one right approach. Everybody has to decide for themselves what their priority is. Values, morals and opinions have influence over the decision of an individual. In my opinion, it is important to develop alternative methods for animal models and use laboratory animals only for medical purposes and only when it is essential for people's health. However, the use of animals for medical and scientific purposes cannot probably ever be completely replaced.

The history of gene therapy treatment is relatively short compared with other modes of treatment. However, during the past few years various studies have been published about the efficiency of gene therapy in practice. Some of the results have been positive. (Rajecki et al. 2008: 3085). Experimental treatments, like gene therapy, may be suitable for patients with terminal cancer which has spread in spite of traditional treatments. The patient needs to be in good condition and understand the possible risks related to gene therapy. Giving the virus treatment to patients in bad conditions is generally avoided, because in those cases the treatment can be more harmful than helpful to the patient. (Rajecki et al. 2008: 3089-3090.) Some adverse events are related to the virus treatments; such as transient fever, malaise, tiredness, skin reactions and pain at the injection site. (Guse et. al. 2011: 603; Rajecki et al. 2008: 3090).

To be able to increase the safety of therapeutic viral agent, the replication of the virus needs to be tumor-selective. That can be achieved with many mechanistic approaches, including the use of viruses with inherent tumor selectivity, gene deletions, engineering tumor specific promoters into viruses and modification of the viral coat. Virotherapy agents raise unique biosafety and risk management issues. The risks must be assessed not only from the perspective of the treated patient but also from the perspective of the patient's contacts and the general public. Good Laboratory Practice and all precautions are needed when working with viral agents. Virotherapy agents may be less toxic than standard therapeutics, but there will always be a risk of toxicity during clinical testing. (Vile – Ando – Kirn 2002: 1063-1064.)

In gene therapy only somatic cells may be handled. Hence, the treatment does not have an influence to the genotype of the progeny. However, gene therapy can cause unintentional side effects. (Räikkä – Rossi 2002: 63.) The introduction of new treatments is normally based on large randomized studies. Although the safety and the efficiency of some cancer treatments being studied, it is still hard to predict whether the patient benefit from it. (Rajecki et al. 2008: 3091.) The objective of the Finnish gene technology Act is to promote the safe use and development of gene technology in accordance with the precautionary principle and in a way that is ethically acceptable. A further objective is also to protect human and animal health and the environment when carrying out the contained use or deliberate release into the environment of genetically modified organisms (Geenitekniikkalaki 377/1995).

Some safety concerns are related to oncolytic vaccinia virus treatments. Vaccinia viruses have not yet been tested as gene therapy agents in large populations to determine reliably the occurrence of adverse events. However, vaccinia viruses have generally shown only mild toxicity in clinical cancer trials. The characters of the adverse events depend on the strain of the used. Strains with high oncolytic potential are likely to have more serious side effects. One safety concern is also the contagiousness of the virus. Several cases are reported where recently vaccinated subjects have transmitted a vaccinia virus infection to others. Most of these have occurred in hospitals and in almost all cases the individual has been naive to vaccinia virus. However, this contagiousness refers to strains that were/are used in smallpox vaccination purposes. (Guse et al. 2011: 603.). To be able to increase the safety of therapeutic viral agent, the replication of the virus needs to be tumor-selective. Oncolytic viruses are genetically modified and they do not replicate in normal cells (Rajecki et al. 2008: 3090).

Gene therapy holds great promise for effective therapies for cancer. However, the proof of efficacy and safety still need to be provided by randomized clinical trials. (Guse et al. 2011: 603.) It is also important to avoid raising the hopes of patients too high as this may result in disappointment. Furthermore, gene therapy needs to be available for all patients so that inequality in society is avoided (Räikkä – Rossi 2002: 66). Most importantly, it is essential that the treatments cause more advantages than disadvantages to the patients.

6.2   Reflection

This final project was a brief subproject which related to one more extensive on-going project of CGTG. As a product of this project I was able to carry out two successful vvD-GFP preparations without any major problems or surprises. These virus preparations will play a role in an on-going "Safety and efficacy of vvd-TRAS virus pre-clinically" -animal experiment. In that experiment, the vvD-GFP viruses from the preparations of this project will function as a negative control to vvd-TRAS viruses. In this way, although this final project was rather brief, it will have a small, but still notable role in the research work of CGTG.

Along with this project, I updated the work instructions of VV preparation process and made some supplements into them. I compiled the work instructions from biomedical laboratory technologist's view, and built them so that person with only a little experience is able to follow them. I hope that these instructions are helpful in orientation of a new staff member or a student, and can be used as a note by current staff. Because this VV preparation process was new to me, I think that I was able to compile practical and illustrative work instructions. This work instruction process was extremely instructive and advantageous, because while learning new techniques and methods, I had to explore and understand them more deeply. In addition, this process developed my skills in information retrieval and gave me confidence to work more independently.

Some different approaches were tested for studying the cytotoxity of serum-free medium on A549 cell cultures. First, I tried to inoculate the cells to the 96-well culture plates both in serum-free medium and in medium with 10% FBS, but soon I noticed that the cells in serum-free medium started to suffer and died quickly. It seemed that the cells did not attach properly to the bottom of the wells in serum-free medium and therefore it was not possible to carry out the cell viability assay. Thus, the approach needed to be changed. It was more successful to plate the cells first in medium with 10% FBS and then change the medium on the next day and start the three days lasting cell incubation in different serum concentrations. The cell viability of A549 cells was measured on each day by MTS assay.

According to the results from cell viability measurements, it was possible to find out that serum-free growth medium was more disadvantageous to the cells than medium with 10% serum. However, VV preparation was possible to carry out successfully in serum-

free culture, but the virus titer was lower than in culture with 2% serum. Therefore, it is possible to carry out VV preparations in serum-free cell cultures, if slightly lower virus titer is acceptable. On the other hand, serum-free cell culture has a number of other advantages. To be able to see the truthful influence of serum-free culture to VV titer and make reliable conclusions, more serum-free VV preparations need to be carried out. In addition, new cell viability measurement should be carried out with medium containing 2% serum, because the infection of VV is normally carried out in such serum concentration. In that way, the cell growth and the virus yield could be more in proportion with each other.

Finally, based on the titer results of this project; plaque assays yielded lower virus titers than $TCID_{50}$ assays. Because both tittering assays were made twice, it was noticed that at each time both methods gave similar results. However, the level of the virus titer was different between these two methods. According to the project's results, $TCID_{50}$ assay gave more than 50% higher titers than common plaque assay. Hence, only one method can be in use in the process at the same time, and the titers must be determined using the same method, in order for them to be comparable with each other. However, to be able to compare these titering methods reliably and to be able to evaluate the statistical significance of the results, more data is needed. The results from this study are only suggestive.

This whole final project was very interesting, challenging and educational experience. I learned a variety of different techniques and methods and increased my theoretical knowledge. I had to tolerate uncertainty, find answers and ask for help, but as a reward I had experiences of success. I am very satisfied with my thirst of knowledge, with my accuracy in work and with my persistence in this process. I feel that I have received good supplies for the future. However, I would have needed more practice in my English language skills, in mathematical statistics and skills in use of MS Excel. I feel lucky and privileged that I was able to be a part of the valuable work of Cancer Gene Therapy Group and meet the people working there. I believe that I will become a qualified and enthusiastic biomedical laboratory technologist.

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Preliminary Preparation Instructions for VV

1/2

VACCINIA VIRUS   **BEFORE STARTING THE PREPARATION**

. . . .

Prepare the material below or make sure that they are available before starting the virus preparation!

- **Growth medium**(GM) for A549 cells and Vero cells:

A549 for virus preparation	**Vero** for titration
DMEM 1,0 g/L glucose 500 ml	**DMEM** high glucose 4,5 g/L 500 ml
L-glutamine 5 ml (1%)	**L-glutamine** 5 ml (1%)
Penicillin/ streptomycin 5 ml (1%)	**Penicillin/ streptomycin** 5 ml (1%)
FBS (fetal bovine serum): **10%** → 50 ml for culturing **2%** → 10 ml for infection	**FBS** (fetal bovine serum): **10%** → 50 ml for culturing **2%** → 10 ml for titration

- **Tris** (Trizma base):
  - 1 mM Tris pH 9,0 (1mM = 0,001 M)
  - molecular weight: 121,14 g/mol (divide by 1000 to get g/mmol)
  - adjust the right pH with HCl or NaOH
  - → autoclave

- **Sucrose**
  - 36 %
  - in Tris
  - → sterile filter (sucrose decrades in autoclave)

- **Bactoagar**
  - 1,3 % in water
  - → autoclave

- **PBS**
  - 500 ml mix

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- **Trypsin 1x**
  - dilute 10/10 trypsin to 1/10 trypsin
  - take two bottles of PBS and take 50 ml out of each
  - take one bottle of 10x trypsin (100 ml) and divide it to two PBS bottles (50 ml each)
  - aliquot 1x trypsin to Falcons (50 ml) and store in freezer (write the LOT number to the tubes)

Take the cells for subculture from the freezer. Do this soon enough that you have time to passage the cells 3-4 times before infection.

- **Cells from freezer:**
  - put 10 % GM into one flask (ca. 24 ml)
  - take the cells from the freezer (-140 °C) and thaw quickly in 37 °C water bath
  - add 1 ml of cell suspension to the flask
  - incubate 24 hours and change medium
  - passage when the cells are confluent

- **Harvesting cells:**
  - remove medium from the flask with suction and pasteur pipette
  - put 5-6 ml PBS into the flask and rinse the cells → remove PBS with suction
  - add 5 ml trypsin to the flask and incubate at 37 °C for 6 minutes
  - tap the flask and check with the microscope that cells are detached (incubate longer at 37°C if cells are not detached yet)
  - add 5 ml 10% GM to the flask (to inactivate trypsin)
  - rinse carefully the bottom of the flask with cell suspension and pipette to release all cells and to make the suspension homologous
  - add 5 ml of cell suspension to one or more flask containing ca. 20 ml of 10% GM
  - incubate the flasks at 37 °C with 5% CO_2 atmosphere, check the cells daily with microscope

For large scale vaccinia virus preparation, prepare ten 100% confluent T-175 flasks with A549 cells.

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Large Scale VV Preparation Instructions

1/2

VACCINIA VIRUS   **LARGE SCALE PREPARATION**

· ·

- prepare ten 100% confluent T-175 flasks with A549 cells

INFECTION

- infect cells with vaccinia virus at MOI 0.1
  - put virus in 5 ml of 2% GM per flask on the cells → incubate 1 hour at 37 °C
  - add 25 ml 2% GM
    - incubate circa 3 days at 37 °C with 5% CO_2 until extensive CPE is visible

How to calculate the volume of virus for infection?

- MOI = multiplicity of infection; ratio of infectious agents (virus) titer to number of infection targets (cell)
- 1 MOI = 1 infectious virus/ cell
- 0.1 MOI = 0.1 infectious virus/ cell
  - one T-175 flask of A549 cells contains ca. 10 million cells → 10^7 x 0.1 (01 MOI) = 10^6
  - 0.1 MOI = 1 million infectious viruses

- e.g. the titer of virus for infection: 5,5 x 10^8 PFU/ml → 5,5 x 10^5 PFU/µl (divide by 1000)
  - (1 x 10^6 PFU / 5.5 x 10^5 PFU/ µl) = 1.8 µl
  - ten flasks → (10 x 1.8 µl =) 18 µl of virus + 50 ml 2% GM

AFTER VISIBLE CPE

- scrape cells from the flasks and put the suspension in 50 ml falcons (5-6 falcons)
- spin down 1000 rpm for 10 minutes at 4°C, discard supernatant
- resuspend pelleted cells with Tris, total volume (all tubes together) 30 ml
- freeze thaw three time (-80°C), vortex after every thaw
  - or store at -80°C and continue with freeze thaw when possible

PURIFICATION

- after third thaw, centrifuge 3000 rpm for 10 minutes at 4 °C (to pellet the cell debris)
- collect and save the supernatant, put on ice (virus is in the supernatant)
- add 10 ml Tris to cell pellet, vortex, and centrifuge again 3000 rpm for 10 minutes at 4 °C
- collect supernatant and combine with earlier collected supernatant (total volume 40 ml)

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- **Ultracentrifuging**: two ultracentrifuge tubes are needed
- put 10 ml sucrose (36%) in each ultracentrifuge tube
- layer 20 ml of viral sample on top of the sucrose cushion in each tube
  - be careful, do not disturb the interface between sample and cushion
  - pipette slowly along the inner side of the tube
  - balance the tubes accurately!
- ultracentrifuge 20,000 g (12K rpm) for 90 minutes at 4 °C
  - Beckman SW28.1 rotor; swinging bucket

- prepare Eppendorf tubes and tags for virus storage
  - write on tag: name of virus, date, your initials
- aspirate/ pour immediately all supernatant being careful not to disturb the pellet
- resuspend each viral pellet in 1 ml Tris
- combine both suspensions
- aliquot the viral suspension to **10 µl**, **20 µl**, 50 µl and 100 µl in Eppendorf tubes and store at -80 °C

Balance of ultracentrifuge tube buckets:
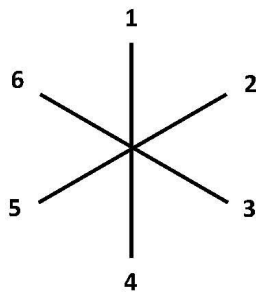
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Plaque Assay instruction

VACCINIA VIRUS   **TITRATION: Plaque Assay**

. . . .

1st DAY: SEEDING

- plate two 6-well plates with Vero cells
  - trypsinize Vero cells and count the cells with a hemocytometer
  - make 26 ml of suspension having 1 000 000 cells in 2 ml of 10% DMEM (altogether 13 million cells in 26 ml)
  - pipette 2 ml of cell suspension into each well on 6-well (1 million cells / well)
- incubate the plates 24 hours at 37 °C in 5% CO_2 atmosphere

2nd DAY: INFECTION

- make the virus dilution series in 2.5 ml total volume 2% DMEM:
  - first dilution: put 5 µl virus in 495 µl 0% DMEM → vortex vigorously

Dilution	Virus/ prev.dilution	2% DMEM
10^{-2}	5 µl	495 µl (0%)
10^{-3}	50 µl	450 µl
10^{-4}	50 µl	450 µl
10^{-5}	250 µl	2,25 ml
10^{-6}	250 µl	2,25 ml
10^{-7}	250 µl	2.25 ml
10^{-8}	250 µl	2.25 ml
10^{-9}	250 µl	2.25 ml

- aspirate medium from the 6-well plates and put 1 ml of each virus dilution to one well (10^{-5}-10^{-9})
  - put also 1 ml of 2% DMEM to one well as **negative control** (mock), work quickly to ensure that the cells are not drying out while they are without medium
- incubate 1 hour at 37 °C in 5% CO_2 atmosphere
  e.g.
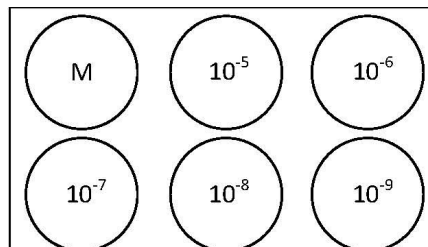
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- **Agarose overlay:**
    - prepare and warm up 2xDMEM (douple the ingredients of 10% GM to 2xDMEM)
        - 50 ml is enough for 2 plates
    - melt bactoagar (1,3%) carefully in microwawe (don´t let it boil!)

    - aspirate the virus solution from wells
    - add carefully 2 ml of agarose to each well (bactoagar and 2xDMEM in proportion 1:1)
        - aliquot 5 ml of 2xDMEM in 15 ml falcons
        - pipette 5 ml of bactoagar and suspend it with 5 ml of 2xDMEM
            - 10 ml of agarose is enough for 4 wells
    - let the plate stand in the hood to cool down and solidify (ca. 15 min)
    - incubate at 37 °C in CO_2 atmosphere for 2-3 days

WHEN PLAQUES ARE VISIBLE

Fixation and staining

- cover the agarose in each well with methanol-acetic acid fixative (ca. 1 ml)
    - incubate at room temperature for 1 hour
- rinse the agarose with water, so that the gel detaches (remember to use a colander!)
- put 0,5 ml Coomassie® staining solution per well (so that the bottom covers)
    - incubate for at least 1 hour
- rinse with water, leave the plates to dry

Calculation

- count the plaques
    - count the plaques from wells that have ca. 100 plaques
    - count the next higher diluted well and take the average (transform in same dilution!)
    - take the average from both plates
    - calculate PFU/ml

e.g.

1. 84×10^7 $\rightarrow 8.4 \times 10^8$

2. 7×10^8

$(7 \times 10^8 + 8.4 \times 10^8) / 2 = 7.7 \times 10^8$ $= 7.7 \times 10^8$ PFU/ml

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RECIPES

Methanol-acetic acid fixative (3:1)

- 3 parts methanol   e.g. 375 ml
- 1 parts acetic acid   e.g. 125 ml

Coomassie® staining solution

- 250 ml of 40 % methanol   e.g. 100 ml methanol + 150 ml mQ H_2O
- 250 ml of 20 % acetic acid   e.g. 50 ml acetic acid + 200 ml mQ H_2O
- 0,5 g Coomassie Briliant Blue
    - BIO-RAD: Coomassie Brilliant Blue R-250/ G-250

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TCID$_{50}$ Instruction

1/2

VACCINIA VIRUS   **TITRATION: TCID$_{50}$**

▪ ▪

1st DAY: SEEDING

- plate two 96-well plates with Vero cells
  - trypsinize Vero cells and count the cells with a hemocytometer
  - make 25 ml of suspension having 100,000 cells in 1 ml of 2% DMEM (altogether 2.5 million cells in 25 ml)
  - pipette 100 µl of cell suspension into each well on 96-well plate using multichannel pipette (10,000 cell / well)
- incubate the plates 24 hour at 37 °C in 5% CO$_2$ atmosphere

2nd DAY: INFECTION

- make the virus dilutions for infection (two plates):

Dilution	Volume	Virus/ prev.dilution	2% DMEM
10^{-1}	1x	10 µl	990 µl
10^{-2}	1x	200 µl	1,8 ml
10^{-3}	1x	200 µl	1.8 ml
10^{-4}	1x	200 µl	1.8 ml
10^{-5}	2x	400 µl	3.6 ml
10^{-6}	2x	400 µl	3.6 ml
10^{-7}	2x	400 µl	3.6 ml
10^{-8}	2x	400 µl	3.6 ml
10^{-9}	2x	400 µl	3.6 ml
10^{-10}	2x	400 µl	3.6 ml
10^{-11}	2x	400 µl	3.6 ml
10^{-12}	2x	400 µl	3.6 ml

- pipette 100 µl of virus dilution to each well so that each column has all dilutions (10^{-5} - 10^{-12})
  - add the dilutions so that the lowest dilution (10^{-5}) is on the lowest row and the strongest dilution (10^{-12}) on the highest row
  - **use the two last columns for negative control** (mock): do not add virus dilution into these wells but plain medium

- incubate the plates for **10 days** at 37 °C in 5% CO$_2$ atmosphere before reading the results
  - check the cells several times during the incubation period by microscope

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Reading the TCID$_{50}$ results

- after 10 days, observe the plates with microscope, check every well for CPE
- a well is counted positive if only a small spot or even few cells show CPE, otherwise the well is negative
- determine the ratio of positive wells per row

Test is valid if:
- negative controls do not show CPE
- lowest dilution shows 100% CPE (10/10)
- highest dilution shows 0% CPE (0/10)

e.g.

		1	2	3	4	5	6	7	8	9	10	11	12	Ratio:
10^{-12}	A											M	M	= 0
10^{-11}	B											M	M	= 0
10^{-10}	C		CPE									M	M	= 0.1
10^{-9}	D			CPE	CPE	CPE			CPE			M	M	= 0.4
10^{-8}	E	CPE	CPE	CPE	CPE	CPE	CPE	CPE	CPE	CPE	CPE	M	M	= 1
10^{-7}	F	CPE	CPE	CPE	CPE	CPE	CPE	CPE	CPE	CPE	CPE	M	M	= 1
10^{-6}	G	CPE	CPE	CPE	CPE	CPE	CPE	CPE	CPE	CPE	CPE	M	M	= 1
10^{-5}	H	CPE	CPE	CPE	CPE	CPE	CPE	CPE	CPE	CPE	CPE	M	M	= 1

- sum the ratios
  - start always from the **first** dilution (10^{-1})
  - e.g.   S= 1+1+1+1+1+1+1+1+0,4+0,1 = 8,5

- calculate the titer: T= $10^{1+1(S-0.5)}$ (for 100 µl dilution!)

  - e.g. T= $10^{1+1(8.5-0.5)}$ = 10^{9} TCID$_{50}$/ 100 µl

- transform TCID$_{50}$ / 100 µl in PFU/ml
  - 10^{9} TCID$_{50}$ → 10^{10} TCID$_{50}$/ml (multiply by 10)
  - subtract 0.7 from the log → $10^{10-0.7}$ = $10^{9.3}$ PFU/ml
  - $10^{9.3}$ ≈ 2 x 10^{9} PFU/ml

- repeat the same calculations for the second plate and take the average

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