INTEGRATED VIRUS DISCOVERY, ISOLATION AND VACCINE PRODUCTION SYSTEM: BIG SCIENCE IN AN AGE OF GLOBAL PANDEMICS

Plenty of COVID-19 Virus Is Available Now: Why Not a Killed Vaccine?

By Norman G. Anderson

Viral Defense Foundation

With a short introduction[1] by N. Leigh Anderson and Elizabeth A. Anderson

Quantities of COVID-19 virus sufficient to prepare thousands of doses of a first-generation killed vaccine could be rapidly isolated from lungs of disease victims using existing large-scale virus isolation technology (ultracentrifugation). Such a killed vaccine, producible within weeks using classical methods of virus inactivation, would allow vaccination of at least some healthcare and other essential service personnel. Just as the blood of recovered patient can contribute antibodies for the treatment of others, so there is a case in the present emergency for using virus harvested from the corpses of victims to inoculate those in greatest danger.

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Plenty of COVID-19 Virus Is Available Now: Why Not a Killed Vaccine?

A short introduction[2] to notes developed by Norman G. Anderson circa 2016 regarding an "INTEGRATED VIRUS DISCOVERY, ISOLATION AND VACCINE PRODUCTION SYSTEM: BIG SCIENCE IN AN AGE OF GLOBAL PANDEMICS"

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It is not widely appreciated that the physical quantity of virus in a dose of current commercial killed vaccines is very small: on the order of 15-50 micrograms. Combining published measurements of COVID-2 genome copies in the lungs of sub-lethally infected rhesus monkeys (~10^8 copies/g; [1]) and an approximate virus mass of 10^8 daltons, one can estimate the viral content of a pair of sub-lethally-infected human lungs as >20ug of virus, with a much higher yield likely in lethal-infected cases. Thus in a conservative case, each fatality from COVID-19 could provide killed vaccine to inoculate one or more persons.

METHOD SUMMARY

The proposed approach is not specific to COVID-19 and relies on technologies and methods with a long history in the viral vaccine field. The following steps would be required:

1. **Virus acquisition**. Tissue and fluid samples derived from deceased patients containing virus are harvested by medical staff and inactivated by ionizing radiation to make all subsequent operations safe for transport and subsequent processes.

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2. **Virus isolation**. Virus is purified from infected tissue homogenates by high-resolution two-dimensional zonal centrifugation (S-rho) using existing K-II (CC40) ultracentrifuges currently used to purify the influenza virus in annual vaccines.

3. **Vaccine preparation**. Limited quantities of a killed vaccine are rapidly produced using established methods of virus inactivation for vaccine use (e.g., radiation or formalin).

4. **Human Trials**. There are obvious ethical concerns with the administration of a novel vaccine to healthy subjects, given the untested efficacy, potential for harm (e.g., by super-sensitization), and limited availability of such a pilot vaccine. However, in the absence of effective alternatives, the approach outlined here could buy time for alternative killed virus sources and alternative vaccine approaches to be developed.

5. **Large scale production**. Given time, large-scale culture of the virus (analogous to production of influenza virus in chicken eggs) can be accomplished, and the methods used above can be scaled up to vaccinate large populations.

TECHNOLOGY BACKGROUND

The technologies for virus discovery, isolation, and vaccine preparation described here originated in the multi-laboratory facilities of the Manhattan project at Oak Ridge TN.

In the mid 60's, a Joint NIH-AEC Zonal Centrifuge Development Program led by Dr. Norman G. Anderson adapted technology from the classified gas centrifuge project at Oak Ridge (for uranium enrichment) for purposes of virus isolation. The culmination of this project, the very-large-scale K-II continuous-flow zonal ultracentrifuge, was tested initially through collaboration with Dr. Charles Reimer of Eli Lilly & Co. on a commercial influenza vaccine. The resulting purification of flu virus from egg protein contaminants has eliminated the many deaths previously occurring each year from anaphylactic reactions to flu shots.

Current versions of the K-II centrifuge are manufactured by Hitachi-Koki in Japan (as the CC40), and by the Italian firm of AlfaWassermann (as the K-II). Several hundred machines have been produced, and most are still in use processing ~100 liters of virus source material per day.

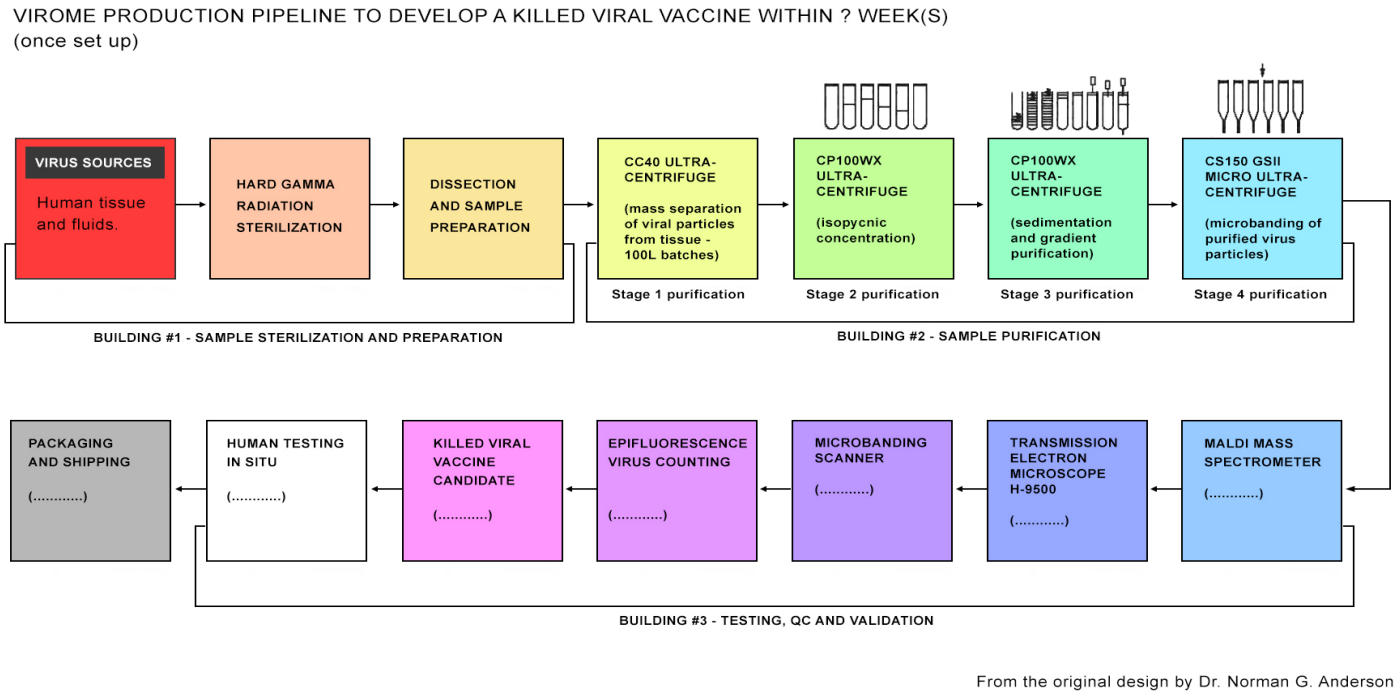

Figure 1. Diagrammatic Outline of Virome Project Laboratory.

1. Munster VJ, Feldmann F, Williamson BN, *et al.* Respiratory disease and virus shedding in rhesus macaques inoculated with SARS-CoV-2 [Internet]. Microbiology. Available from: http://biorxiv.org/lookup/doi/10.1101/2020.03.21.001628.

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INTEGRATED VIRUS DISCOVERY, ISOLATION AND VACCINE PRODUCTION SYSTEM: BIG SCIENCE IN AN AGE OF GLOBAL PANDEMICS

Norman G. Anderson

Abstract

The greatest single threat to the future of mankind is the emergence of a new or highly modified, lethal, and rapidly spreading virus for which we have no vaccine or effective antiviral drug. Such a virus can be provided by nature, or can now be synthesized in the laboratory. With modern transportation, such a virus can spread world-wide in as little as a month. Authoritative estimates for the time required to make and distribute an effective vaccine against a new virus vary from 10 to 20 years. There is, therefore, no existing credible, effective, deployable and tested defense system, or, until this document, a detailed plan for providing one. Unlike all other defense systems, there is no "proving ground" where start-to-finish performance is evaluated.

Initially we review the background of this project. We then ask whether the threat is real. Thirdly, we review briefly the status of present biodefense efforts. Fourth, we present a system and plan for an effective, detailed real-time defense based on properties that are common to all viruses. Fifth, we propose how this plan can be implemented now.

Many different biodefense plans are possible. What is essential is to have at least one that is presented in sufficient detail to be immediately implemented.

This is a defense project, keyed to the maximum credible threat, with all operations carried out at the maximum possible speed in one place. The unit of operation is a complete integrated laboratory that is in constant operation and always in a high state of readiness.

1. Background

The technology for virus discovery, isolation, and vaccine preparation described here originated in laboratories of the Manhattan project at Oak Ridge.

One of the main technologies explored in the Manhattan Project was to separate uranium isotopes by preparative mass spectrometry. Over a thousand giant Calutrons were assembled operated by several thousand people. After WWII this facility was used to go straight through the atomic table to separate and characterize all of the stable isotopes. What had been "pure" research was "industrialized" on a large scale: This gave the author the idea that a plant could be built to systematically isolate all of the components of human cells. The Manhattan Project was based on the development and scaling up of separations technologies including, among others, ion exchange, diffusion, centrifugation, crystallization, and mass spectrometry. A basic factor in the entire project was time. The original objective was to win WWII. Almost no pilot plants were built,

rather giant facilities, complete with all the required supporting facilities, were assembled even if these involved extensive duplication.

The staff of these facilities discovered that crash projects were often more efficient that conventional approaches. If this approach were to be taken in the biomedical sciences some urgent objective is required beside the survival of wartime facilities, and one was found.

Many types of animal cancer had been found to be caused by viruses that could be isolated and used to make vaccines that could prevent these cancers from forming. However, despite extensive effort, no major human cancer virus had been found using classical virus discovery methods. I proposed that major human cancer virus might exist, but simply did not grow under laboratory conditions. I proposed that purely biophysical search and isolation methods might solve this problem.

The same technology is here shown to be directly applicable to the central problem of biodefense, which is to rapidly isolate and sequence any and all new pandemic viral agents that might appear, and to make and distribute a protective vaccines before a pandemic has reached its peak. i.e., to solve the central problem of current biosecurity.

The early project was the Joint NIH-AEC Zonal Centrifuge Development Program of the 1960s. The project grew to include other NIH institutes (NIGMS and NIAID) and NASA. Much of the work was done in close collaboration with the gas centrifuge project of the Separations Systems Division of the Oak Ridge Gaseous Diffusion Plant.

Technically the joint program was hugely successful. The zonal centrifuge, the centrifugal fast clinical analyzer, and two-dimensional continuous-sample-flow-with-banding centrifugation for large-scale vaccine purification were invented, and were reduced to practice and commercial production. The problems of finding unculturable human viral pathogens was therefor solved, as was the problem of purifying viruses in large commercial quantities. Unfortunately, and contrary to all expectations, no viruses causing any major human cancers were found, despite an intense effort. The major force behind the project thus disappeared, although the technology succeeded.

The joint project was part of the Molecular Anatomy Program which was set up a laboratory in the K-703 Building (the K-25 power house) in the K-25 area of the Oak Ridge reservation.

The most technically difficult part of the program was large-scale vaccine purification. It was difficult to design and develop a centrifuge for doing this in the absence of any data on a cancer virus or other viruses to work with. Work with small prototype centrifuges was not promising. I decided to find a vaccine actually in production and design a full-scale system around it. This was in keeping with the Manhattan Project philosophy of skipping from early bench scale or even an idea itself, to full-scale production.

Dr. Charles Reimer from Eli Lilly & Co. suggested that we tackle the problem of purifying commercial influenza vaccine as a model problem. The batch size was 100 liters which should be processed in one working day. I set the design which involved flowing the vaccine in a 1 mm thick sheet over an immobilized liquid density gradient only 10 mm deep, all in a large tubular rotor. The virus was expected to sediment out of the stream and band isopycnically i.e., be captured at their density levels, in the gradient.

This was to happen in a rotor spinning at up to 41,000 rpm in a high vacuum inside heavy HY80 naval turret armor with refrigeration to control temperature. Seals were required to flow liquids in and out at speed without burning up. When all was done, and the virus was captured as a layer less than 1 mm thick in a spinning gradient, which must then be recovered at rest without loss of resolution. The portion of the project was funded partly by the vaccine development board of NIAID who was interested in eliminating the many reactions and deaths that occurred each year from flu shots, and which had required that they be given under close supervision in case of anaphylactic shock due to egg protein contaminants.

Most of this joint program escaped any detailed review. An exception occurred here and the board concluded that the system could never work and withdrew funding. NCI however agreed to continue, the centrifuge was made and tested at Oak Ridge, tested and installed at Lilly, and was a complete success, as subsequently described in this paper. It is manufactured by Hitachi-Koki in Japan as the CC40, and the Italian firm of AlfaWassermann as the K-II. It has been used world wide with little change in rotor design, since its inception, although there are many that should be made. I can find no record of the incidence of vaccine deaths prior to its introduction so no estimates of the number of lives saved since 1968 can be made now. This machine largely accounts for the fact that flu shots can now be given in supermarkets. The majority of all virus ever isolated on earth has gone through these machines.

The interval from my conception of the K-II to the announcement by Lilly that it was safe and effective, and the decision by the AEC to make it immediately available to members of congress was a little more than four years.

This S-rho two-dimensional technology was initially demonstrated using bacteriophage as test viruses, and animal tissues. In studies on human blood a virus particle and an unusual elongated particle were discovered in a patient who had hepatitis and the results published. This work was reported to the Assistant Director of NIAID who indicated that these were not hepatitis related because an extensive search at Yale had indicate that there were no virus particles in blood associated with hepatis B. Blumberg found these particles later and received a Nobel Prize in for it 1975.

Over 50 different rotor designs were constructed and tested at Oak Ridge. Two of the designs are in commercial production and use to this day.

Beyond this one case, no new viruses were found by the project during the Oak Ridge years. It should be remembered that this was before nucleic acid sequencing, and PCR.

The centrifugal fast analyzer, originally called the GeMSAEC clinical analyzer was a fluke. The National Institute of General Medical Sciences realized that clinical chemistry was largely a monopoly held by the Technicon Corporation, and held a meeting at Duke addressed to this problem to which I was invited. The central problem was to design an analyzer that was natively controlled by a computer, did multiple analyses in parallel, provided data at a high rate, and was simple and compact. The result was the GeMSAEC. It was manufactured by four different companies, and was the first analyzer produced by Roche Diagnostics. World-wide sale and tests were valued at over $400M before it was supplanted by other technologies. AEC licensed the patent abroad and royalties to the AEC, when last recorded, were $3M. (During the entire period of this project, inventors were paid $1 per patent).

Just before this project was completed at Oak Ridge, NIGMS withdrew support. The term GeMSAEC was preserved as a cautionary tale.

Five things terminated the Molecular Anatomy Program at Oak Ridge. The first was the failure of the cancer virus concept. The second was the completion and commercialization of the K-II, GeMSAEC, and zonal centrifuge programs as initially conceived. And the third was a company-wide management decision by Union Carbide Corporation, who managed Oak Ridge at the time, to eliminate all satellite laboratories. A number of "off site" and unauthorized, and otherwise unknown to the board enterprises had been discovered by top management, and had been summarily order to close.

Among these was a satellite laboratory funded by NIAID to transfer technology from Oak Ridge directly to NIAID programs. I set this up and operated it from Oak Ridge, and it was under the local direction of Dr. John Gerin, an Oak Ridge employee. After its termination by Carbide, it was transferred to Georgetown University and lost all contact with Oak Ridge. In the mid 90s a decision was made by NIAID to gradually terminate this project, and it was closed in 2007.

During the last four years I was allowed work there at no cost to NIAID, and provided that I bought my own supplies. When the laboratory was closed, it was found that there was insufficient space at Georgetown to store some of the equipment, and it was loaned to me to work in a laboratory in rented space set up with my son, Dr. Leigh Anderson, to pursue both of our interests, but at our own expense. Much of the ultramicro-banding work described here was done in that laboratory, which was closed on September 4, 2012. To my knowledge there does not now exist a laboratory set up to exploit S-rho technology for biodefense as described here anywhere in the world.

The last reason was the termination of the gas centrifuge project itself at Oak Ridge which ended the work on biological centrifuges associated with virus isolation, and replaced it with the Atomic Vapor Laser Isolation Project at Lawrence Livermore. Gas centrifugation in the US was privatized and has not biological interests.

The initial objective here is to describe for patent purposes a complete system which has a high probability of aborting any new lethal highly infectious outbreak before mass death occurs. All the components are to be optimized to work together to detect and stop an outbreak in the shortest possible time. It is essential that all components be physically present together, be fully and continuously operational, including the manufacture of vaccines. No trace of the original zonal centrifuge survives at Oak Ridge.

2. Is the Threat Real?

Viruses are promiscuous molecular machines. As far as can be determined all living forms are infected with them. Many species, including man, are infected with a hundred or more different viruses during a lifetime. Viruses normally cause a large fraction of all human deaths world-wide, and they can now be synthesized from scratch in the laboratory.

Viruses are everywhere. I first discovered in 1966 that the concentration of viruses in the ocean is very high (1), and is about equal to the levels observed in human blood during many viral infections .

I proposed that, since viruses can exchange genes between themselves and their hosts, the reason for their continued existence was that they mediated gene transfer

between different life forms (2), a process essential to evolution itself. This conclusion, originally considered heretical, is now accepted doctrine.

The viral load of the world's oceans is now estimated in billions of metric tons. Most marine viruses have a turnover rate of about one day, and are believed to keep the algal population of the ocean in check. Without them the oceans would be a deep messy green. Since viruses cross both species and phylum barriers, viruses constitute a gene FedEx for slowly transmitting genetic information from the astronomically large mutation engine provided by the oceans of the world, to all other life forms. And since, in a cell infected by two viruses, genetic information can be and is exchanged to yield a new viral construct, the means for making and distributing new viruses is essentially unlimited.

The results are visible to all. Within living memory, the influenza pandemic of 1918 killed between 50 and 100 million (more than perished from WW I), and HIV has appeared, spread unchecked. And has killed an estimated 30 million with 60 million infected. SARS, which had a case mortality rate of ~15%, could have killed an estimated 39 million in the United States if its infectivity had not been low, the average patient infecting only three people, as compared, for example, with ~12 for measles.

This is all made more serious by the discovery that an innocuous version of a mouse pox virus could be rendered lethal by adding to it a normal mouse gene that affects the immune system. This raises the possibility that innocuous viruses in general circulation in man could be rendered lethal by picking up a normal human gene, which they appear to do on occasion.

In general, it is accepted that the rate of appearance of new viral pathogens in a given population is proportional to population size, and to the rate of exchange of viral genes across that population. From 1800 to the present the worlds population is estimated to have grown by a factor of seven and will continue to grow. Travel to all parts of the earth is now fast and routine. People interact with, and eat an ever-larger proportion of all plants and animals on the planet. More new lethal human viral pathogens will inevitably emerge, and at a more rapid rate.

That mankind has experienced many viral infections in the past is demonstrated by the discovery that a large fraction of the entire human genome is composed of viral fragments, fossil bits left from ancient virus infections.

It is estimated that over 99.9% of all life forms that have occurred on earth are extinct. It is logical to assume that viruses were involved may have killed off many of them. Man may not be exempt from this process.

In fact, Martin Rees, former President of the Royal Society and Astronomer Royal in Britain, has wagered an even chance the human race will disappear before the end of this century, most likely from an infectious agent.

Two things make this document of crucial importance now. The first is that the collection of genes that comprise a virus can be so easily exchanged. Cut and paste virology is essential to molecular biology. All human genes have been inserted into viral vectors and transferred into living cells as an essential step in the Human Genome Project. And, as the vast majority of human viral pathogens are sequenced and can be synthesized, we now possess a vast tool box for making new human viral pathogens. Cutting and pasting these at random can now be easily done, to make thousands of different potentially lethal viruses. Injecting these into a human volunteer is a simple way

to sort out the virions that are lethal from all the rest using suicide volunteers such as are available in many parts of the world. Mutually assured destruction would appear to prevent this from being done.

Unless there was a rather simple way to make a live vaccine against the new BW virus which is now the case.

The technology for rapidly making live vaccines for their sequenced lethal counterparts derives from the fact that genes are written in a triplet code, i.e., each of the 20 amino acids which comprise a protein are coded as a set of three bases, of which there are four. These triplets are called codons.

This means that there are 64 possible codons in which the 20 amino acids could be coded. Only methionine and tryptophan have only one codon. The others have either two, four or six, except leucine which has three.

The different codons for one amino acid are called synonymous codons, because they are translated into same amino acid, but at different rates or efficiencies.

When virus infection occurs, there is a race between rate of virus multiplication, pathogenicity, and immune-mediated recovery. If a version of the virus could be made in which the steps involved in pathogenicity could be slowed down, and/or the rate at which immunity appears speeded up, then one would have a live vaccine. By systematic synonymous recoding of a viral genome, the rates at which genes are expressed can be altered to yield a "live vaccine virus" which induces an immune response before a pathological one. None of the gene products (proteins) would be altered, however.

Recoding may be done rapidly on any virus with a high probability of working providing one that has the required genomic properties. With further research additional specific recoding can be done to change not only gene expression, but gene function.

This means that, for the first time, deploying, or threatening to deploy a viral BW pathogen is conceivable. The simultaneous combination of a sword and a shield could be used either offensively or defensively. The mere fact that it is possible affects all defense planning. Regardless, the world requires a reliable defense against this possibility.

Only a complete nuclear exchange of all nuclear warheads on earth equals the in seriousness the viral threat. The major difference between the two is that a nuclear exchange can be prevented, and is not inevitable. Viral extinctions can be the product of either man or nature, may be inevitable in any cases, but could be stopped midcourse, by decisive, preplanned human intervention, as is suggested here.

Mass migrations with minimal medical attention are a new potential source of human pathogens.

The purpose of this provisional patent application is to propose, for the first time, a real-time relatively complete viral defense plan, including a complete system for rapidly isolating intact viruses from multiple samples in parallel. These including serum, plasma, urine, bile, lymph, tissues extracts and tissue culture fluids, concentrating them into pellets, and transferring these quantitatively using micro-ultracentrifuges and other devices, including electron microscopy, epifluoresence microscopy, and immune-analyses of various types to sequence, identify and characterize

Additional purposes are to identify high concentration sources of virus in patient and animal derived materials, to isolate from these sources sufficient viral mass to make killed vaccines to immunize health care workers immediately at risk, to develop and evaluate sources of high-intensity ionizing radiation to inactivate virus sources and

isolated viral masses routinely, and to use sequence data to manufacture and distribute live vaccine by reverse genetics and synonymous codon substitution.

These aims would be accomplished in complete turn-key laboratories that are commercially supplied and maintained to make viral defense a reality in any country of the world.

3. Present Status of Biodefense

The paradigms underlying present antiviral defense strategies assume that each new viral pathogen poses new and unique problems. An idiosyncratic response is therefore required that can only be implemented ***after*** a pandemic has begun. Given the speed with which a new virus can spread world wide, the target time between initial alarm and a distributed vaccine must be measured in weeks, not years. This objective cannot be met under present paradigms.

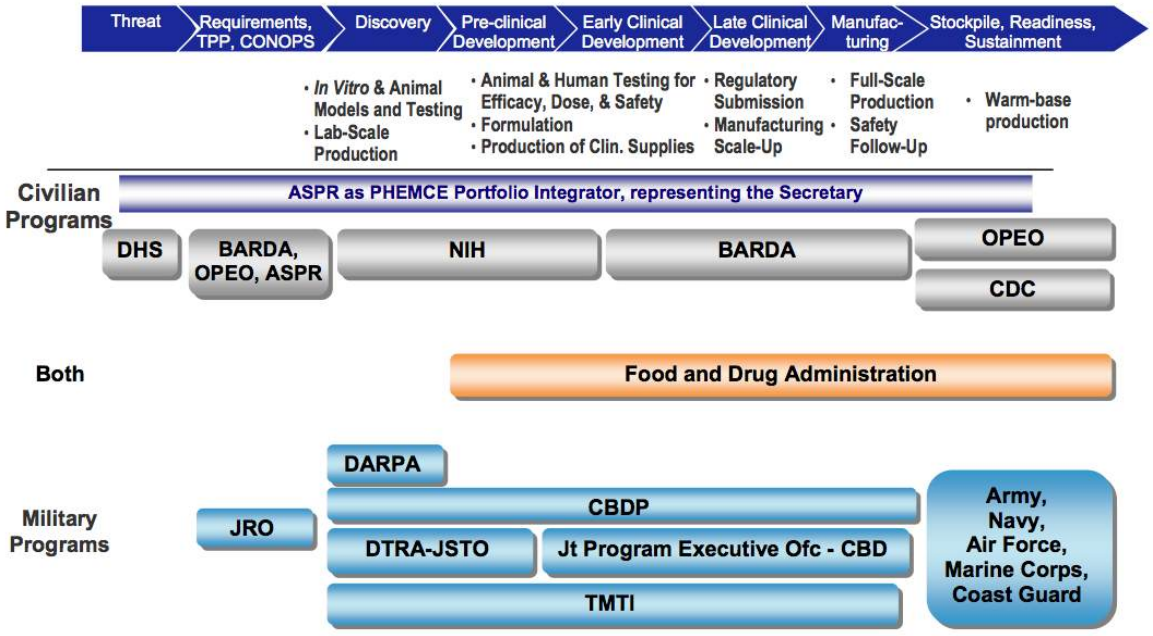
To quote from Schuler et al (3), " *Biodefense activities are spread throughout the federal government, with more than 26 biodefense positions that are appointed by the president and confirmed by the Senate located in more than a dozen government agencies and government organizations Together, these programs administered more than $5.5 billion in funding in FY2004.1 This dispersion of responsibility across multiple agencies differs from other large government national security initiatives, such as the Missile Defense Agency (MDA), for example. MDA ' s head, who is appointed by the Secretary of the Department of Defense, commanded a budget of approximately $7.7 billion in FY2004.1 The budget for the Missile Defense Agency is approved by the Undersecretary for Acquisitions, Technology, and Logistics, a presidentially appointed, Senate-confirmed position.*

The comparative diffusion of biodefense programs reflects the multifaceted nature of biodefense activities, but this organizational structure also presents particular challenges to efforts to design, implement, and oversee a coherent, coordinated, and efficient biodefense strategy."

A more recent review of biodefense, entitled ominously, "**Where Are the Countermeasures?**" published in 2010 by the National Biodefense Science Board gives the following situational assessment:

"The U.S. Government workers involved in MCM discovery, development, acquisition, and fielding are doing good and important work. But they are not synchronized, their projects are not prioritized, and oversight from the highest levels of Government is neither consistent nor evident. These inefficiencies are prolonging America's vulnerabilities".

Table 1 below from this report summarizes the entire detection-to-response process. Super imposed on this process are the nine steps in the technology readiness assessment process.



Key:

ASPR	Assistant Secretary for Preparedness and Response
BARDA	Biomedical Advanced Research Development Authority
CDC	Centers for Disease Control and Prevention
CBDP	Chemical and Biological Defense Program
CONOPS	Concept of Operations
DARPA	Defense Advanced Research Projects Agency
DHS	U.S. Department of Homeland Security
DOD	U.S. Department of Defense
JPEO-CBD	Joint Program Executive Office for Chemical/Biological Defense
JRO	Joint Research Office
JSTO	Joint Science and Technology Office
NIAID	National Institute for Allergy and Infectious Diseases
NIH	National Institutes of Health
OPEO	Office of Preparedness and Emergency Operations
PHEMCE	Public Health Emergency Medical Countermeasures Enterprise
TMTI	Transformational Medical Technologies Initiative
TPP	Target Product Profiles

Sources: U.S. Department of Health and Human Services and U.S. Department of Defense

Table 1. Phases of Medical Countermeasure Development and Federal Agencies Responsible for Activities During Those Phases.

(From: A Report of the National Biodefense Science Board entitled, "Where Are the Countermeasures? Protecting America's Health from CBRN Threats" March 2010, Page 22)

Nine major reviews of biodefense together with a short summary of each are shown in Table 1 [taken from study by Russell and Gronvall (4)].

Table 1. Selected Reports and Articles Chronicling Need for Improvement in Medical Countermeasures

Year	*Report Authors*	*Summary of Findings*
2000	Independent experts tasked by the Deputy Secretary of Defense	*Top Report: Report to the Deputy Secretary of Defense by the Independent Panel of Experts* *Summary:* Found that the DoD vaccine acquisition approach was "insufficient and will fail." Recommended that DoD consolidate its vaccine programs for bioweapons and natural disease protection; develop a distinct acquisition process to resemble best industry practice for vaccines; and hire qualified staff at all levels. Recommended forming a government-owned contractor-operated vaccine production facility.
2002	Institute of Medicine	*Protecting Our Forces: Improving Vaccine Acquisition and Availability in the U.S. Military* *Summary:* Recommended that DoD merge vaccine efforts for BW threats and for natural diseases and restructure their acquisition process to be tailored to the vaccine development process. Urged greater coordination with the civilian sector.
2004	Institute of Medicine and National Research Council of the National Academies	*Giving Full Measure to Countermeasures: Addressing Problems in the DoD Program to Develop Medical Countermeasures Against Biological Warfare Agents* *Summary:* Producing MCMs has not been given sufficient priority. Management of the effort was disjointed, and DoD leadership lacked "an adequate grasp of the commitment, time, scientific expertise, organizational structure and financial resources required for success in developing vaccines and other pharmaceutical products." Documented 10 years of failure to produce vaccines, starting from the 1990-91 Gulf War. Recommended a complete reorganization of the program, with regular external review, staffed and led by people with industry experience, consolidated into a separate DoD agency.
2010	Commission on the Prevention of Weapons of Mass Destruction Proliferation and Terrorism	*Prevention of WMD Proliferation and Terrorism Report Card, An Assessment of the U.S. Government's Progress in Protecting the United States from Weapons of Mass Destruction Proliferation and Terrorism* *Summary:* The nation received an "F" grade for failing to enhance the nation's capabilities for rapid response to prevent biological attacks from inflicting mass casualties, particularly "the lack of priority given to the development of medical countermeasures." The Chair, Senator Bob Graham, and Vice-Chair, Senator Jim Talent, believed there was inadequate expertise and investment in countermeasures, "a symptom of a failure of the U.S. government to grasp the threat of biological weapons."
2010	National Biodefense Science Board	*Optimizing Industrial Involvement in Medical Countermeasure Development* *Summary:* Identified barriers between government and industry that hamper progress, including inconsistent funding; slow, unwieldy contracting; lack of clarity about MCM requirements; and lack of coordination between federal partners. They also found that government had an inadequate understanding of how industry makes drugs and vaccines. Recommended that MCM leadership be centralized and deficiencies addressed.
2010	National Biodefense Science Board	*Where Are the Countermeasures? Protecting America's Health from CBRN Threats* *Summary:* Thought the MCM development effort required more prioritization, synchronization between government agencies, and leadership. Found that the various gov't agencies are not coordinating, and that there is no unifying strategy for developing and using CBRN MCMs. Recommended that there be increased accountability in the MCM process and a clarified regulatory process, and recommended coordination versus reorganization.
2010	Department of Health and Human Services	*The Public Health Emergency Medical Countermeasures Enterprise Review: Transforming the Enterprise to Meet Long-Range National Needs* *Summary:* Stated that the nation should prepare for known and novel threats, man-made or naturally occurring. Strategy addressed shortfalls in MCM development, which should include increased focus on regulatory science; plans to build a flexible manufacturing capability; adding early development teams to assist in translating basic research to eventual products; establishing an "MCM development leader"; setting up an Enterprise Senior Council, replacing the Enterprise Governance Board as a decision forum for MCM policy and implementation. Recommended that ASPR/BARDA increase contracting speed.

(*continued*)

Year	*Report Authors*	*Summary of Findings*
2011	Bipartisan WMD Terrorism Research Center	*Bio-Response Report Card* *Summary:* The nation received a "D" grade; there still existed a lack of clearly defined requirements for MCMs, common set of prioritized R&D goals, coordination with budget requests, and sufficient and sustained funding. Noted that external advice given to HHS in March 2010 by the National Biodefense Science Board was not taken.
2011	Government Accountability Office	*Improvements Needed for Acquiring Medical Countermeasures to Threats from Terrorism and Other Sources* *Summary:* Credited HHS for beginning to address recommendations from its internal 2010 review, but faulted them for not developing "an adequate strategy to monitor implementation" and stated that their process will not allow for measuring progress across medical countermeasure initiatives. They also pointed out that the 2007 HHS plan for countermeasure priorities has not been updated biennially as promised.

The final conclusion, however, was that between 10 and 20 years are required to make, test, and distribute a vaccine against a new virus.

All studies on biodefense run counter to experience in the Manhattan Project, the Space Program, missile defense, and military R&D, especially project management as outlined in Project Hindsight. To this point, the HHS Barda project is based on the following paradigms and assumptions:

1. Each outbreak is treated as a unique event, requiring a complex idiosyncratic response that can only be assembled after a pandemic starts.
2. This means that the prudent solution to this problem is to attempt to organize as many different laboratories, centers of excellence, companies, and agencies as possible to that every conceivable alternative is available in an emergency.
3. This requires an exponentially growing overlay of communication and management.
4. This approach cannot be tested in the field, nor can throughput time be estimated in advance of an actual pandemic.
5. No true experimental model of this approach is possible if for no other reason than that the disconnected critical set of operators essential to providing an effective countermeasure cannot and will not all work on a new lethal agent at the same time.
6. There is no plan or interest in concentrating in one place the critical mass of talent and facilities requires to assemble a complete system, no matter what that system turns out to be.

The decision not to treat biodefense as so called "big science" precludes success. This is a default decision because the cadre of scientists involved and institutionally and personally are against the concept. All managers with no one in command will not work, especially in times of fear and stress.

The intercommunication formula, n(n-1)/2, calculates the number of inter-communicators required to make a multicomponent system work. This number runs into the thousands in practice under present paradigms.

4.1 Viral Defense Technology

Here we propose a radically different "one size fits all" approach that employs initially only properties and technologies that apply to all viruses, and which can therefore be implemented *before* a pandemic occurs. It is illustrated diagrammatically in Figure 1.

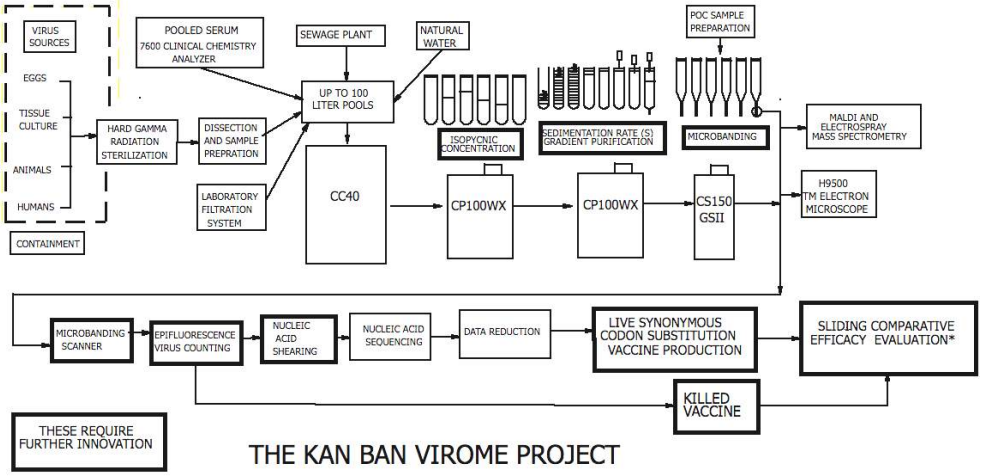


Figure 1. Diagrammatic Outline of Virome Project Laboratory.

The basic concept is that there must be in place a facility that proceeds from detection to vaccination using initially the simplest approach, which is a killed vaccine.

The basic paradigm underlying this approach states that a killed vaccine can be made against any and all viruses from which some humans recover. Any given killed vaccine may require multiple injections at multiple sites, but can be made to work. A second paradigm states that all high purity killed vaccines are safe to inject without extensive testing, and can be used as soon as prepared. This means that that ring vaccination can begin in time to actually stop virus transmission.

4.2 Separations

The separations are based on the size and density of viruses, their resistance to selected enzymes that digest non-viral contaminants, on changes in viral isopycnic banding density produced by changes in ionic composition, and on the use of gradient zones to transiently expose sedimenting viruses to less rapidly sedimenting reagents. The underlying technology is high-resolution two-dimensional zonal ultracentrifugation.

Detection is by fluorescence using nucleic-acid specific fluorescent stains and light scattering, and quantitation is by volumetric measurement, optical counting, or mass fluorescence.

Identification is by single molecule sequencing, immunobinding, electron microscopy, mass spectrometry, and precision isopycnometry.

Thus, initial vaccination is done with highly purified radiation-killed viral loads isolated from patient sources, followed by live vaccines prepared by synonymous codon recoding and by other synthetic genomics methods.

The overall strategy is to provide the technologies described as complete turn-key laboratories that can be placed anywhere in the world, which are in communication with each other and the central provider, and which are constantly updated. These laboratories would be in continuous operation, be constantly improved, and would make and test new vaccines on a regular basis, even if few of these are ever used.

Thus would be formed a defense system in a true military sense, tested, manned, undergoing constant improvement, ready for instant deployment and providing a flexible response.

4.3. Technology Summary

A variety of technologies are required which include:

Global Screening for Early Pathogen Discovery:- High-resolution two-dimensional zonal centrifugation (S-rho) technology is embodied in a series of centrifuges that range from the K-II (CC40) ultracentrifuge to microbanding ultracentrifuges described in this document. The K-II (CC40) concentrates the viral load from up to 100-liter pooled batches of excess diagnostic serum or from other samples including urine, obtained from febrile patients, preferably obtained on admission. The fractions recovered are subject to S-rho processing in successively smaller versions until the complete viral loads are reduced to a volume equal to or less than that of individual patient concentrations. Thus when a 100-liter pool of 0.5 ml samples is reduced to a 0.5 ml concentrate, it contains at least the same concentration of a given virus as was originally present in a contributed sample. Thus a 100-liter pool, if assembled from 0.5 ml samples, screens 200,000 individuals. The process would be repeated at short intervals, providing a running inventory of viruses circulating in man. Properly done, there is a reasonable chance of providing both the first notification of a new virus, and a much greater chance of providing a real-time map of the spread of a pandemic.

Containment and Radiation Sterilization: Two sample steams are envisioned, the first being inactivated by ionizing radiation to make all subsequent operations safe for non-contained procedures including sequencing. The second stream is processed in remotely controlled and automated “hot” cells to provide live viruses when these may be required.

Rapid production of limited quantities of a killed vaccine:- The mass of virus required to make one dose of a commercial killed vaccine varies from ~15 - ~50 micrograms. Many infected tissues or body fluids are known which contain sufficient viral mass to make from 1 to several hundred doses per gram. Many viruses prepared using S-rho methods are as pure as can be prepared, and can hence can be used for immediate vaccination. Initially S-rho and epifluorescence microscopy are used to identify the location of high viral loads in patient-derived samples of all types. When the best sources are discovered, arrangements can be made to isolate experimental doses of a killed vaccine.

Point-of-Care Diagnostic Virology:- Using microbanding tubes designed to use both S-rho and transient zonal digestion, with epifluorescence detection of virus packed

onto a dense fluorocarbon, the viral masses actually isolated can be estimated, and characterized by EM, MS, and sequencing. Fluorescence emission wavelength measurements distinguish ss and ds DNA and RNA viruses.

Sequencing:- Single molecule sequencing is planned for, realizing that this is a rapidly moving field. Point-of-care sequencing may require a special sequencer to rapidly sequence one or a few viral species at a point-of-care location.

Live Virus Synthesis:- Synthetic attenuated virus engineering (SAVE) uses synonymous codon substitution to make viral genomes that code for the same proteins as wild type genes, but with genes expressed at different rates, so that an immune response precedes pathenogenesis. This and other techniques allow candidate live virus vaccines to be rapidly made and tested.

Ancillary Projects:- To make each step operational a variety of ancillary instruments and systems are required that are outlined in the subsequent text.

Human Trials:- The original requirement that led to my invention and development of the K-II (CC40) centrifuge was production of a pure vaccine on a large scale. The FDA would not approve an anti-cancer vaccine made in cancer cells unless it was of extraordinary purity. The S-rho technology provides the highest resolution separation that can be made at this time. If the product of this process causes a side effect that renders it unacceptable, that component is an unavoidable component of the virus itself. In the chaos and panic of a true pandemic, it is ethical and rational to initiate human trials of a highly purified radiation-inactivated viral vaccine as rapidly as possible. If the initial killed vaccines fail to protect, this is an essential fact to know, because then forceful quarantine measures, that may then be our only defense, may be put in place.

Organization:- To compress the throughput time to eight weeks or less, it is essential to have one or more initial beta test laboratories that are complete, start-to-finish, and which are continuously developing and testing new vaccines both from animals and human sources.

Experience has shown that redirection of existing laboratories to do new and complex projects that are urgent and time-constrained rarely succeed. What can be done is for a new centralized facility to subcontract elements of the project, preferably to more than one contractor to provide competition. A provisional diagrammatic outline of such a laboratory is shown in Figure 1.

4.4 Common Properties to be Exploited

The properties of viruses which, taken together, could distinguish them from all other particles that might be present in infected samples include (a) size, (b) density, (c) responsiveness to changes in counter-cations causing alterations in isopycnic banding density, (d) resistance to specific enzymes and other reagents which can digest contaminants, (e) permeability to and staining by nuclei-acid-specific fluorescent dyes, and (f) distinctive morphology detectable by electron microscopy. Viruses also contain proteins that are physically distinguishable from all other proteins that may be present by mass spectrometry. In the end, however, the most specific biophysical property is a unique genomic sequence, which distinguishes one viral genome from all other genomes on earth. With the emergence of rapid sequencing, sequencing itself may provides the best analytical tool to guide and evaluate detection, purification and isolation procedures.

Viruses generally survive passage through the gut, suggesting that viruses, like living cells, are resistant to nucleases and proteases (5) which readily digest contaminating sub-cellular components that may be present (6). Hence by adding enzymes and other reagents to zones in density gradients as subsequently described, viruses may be transiently exposed to these reagents as they sediment through zones containing them, washed in subsequent zones, and then banded or pelleted. Thus the steps in virus processing can be carried out by simply sedimenting them through cleverly constructed zones in a density gradient.

Nucleases have previously been used to remove non-viral nucleic acids during virus discovery (7), and proteases have been employed in large scale virus isolation of, for example, hepatitis mouse leukemia virus and Lucke kidney tumor herpesvirus (8-10).

A wide range of nucleic-acid specific fluorescent stains are available which have been shown to penetrate and stain many different viruses (11).

4.5 Separations Based on Centrifugation

The first problem is to develop universal methods for virus isolation and quantitation.

The most widely used method employs differential centrifugation and may be done in swinging bucket or angle head rotors. As shown diagrammatically in Figure 2, the process starts with a tube full of a mixture of particles A, and is centrifuged to sediment particles differentially into a layered pellet. Only the last particle species sedimenting can be obtained in a pure state, and only part of it. In practice tubes are repeatedly centrifuged for increasing periods of time, and the supernatant and pellet recovered at the end of each run. By repeating this process, discarding lower speed pellets, and recovering and recentrifuging high speed pellets, useful separations of subcellular components have been made. To achieve high purity large starting samples, and many centrifugal steps are required. The method has never been successfully applied to the recovery of trace viruses in high purity from mammalian tissues.

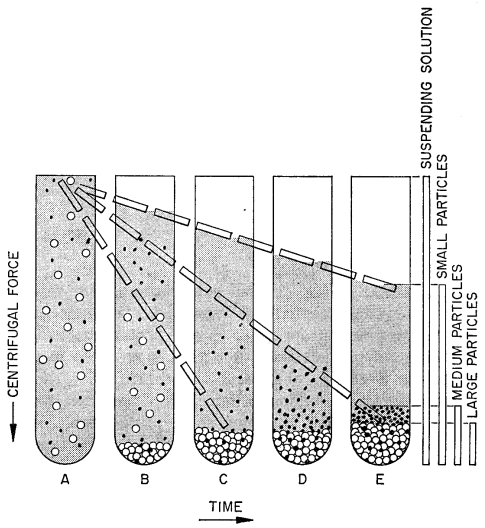


Figure 2. Differential Centrifugation of a Tissue Homogenate

The invention of density gradient centrifugation by Brakke (12) marked a turning point in cell fractionation and virus isolation. The technique, as employed with swinging bucket rotors, is illustrated diagrammatically in Figure 3.

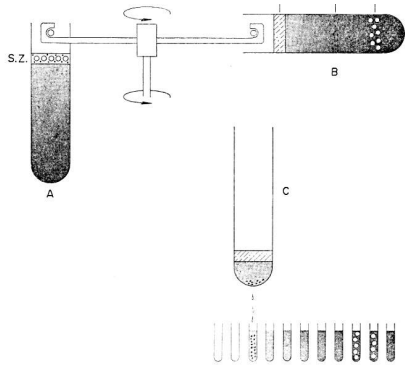


Figure 3. Rate-Zonal Centrifugation in a Swinging Bucket Rotor

A. Centrifuge tube containing a liquid density gradient and sample zone at rest. B. Rotor accelerated to operating speed, and particles separate on the basis of sedimentation rate. C, Tube is decelerated to rest, and gradient recovered as a series of samples.

This is not an equilibrium process and particles will sediment further if centrifuged longer, hence the term, "Rate zonal centrifugation".

The resolution that may be obtained with this process is quite remarkable. Resolution depends on the thickness of the sample zone, the concentration of particles in the gradient, the steepness of the gradient, on absence of stirring at all times, and on the number of fractions collected on gradient.t recovery. In the end, however, the particle mass to be fractionated is very much smaller than in differential centrifugation. In fact, as in nearly all of physical chemistry, one extrapolates to zero concentration to obtain the "ideal" values particles have when they are not there. So, one has neatly defeated oneself, unless some means can be found for making, spinning, and recovering very large-volume gradients without loss of resolution. This is not possible in swinging bucket rotors, which are already designed to spin as fast as their construction materials allow.

There is, fortunately, a different parameter which can be exploited which has different advantages and limitations. This is physical density and the process is termed isopycnic banding. This may be exploited in several ways, two of which are shown in Figure 4.

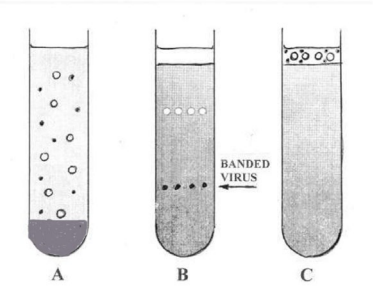


Figure 4. Isopycnic Banding of Particle Mixtures

In A, a centrifuge tube filled with a particle mixture including a virus is layered over solid CsCl. During centrifugation, CsCl dissolves and forms a gradient as the salt diffuses centripetally, yielding the equilibrium condition shown in B. Alternatively, a particle mixture containing a virus is layered over a gradient as shown in C, which, after centrifugation, also yields the equilibrium condition shown in B. Note the a much greater sample mass in present in A as compared with C.

Isopycnic banding is an equilibrium process and the centrifugation time is limited. Capacity in a given gradient is, however, high.

These two processes, rate-zonal centrifugation and isopycnic banding, may be combined to give a high-resolution two-dimensional process as shown in Figure 5.

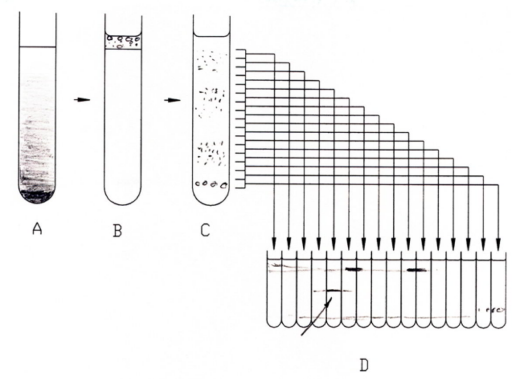


Figure 5. Schematic Diagram of High-Resolution Two-Dimensional Centrifugation

High resolution two-dimensional or S-rho centrifugation involves a separation where a density gradient is pre-formed in tube A, a sample is set in place on top of gradient in as shown in B, and then centrifuged in C to give a separation based on sedimentation rate, S. Each fraction recovered from this rate-zonal separation is then recovered and isopycnically banded in separate tubes in D. These isopycnic banding tubes are then lined up and photographed to produce an image which closely resembles the plot of Figure 7 below. Virus added to the starting sample gives a faint band

indicated by the arrow in D. Experimental studies have shown that viruses can be recovered directly from many different types of tissue in a high state of purity with this technique, provided that enough virus is present to detect by light scattering. This is the first two-dimensional high-resolution separation method ever developed.

Developing some method for achieving the separations predicted from Figure 5, but in large fluid volumes at very high speeds has, therefore, been an essential aim or this project.

The first major breakthrough was the invention of the basic zonal centrifuge design, which employed a hollow bowl configuration as shown in Figure 6.

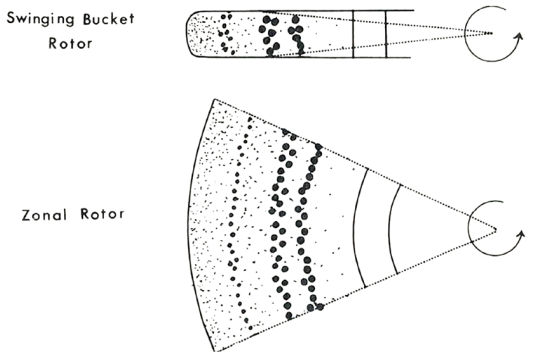


Figure 6. Comparison of Swinging Buckets and Zonal Rotors

Swing bucket separation shown at top. Since all particles sediment along radial lines, those outside the radial lines shown hit the wall at some time.

Section of zonal rotor shown in bottom image. Compartment is sector-shaped and all particles can sediment without hitting the walls. In practice, this shape is continued around the full circumference as shown in Figures 16 and 17 .

The second was the development of fluid line seals that allowed liquids to be pumped into and out of the rotor during centrifugation. This allowed swinging buckets to be dispensed with, and centrifugal force used to keep all elements of a gradient in position during both loading and unloading.

The third was the use of internal surfaces that, in spinning polar coordinates, funnel large cross-section zones with surface areas of hundreds of square centimeters to fluid lines a few mm in diameter over a radius of a less than a cm, all without loss of resolution.

This project originally depended on engineering expertise from the gas centrifuge project for isotopic separations, which in turn is based on the atomic table including stable isotopes. Many theoretical calculations in physics depend on such date. In the Virome Project, analogous information on the biophysical properties of the spectrum of biological particles, including viruses, that constitute our basic data. We must know how viruses differ from each other, and from all other particles that we may encounter.

It is only with such data that we can begin to devise the separations and analytical methods that will be needed.

When the sedimentation coefficients of major subcellular particles and 52 different viruses were plotted against their isopycnic banding densities, it was discovered, as shown in Figure 7A, that most viruses fall in an otherwise almost vacant area of the plot, indicated by a blue circle in the middle of the plot, which I have called the "virus or virion window" (13). Isolation of the entire "window" area for shotgun sequencing is an essential element of global screening, as is described below.

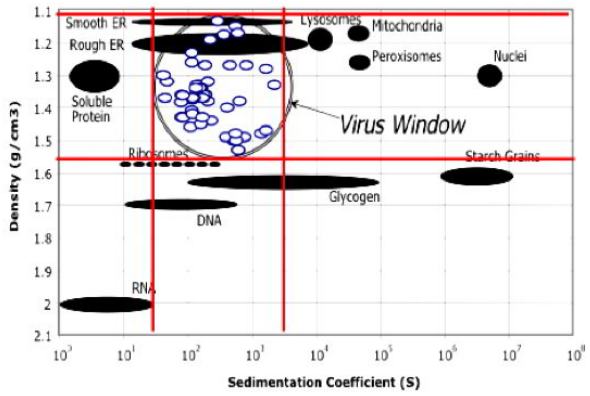
The major objective, however, is to increase the resolution of both sedimentation rate and isopycnic banding techniques, aiming to be able to isolate one virion type as shown in the red square of Figure 7B.

Data for Figure 7 were obtained from the literature, using a variety of instruments, systems, and conditions. A long-term objective of this project, therefore, is to re-determine the values for S for and ρ experimentally for as many known viruses as possible under standardized conditions. From such data the experimental conditions for isolating one viral species may be calculated, and conversely, viruses may be identified clinically from microanalytical determinations of these values. Many viruses are homogeneous with respect to many biophysical properties, others are pleomorphic, in which case one aims to isolate the predominant or "peak" form.

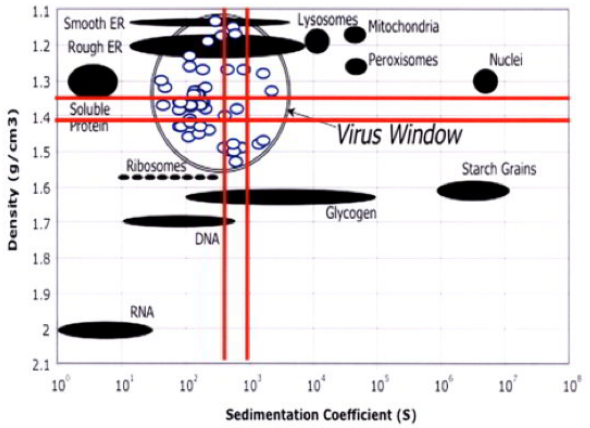
The resolution indicated in the lower plot has been achieved in practice. These plots, therefore, provide the basic information required to design and develop a systematic method for virus isolation based on sedimentation rate (S) and on banding density (ρ).

Rate zonal separations in density gradients provide unique advantages in virus isolation and production. Different slowly or almost non-sedimenting reagents may be included in different zones. Thus a virus may pass through a zone containing lytic enzymes (or other specific very slowly sedimenting reagents), leaving digested contaminant fragments behind as the viruses continue to sediment on through wash zones, to finally reach a zone where they band and may be detected and/or collected.

Thus a multistep process is efficiently carried out in one gradient.



A



B

Figure 7. S-ρ Plots of Major Sub-cellular Particles and Viruses.

A. Major sub-cellular particles are shown in black, and 52 different viruses are shown a small blue circles. The large circle encloses the virus window. Red lines indicate the S and ρ values used to design separations. Region to be isolated for global virus screening is shown in the red square

B. When the sedimentation coefficients (S) and banding densities (ρ) are known, a precision separation can be made. Experimentally the resolution indicated in this figure by the red square has been achieved. (Virus data provided by Dr. Peter Spragg. Modified from Anderson et al, (13)).

4.6 Basic Data for S-ρ Separations

The sedimentation coefficients of viruses range from ~90 to ~3,000 S and their isopycnic banding densities, ρ, range from ~1.12 to ~1.55 g/ml depending on the gradient solute used. In addition, viral isopycnic banding densities may be manipulated in ways that allow them to be separated from many non-nucleic-acid-containing non-viral

particles. For example, if a virus banded in iodixanol is found to be contaminated with membranous particles, rebanding in CsCl will usually resolve them.

Viral densities may be altered by changing from a cation such as Na+ or K+ to Cs+, and even a uranium salt. These do not alter the banding densities of those glycoproteins that may contaminate the virus window.

4.7 The Development of Biophysical Separation Systems for Viruses

While S-ρ gradient separations provide high resolution, it is at a high cost in particle capacity. Whereas differential centrifugation can be done with the tubes full of a sample which may be quite concentrated, rate zonal separations require small-width sample zones of limited particle concentration. I invented zonal rotors, as will be described, to solve this sample capacity problem without decreasing rotor speed or g forces.

The basic idea of a zonal rotor, as illustrated in Figure 6, is to carry out separations in a hollow bowl that is divided internally into sector-shaped compartments. The entire volume of the rotor may be loaded with a density gradient during rotation through fluid line seals, with the gradient kept in its expected positions by centrifugal force.

In some versions the seals are removed during high-speed operation. In other versions of the zonal rotor concept, the gradients are run into a rotor at rest with the gradient planes in a horizontal configuration, reoriented to a vertical configuration for high speed operation, decelerated to the original rest configuration, and then unloaded by gravity.

We review four different classes of S-ρ zonal centrifugal systems developed under this program. The largest, to which I gave the designation K-II, is designed to process samples of up to 100 liters in volume, or homogenates of this volume prepared from kilograms of tissues, using a complex technique termed continuous-sample-flow-with-banding. The K-II may also be used in a batch process using rate-zonal reorienting-gradient centrifugation with a gradient volume of up to 8 liters. These rotors have an axial ratio (length/diameter) much higher than 1.

The second class includes so called zonal centrifuges having gradients that range from ~250 to 1.7 liter ranges (14-23), and which are usually loaded and unloaded while rotating. These rotors have an axial ratio less than one. Note that rotors with a ratio near one tend to be unstable.

The third and more conventional class is designed for the recovery of the viruses and other particles using gradient volumes ranging from ~30 ml down to a few milliliters using conventional swinging bucket ultracentrifuge rotors and tubes, but with special floats for making gradients.

The fourth class is designed for concentration of viruses down to volumes in the ul and nl range and is intended to interface with single molecule sequencers and mass spectrometers.

These centrifuges and centrifugal systems are designed to comprise a continuous series in which viruses recovered from the largest systems may be further purified,

concentrated and transferred between systems without appreciable loss until, in the last step, the viral load is packed into a volume approaching that of a pure virus preparation. Ancillary requirements are for methods for physical virus counting, determination of the mass of virus pellets by fluorescence, for estimating the mass of viral pellets volumetrically, for shearing nucleic acids for single molecule sequencing, using very small numbers of molecules, and for making and recovering gradients over a very large range of volumes.

Note that it is an almost universal experience that as small and smaller pellets are dealt with, they tend to be lost because of the large surface areas involved, and because of small irregularities in them.

4.7 Global Screening for Human Viral Pathogens

If a pandemic is initiated by a singular event such as a mutation or an assortment, and if the symptoms are sufficiently unique, there is almost no chance that the index case will be identified unless the symptoms are very unusual. In real life, therefore, it must always be assumed that several cycles of infection will have occurred before a pandemic is recognized and announced. There is also no way to predict where in the world where this will occur.

Viremias peak generally with fever and usually rapidly subside as antibodies are formed. Both from the viewpoint of detecting pandemics early, of deciding on therapy, and for maximizing the possibility of obtaining samples with a high viral load, it is important of obtaining samples early.

Note that the difficult problem of obtaining large numbers of samples has already been solved. Plasma and serum samples are now routinely obtained nation-wide from very large numbers of people, are shipped to central locations for overnight analysis. The excess diagnostic serum now discarded amounts to hundreds of liters per week. We have previously proposed in detail how excess diagnostic serum collected nationally can be pooled, processed through the K-II centrifuge, further concentrated, purified, shotgun sequenced in depth (24), and the sequences from viruses found and assembled. Most of the technology required has been worked out for oceanographic studies using filtration for virus concentrations (25-28). To date, these filtration concentration methods have not been successfully applied to large volume of serum or plasma. Computer programs for metagenomic analysis of viral mixtures are now available (29), and long read lengths are advantageous in assembling sequence data (30). It is difficult to understand why this project has not already been done.

As global screening matures it will be possible to use sequence data to rapidly produce probes for new viruses for PCR analysis, and to make immunosubtractive columns to efficiently remove known and non-pathogenic viruses.

A separate sampling line may also be built into current large diagnostic machines to continuously remove small serum sub-samples from large sets of samples, and pool these for sequencing. Note the very small sub-samples of pools may be retained to be able to trace a virus back to a geographical area or a machine.

There is a reasonable chance that a new lethal pandemic virus can be detected and sequenced using global screening as proposed here, before it was discovered in the clinic. Conversely, if a new virus is discovered clinically and PCR reagents prepared, all

previous global concentrates could then be interrogated to see if it has appeared before and had been missed.

If correlations are suspected or discovered between early viral infection and a late-onset disease, then serial back analysis of screening aliquots stored over extended periods of time should confirm these.

Some human pathogens have been reported to be absent from blood during disease. It is important to check this information with sensitive PCR methods to find out if this is really true for all stages of infection. Since it may be true, the methods described should also be applied to urine, phlegm, and tissues as they become available. As subsequently discussed, the search for large viral loads is important to killed-virus vaccine preparation

4.8 Large Scale Virus Isolation

The K-II was designed to isolate viruses from 100-liter batches of serum or crude vaccine using a unique process termed “continuous sample flow with banding”. It is designed to make a two-dimensional separation in a continuous process in one rotor.

As noted, when used for screening, one 100-liter batch, if each patient contributed 0.5 ml, would represent 200,000 individuals, and five runs would screen one million. If an effort were made to include only samples from all febrile patients on admission, the percent of positives would be increased.

As shown in Figure 8-A the K-II rotor is loaded with a two-step gradient, which is slowly accelerated and reoriented to a vertical configuration, shown in B, and flow of sample through the rotor is started in C , when the rotor is spinning at top speed. The flowing virus-laden stream is less dense than the gradient, and the sample stream flows inboard of the gradient along the core and out the bottom of the rotor. During this process the virus sediments out of the stream and bands in the gradient that remains imprisoned in the rotor. When the entire sample batch has run through the rotor, as shown in C, rotation is continued to band the virus sharply in the gradient D, after which the rotor is slowly decelerated to rest, gradually reorienting the gradient to the rest position E. The gradient is then slowly drained out through the bottom F by gravity, through a recording spectrophotometer G, and into a fraction collector H. It is evident that the collected fractions shown in I each have a physical liquid density approximately equal to that of the particles in it. If these fractions are each centrifuged separately at high speed, they will form new gradients due the centrifugal force itself and will further concentrate virus and contaminants into bands which may be collected separately. This isopycnic banding step may be done in angle-head rotors to reach speeds and centrifugal forces much higher than existed in the K rotor. Thus virus may be concentrated from large volumes into very small ones without pelleting.

.The K-II is now in routine commercial use to purify commercial influenza vaccine throughout the world (31) (32), and has essentially eliminated vaccination deaths occurring due to anaphylactic shock caused by egg proteins, saving hundreds of lives. It has also been used to produce a variety of other vaccines, including the original hepatitis

vaccine prepared from HBV infected human serum (33). This work conclusively demonstrates the power of the S-ρ process in routine use, and further indicates that much of the technology required to make new human vaccines very rapidly exists.

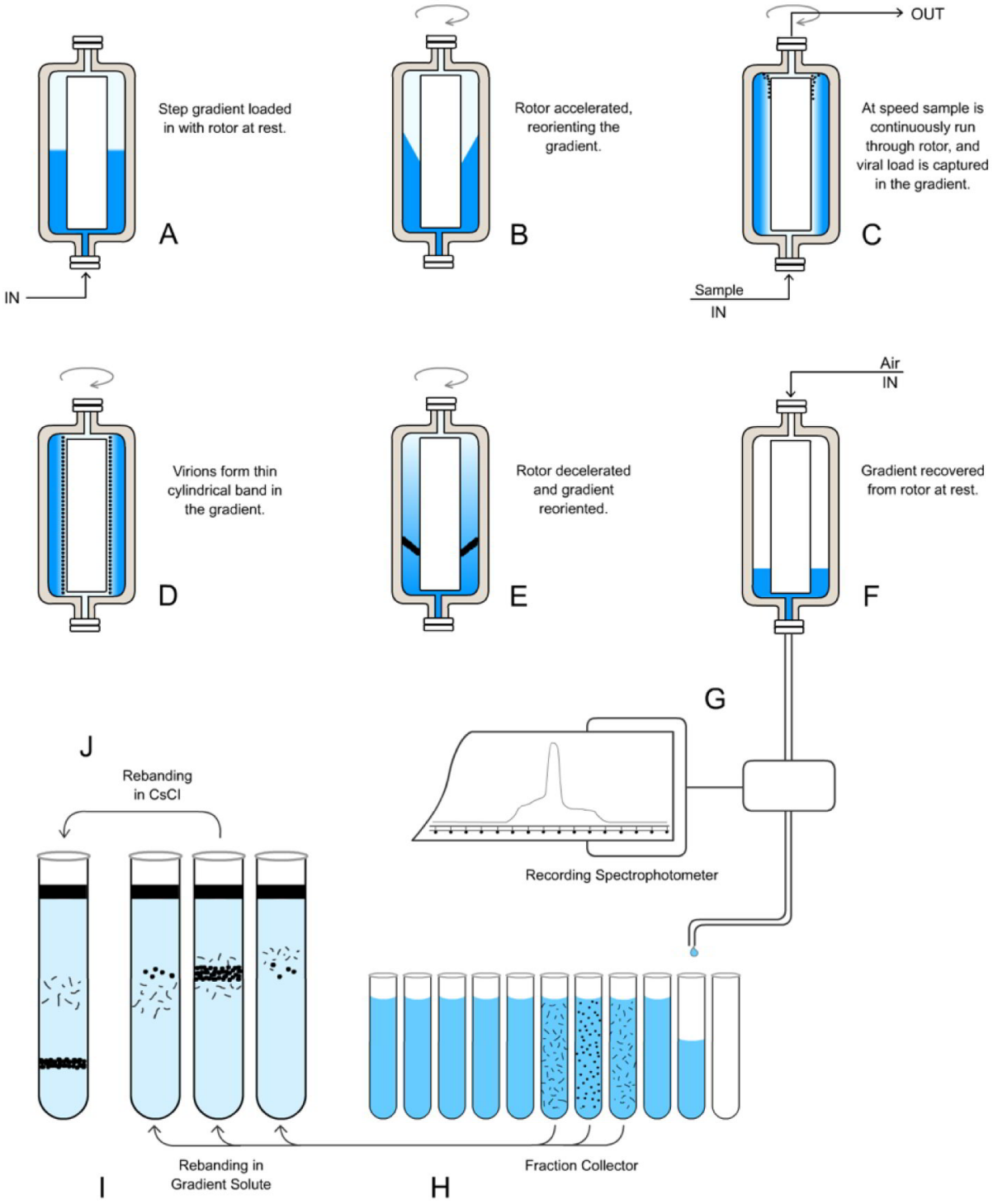


Figure 8. Schematic Diagram of Operation of K-II Centrifuge System

A. Rotor filled at rest with two-step gradient consisting, first, of a light buffer solution. followed by the dense end of the gradient to be used.
B. Rotor is slowly accelerated to reorient the gradient as shown, and then rapidly accelerated to operating speed. During this period diffusion forms a gradient.
C. Sample flow is initiated through either the bottom or top line, and effluent is discarded.
D. After flow is complete, centrifugation is continued to complete isopycnic banding of the viral load.
E. The rotor is then slowly decelerated and gradient is reoriented to rest configuration.
F. Gradient is allowed to flow out under gravity, or can be pumped out displacing it with a light buffer solution from the top.
G. Gradient flows through recording spectrophotometer or fluorimeter to locate bands. Physical density or refractive index may also be recorded.
H. Gradient is collected as large fractions.
I. To concentrate and further purify virus, tubes containing virus are re-ultra-centrifuged in angle-head rotor tubes, and viruses are concentrated in self-forming gradients.
J. For further purification, CsCl may be added to the most virus-rich tube to increase density. Tube is re-centrifuged to further separate virus from nucleic-acid-free contaminants based on the density change produced by CsCl.

In some cases, the recovered virus may be contaminated by membrane fragments containing polysaccharides which may make the membranes as dense as viruses. This is especially true if the K-II gradient is made with sucrose. If further purification is required, one or more fractions from image I may be re-banded after the addition of small amounts of dry CsCl to increase the viral banding density as shown as a single tube. CsCl binds to nucleic acid to increase viral banding density without changing that of majority of non-nucleic acid contaminants.

The K-II is the first totally new, from top to bottom, ultracentrifuge system in the last half century.

Figure 9. Commercial Installation of the CC-40 Centrifuge

An installation of a CC-40 version of the K-II centrifuge is shown in Figure 9, and the original titanium rotor built at Oak Ridge is shown in Figure 10. Examples of purification of six different strains of influenza are shown in Figure 11, and electron microscope pictures of crude and K-II purified influenza vaccines are shown in Figure 1

The armored centrifuge system is shown to the left, and electronic and display are shown to the right.

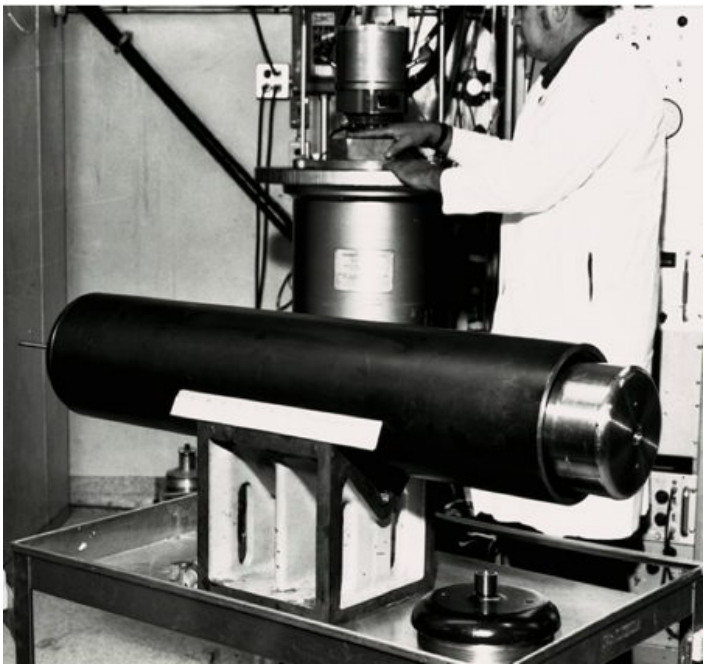


Figure 10. Original Titanium K-II Rotor at Oak Ridge

Rotor with core extending out is shown in the foreground, while the cylindrical armor and air drive is shown in the background.

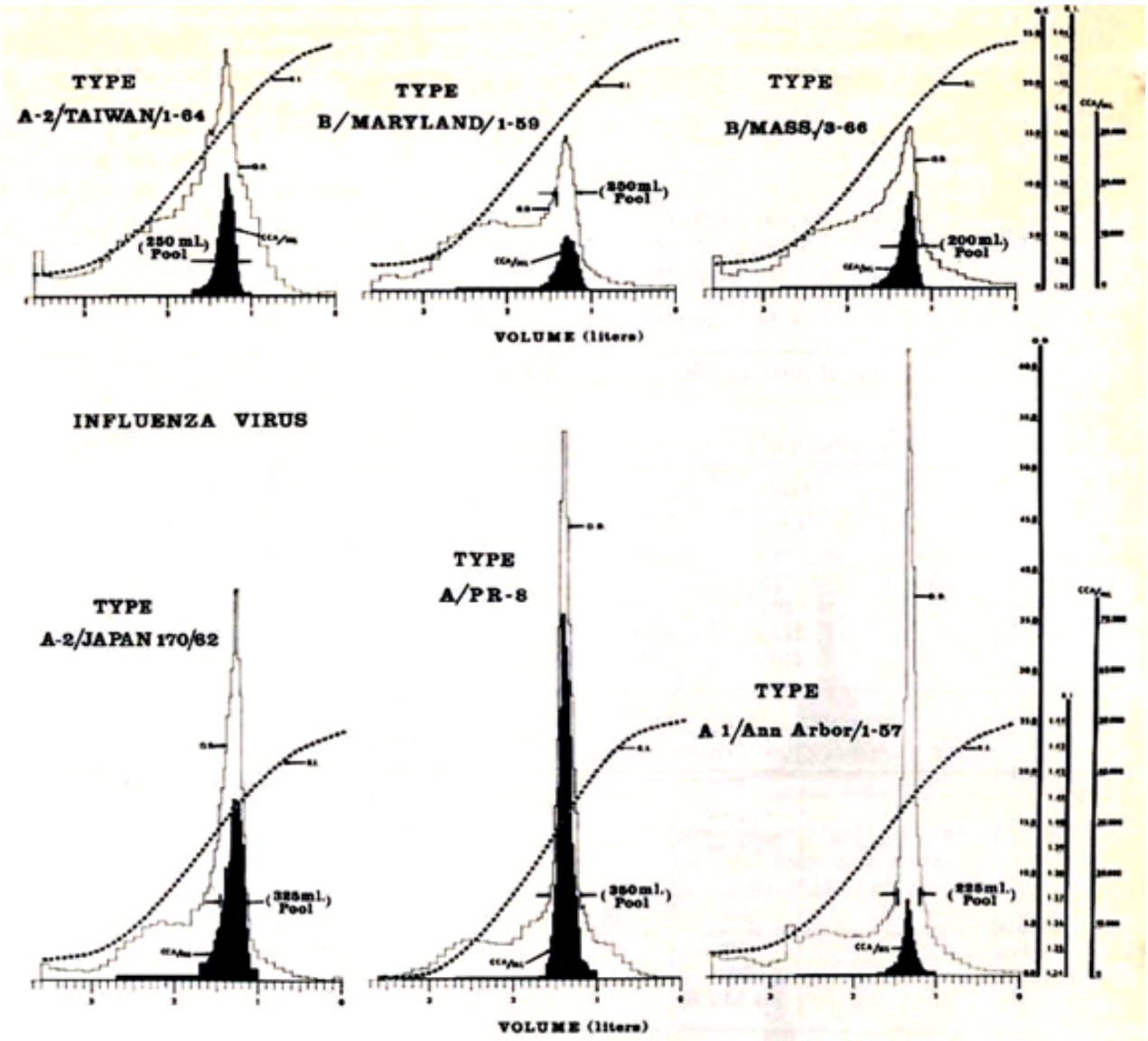


FIG. 5. *Hemmagglutinin and optical density profiles of influenza virus purified by the combine process: $BaSO_4$ absorption-elution, followed by isopycnic banding in the K-II zonal ultracentrifuge.*

Figure 11. . Recovery of Influenza Viruses Using a K-II Rotor.

Purification of six different strains of influenza in the K-II system is shown. Recovered virus zones are shown in solid black, density of gradient shown in as dotted line, and optical absorbancy is shown is shown as faint black line. From (32)

The success of the K-II (CC40) purified influenza vaccine in commercial use was surprising since nearly all side effects disappeared. The process was quickly adopted word wide.

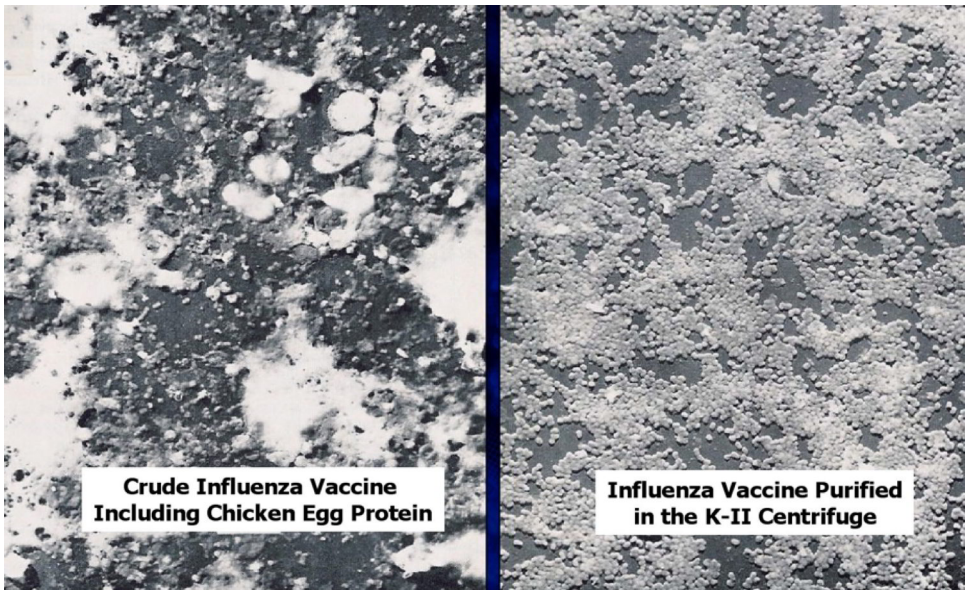


Figure 12. Purification of Virus from a Crude Influenza Vaccine. From (34)

From the purification illustrated in Figure 12 it was concluded , "*That the inherent toxicity of purified vaccine strains and combinations thereof is uniformly low, and that the variable side effects frequently seen with conventionally produced vaccines are not virus related. Purified vaccines are equal in antigenicity to equivalent doses of conventional vaccines, demonstrating that non-viral components of conventional vaccines have no beneficial effects on vaccination antigenicity. Purified vaccine of twice normal potency is no more reactogenic than the same vaccine in standard doses, indicating that future vaccines of even higher potency and low reactivity are well within the realm of possibility* (34).

I can, unfortunately, find no data on the mortality before and after the K-II (CC-40) was introduced indicating how many lives may have been saved in the decades during which it has been used. This centrifuge largely accounts for fact that vaccination can now be done in supermarkets with minimal medical supervision. Few virologists known in detail how it works, that its development was never finished, or that the gas centrifuge project which provide me with technical support was disbanded in 1986 and has not been fully reconstituted. Very early attempts to apply this technology to HIV discovery, though planned in detail, could therefore not be made, nor can the improvements originally envisioned in the original design, be completed to this date. This delayed HIV research by about two years.

As The K-II (CC-40) centrifuge design represents a closely calculated balance between rotor speed, radius, length, strength of materials, flow rate, thickness of the flowing steam, thickness and slope of the density gradient, and loss of resolution during gradient recovery at the end of a run. To maximize centrifugal force, the liquid volume should be as narrow and at as great a radius as possible.

While a smaller prototype of this design was developed and tested, the concept for the final design was not accepted by the Vaccine Development Board of NIAID, and funding from that source ceased but was replaced temporarily by the AEC and NCI.

The argument and problem were that the gradient would be only 10 mm thick, have an inner surface 2,680 cm^2, over which was to flow up to 100 liters of crude vaccine with a thickness of only 1 mm. No one had ever done banding with 10 mm long gradients. Obviously, the gradient would diffuse away during the hours required for a run. Further, if the core had to have a taper to allow for dynamic unloading, that would mean that the part of the stream would be in a smaller centrifugal field. We knew about gradient reorientation, but with 100-liter samples costing nearly $100,000 each, were afraid to try it.

An accidental power failure at the end of an actual influenza vaccine run at the Lilly laboratories, which resulted in the rotor slowly coasting to rest, showed that the gradient reoriented with very little loss of resolution. Core taper could therefore be eliminated. With the development of equilibrium centrifugation, it was realized that centrifugal force continued to sediment solute back into the gradient to keep the gradient steep, partially accounting for the high performance observed.

Resolution is, fortunately amazing. For example, the Type A/PR-8 virus peak shown in Figure 11 has a width in the rotor of slightly more than 1.5 mm. When dynamic unloading was compared with reorientation to rest the peak resolution was found to be the same.

It is concluded that there is a high probability that a killed vaccine capable of protecting thousands of individuals could be obtained from patient or animal-derived material within less than one week after the initial outbreak is detected and before major fatalities have occurred. One would aim to have available the option of using either human or animal virus sources.

4.9 Virus Isolation from Large Tissue Masses with the K-II (CC-40)

The K-II (CC-40) opens up many interesting possibilities for rapid virus isolation in the earliest stages of pandemic.

The largest single virus isolation studies to date have involved nucleopolyhydosis viruses that attack and kill the larvae of many forest insects. Kilogram amounts of infected larvae have been processed using two cascaded centrifuges to produce ~1-100 grams of concentrated polyhydrosis inclusion bodies (PIB) each of which contain varying numbers of virions (35, 36). Recovered PIB zones can have the consistency of milk shakes. These, we believe, are the largest virus samples ever seen, and the purification steps may be completed in a few days. A virus-rich gradient recovered from a K-type rotor is shown in Figure 13.

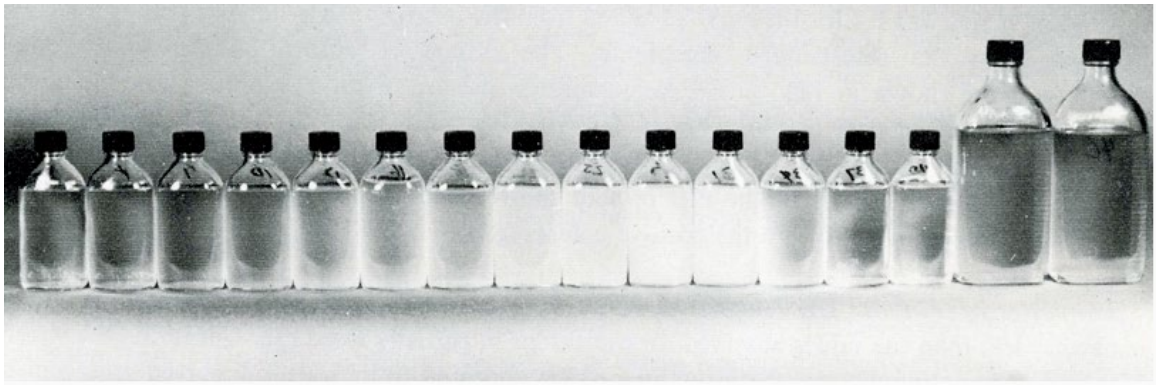


Figure 13. Very Large-Scale Isolation of Tussock Moth Virus for Forest Insect Control (37)

This work makes possible the most direct approach to vaccination which is to isolate the viral load directly from irradiated human tissues to make a killed vaccine.

The average weight of an adult human liver is ~1 kg and a 5% homogenate would have a volume of 20 liters, and 5 livers would yield 100 liters. Existing homogenization systems can produce homogenates of this volume, and existing filtration and commercial continuous flow clarifiers can remove particles down to the viral range from such samples. The K-II (CC-40) can then do a type of S-ρ separation that yields banded virus. Theoretically this could be done in a long workday recovering the viral load from ~ 4 x 10^{11} cells. The volume of the viral band is in the range of 200 ml.

If 500 virions were present per cell in the average tissue, and the average virus weighed 0.4 x 10^{-15} grams, then the viral mass would be 80 ~mg, or ~5,000 doses.

Note that as many as 15 K-II (CC-40) centrifuges have been operated in parallel for commercial vaccine production from human sources in the commercial production of hepatitis B vaccines (33) (38).

The point to be made is that using direct virus counting methods and existing technology it is possible to produce vaccines on a useful scale from almost any biological source.

As previously noted, no high purity killed vaccine has ever killed anyone. All of the technology required to make high-purity killed Ebola vaccines have existed since the start of the current outbreak. The reagents required to demonstrate both humoral and cell mediated that such vaccines produce both types of response have also been available. The tens of thousands of deaths occurring during this outbreak are best described as unnecessary Fear of Death Associated (FDA) killings.

4.10 Modifications to the K-II (CC40)

The K-II (CC40) centrifuge moved straight into production, with some armor modifications, and with a change from aluminum to titanium as the rotor material.

Aside from substituting an electric drive for an air turbine and improvement in controls, no major changes have been made, although many were initially envisioned. Among these are an improved valving system, improved leak detection, and redesign of

the core to improve preclarification. A proposed valving system is shown in Figures 14 and 15.

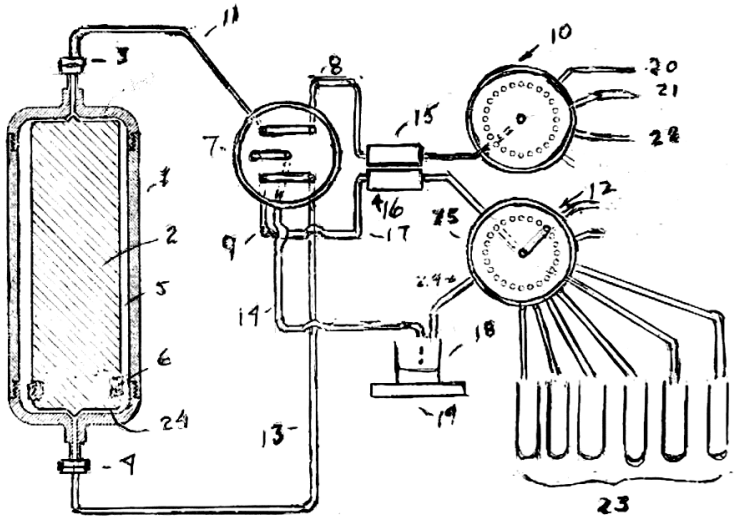


Figure 14. Valving System for CC40 or K-II.

1. Rotor, 2. Rotor Core, 3. Upper fluid line seal, 4. Lower fluid line seal, 5. Rotor Chamber, 6. Compressible ring for fluid volume reduction. 7. Reversing valve, 8. Fluid line from feed valve, 9. Line from reversing valve to collecting valve, 10. Feed valve, 12. Collection valve, 14. Drain line, 15. Upper line sensor, 16. Lower line sensor, 17. Line for lower sensor to reversing valve, 18. Drain collection, 19. Drain balance , 20-21. Feed lines, 22. Wash line, 23. Collection vessels, 24. Radial line in rotor that are free of liquid at operating speed.

The valve is specially designed to adapt the K-II(CC40) to global screening for human viral pathogens. For vaccine use, this centrifuge is specifically designed to isolate one virus, and collection therefore can be done in a relatively few fractions. When a very complex mixture of virus is to be recovered, then it is important to collect it in many fractions to improve coverage of rare viruses. It may also be necessary to maintain a steeper gradient or a multicomponent gradient from the outset. When working with infectious materials it is also important to have more facile remote operation through servomotor-controlled valving.

It is also important to have fluid flowing through the rotor at all times during high-speed operation. If flow is stopped, solute diffusing into the flow space will increase back pressure. If moving between input lines it is important to do this quickly, as can be done with this valve.

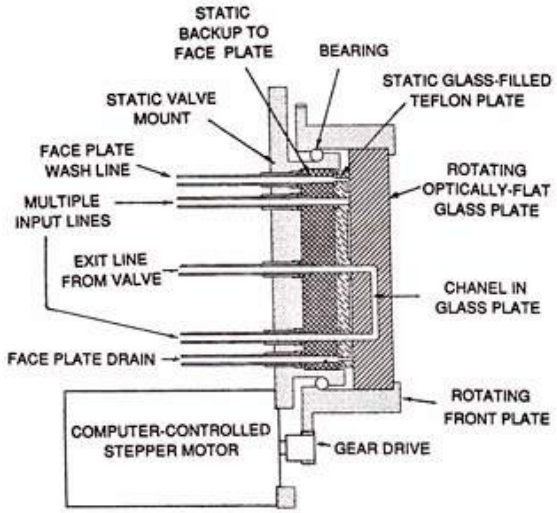
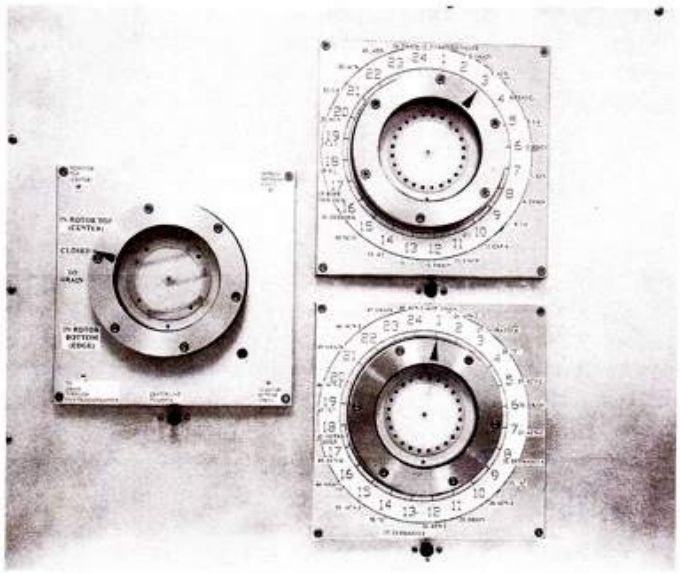


Diagram of side view of 24-port rotary reagent selection valve. Rotating front plate and attached rotating optically flat glass plate are the moving parts.



Photograph of three rotary valves used to control fluid flow. Valve to the left is the reversing valve, and two valves on the right are reagent valves.

15. Reversing and Multiport Valve

Note that rotating glass face plate in upper figure with U shaped hole is one solid piece of glass. From Anderson, et al, Large scale oligonucleotide synthesizers, 1995 (39)

Note that that feed valve 10 is also a versatile gradient maker. The version shown provides for 24 consecutive solutions, but can be designed to provide more or fewer steps. This means that density gradients with many different reagents or wash steps can be provided. When large numbers of steps are included, diffusion soon smooths the

gradient out in a spinning rotor. This means that many different gradient shapes can be readily provided

An additional advantage is that liquid flowing through the system is visible, and air bubbles are easily seen.

The K-II drive system can spin a variety of different rotor configurations including analytical rotors, swinging bucket rotors, angle head rotors, and vertical tube rotors. This means that large rate zonal separations or continuous-sample flow can be made and the collected gradients, isopycnically concentrated in No. 30 rotor tubes as shown subsequently in Figure 18. For high resolution work three rotors are required which would require three ultracentrifuges if the analyses are to be made as rapidly as possible. The alternative is to design three No. 30 rotors to be screwed together and spun by the same K drive used to make the initial concentration. If two No. 30 rotors are to be used, these may be attached together by a short vertical tube giving dumbbell configuration that that is stable at high speed. Note that in the analytical configuration an optical train is included.

4.11 Intermediate-Scale Zonal Centrifuges

The K-II (CC-40) systems give confidence that large scale vaccine isolation is feasible, but is too large for pilot small scale studies. Hence there is a requirement for intermediate- and small-scale centrifugal systems that work on the same principle so that results can be translated up and down the whole series. They are also necessary for concentrating the relatively large fractions from the K system

The initial series of intermediate-scale zonal rotors produced by the Joint NIH-AEC Zonal Centrifuge Development Program (the A and B Series) were designed to operate in conventional commercial centrifuges with modifications that allowed tubular rotors with an upper bearing and coaxial seal constantly in place. However, this allowed the sealing surfaces to dry and heat up. Hence a new design was adopted in which the upper coaxial seal was in place only during dynamic loading and unloading at low speed, but that was removed and replaced with a cap before acceleration and the coaxial seal reattached after deceleration to low speed for unloading.

B series zonal rotors were designed specifically for virus work and were initially designed to be loaded and unloaded dynamically through the center line. This design was abandoned and later designs allowed loading and unloading steps to be done optionally either through the center or the edge lines. The operation of such rotors is shown schematically in Figure 16, and an actual rotor core and lower half of the rotor are shown in Figure 17.

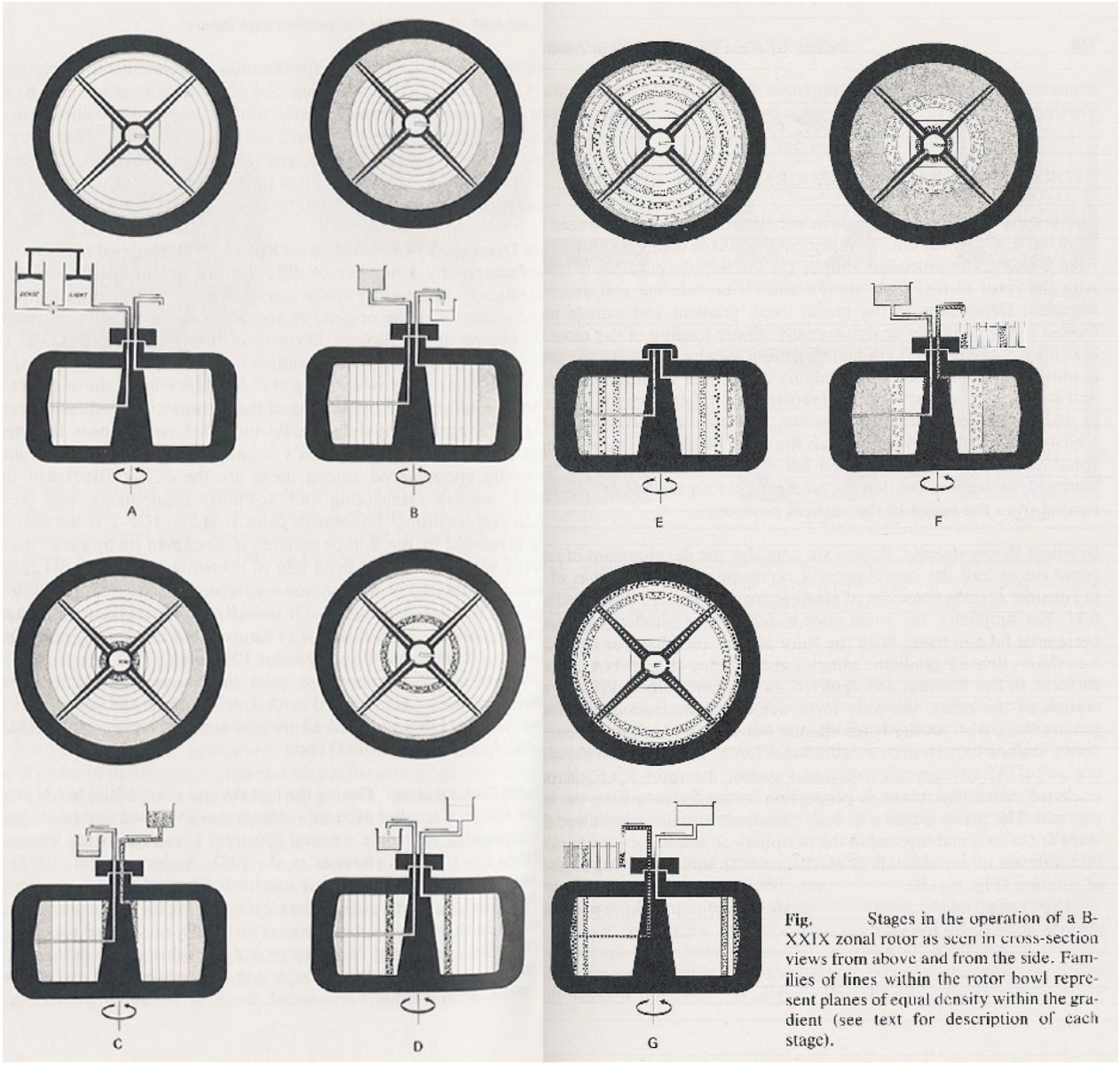


Fig. Stages in the operation of a B-XXIX zonal rotor as seen in cross-section views from above and from the side. Families of lines within the rotor bowl represent planes of equal density within the gradient (see text for description of each stage).

Figure 16. Operation of B Series Zonal Centrifuge Through the Edge Line

This shows the operation of Oak Ridge B-XXIX rotor which is designed for loading and unloading from either the rotor center or the edge. At each stage side and top views are presented. All operations carried out while the rotor is spinning.

A. Gradient is pumped into rotor through the edge line, light end first.

B. After gradient is, in it is followed by a small volume of a dense "cushion" to move the gradient away from the rotor wall.

C. Flow through the rotor is reversed and the sample is pumped in through the center line.

D .A continuing flow of a light overlay is pumped in through the center line to move the sample radially out clear of the rotor core.

E. The static upper seal then is removed and the rotor is accelerated to speed to move particles out to form separated particle zones at speed. The rate separation is a dynamic one, and particles would sediment further until they reach their isopycnic level, provided that level is in the gradient.

F. When the rate separation is complete, the rotor is decelerated to load-unload speed (~3,000 rpm), the static upper seal is re-attached, and a light solution, usually water or a dilute buffer, is pumped in through the center line displacing the gradient through the edge line ,back to the rotor center, and out into a fraction collector.

G. The light displacing solution is pumped in until the gradient has been completely displaced and collected.

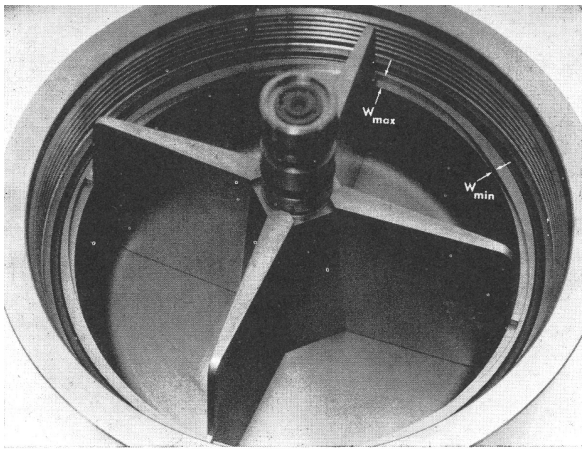


Figure 17. B-XXIX Titanium Zonal Rotor Designed for Either Center or Edge Dynamic Loading or Unloading

Figure 17 shows the core and lower half of the rotor. The internal volume is divided into sector-shaped compartments by vanes that contain the four center-to-edge lines.

Much of the sophistication of this system lies in the use of specially angled surfaces which, in polar coordinates and in high centrifugal fields, combine to vector gradients in and out of rotors during rotation with minimal loss of resolution.

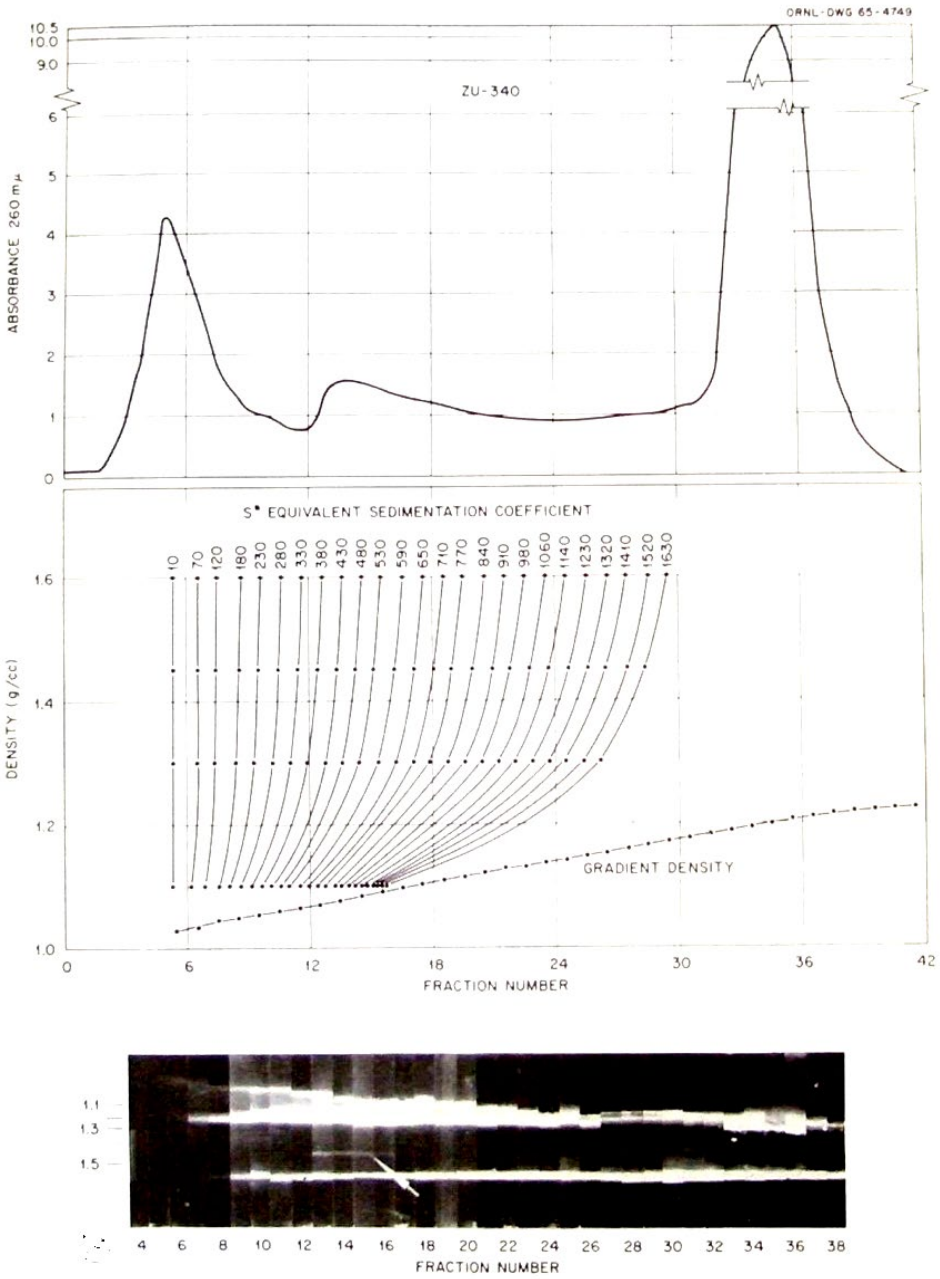


Figure 18. Recovery of Added T3 Phage from a Whole Rat Liver Homogenate Using The S-ρ Technique.

Phage was recovered from band indicated by the arrow. Rest of the pattern is described in the text. From Anderson, N.G .et al Nat. Canc. Inst. Monogr. 21, 1966 (13)

To demonstrate that the separation suggested by the S-ρ plot of Figure 7 could be actually achieved in practices, a rat liver homogenate was prepared and fractionated by rate zonal centrifugation as shown in the upper two panels of Figure 18 and by isopycnic banding in the composite bottom panel. T3 phage was added to the sample homogenate

initially and is recovered and indicated by the arrow in the lower panel. It was shown to be in a high state of purity by electron microscopy. Comparison between the bottom panel in Figure 18 and the S-ρ plot in Figure 7 show that the basic concept works. A variety of further experimental studies support this conclusion.

To do isopycnic banding of fractions from rate-zonal separations without losing much resolution, some very simple means are required for making multiple gradients and for observing and recovering banded viruses at different level in sucrose or CsCl gradients, without either diluting the recovered fractions or pelleting the viruses before banding.

To make full use of the preparative aspects of this system, each fraction of the rate-zonal gradient to be banded should have approximately the same volume as the tubes used for isopycnic banding. The volumes of such tubes range from a few ml for small gradients to 39 to 94 ml for large ones. In order to combine efficiently banding with gradient collection, dry CsCl can be introduced into each collecting tube, and the rate gradients are collected into those tubes in ~30 ml fractions. Sucrose used in the first S rate separation is not removed. The tubes are then sealed, centrifuged, lined up, and photographed using scattered light. In this process the virus sediments down onto a sharp gradient formed as the solid CsCl dissolves and then diffuses upwardly. An example of this is shown in Figure 19.This is an excellent general method for combining concentrations by sedimentation with isopycnic banding.

This and other work described below demonstrates that viruses can be readily isolated from a variety of tissues and that a complete bench-scale series of centrifuges exists, partly in prototype form, for working with small animal tissues, human and large animal organs or pieces of them to produce vaccines. Depending on the original sample load, this can yield hundreds to thousands of doses of killed vaccines when in continuous operation.

Note that over fifty different rotor rotors were designed, built and tested during the course of the Joint NIH AEC Zonal Centrifuge Program in Oak Ridge. Figure 20 illustrates the resolution obtainable by rate-zonal centrifugation in a 1.7 liter B-IV zonal centrifuge (18). (This rotor has been superseded by the B-XIV and B-XV, and by the B XXIX and B XXX rotors). Note that the width of the virus peak at half height, measured in Svedberg units, is about the same width as the diameter of the virus circles shown in Figure 7B, demonstrating that rate zonal centrifugation resolution can be very high.

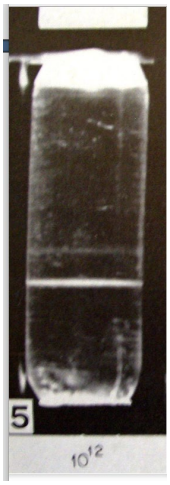


Figure 19. T3 Phage Banded in Diffusion Generated CsCl in Angle Head Rotor

The band contains ~10^{11}page particles. Rate-zonal centrifugation to make the initial rate separation was done in a B-IV zonal centrifuge. From: NCI Monogr.21 1966, p 275 (13)

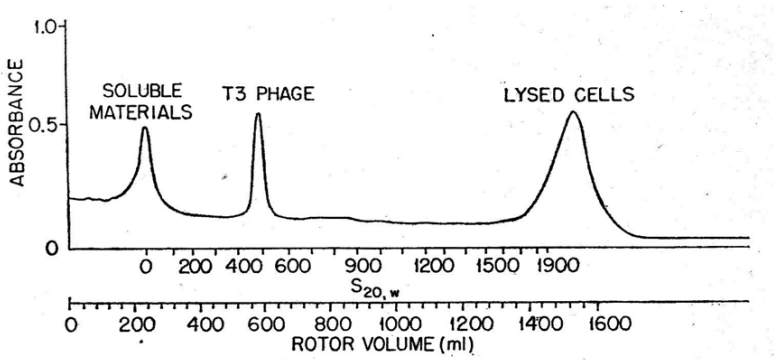


Figure 20. T3 Phage Lysate Separated by Rate Zonal Centrifugation

Width of T3 phage peak is equal to ~ 60 ml at half height. The range of s values at half height is approximately 50 s.

In Figures 21 and 22 an experiment was done with a T3 lysate believed to contain only one phage type. (As in Figure 13 above, the top panel shows the optical density of the gradient at 260 nm and 1 cm path length and the center panel plots the equivalent sedimentation rates for particles reaching that position in the gradient as a function of their density. The bottom panels show a montage the isopycnic banding tubes. This panel is assembled from photographs of all these tubes pasted together.

In Figure 21 the position of T3 is indicated at the tip of the left arrow, while the right arrow shows the position of T4, which was faintly visible to the naked eye. Round dots are density beads of different densities (40).

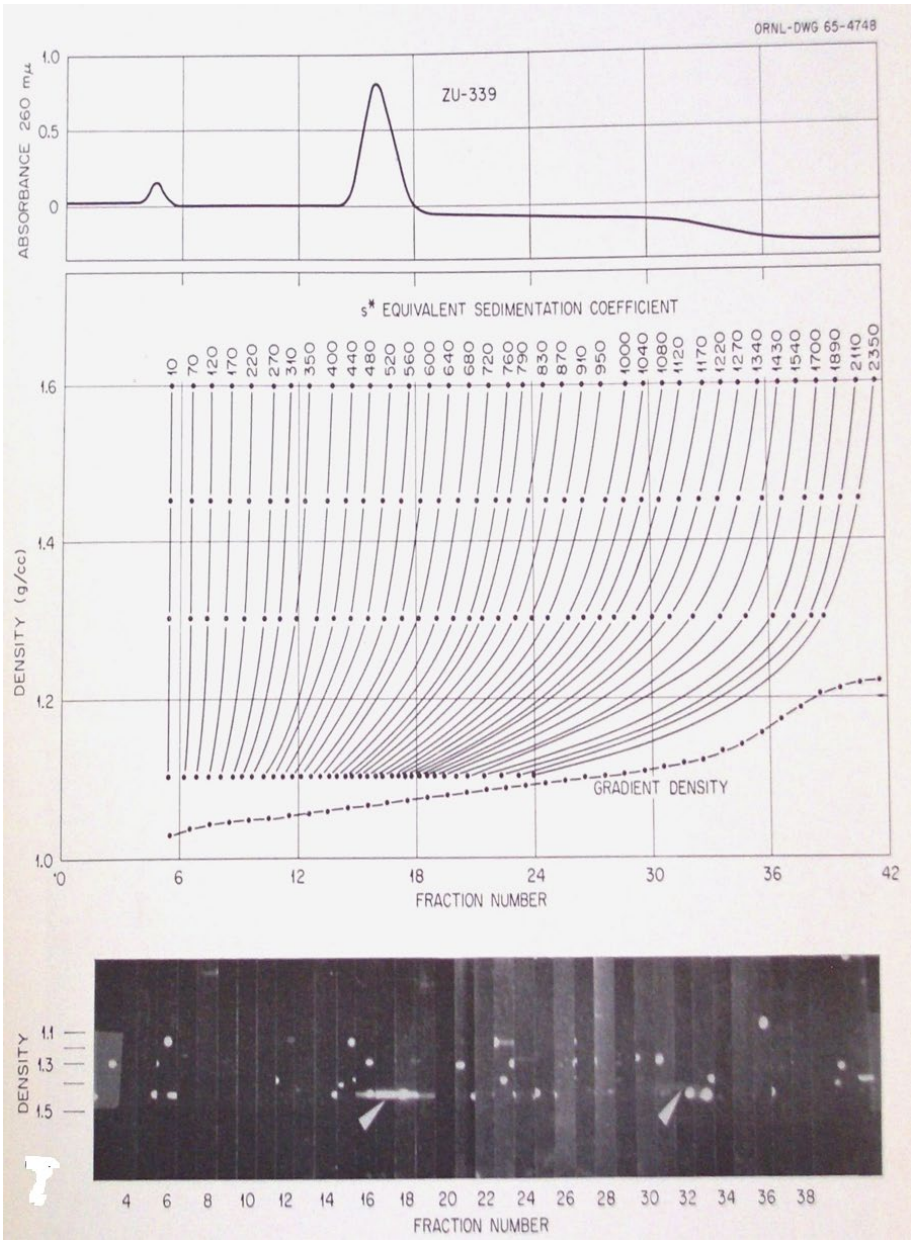


Figure 21. Combined Rate-Zonal and Isopycnic Zonal Separation of T3 Phage Preparation Contaminated with T2 Phage.

The positions of the two virus bands indicated by the two arrows, T3 on the left, and T3 on the right. The floating round particles are density beads, while the horizontal bands are the phage. Monogr. 21, 1966, p 277.(13)

To our surprise, the second band had the morphology of T3 phage. Electron micrographs of the two bands from Figure 21 are shown in Figure 22.

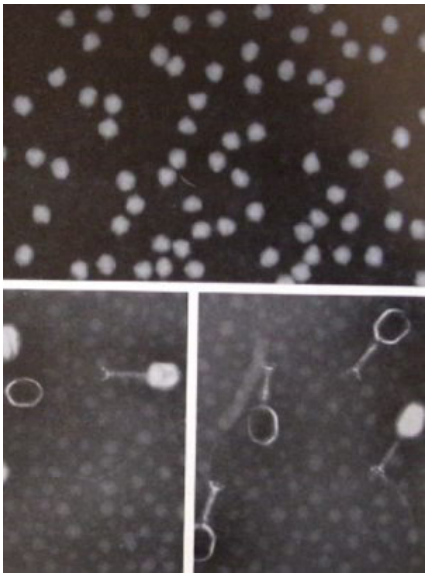


Figure 22 Electron Micrographs of T3 and T4 Bacteriophage from the Experiment Shown in Figure 21

Upper micrograph shows T3 phage from Figure 21, while lower micrograph shows recovered T2 phage. Monograph 21, p 278 (13).

4.12 S-rho Centrifugation in Conventional Tubes

In the series of centrifuges employed here, conventional swinging bucket centrifuges fall in the middle. A major barrier to their use, both for research and clinical studies, has been the time and effort required to make, handle, and recover the gradients required. A simple small-scale solution to this problem is shown In Figures 23.

This method makes use of reagents stored in large sealed transfer pipettes that can be prepared in advance, deaerated and stored frozen. After the first and densest zone is in place, a float is inserted that fits the centrifuge tube closely, and slows the liquid travel past the float, allowing zones to be made slowly and without mixing.

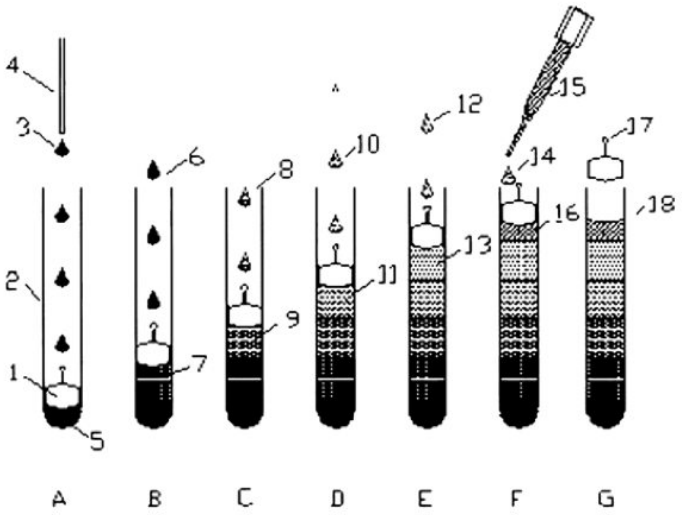


Figure 23. Use of Floats to Make Step Gradients.

In A, float 1 is initially supported on the densest gradient zone 5 in ultracentrifuge tube 2. Drops of the next zone 3 are dropped on top of float from pipet 4. In B the float has risen as the second zone as liquid 6 is introduced, forming zone 7. This process is repeated in C to E. In F the sample zone 16 is introduced, and float 17 is lifted out in G.

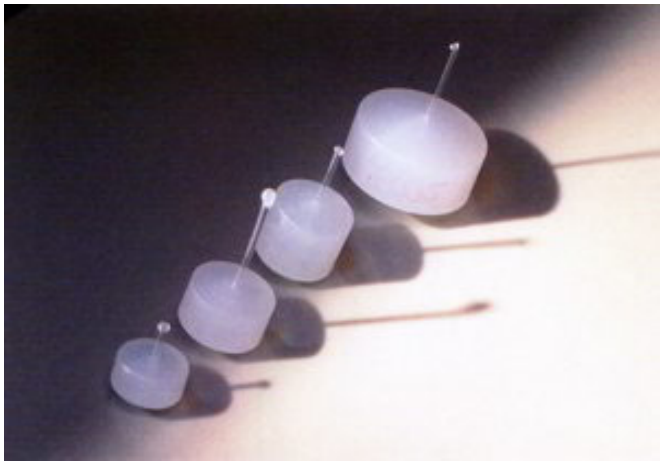


Figure 24. Polypropylene Floats for Constructing Liquid Density Gradients in Different Sized Centrifuge Tubes.

Polypropylene has a density of 0.85 g/ml, and therefore always floats on water or density gradients used. Whiskers allow floats to be recovered.

Figure 24 shows a series of polypropylene floats for different swinging bucket rotor tubes, and a 20-step gradient in a Beckman SW 20 rotor tube made with a float is shown in Figure 25.

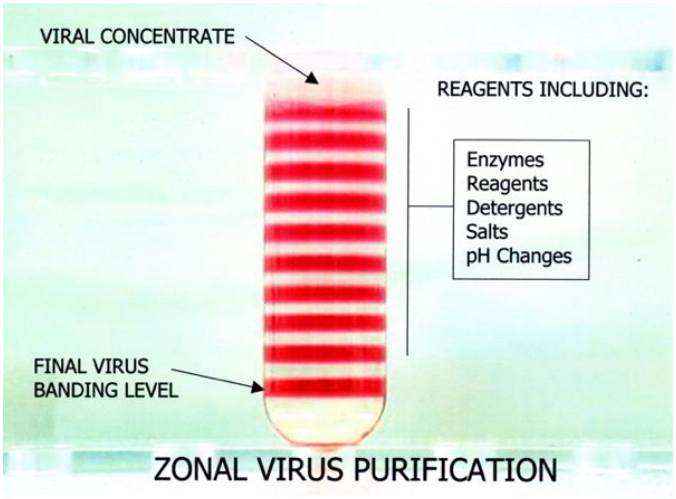


Figure 25. Liquid density gradient in Beckman 28 Rotor Tube.

In this 20-step gradient, cobalamin has been used to mark every other step. Image taken just after gradient was formed. After a few hours the gradient will become almost linear.

This convenient method for making density gradients makes it possible to have different reagents in different layers. Sedimenting particles travel through these layers, which include wash layers, so that virions separated from small molecules in the starting sample, are washed, are sequentially exposed to different reagents, washed again, and then pelleted or banded. The objective is to have a convenient standard method for S-ρ separations.

Sealing swinging-bucket ultracentrifuge tubes and recovering gradients have always posed problems. We have developed a mixture of waxes that will flow to form flat disks during ultracentrifugation, that will prevent outflow when the tubes are pierced from the bottom, and can likewise be pierced with a fine needle from the top as shown in Figure 26. When this needle is partially withdrawn it serves as a fine control needle valve that can be used control the rate at which drops are collected by gravity. If all steps are done on a timed schedule, including the time of gradient diffusion, and if temperature is carefully controlled, reproducible results can be obtained.

Figure 26. Wax Closure in SW 41 Ti Swinging Bucket Rotor Tube

Micromovement lowers fine needle into wax closure until tip emerges from bottom of the wax. Tip is then slowly withdrawn to allow air to flow into the top, giving controlled outflow.

The rate of droplet flow can be very finely controlled and the factions collected in a microtiter plate, as shown in Figure 27, thus providing a disposable means for handling infectious materials and a simple solution to a very long-standing problem.

This system can be used in a micro-version subsequently described where the needle serves as a piston to extrude micro- and nano-liter droplet volumes.

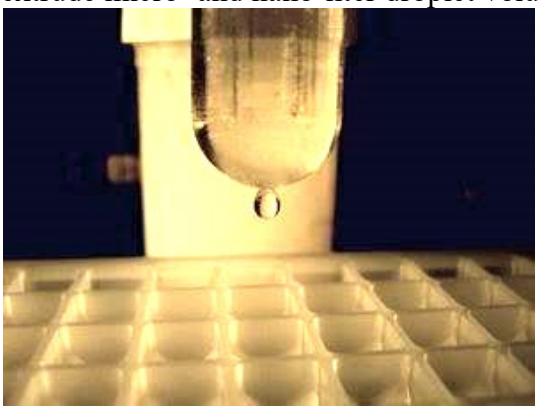


Figure 27. Direct Droplet Fraction Collection Using A Microtiter Plate

4.13 S-Rho Micro-banding Tubes

Microbanding tubes were conceived as a general method for isolating and concentrating viruses from samples in the ml range with isopycnic banding in narrow capillary tubes. At the start of this project it was assumed that very strong plastic materials would be required, and a set of tubes injection molded from polycarbonate was prepared as shown in Figure 28. These were supported in the swinging buckets by Delrin inserts for use at speeds up to 41.000 rpm (288.000 g_{max}). These tubes could not, however, be readily sectioned. It was found experimentally very difficult to keep the mold pin defining the center of the capillary in place when the tubes were made because they were bent by the very viscous molten polycarbonate during injection molding. This made it impossible to make the very small capillaries necessary to detect and recovery very small viral loads.

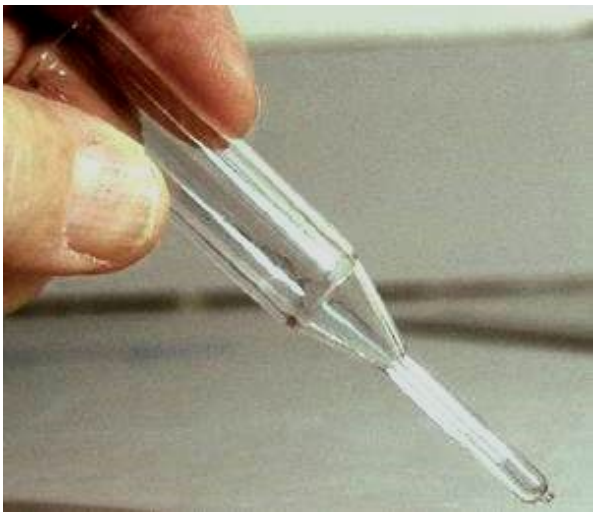


Figure 28. Injection Molded Polycarbonate Micro-Banding Tubes.

Figure 29 shows Phi X 174 phage banded in Iodixanol in a polycarbonate tube illuminated with 488 nm laser light but without a narrow band pass filter so that the outline of the tube may be seen.

A sensitive detection method is required which involves the use of a laser for monochromatic fluorescence excitation, and narrow band pass filters for detecting emitted fluorescent light, which also includes a micro-alignment system such as is shown in Figure 30 to align the laser beam and the capillary.

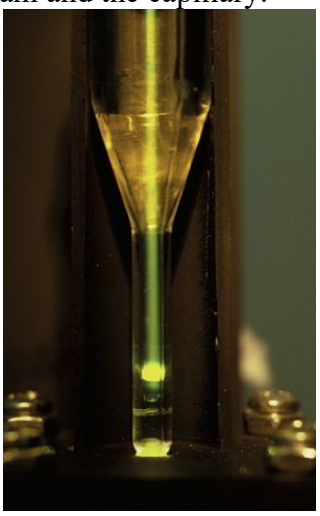


Figure 29. Banded Phi X 174 Phage Illuminated by 488mm Laser Light from the Top.

Virus was stained with Sybr Gold and banded in Iodixanol® in a polycarbonate tube.

Figures 31 illustrates the data obtainable from the type of scanner shown in Figure 30. The argon-ion laser provides lines at 458, 488, and 514 nm, and excitation filters are provided to select only one line at a time. The emission filters include 488, 510, 520, 535, 550, 580, 600 nm, all with half maximum band width of 10 nm.

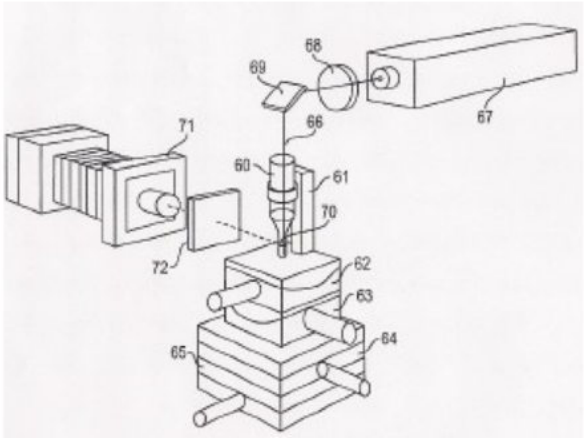


Figure 30. Schematic for Scanning Microbanding Tubes Including a Goniometer

Components are: 60 Microbanding tube, 61 microbanding tube holder,62 and 63, goniometers to alignment tube with laser beam, 64 and 65 X and Y movements to further aligned tubes, 66 laser beam, 67 argon ion laser, 68 interference filter if needed to isolate one wavelength, 69 front surface mirror, 70 narrow portion of microbanding tube with aligned laser beam, 71 digital color camera, 72 emission interference filter. The goniometer is required to align microbanding section of the tube with the laser beam.

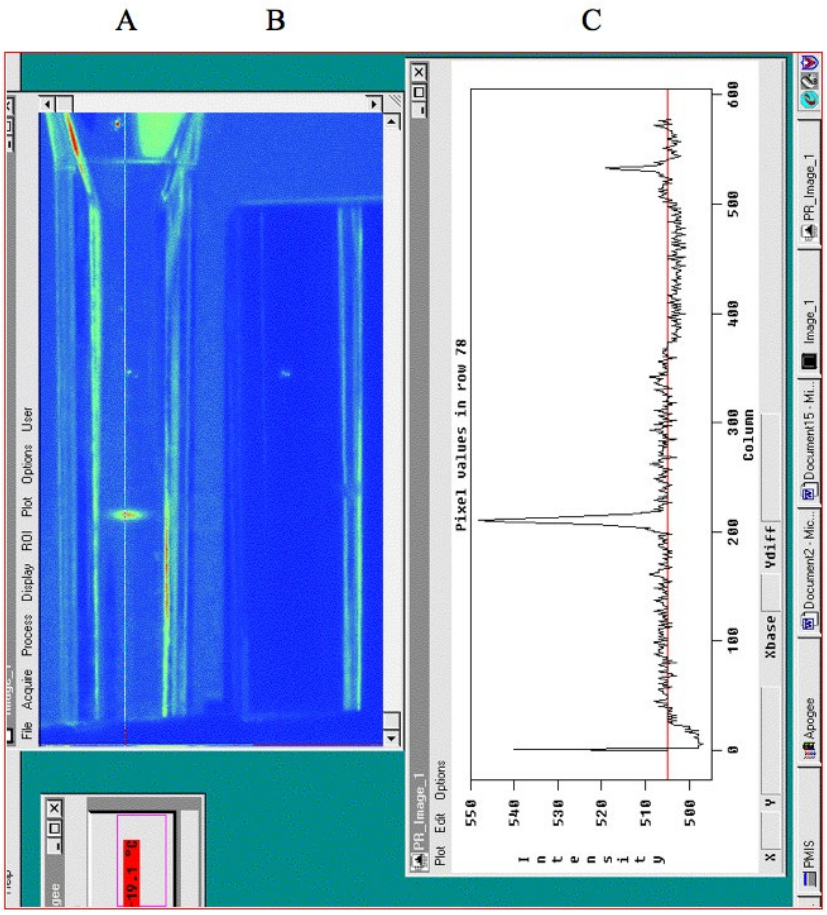


Figure 31 Isopycnic banding of Lambda Phage in Iodixanol-CsCl Gradient in a Microbanding Tube as Detected by Scattered Light at 456 nm.

Two views of the microbanding tube are incorporated into the same image during the same exposure to indicate the effect of polarization on scatter. Microbanding tube is shown in image A to the left and is positioned normal to the plane of the electrical vector of the laser illumination, and to the right B , normal to plane of the magnetic vector of the polarized laser beam. Image C shows intensity of the scattered light along the center of the tube image A. Image B is blank because all scattered light is polarized in one plane.

An image of lambda phage in a scanner similar to that shown in Figure 30 is shown in Figure 31. In A is an image of the banded virus, while B shows an image of the tube using a mirror to view the same tube from the side, thus demonstrating the absence of fluorescence at a right angle from the emitted light. C is the absorbance along the center line of the narrow section of the tube.

Plastics such a methylmethacrylate form bubbles as they are pulled out from thick-walled tubing on a vertical glass lathe, and also end up with very thin walls. However, glass can be pulled out to give an internal taper whose ends are below the level of detection with the light microscope, thus getting down to the limits of a few virions. The wall thickness of the glass at the tip can be controlled by slow pulling and careful control of the temperature.

We found that such tubes could be floated in 80% saturated CsCl at room temperature and spun to about 25,000 rpm, providing they were free of scratches. Above that speed they shattered into a powder. Lambda phage packed in the tip of a borosilicate glass tube is shown in Figure 32.

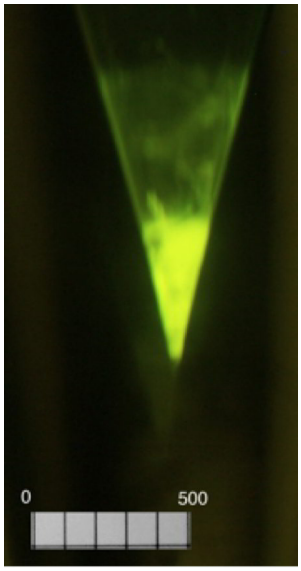


Figure 32 Lambda Phage Packed into Tip of Glass Microbanding Tube.

Estimated 10^9 virions in pack. Scale is in microns. This illustrates what packed viruses look like. Centrifuged for four hours at 25,000 rpm at 25 C with the glass tube floating in 85% RT saturated CsCl. Scale is in microns

In experiments with glass with sharp internal tips it was found that the lower limit of detection is largely set by the mass of extraneous particulate material present in sample and/or gradient solutions. If this technology is to come into general use at the limits of detection, particle-free reagents will be required, and we have developed centrifugal methods for ultracentrifuging reagents in inverted sealed transfer pipets, cutting off the tip with the contaminants, and then resealing them and storing them frozen. The tubes are not cut open until used. Particle-free solutions can then be transferred directly into microbanding tubes.

These experiments suggested that microbanding tubes might be fabricated from relatively soft plastic such as polypropylene (density 0.855 to 0.945 g/cc) and might survive ultracentrifugation floating on water especially if a very small amount of a dense fluorocarbon were placed in the bottom to keep the tubes oriented.

Prototypes of these are shown in Figures 33 and 34, and are translucent and hence scatter rather large amounts of light. In the molten state polypropylene is clear, and as it cools, crystallization occurs yielding a surface that is microscopically very uneven. This is the case with nearly all adjustable pipette tips. Hence some virus would be expected to be trapped as they are sedimented against tapered walls.

Additives such as MILLAD NX 8000 UC are now available that yield very small crystals in the nanometer range, very smooth surfaces, and are very transparent. Since individual virions may be detected using either epifluorescence microscopy or cell sorting, it may prove possible to detect individual virions banded in more transparent tubes. If so, the detection range nay equal that of PCR.

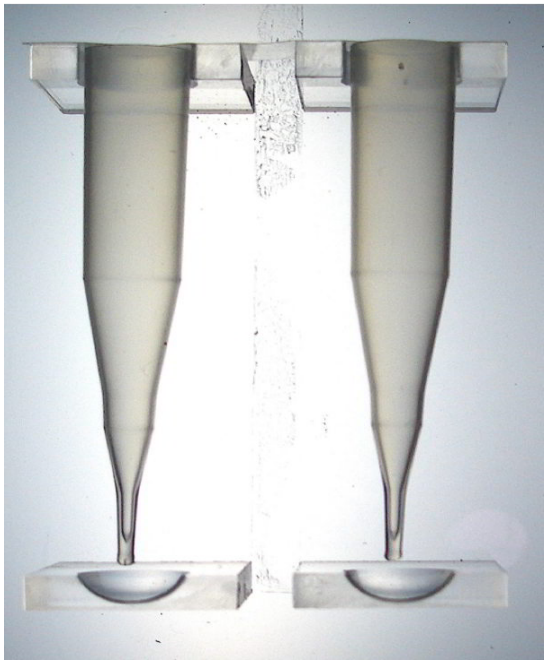


Figure 33. Floating Polypropylene Microbanding Tubes

Tubes kindly fabricated by Thermo-Fisher in Finland. The internal surface of polypropylene is rough due to the presence of small crystalline structures. These may possibly be eliminated by using syndiotatic polypropylene or other clear plastic.

Figure 34. Tip of Tube Shown in Figure 33

This illustrates sharp internal tip which may serve for volumetric measurements of viruses and other small particles. Scale is in mm.

One design for a multipurpose polypropylene micro-banding tube is shown in Figure 35, which includes a float for control of zone loading in a tapered section, a sharp

taper tip for maximum detection sensitivity, a capillary section for either pelleting against a fluorocarbon or isopycnic banding, and the option of diagonally sectioning the capillary to directly inject killed concentrated virus. Note that polypropylene additives that promote transparency greatly increase strength and stiffness, making possible this dual isolation/injection use.

The major technical problem with this design is molding the tip.

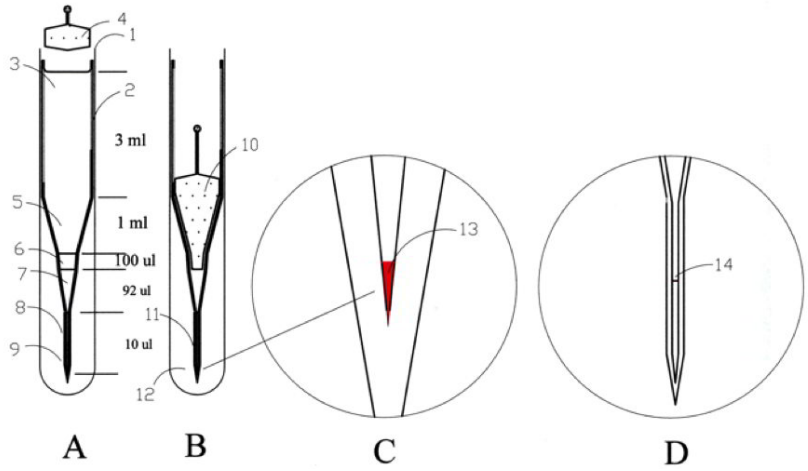


Figure 35. Design of Microbanding Tube for Pellet Volume Measurement and For Direct Vaccine Injection

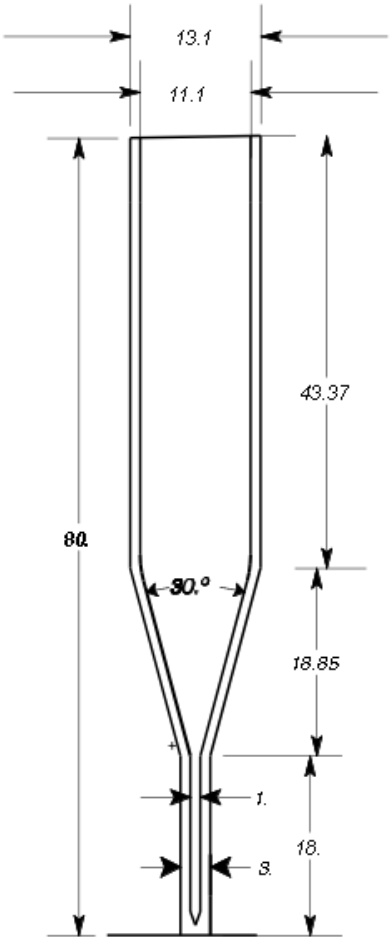
A. *1. Ultraclear centrifuge tube. 2. Upper section of microbanding tube. 3. Sample section, 4. Float for upper section. 5. Upper zone (1 ml), 6. Middle zone (100 ul), 7. Bottom. zone 92 ul, 8. Narrow banding section (10 ul).*

B. *9. Sharp tip. 10. Float for tapered section. 12. Internally tapered section for pelleting.*

C. *13. Inverted conical pellet.*

D. *14.Virus sedimented onto a fluorocarbon. The entire tube may be irradiated with X-gamma rays or electrons to sterilize the tube and contents, and to inactivate the virus. The tip of this section may be diagonally cut off to make it into an injection needle, the whole section connected to a syringe, and the virus and fluorocarbon injected as a killed vaccine.*

A simpler and more straight forward design is shown in Figure 36.



ULTRACENTRIFUGE MICROBANDING TUBE

TYPE FOUR

Wall Thickness = 1 mm

Viral Defense Foundation

36. Polypropylene Microbanding Tube

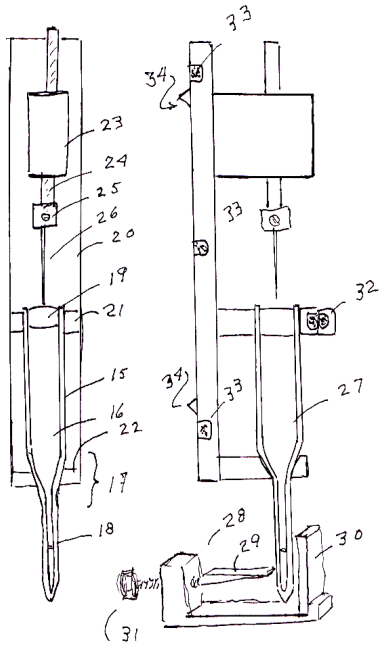


Figure 37. Device for recovery of pellets or band from microbanding tubes using wax for plastic closures.

A convenient and inexpensive device for recovering virus pellets or bands from tube such as are shown in Figures 35 and 36 is shown in Figure 37. It includes microbanding tube 15 containing sample area 16, gradient area 17, and virus 18 pelleted against a dense fluorocarbon, and a wax or plastic foam closure 19 mounted on extruder panel 20 and held on forks 21 and 22. Vertical movement 23 moves rod 24 and attachment 25 to move long sharp needle 26 into and through closure 19. To the right is a side view of the same device in which the tip of tube 27 is cut off by blade 29, which is driven by movement 31 against vertical glass surface 30. The wax or plastic foam closure prevents fluid from flowing out when this is done. The cutting device may then be removed and the pellet extruded in one of two ways. The needle may be slowly moved downward to extrude the pellet, which is observed by fluorescence. The pellet may be extruded onto a fluorinated surface as described below, which has a hydrophilic spot in the center to keep the drop centered. Extruder panel may include magnetic snap on closure 32, magnetic attachments for the whole device 33, and alignment pins 34.

Since the capillary microbanding section is difficult to mold as part of a much larger tube, we have explored the possibility of making it as a separate and detachable part as shown diagrammatically in Figure 38.

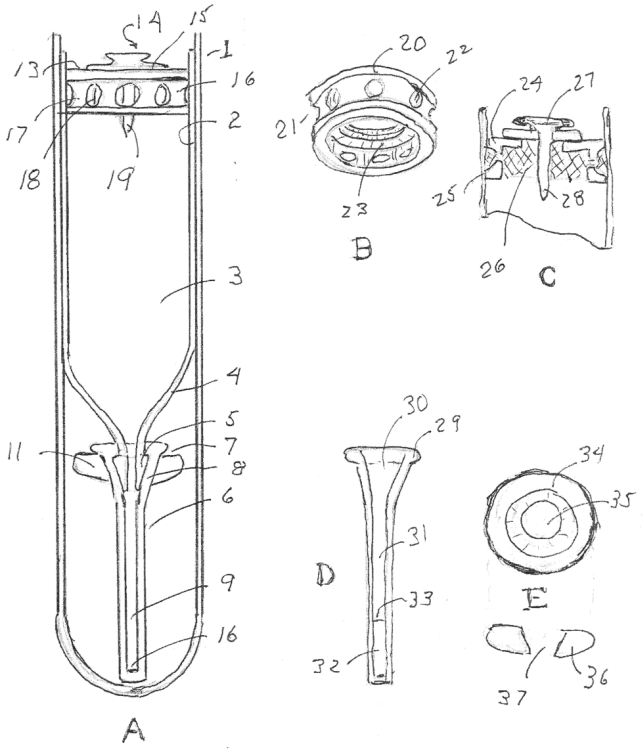


Figure 38. Multipart Microbanding Tube

Components include: In A which is a side view of the assembled tube, 1.Transparent swinging bucket rotor tube, 2. Upper concentrating section, 3. Sample chamber, 4.Upper tapered section, 5. Upper tapered capillary section, 6. Capillary section, 8. Tapered joint, 9. Capillary, 10. Sealed tip, 11. Floating support ring, 13. Polypropylene ring, 14. Sealing screw, 15. Flange on sealing screw, 16. Recessed groove, 17 and 18, Holes through which wax moves during high speed centrifugation, 19. Tip of sealing screw.

In B, 20. Upper sealing flange, 21. Circumferential groove contain wax, 22. Holes through which wax flows at high speed, 23. Flanging in C which is a side view of wax closure, 24. Polypropylene wax closure ring, 25. Holes through which wax flows during high speed centrifugation, 26. Wax. 27. Sealing and flow control screw, 28. Tip of threaded screw.

In D, which is a side view of the removable capillary section, 29. Lip against which the supporting ring pushes, 30. Tapered section to fit upper concentrating section, 31. Capillary. 32. Supporting dense fluorocarbon, 33. Meniscus of fluorocarbon on which virus sediments.

In E. Floating supporting ring with hole 35 and 37, Taper 34, and 36. Ring with sufficient volume to support capillary by floatation. This ring is fabricated from a light plastic which is driven upward in response to centrifugal force to seal the connection with the section above.

The removable capillary section of Figure 39 may be designed in a variety of ways some of which are illustrated in Figure 40.

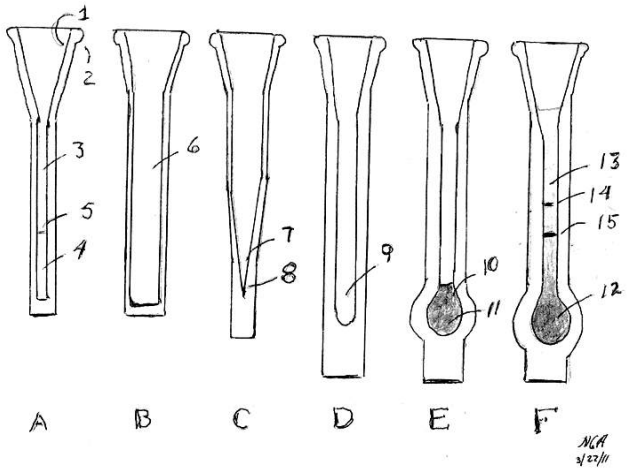


Figure 39. Versions of Removable Capillary Tubes

A: 1. Taper, 2. Supporting lip, 3. Capillary, 4. Fluorocarbon, 5. Pelleted Virus.
B. Large volume capillary.
C. 7. Taper tip, 8. Conical collecting end for estimation of viral volume.
D. 9. Large volume for pre-prepared banding gradients.
E. 10. Capillary with bulb 11 filled solid solute or concentrated solute.
F. Formation of isopycnic banding gradient while virus is sedimenting. 13. Light end of diffusion-formed gradient. 14 and 15. Two different viruses isopycnically banded in diffusion-formed gradient.

The separation shown in Figure 39 F is extremely useful for both concentrating and fractionating virus mixtures such as are or obtained during global screening for human viral pathogens by ultracentrifugation as described in an earlier section.

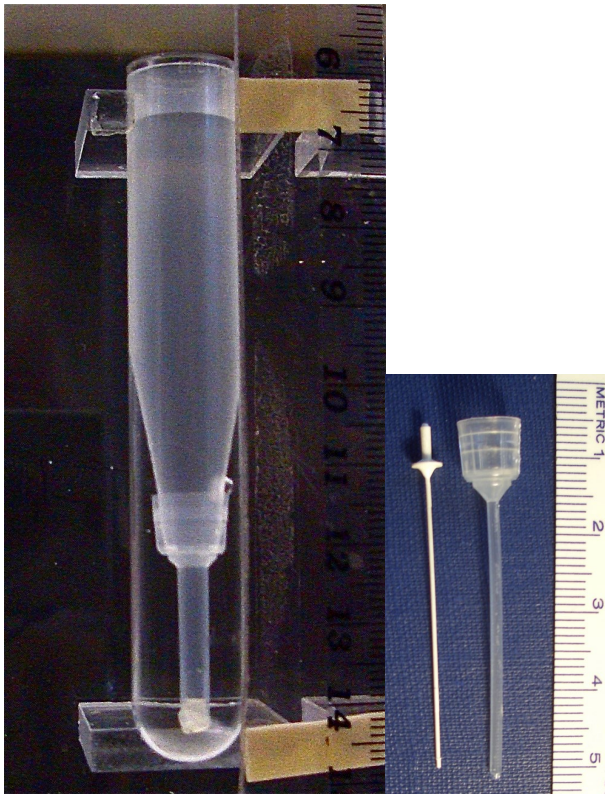


Figure 40. Two Part Microbanding Tubes

A prototype of a two-part microbanding tube is shown in Figure 40. The bottom section for the left image is the capillary section from a 1 ml capillary piston. On the right is the piston and capillary (CP 10) with a total volume of 10 uL, both supplied by Gilson. These are prototypes of tubes to be developed. As supplied these capillaries are open at the bottom. For this use the tips are heat sealed before use.

4.15 Preparation of Serum or Plasma for Virus Isolation

Until very large numbers of human sera, both virus infected and non-infected, and from individuals with a wide variety of diseases are examines using the microbanding methods described here, the potential background of particles that might possibly be confused with virions, but are not virions, will not be known.

It is therefore important to have some standard centrifugal method for pre-centrifuging experimental sera individually, such as is illustrated to Figure 41.

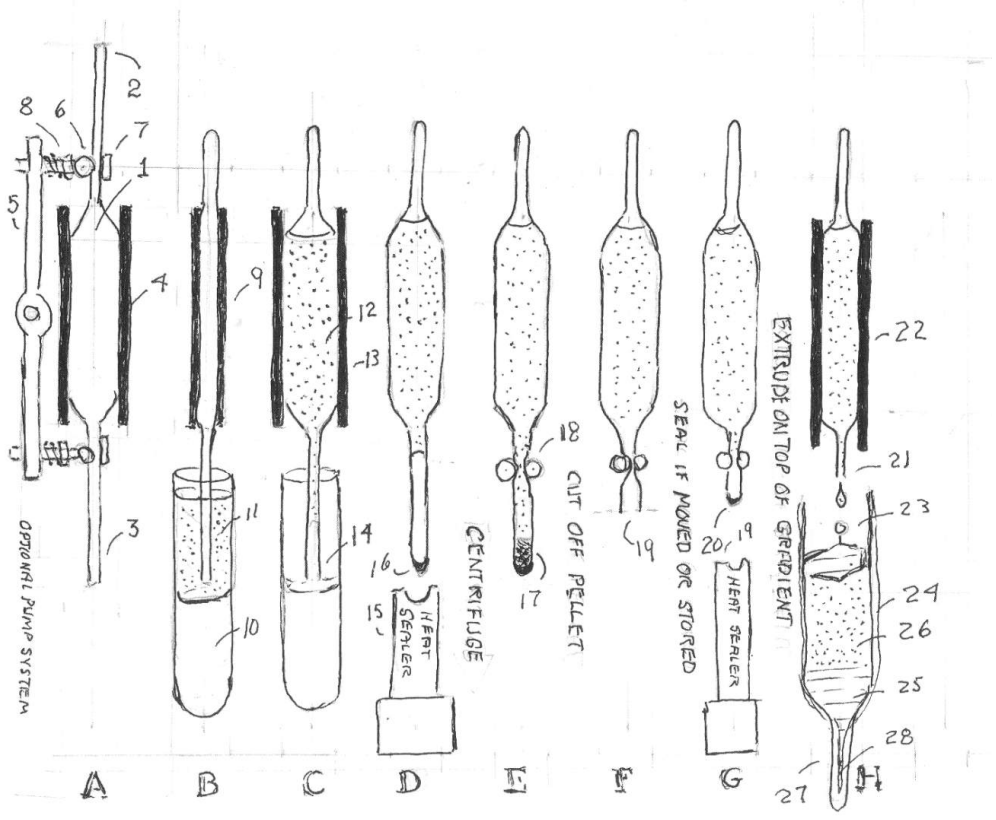


Figure 41 System for Pre-centrifuging Serum Before Microbanding

A. Method for making pump using modified open transfer pipet 1 open at the top 2 and bottom 3 sandwiched between a two-plate squeezer 4 and arranged so that part 5 can alternatively close upper closure 6-8 and corresponding lower part, with plate 4 moving to squeeze pipet 1 between these motions.

B. Transfer pipet squeezed to remove air and dipped into serum 11 on top of packed RBC and WBC 10.

C. Squeeze plate 13 is opened and serum 12 intake is continued until some air is sucked into lower end of pipet 14.

D. Lower tip of pipet is heat sealed 16 by sealer 15. The tube is then centrifuged to remove particles having sedimentation coefficients larger than the smallest virus.

E. The lower tip 17 containing sedimented material is then clamped off.

F. The lower tip 17 is then cut off.

G. The low end 20 may then be heat sealed by heat 20.

H. To transfer the serum to a microbanding tube, squeeze plate 22 empties serum 21 onto float 23 in microbanding tube 24 producing sample zone 26 floating on reagent zone 25 atop fluorocarbon 28 in capillary 27.

4.16 Use of Conventional Gel Loading Pipets

We found that conventional gel loading pipettes can be sealed by carefully dipping the tip into molten polypropylene for a few seconds and quickly withdrawing

them. These tubes fortunately float in water. Lambda phage sedimented pelleted against a dense fluorocarbon (PF 5050) is shown in Figure 43 (red arrows). One of the very tiny viral bands as seen under a modified epifluorescence microscope is shown in Figure 44. Note that the volume of this pellets may be estimated to yield a "virocrit" provided that the volume of packed individual virions is known.

Since individual stained virions may be counted in flow cytometers (41), we believe that individual virions can be detected with suitable optics in these micro-banding tubes.

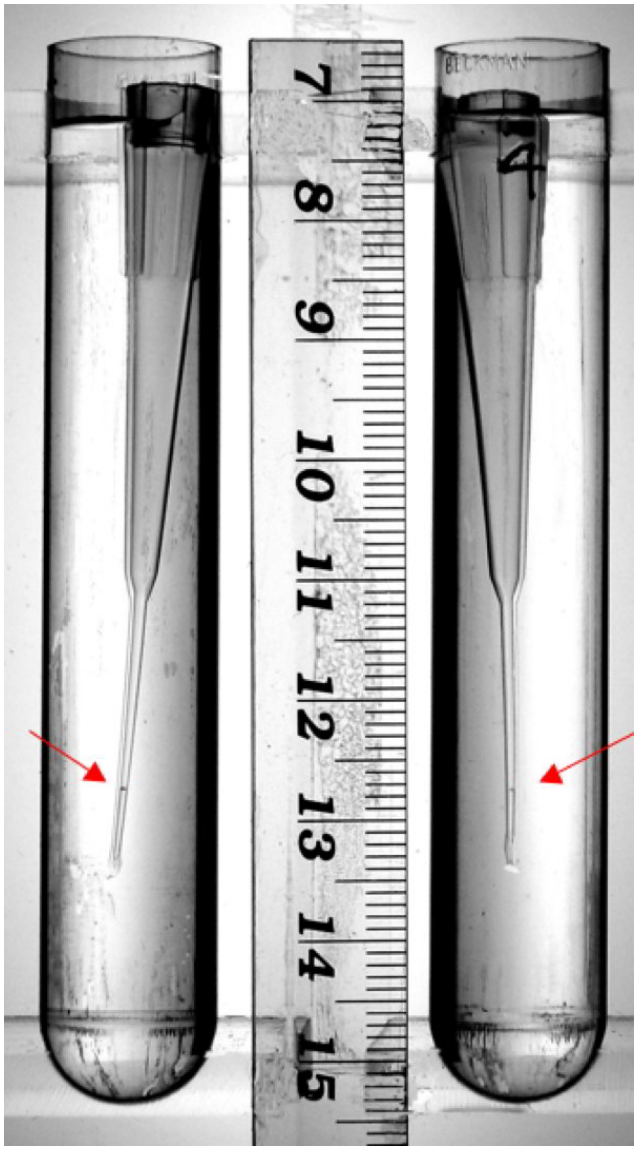


Figure 42. Lambda Phage Sedimented Against PF 5050 Fluorocarbon.

Microbanding tubes made from gel loading pipettes are shown floating in water. Lambda phage are at the level of 12.8 cm as indicated by arrows, and are barely visible in each tube. Scale is radius from the axis or rotation. Tubes were centrifuged in a

Beckman SW 41 Ti rotor at 41,000 rpm. Phage were pre-stained with Sybr gold. Each virus disc contains ~1 x 10^9 virions, the usual viral mass for one dose of a killed vaccine.

Figure 43. Epifluorescence Microscope Image of Virus Band in Figure 42.

Lambda phage was stained with Sybr gold, and is sedimented against PF 5050.

Note that if sufficient viral mass is present to be detected by fluorescence, there is sufficient material for real time sequencing of both DNA and RNA (42).

Figure 44 illustrates that this technique can be applied equally well to large proteins such as ferritin for mass spectrometric analysis.

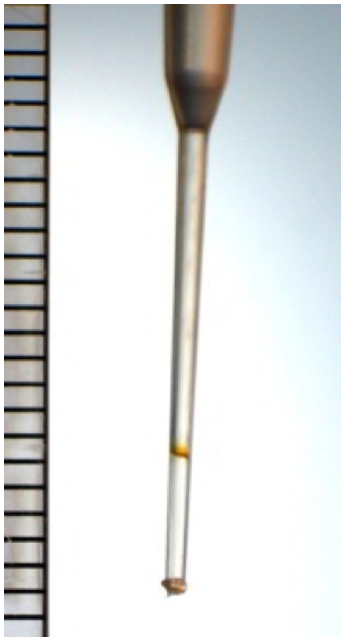


Figure 44. Ferritin Sedimented Against Fluorocarbon PF 5050

Centrifuged for 4 hours at 41,000 rpm, 288,000 x g_{max} in SW 41 Ti rotor. Scale is in millimeters.

The entire process is shown diagrammatically in Figure 45. The gel loading pipettes (GLPs) are initially heat sealed at the tip, loaded with very small amounts of a dense saturated fluorocarbon, and then serially loaded from the top with small amounts of gradient zones which are then each centrifuged down in place. The sample of serum (up to 200 ul) is then pipetted in, and the whole sealed with a small amount of a special wax that flows during centrifugation to provide a flat upper seal for the tubes. After centrifugation, the tips of the GLPs may be cut off. No liquid flows out because the tubes are sealed with the wax at the top.

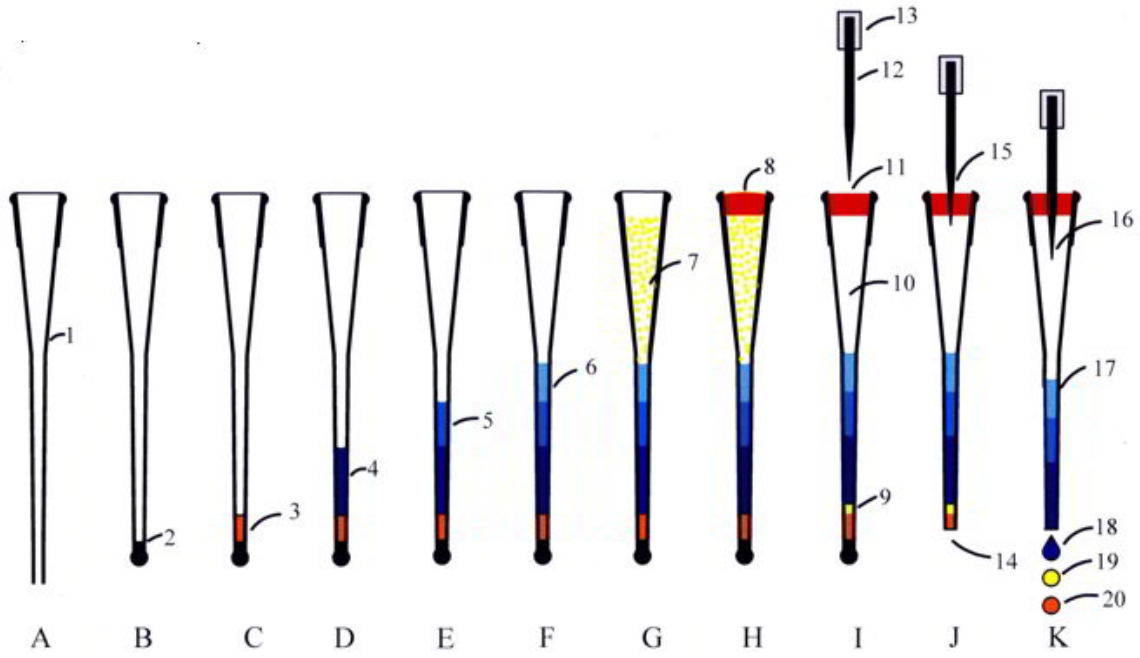


Figure 45. Procedure for Loading, Centrifuging and Recovering Virus Bands from Microbanding Tubes.

A1: Original gel loading pipet (GLP). B2: Tip of heat sealed GLP. C3. Fluorocarbon is introduced to the bottom of the tip, and centrifuged down into position. The level of the meniscus can be controlled by allowing the fluoro-carbon to evaporate to a controlled level. D4: Densest step of an aqueous gradient is introduced and centrifuged down at low speed. E5 and F6: Next steps in which gradient zones are introduced and spun down. G7: ~200 ul sample is introduced and spun down to remove air bubbles if necessary. H8: Soft wax seal put in place. I: Microbanding tube ultracentrifuged, clearing virus I9 from sample volume 10, leaving flat wax plug 11 with no air bubbles under it, and with needle 12 attached to movement 13 poised to be inserted into wax. J15: The tip14 is cut off, and the needle is slowly inserted into wax cap, to begin extrusion of the fluorocarbon. K: Step gradient 17 is gradually moved down, extruding droplets 18-20. Sub-droplet sized volumes may be recovered by touching to a glass slide, or a Teflon surface. This may be controlled electronically if the GLP and the capture surface are conductive. If the virions have been stained, the virions in the dried droplets may be counted, and/or recovered for sequencing.

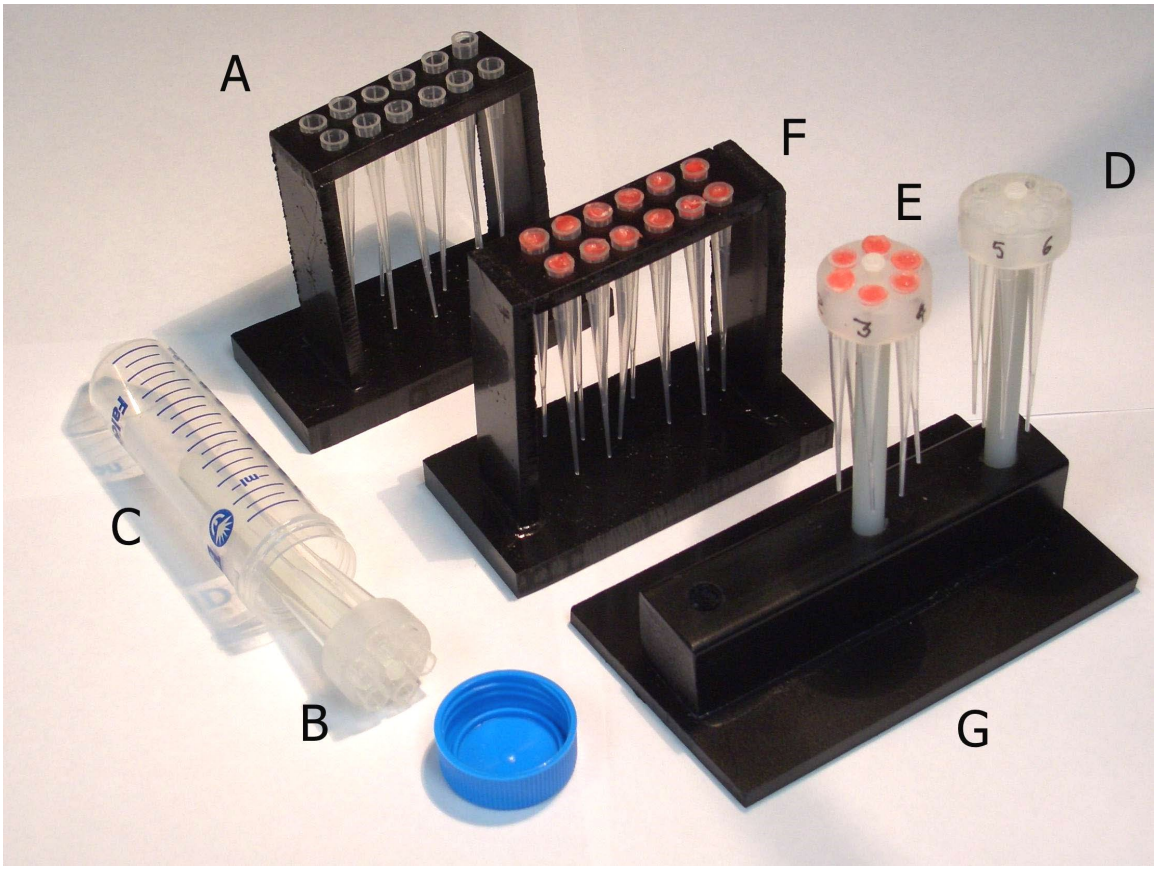


Figure 46. Holders for Preloading Microbanding Tubes Shown in Figure 45

A. Sealed microbanding tubes on rack. B. Microbanding tubes after addition of fluorocarbon, and ready for centrifugation in tube C. D. Microbanding tubes on adapter used to centrifuge down in succession fluorocarbon, and densest portion of step gradient, followed by sample layer. E. Tubes sealed with wax closure. F. Set of tubes ready for ultracentrifugation as shown in Figure 46H.

To extrude the gradient and collect the viral zone(s), a very small microliter micropump is needed. These are unfortunately very expensive. However, a very simple solution to this problem is to move a very small sewing needle through the wax as shown in Figure 46 I-K. This movement can be controlled using an arrangement such as that shown in Figure 47. The gradient may be recovered as droplets as shown, or in smaller amounts deposited as the end of the tip touches a surface as shown in Figure 48. e that the configuration shown in Figure 45 K is ideal for electrospray mass spectrometric analysis in that the extrusion needle can be used as an electrode.

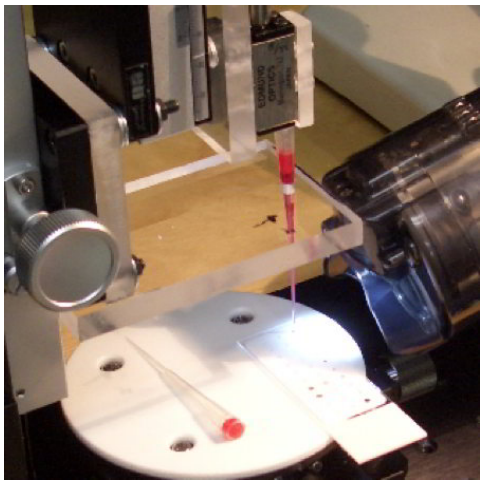


Figure 47. Spot Fraction Collection Using Teflon-Masked Slides

The XYZ movement is shown in center, the TV camera is to the right, and a second wax-sealed microbanding tube is on circular collecting plate to the left.

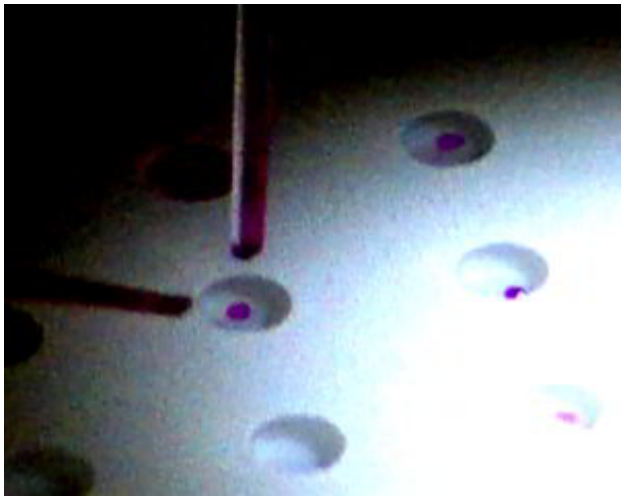


Figure 48. Extrusion of Nanoliter Volume Fractions Onto Teflon-Masked Slide

The effect of laminar flow in small capillaries on resolution using the extrusion system described is not known. An alternative was therefore developed in the form of a precise system for cutting the micro-banding capillaries as shown in Figure 49.

A capillary to be sectioned is shown in Figure 49 attached to a glass plate. Razor blade moves back and forth against capillary to section it, and also moves sideways leaving tiny sections on the blade as shown. Between cuts the blade is moved downward. Location of virus disc may be discerned under UV light.

The Z movement was controlled by hand, accounting for the differences in length of the tube sections recovered shown in Figure 50. Comparison of these two systems, extrusion vs sectioning will indicate which is superior.

Figure 49. Microtome (Viratome) for Sectioning Ultramicro-banding Tubes

Figure 50 GLP Microbanding Sections Still Attached to a Razor Blade

When the sections are placed in microfuge tubes and centrifuged at high speed, the sections float partially above the aqueous liquid, and the viruses float out and can be recovered in the solution or pelleted.

Note that the microbanding system is designed for isolating viruses from individual patient plasma or serum samples. It will be of considerable interest in pediatric

diagnostic virology. It may also be useful for virus isolation from pediatric urine samples, for example, for searching for a virus associated with Kawasaki's disease.

4.17 Density and Sedimentation Rate Calibration Beads

There have been few systematic studies on the exact banding density of different viral pathogens. Some have exact densities, others may be "pleopycnic". With proper equipment, precise measurements may be made, and the clinical usefulness of isopycnometric measurements for virus identification evaluated.

Individually visible density marker beads were first described in 1966 (40), however a wide ranging set is not, at present, available. A limited set is shown in Figure 51. Although the resolution of the gradient is very high, it may be difficult to make accurate identifying density measurements with them unless the beads are very small, which makes them difficult to handle.

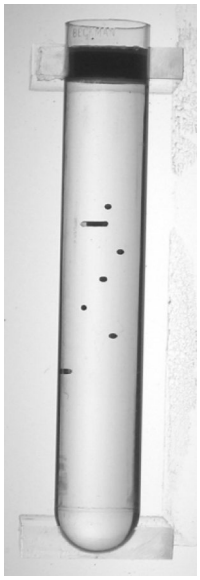


Figure 51. Density Markers in a CsCl Diffusion Gradient

Five round density marker beads in a cesium chloride gradient. Densities, from the top, are 1.1, 1.2, 1.3, 1.4, 1.5 and 1.6 g/ml. Density of bar is 1.15 g/ml. Note that beads next to the wall in either horizontal direction may be difficult to see, demonstrating the advantage of using bars in place of spheres. The density beads shown were made in the early 1970s and are no longer available. Note the presence of a wax plug closure at the top.

This suggests that they should be made as cheaply as possible, be available in sets, and be disposable. Round density beads may block the pinhole used to recover the gradient, suggesting that they be made as short rods like the one shown for one marker in Figure 51.

In prototype contract studies we have had synthesized fluorescent polystyrene latex beads of viral dimensions, using various ratios of styrene (ρ = 0.909 g/ml and bromostyrene (ρ = 1.46 g/ml) to make the markers shown in Figure 52. We plan to extend this work to also make sedimentation rate calibration beads.

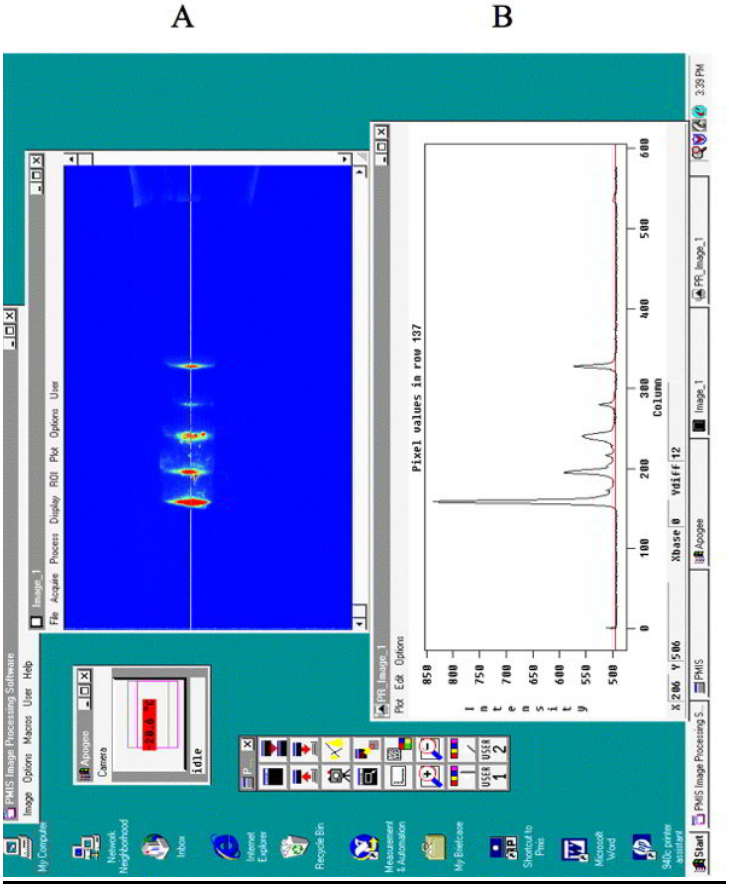


Figure 52 Banding of Density Indicating Beads in Iodixanol-CsCl Gradient.

A. Microbanding tube (left blue image) showing fluorescence of density markers. Densities of beads are, from the top: 1.073, 1.114, 1.188, 1.373, and 1.53 g/mL.

B. Corresponding strip chart recording of fluorescence. Note that the image has been rotated so that the tube is vertical.

Accurate determination of viral physical density may be very useful for rapid preliminary virus identification. As shown in a subsequent Figure 52 differences as little as $\pm$ 0.01 g/cc are readily detected. Thus for viruses that are not pleomorphic, we estimate that over 40 different viruses could, in theory, be distinguished from each other on the basis of banding density alone.

A set of density beads ranging from 1.019 to 1.188 g/cc is available from GE Health care, and beads ranging from 1.00 to 1.12 is supplied by Cospheric Inc. the latter are hydrophobic and require the addition of a detergent to prevent aggregation.

A variety of methods are available to functionalize latex particle surfaces to make them more hydrophilic (43).

4.18 Multiple-Parallel Microbanding Rotor

When small numbers of samples are involved, microbanding tubes are sealed with wax then centrifuged at high speed in swinging bucket rotors.

To process larger numbers of tubes, these may be arranged in sets as shown in figure 53, and then are placed in large zonal rotors partially filled with water. This allows them to reorient to a horizontal configuration on acceleration and back to a vertical one after deceleration. This process is illustrated diagrammatically in a B-15 rotor in Figure 53. Using a K type rotor, which has a volume of 8 liters, much larger numbers of micro-banding tubes (or even larger ultracentrifuge tubes) may be run.

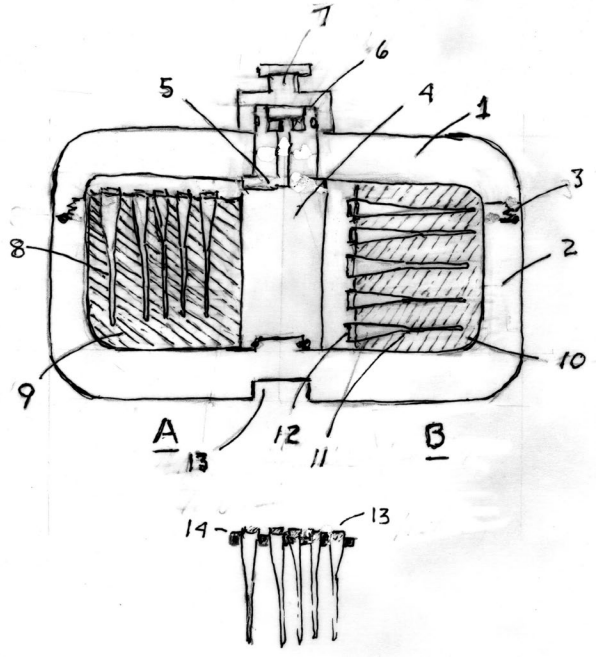


Figure 53. Reorienting Micro-banding Tubes in Ti-15 Zonal Rotor.

1. Upper cap of rotor. 2. Lower section. 3. Buttress threads. 4. Rotor core. 5. Fluid line to rotor center. 6. Lower section of rotating seal. 7. Upper cap attached during rotation. 8. Micro-banding tubes in vertical position at rest. 9. Supporting fluid. 10. Supporting fluid at speed. 11. Microbanding tubes in horizontal position at speed. 12. Sealed top of tubes. 13. Microbanding tubes in numbered holder 14. View A is at rest, View B is at speed.

4.19 Sterilization with Ionizing Radiation

Chemically inactivated virions can mediate a humoral response, the while radiation killed viruses generally can mediate both the a humoral and a cell mediated response (44). Surprisingly, this type of response can also produce cross-protective immunity. Chemically inactivated influenza virus produces strain protective immunity,

while gamma ray killed influenza of almost any strain may produces cross-protective immunity against other flu strains (45, 46).

Thus in this project, we have the amazing good fortune of having selected ionizing radiation inactivation, only to discover that such inactivation gives a much improved vaccine, which can be prepared and stored in a frozen state, and which can be given intra-nasally in the instances where this has been tried, and which can yield cross protection against different viral strains (47).

Therefore, a major part of this effort will be expended in developing radiation facilities that can be widely used, and which can deliver doses of up to 50 kGy range in a reasonable time.

In all the work described here biological safety is a central concern. At a very early stage in all processes involving killed vaccines, all samples can be sterilized using ionizing radiation, including gamma rays, x-rays, or electron beam radiation. Standardization on ionizing radiation has the advantage of sterilizing both large and small objects, of being physically standardized, and of killing viruses in small cracks. Note that the effects of thermal and ionizing radiation induced inactivation are synergistic (48).

Complete sterilization without appreciable loss of immunogenicity has been reported for mouse brain rabies vaccine using an electron beam and a dose of 8.5 x 10^5 reps (49) (50) . Purified Venezuelan equine encephalitis tissue culture vaccine has been inactivated with Cobalt 60 gamma rays 6 x 10^6 r, and antigenicity was retained (51, 52).

Isolated DNA from pMC1 plasmids was found to give 5 X 10^{-7} double strand breaks/nucleotide/rad with ^{60}Co irradiation (53). While ss or ds DNA compressed into viruses may have a different radiation sensitivity, radiation required to inactivate viruses may be found to give oligos in the same length range required for long-read sequencing, eliminating the requirement for shearing. Experimental studies are required to find out if this is true.

It may prove possible to routinely fully irradiate cadavers before autopsy when death is due to a highly infectious agent, making post-irradiation body handling safe (54). This may prove to be a useful source of live vaccines. Note that there is a precedent for making vaccines from human sources. HeptaVax, Merck's original commercial hepatitis B vaccine was prepared from human serum using K-II centrifuges.

Thus radiation inactivation may yield material safe to use without containment and may simultaneously also yield killed vaccines that can be used directly, without chemical inactivation.

4.20 Optical Virus Counting

Rapid biophysical methods for detecting and/or counting any and all viruses over a wide dynamic range are essential to this project.

Individual fluorescently stained virions may be counted by epifluorescence microscopy, and the viral load in sea water is routinely measured in this way on inorganic filters (11, 55-57). This method has not been gotten to work directly and routinely on serum to our knowledge. However, using the purification methods described here, counting virions from serum on filters, as done for sea water, may be possible, and should be explored.

This work is based on the assumption that all viruses are permeable to nucleic acid-specific fluorescent dyes. Some viruses, however, such as polio, may be less permeable than others (58).

Various fluorescent dyes have been reported to be differentially bound to ssDNA, ds DNA, ss RNA, and ds RNA, but these putative properties have not been quantitatively explored with intact viruses. The recently developed fluorescent dye specific for quadraplex DNA may give insights into DNA packing in DNA viruses (59).

The objective here is to both purify and concentrate viruses to provide very small samples that are sufficiently pure to count directly by either epifluorescence or electron microscopy. Figure 54 shows a prototype closed system in which virions are sedimented out of serum through a gradient including zones containing enzymes and/or other reagents, though wash zones, coming to rest on an activated glass cover slip supported by a flat sapphire disk, both supported on a Delrin® plastic plug denser than any liquid in the system. Sedimented virions become covalently bound to the glass surface.

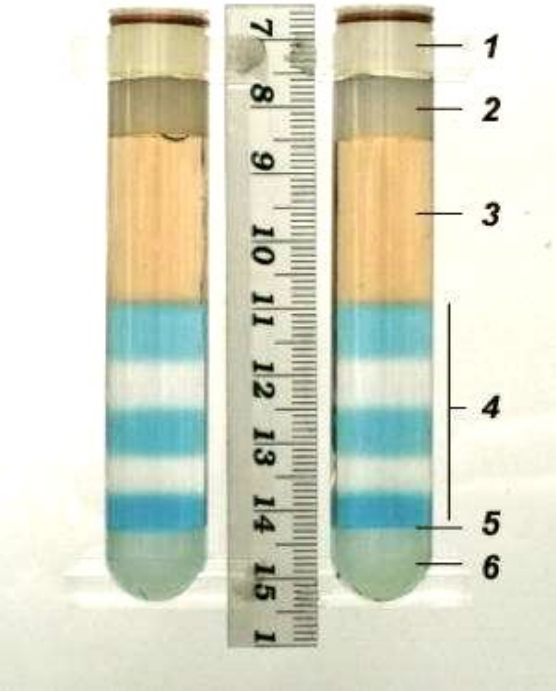


Figure 54. Virion Counting (ViCount) Tube.

1. Polypropylene closure which floats on gradient, and has a Nylon® removable screw for both introducing a step gradient, and for removing gradient after centrifugation. 2. Float for making step gradient. 3. Serum sample. 4. Five step gradient including zones containing lytic enzymes, wash zones, and a zone containing glutaraldehyde. 5. Glass cover slip activated with aminopropylsilane and supported by a sapphire disc, 6. Delrin® support.

Alternatively, the aminopropylsilane on the glass surface may be preactivated with glutaraldehyde so that sedimenting viruses are bound directly to the surface as they contact it.

This design provides a complete system which may be sterilized before use, and re-sterilized externally after centrifugation. Gradients may be preformed by adding steps one at a time using the float 2 included in the system. Sedimenting virus may optionally be focused down to a small area using a tapered insert, thus greatly increasing the sensitivity of the system.

Lambda phage and bacteria (included for calibration purposes) covalently attached to an activated glass cover slip are shown in Figure 55. Virions in these images may be counted using open source program Image J. The apparent size of the virions on the micrograph reflect the optical circle of confusion and pixel size in the camera used. Note that individual virions are detected. The detection range for viruses therefore is, in theory, down to one virion, and ten or more in actual practice depending on the concentration system used.

The challenge here, and in subsequent micro-banding studies, is to detect viruses by fluorescence over the same range as is encompassed by PCR but without the requirement for special reagents or thermocycling.

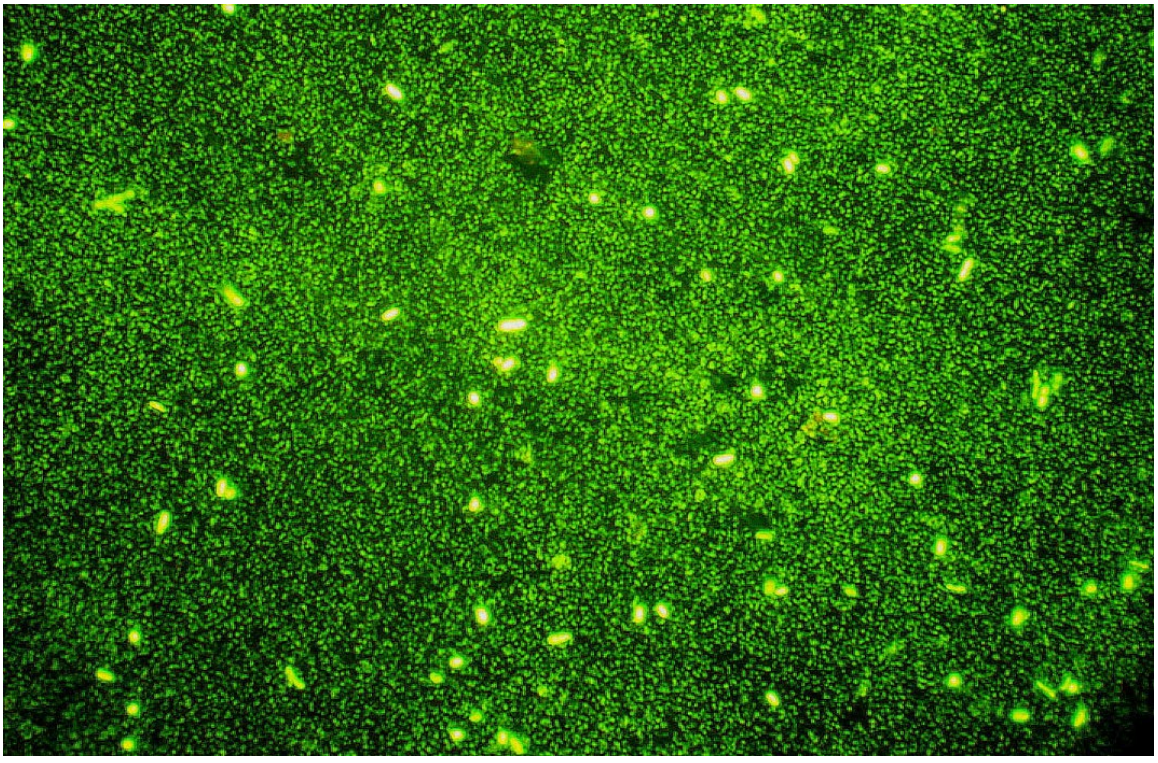


Figure 55. Fluorescently Labelled Lambda Phage Covalently Attached to a Glass Cover Slip.

Small particles are phage. Stained killed bacteria (larger particles) provide an internal standard for counting. Virions and cells stained with Sybr Gold. Centrifuged 2 hours at 41,000 rpm (288,000 x gmax) in a SW 41 Ti rotor.

4.21 Reference Standard Virus Samples

For the systematic development of methods for isolating and characterizing viruses it is essential to have readily available frozen aliquots of known viruses in known concentrations. For initial studies the following have were prepared under contract:

1. Lambda phage (dsDNA) 1.5×10^{10} pfu/ml,
2. M13 phage (ssDNA) 1.0×10^{10} pfu/ml
3. MS2 phage (ssRNA) 3×10^{10} pfu/ml
4. Phi 6 (dsRNA) 1.36×10^{9} pfu/ml

This includes ss and ds DNA and ss and ds RNA. This list needs to be extended, and standardized in collaboration with the National Institute of Standards and Technology.

4.22 Mass Spectrometric Analysis of Viruses

Nearly all virions contain genes coding for proteins in the mass range ideally suited for identification in their intact state by either MALDI or electrospray mass spectrometric analysis, and all larger viral proteins can be more completely analyzed after tryptic digestion. The speed with which this can be done is impressive (60). Numerous examples of MS analysis of individual viruses have been published (61-66) , and tobacco mosaic virus is shown in Figure 56 as an example.

While MS is a necessary and obvious component of this project, clinical advances in this field have been impeded by the lack of methods for isolating viruses in a high state of purity, absence of reasonably complete reference data base, the dangers of working with infectious materials that can survive passage through a mass spectrometer, and the relative unavailability of purified viruses of many types for use in the development of systems and procedures. Few mass spectrometrists desire to prepare and isolate viruses, to determine their purity, and then handle them in an infectious state, especially since live virion have been recovered from MS detectors. One answer is the inactivate all samples with gamma radiation before examination by MS or sequencing.

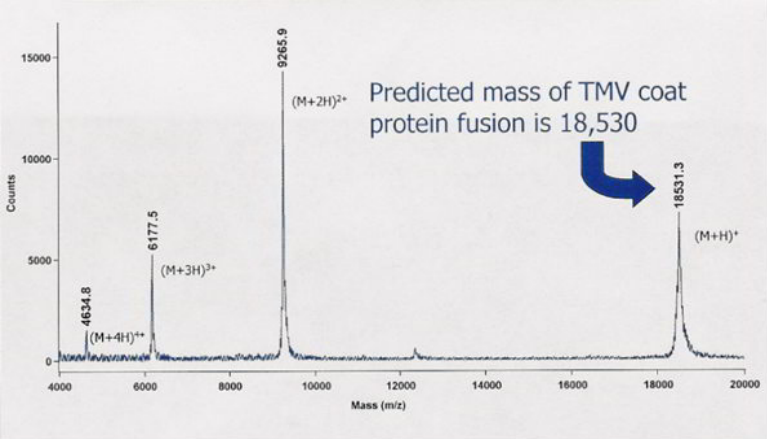


Figure 56. Mass Spectrometric Analysis of Tobacco Mosaic Virus Subunits

Given the extraordinary precision, sensitivity and speed of mass spectrometric analyses, there is every reason to believe that this technology will in the future play a central role in virus detection and isolation.

It is probable that sterilization by ionizing radiation will not materially affect MS analysis. Should this be found to be true experimentally, mass spectrometry will assume a very large role in quantitative virology.

4.23 Electron Microscopic Identification of Viruses

While an extensive literature on virus detection and counting by electron microscopy exists (67-69), the use of EM in clinical virology is not routine , but is quite common in veterinary virology (70). For example, in a series of more than 1800 cases of suspected poultry enteritis virus (PEV), and over 1500 were positive for one or more viruses (71). Examples of the types of images acquired are shown in Figure 57.

EM studies on virus-infected tissues often leave the impression that high numbers (>100) virions may be present per cell. At 80,000 x magnification 20 x 25 cm images of 25 nm thick tissue sections represent ~2 x 10^{-12} grams of tissue. Assuming 3 x 10^{8} cells per gram, one cell would weigh ~0.33 x 10^{-8} g, and ~1,700 images are required to image one cell. If there is one virion per image, there would be ~5.3 x 10^{12} virions per gram, which, for an average virus weighing 0.4 x 10^{-15} grams would be 2.1 mg, or 140 doses/gm or 149,000 doses/kg. This supports the general view that viral masses sufficient for large- scale vaccine production can be found in the tissues of available large animals and humans. Computerized EM tomography will allow more accurate measurements of viral load in tissues to be made (72, 73).

Relatively pure virus suspensions can be counted by sedimenting them against electron microcopy grids and examining them by EM. The lower limit for counting has been ~10^{5} virions per ml (74-77).

For electron microscopy to become routine, a large library of images of know viruses should be prepared under very standard conditions. Then each new isolate can be compared with library images, and, using image analysis software, an identification can be made, together with an estimate of the probability that the identification is correct.

All techniques for virus concentration described here can be interfaced with the stable isotope standards and capture by anti-peptide antibodies (SISCAPA) method for trace protein analysis (78). This method can be multiplexed to detect up to fifty different viruses in one sample.

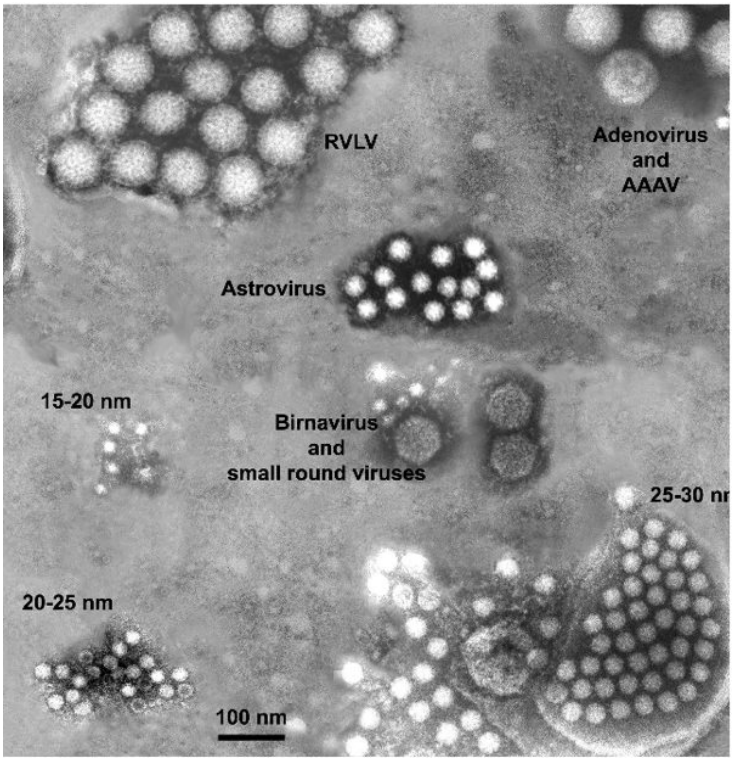


Figure 57. Montage of viruses observed by negative stain EM in cases of suspected poultry enteritis. (From Woodcock, PR, and Shivaprasad, Avian Dis 2008. 52: 209-213, with permission.)

In practice individual patient serum, plasma, or urine samples would be centrifuged in gradient micro-banding tubes which included nucleic-acid-specific fluorescent dyes. Information on viral mass and type of nucleic acid present would be obtained by fluorescence measurements of the viral mass extruded for both EM characterization, and epifluorescence counting, and then subjected to MS analysis, possibly after enzymatic digestion.

Determination of isopycnic banding density would provide addition data for identification. On the basis of these data, a decision whether or not to sequence can be made. The initial objective is to decide very rapidly between bacterial and viral infections, and to choose the optimal therapy as close to initial patient presentation as possible.

4.24 Aggregation of Viruses

Viruses tend to aggregate, as is illustrated by the fact that they can be crystallized. In nearly all studies on virus isolation aggregation has been found to increase with time and as purity increases (79). From the first studies comparing virus titers with viral mass, aggregation has been a problem, but there has been little systematic work on methods for eliminating it. The recently introduced NanoSight system for detecting and sizing virus-size particles should solve the problem of measuring aggregation rates as a function of time and experimental conditions. It may be found that one or more proteins must be included at all stages in purification to prevent aggregation. Alternatively, polymers with alternating hydrophobic and charged sections, such as is the case with partially hydrolyzed polyvinyl pyrolidone, may be useful.

Aggregation is always a function of time. Hence all processes involved in a virus-isolation technology must be carried out with as little time delay between them as possible.

4.25 Alternative Rapid Routine Point-of-Care Immunochemical Virus Identification

To this point we have been concerned with methods which are applicable to all viruses, known and unknown. Of immediate clinical concern, however, is in detecting and quantitating known viruses.

When viruses are present in a sufficiently concentrated form and have been fluorescently labeled, the possibility exists of very rapidly identifying them by bringing each and every one of them in contact with an array of immobilized antibodies. This requires that the viruses are known, and that one has a set of high affinity antibodies available.

A limiting factor soon becomes evident, however, with is that viruses diffuse slowly, and one therefore needs some way of moving the virions across separate dots or stripes of immobilized antibody. To be efficient, each virion must roll across, and be in contact with, each different type of immobilized antibody until it reaches the one to which it is bound.

The challenge is to combine, in one ready-to-go system, centrifugal gradient virus isolation, staining of virions with nucleic-acid-specific florescent dyes, and concentration of the virus into a very small stream that moves, under centrifugal force, past a slanting surface having bands of specific immobilized antibodies. This slanting capturing surface is then scanned to locate virions attached to individual bands. The entire process is estimated to require two hours.

The capturing surface could be glass, since the chemistry of binding antibodies and other proteins to it is well worked out. However, this surface is curved in use, and may be flattened out when removed for scanning. Fortunately, efficient commercial methods for coating plastic surfaces with a vey thin glass film have been worked out which leaves them very flexible. Printing antibodies on such surfaces and scanning them while flat is therefore feasible (80) . It is estimated that this method will have a dynamic range of at least six orders of magnitude. We have called this system “La Scala” i.e. the stairs). The system is shown diagrammatically in Figures 58 and 59.

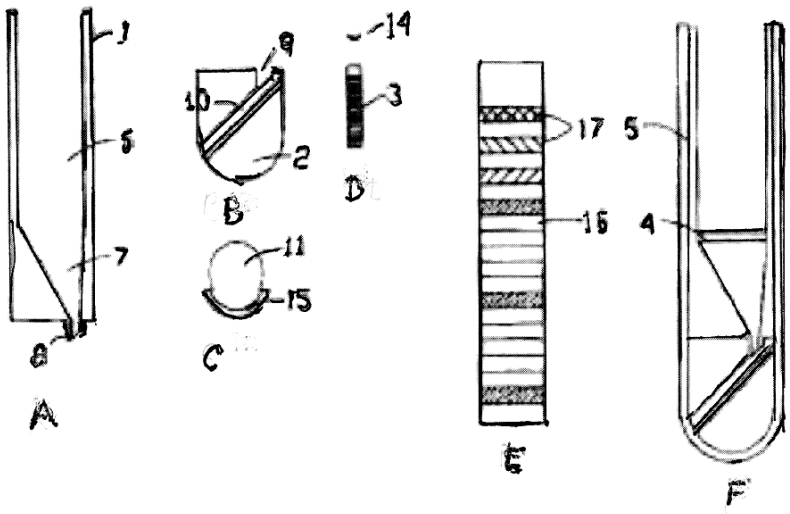


Figure 58. Point-of-Care Multiplexed Centrifugal Virus Isolation and Identification System

1.Upper plastic separable unit A. 2. Lower portion of same unit holding slanted concentration funnel B. 3. Identification strip with horizontal antibody bands C, D, and E. 4. Floating light plastic barrier which allows sample to be introduced without disturbing zone underneath but which floats up during centrifugation. 6. Upper section holding funnel 7. Funnel. 8. Nipple fitting hole 9 in lower section. 10. Slanted capture surface in place.11. Tubular region holding capture strip. 15. Curved capture surface. 17. Immobilized capture bands.

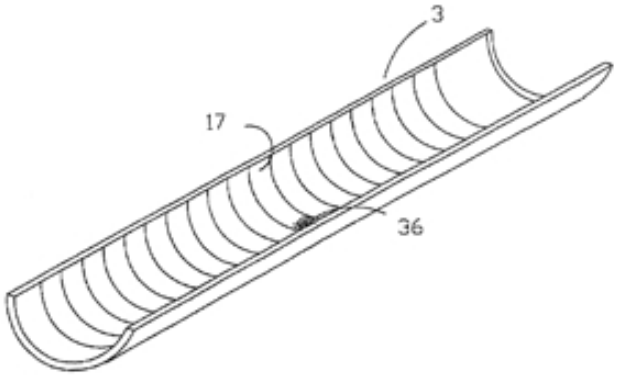


Figure 59. Capture Strip in Curved Form as Used.

3. Capture surface. 17. Immobilized antibody zones. 36. Immobilized fluorescently stained virions. Note that in this configuration the virions slide down the capture surface in a very narrow stream thus concentrating the virus into a narrow line.

4.25 Single Molecule Nucleic Acid Sequencing

Nucleic acid sequencing is undergoing a revolution. Illumina, Complete Genomics, Roche'454 Life Science, and Pacific Biosciences have developed multiple-

parallel sequencers that operate on somewhat different principles, but all use optical readouts. The recently announced Personal Genome Machine (PGM) from Life Technologies detects nucleotide incorporation electrochemically. The heart of the system is a semiconductor chip studded with over one million wells.

Working on a different principle, Oxford Nanopore Technologies does "strand sequencing" by pulling individual molecules through molecular dimension. Electron microscopy of DNA stained with heavy atoms is also being explored.

All of these contribute to mass screening for human viral pathogens as described above and will make it an almost 'real time' effort.

These is, as a result of work described here, a new niche in sequencing, which is rapid and complete sequencing of individual samples which, while desiring long reads, the aim is to quickly sequence only a small number of viral genomes.

It is evident that, especially with microbanding extrusion technology described here, one has a means for rapidly preparing viral DNA or RNA for sequencing using single molecule nucleic acid sequencers such have been recently described (42, 81-84). The challenge provided by single molecule sequencing is to match the sample preparation technology to new sequencers now available so the very small viral mixtures may be sequenced.

4.26 Shearing and Preparing Nucleic Acids for Sequencing

For single molecule sequencers the amount of nucleic acid required for actual sequencing is very small, and, when the read length is very long, it pays to develop means for shearing molecules into matching lengths. The PacBio RS system, for example, is designed to produce read lengths of over 1,000 bases (with instances over 10,000) from ~75,000 single molecules in parallel.

This raises the interesting problem of developing shearing or other fragmentation method that gives sequences longer than those gotten by current shearing devices.

We propose exploration of two method. The first is a new piezo motor driven device that gives a constant and controllable rate of shear in an entire sample micro-volume.

The second, is to explore radiation-induced fragmentation to determine whether it can provide both sterility and controlled fragmentation at the same time.

The first challenge is to determine experimentally how many viral genomes are required reliable data. Complete sequencing with very small numbers of virions would be extremely useful in pediatric studies, and in those viral diseases where the virus may be almost completely absent from blood.

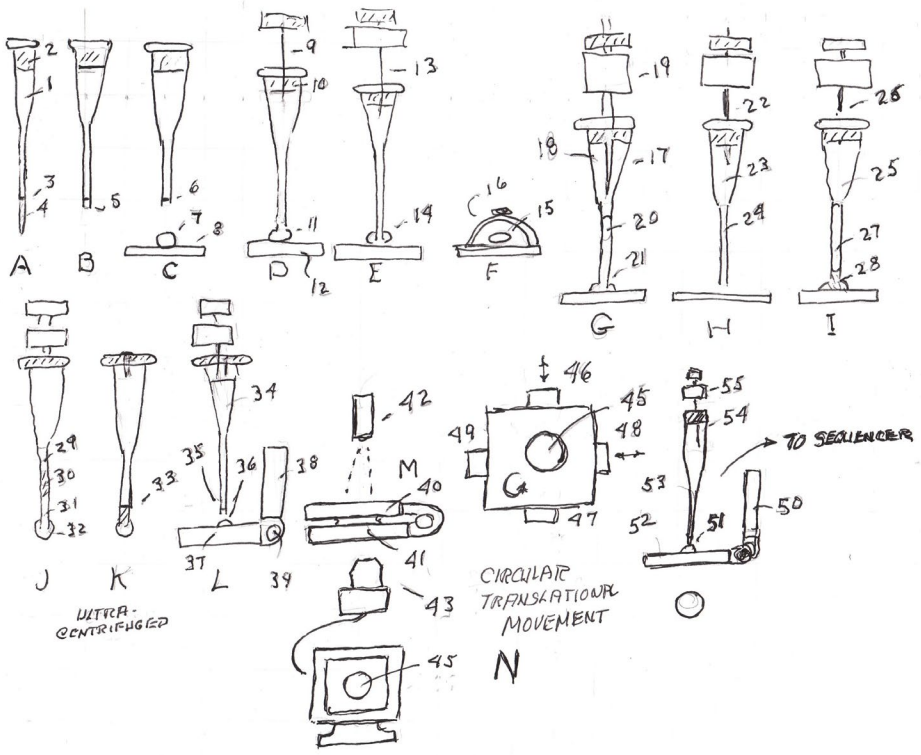


Figure 60. Shearing of Viral Nucleic Acids for Sequencing

The processing system proposed is illustrated in Figure 60 in which the virus is pelleted in a modified gel-loading pipet or other microbanding tube (MBT) A1, is sealed at the top with wax or with synthetic cork-like composites 2, which pipet contains virus 3 pelleted against a fluorocarbon cushion 4. In B the tip of the tube has been cut off 5 below the pellet. No liquid flows out because the tube is sealed at the top. In C the MBT is positioned so that pellet 6 is over a drop 7 of lysis reagent on a hydrophobic glass 8. The drop may be held in position by a small dot of untreated glass or other hydrophilic material. In D a thin needle 9 is driven through closure 10 to extrude the fluorocarbon and then the virus pellet into lysis drop 11 on slide 12. As shown in E, the droplet 14 on the slide may be may be alternately sucked into the MBT and extruded from it using needle 13 to be sure the all the virus is on the slide and the suspension is well mixed. The MBT in F may then be removed, and the droplet 15 covered as shown 16. The lytic reaction frees the nucleic acids and dissolves the proteins. To precipitate the nucleic acids in the drop K it is now drawn up into a new MBT containing methanol or ethanol.

To do this, as shown in G, a fresh MBT 17 is prefilled with alcohol 18, and sealed with wax or a synthetic cork or rubber stopper. A small amount of alcohol expelled using needle 19, and alcohol meniscus is then withdrawn part way up into the capillary section to give an air bubble 20 in the distal end or the capillary. As shown in G, the reaction

drop is then aspirated completely into the capillary 21 pulling it up into the alcohol. The alcohol is then mixed with the drop either by shaking or moving the needle up and down. This produces the condition shown in H in which needle 22 moves alcohol 23 up, leaving a small air bubble 24 in the capillary.

The MBT is then positioned over a drop of a dense fluorocarbon as shown in I, and the fluorocarbon aspirated into the MBT a short distance followed by an air bubble next to the tip (not shown in I). The condition is then that of J with the MBT largely filled with alcohol + nucleic acid over an air bubble 29, atop fluorocarbon 30, above a second air bubble 31, and ending in a tip which is then heat-sealed, and over-sealed with molten polypropylene or polyethylene 32, if necessary.

The needle extrusion apparatus is then removed, the residual hole filled with a small screw-like plastic fitting, and ultracentrifuged to pack the nucleic acids against the fluorocarbon 33 as shown in K.

To shear the nucleic acid precipitated by the alcohol, the MBT is reassembled as a needle-driven syringe a shown in L with the alcohol 34 over the nucleic acid pellet 35, and the pellet extruded into droplet 36, which may be water or dilute buffer, which is on a heavy piece of glass 37 which is, in turn, attached to piezoelectric motors as described below. Glass 37 is attached to cover plate 38 which folds down using hinge 39 to create the condition shown in M. In M, cover plate 40 squashes droplet 41 into a circle of controlled or fixed thickness. The droplet may be illuminated from above by lamp 42 and viewed by electronic microscope camera 43 and displayed as a circle on screen 45. The size of the droplet may be measure on this screen, and, knowing the thickness, the volume may be readily calculated.

In this arrangement N the DNA or RNA may be sheared using the piezoelectric motors 46-49 (so called squiggle motors) may be attached to the sides or the corners. These are phased to drive the upper glass plate with a circular translational motion. Thus every molecule in the sample is identically and continuously sheared, assuming uniform vertical distribution of shear. This assures that all nucleic acid molecules are treated reproducibility. The glass plates are fluorinated except, as shown in O for a tiny spot 51 in the center of one, preferably the bottom, plate 52 which is hydrophilic. When shearing is complete, the shearing cassette O opened, and the droplet retracts to the center of the hydrophilic spot. With careful opening all the liquid volume is thus in one droplet on the lower plate which can be drawn up into capillary 53 of another new MBT using piston needle 25 and transferred to the sequencer.

The shear rate for a fluid flowing between two parallel plates, one moving at a constant speed and the other one stationary, is defined by:

$$\gamma = \nu/h \text{ where:}$$

γ = The shear rate, measured in reciprocal seconds

ν = The velocity of the moving plate, measured in meters per second

h = The distance between the two parallel plates, measured in meters

For the simple case, it is just a gradient of velocity in a flowing material. The SI unit of measurement for shear rate is sec^{-1}, expressed as "reciprocal seconds" or "inverse seconds."

Since 1 ul = 1 mm^3,

$v = \pi r^2 h$, for 1 ul this gives a circle 3.56 mm in diameter and 0.1 mm thick.

We assume that shear length is a function of rate of shear, initial oligo length, time, and the solutions in which the oligos are sheared. If we are to study these quantitatively, we need methods for accurately describing each. . Counterions and pH affect nucleic acid structure in solution, and the effects of these on sheared length under controlled conditions should be determined.

We do not know the ideal target length, but we will assume that it is one kilobase, where the read length is ~700 bases. One base pair = 3.4 Å = 0.34 nm

Assuming that the target length is 1,000 base pairs, this would be 340 nm or 0.34 um, or .34 % of the depth calculated above.

Between two surfaces as close as these, we assume the shear is uniform through the thickness (depth). Using gel electrophoresis, a plot of the sheared length as a function of all the variable, can be determined. This could reduce this part of the sequencing process to a relatively exact science. As read length become longer, and as smaller and smaller samples are to be sequenced, it becomes important to be able to study, control, and use methods such as described here to explore the lower concentration limits of sequencing.

Note that as the read length becomes longer, it will be important to do shearing to produce longer fragments.

4.26 Isolating Very Small Numbers of Virions and Macromolecules

Beyond microbanding, there are few methods for isolating, characterizing, and transferring a very few oligonucleotides in the thousand base pair range or protein molecules. One is provided by isopycnometric methods in which individual molecules may be attached to very small latex particles, and then separated in banding gradients.

The only published method for this is shown in Figure 61 in which immunoglobulin molecules were “weighed”(85). As few as 100 molecules could be weighed, and that increment, if due to oligonucleotides, would be in the range of 250 base pairs. Hence the system could isolate molecules in the kilo base range. This would match the oligo length to the read length of the sequencer.

A variety of methods are available to attaching oligos to latex beads, and recovering and transferring them intact (86).

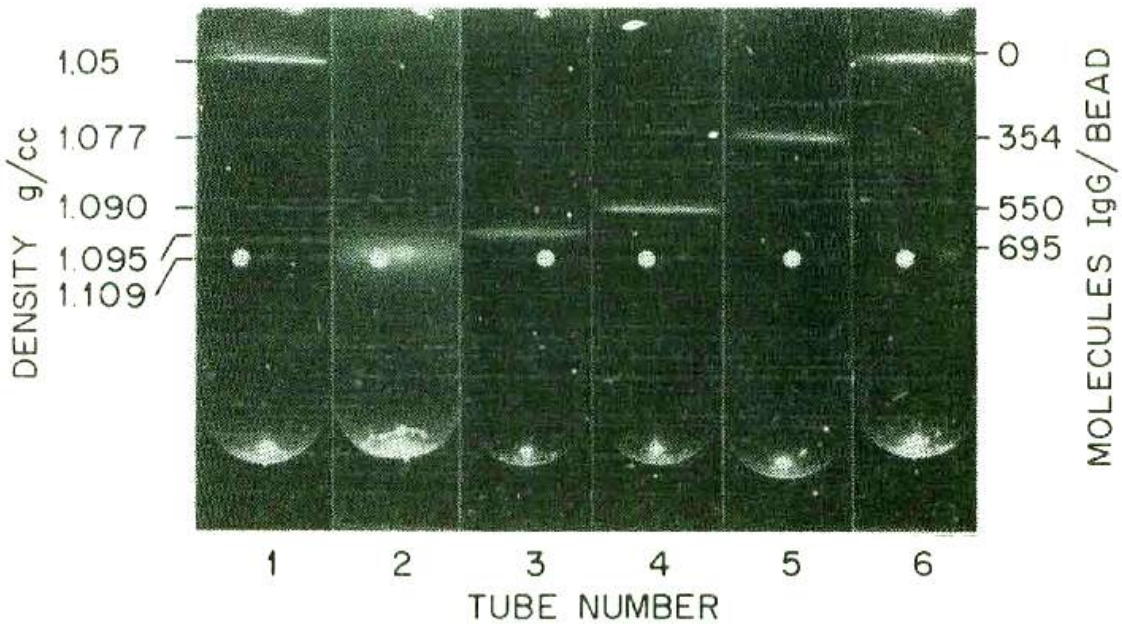


Figure 61. Banding of Polystyrene Latex Beads at Different Densities in a Sucrose as a Function of the Number of IgG Molecules Attached to Each Bead.

Figure 61. *Photograph of six gradients showing banded latex and large reference bead which bands at 1.109 g/cc (identical reference bead was use in each tube). Tube No.1 contains clean beads banding close to 1.05 g/cc, while the remainder contain 0.109 um latex beads incubated for 30 minutes at 56^0C in tenfold dilutions of a solution containing 2,500 ug human IgG (tube 2) and ranging down to 0.25 ug .ml (tube 5). The banding densities and number of IgG molecules bound were respectively for tubes 2-6: 1.099, 695; 1.095, 629; 1.090, 547; 1077, 354; and 1.053 with 31 molecules.*

Round large beads are reference beads having a density of 1.109 g/cc.

This method may provide a useful method for both measuring and handling large oligonucleotides, proteins and viruses, and was used to detect T2 and T4 phage.

Most viruses fall in a size range extending from ~20-300 nm (0.020-0.30 um) while the smallest polystyrene size standards for extend down to 30 um. Viral pathogens range in isopycnic banding density from ~1.12-1.55 g/cc

For use in virus detection and characterization, one would like carrier particles that are either heavier or lighter than all known human viral pathogens. Figure 61 suggests that polystyrene latex particles lightly coated with antiviral antibodies can serve this purpose.

However, synthetic latex particles a generally much larger than viruses.

The alternative is to find carrier particles that a much smaller than synthetic ones. As shown in Figure 18 above, rat liver glycogen exists in S-rho plots as a band extending from <50 S to 4,000 S, but having a very narrow range of isopycnic banding densities that extend from 1.62-1.65 g/cc.

Using zonal centrifuges preparative fractionation of glycogen to yield homogeneous fraction is feasible, and antibodies are readily attached to these. To recover trace amounts of virus from relatively large volumes, carrier glycogen is added to that

volume with the density adjusted to be equal to that of the labeled glycogen and incubated to allow interaction between the carrier and any target virus. A density gradient is then pumped into a zonal rotor, light end first, leaving the carrier glycogen and sample at the rotor edge in a high centrifugal field. Glycogen-virus complexes then move centripetally, and concentrated in so doing, and are detected by fluorescence.

4.27 Future Direction in Rotor Development

Microbanding ultracentrifugation is illustrated in Figures 62, 63 and 64.

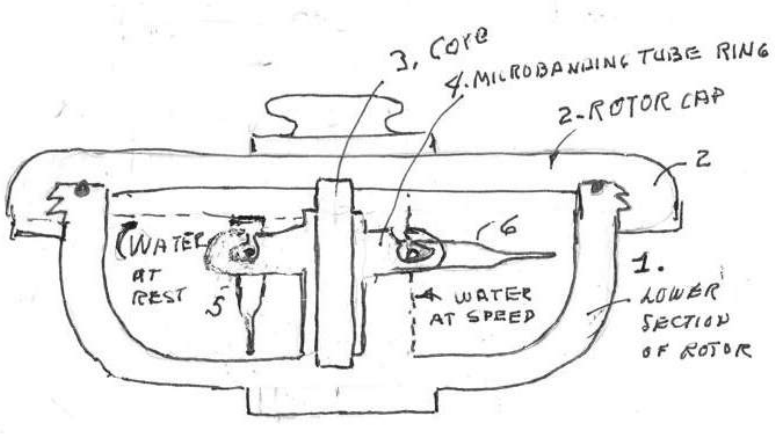


Figure 62. Microbanding in Floating Swinging Buckets in a Zonal Rotor Housing

The basic concept, shown in Figure 62 is to use reorientation of an aqueous liquid in a hollow-bowl rotor, having a lower section 1 and cap 2, such as is used in zonal centrifugation. A central core 3 supports microbanding tube-holding ring 4 which supports the set of microbanding\ tubes. This is usually the same ring shown in Figure 63. The rotor is filled with water or a dilute aqueous solution shown 5, and reorients to the horizontal tube position 6 at speed, and the water with a vertical meniscus as shown. This provides a floating swinging bucket configuration without the necessity for either strong metal buckets, or strong plastic tubes.

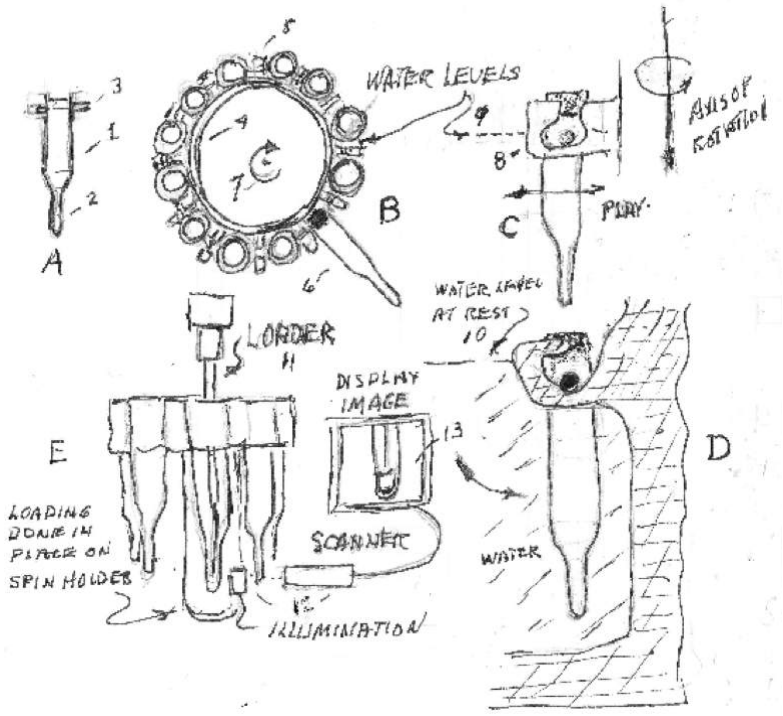


Figure 63. Microbanding in Floating Swinging Buckets in a Zonal Rotor Housing.

Detail of the operation of a floating swinging bucket rotor is shown in Figure 63. A plastic swinging bucket is shown in A, with sample area 1, microbanding region 2, and integral shoulder pin 3 to attach A to ring B in such a manner as to allow movement from vertical positions 5 to horizontal position 6. C and D illustrate that attachment 8 is designed to keep tube attached during reorientation. E illustrates how tubes of B can be scanned to confirm that they have been properly filled.

Unloading the tubes is shown diagrammatically in Figure 64. The support ring 1 is removed from the rotor, dried, and the viruses, when and where present, are recovered. For convenience only one tube 2 and its support 3 are shown. The ends of all tubes are cut off by a device (not shown), and no liquid flows out because the upper end of the tubes are sealed with a moldable wax.

A rod 4 is driven downward onto the wax closure, preferably by a Piezo electric motor 5 held by attachment 6. The rod deforms the wax, essentially without penetrating it, causing small droplets to pass out of tip 7 into microfuge tubes 8, aligned by a flexible connection 9 that can be bent, allowing the tubes do be inserted in order in microfuge rotor 10.

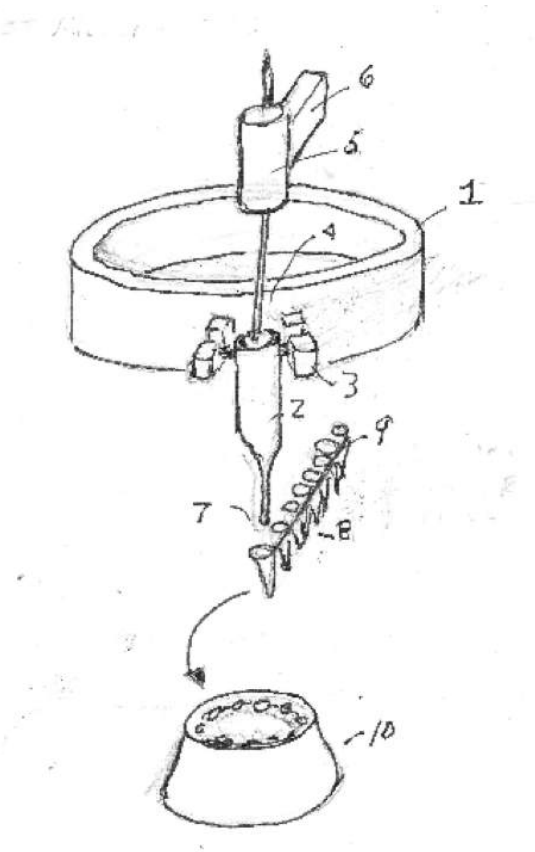


Figure 64. Unloading of Microbanding Tubes

Note that to load a small amount of a fluorocarbon, as is required in the steps of Figure 64 an ink jet type droplet source may be employed, and fluorocarbon sprayed onto an aqueous solution already in the microbanding tube, and before the first centrifugation to remove air bubbles. The fluorocarbon is thus centrifuged down into position more conveniently, and the amount of fluorocarbon dispensed with greater ease and precision. The same concept may be applied to introducing microgradient into capillaries.

4.28 "Natural" Vaccination

It is assumed that those who recover from Ebola infections are immune, and it is also assumed that those who exhibit both humoral and cell mediated immunity without having had hemorrhagic fever are also immune to challenge.

Mysteriously, when 4,349 individuals from 220 randomly selected villages in Gabon were tested for immunity to ZEBOV Ebola virus, the overall virus seroprevalence was 15.3% (87). None of these had a history of Ebola hemorrhagic fever. Additional

studies showed that cell mediated immunity was also present among the smaller number tested. The authors showed that fruit bat saliva can contain live Ebola virus and suggested that the immunizing agent was transmitted through bat-infected fruit. This leaves us with the question, how was live virus converted into such an effective killed vaccine so efficiently in nature?

Swarms of fruit bats are a common sight in Gabon. We suggest that Ebola infected fruit bats would be expected to both urinate and defecate while flying through the air. The net effect would be a continuing shower of dried particles, with the urine droplets ending up as particles with a high concentration of urea, which would inactivate virus as does simple drying.

The internal surface area of a pair of human lungs is extremely large, averaging ~ 92 m^2 (88), and is rich in dendritic cells. In a 24-hour period about 200 liters of air is sampled by one individual, and this continues for a lifetime. The immune system is, in a sense, quantized, in that single antigen presenting cells can activate many T cells, each of which can give rise to >10,000 antibody producing cells. Lifelong exposure to very low levels of dead virus could incrementally produce mass immunization.

The history of killed vaccines, starting with Pasteur, suggest that there is a high probability that such vaccines, given in multiple doses at multiple sites would provide protection, and that a full-blown effort to "make it work" should be initiated for Ebola the outset. If all vaccination fails, then use of killed virus to hyperimmunize recoverees to serve as donors of therapeutic blood and serum would still justify the effort. It would also be advantageous to boost the immunity of those who exhibit unexplained immunity. The experience of building and operating an ultra-rapid vaccine production system for Ebola would also provide a first line of defense against a pandemic caused by a new virus which may have very different properties.

For Ebola virus, and assuming a Mr of 3.8 x 10^8 , a 15 ug dose would contain 2.3 x 10^{10} virions. The first task with the system described here would be to determine the viral load in all infected tissues available, a task that can be readily and safely done using irradiated samples. With such data viral loads can be described in terms of doses that could be prepared.

5.1 Containment, Automation, and Robotics

Biocontainment was essential for early stages of this work done at Oak Ridge and was therefore given special attention. In consultation with Drs. Riley D. Housewright and Howard Glassman Fort Detrick it was discovered that the technology used there, which included stainless steel Blickman Hoods, there was actually borrowed from the Oak Ridge Y12 plant where it was developed for plutonium and uranium-235 parts fabrication. Plutonium dust is extremely toxic and was also very valuable. Uranium and plutonium dust particles were recovered by washing containment interiors with nitric acid and hence these were made of stainless steel and glass. It was obvious that if we did the sterilization with ethylene oxide the containment systems could be constructed of acrylic plastic and the facility shown in Figure 65 was built.

Figure 65. Containment System in Bldg. K-703 at Oak Ridge (Circa 1963)

Containment in biomedical research and in nuclear research have followed radically different paths. In so called "hot cells" used with radioactive materials there is no intention whatsoever that human operators would or ever could entered them during operation. Hence the absolute requirement for remote, and in many cases, automated and/or robotic operation. Also, in high level radiation work, it is simple to determine what is contaminated and to what level. This is not the case with infectious agents.

In the Virome Project, only the initial pre-irradiation steps and some attempts to develop live vaccines need high containment, For irradiation of cadavers (54) and large animals a facility of the type shown in schematically in Figure 48, would suffice. It can be gas sterilized together with all that is in it, opened for walk in adjustment and repair, then sealed and operated remotely. Larger version can be built, if necessary, for doing remote autopsies and dissections. Radiation sterilization can also be considered.

None of this can be improvised on an impromptu basis and done on the fly. Nearly all of the equipment must be "purpose built". Variety of containment systems are possible, and one version is shown in Figure 66.

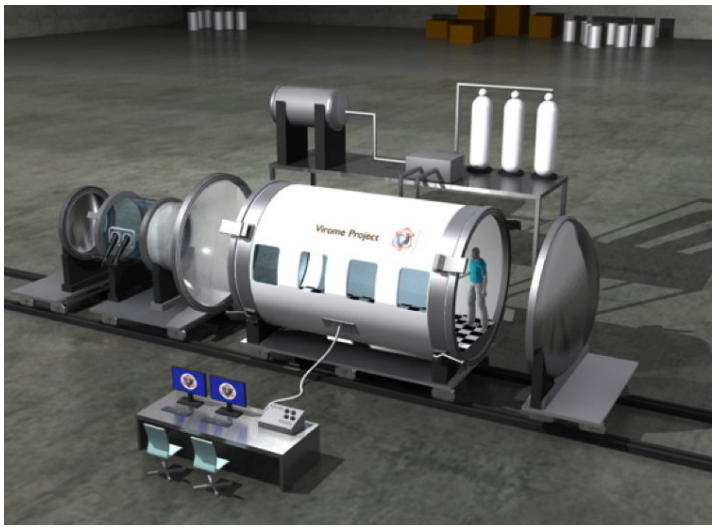


Figure 66. Proposed Remotely Operated BSL 5 System for the Virome Project.

5.2 The Problem of Mass Vaccination

The concern to this point has been with technologies and their development. We now turn to the question of how they could be organized and used in a pandemic.

Rapid universal vaccination is the overall objective, but is generally considered an impossible extrapolation. One would like, however, to have some numerical indication of just how impossible this objective is.

To vaccinating the entire world population of 7.4 billion doses with 0.5 ml vaccine doses of a live vaccine would require a volume of 3.7 million liters of vaccine - equivalent to 25 of the largest tank cars allowed on rails in the US (639,000 liters capacity), together with massive bottling facilities, an uninterrupted international cold chain, and tens of thousands of health care workers to perform the immunizations.

It is for good reasons, therefore, that no present published national or international plan offers the hope of stopping a global disaster of viral origin by rapidly developing a new and effective vaccine and by distributing it to large populations using current technologies and paradigms - all while a pandemic is in progress.

However, the true physical scale of the vaccine problem may actually be quite different as is indicated by the following calculations.

The "world dose" for several live virus vaccines in current use, such as measles, rubella and mumps, in which each 0.5 ml dose will contain around 10^3 infectious virions, would contain a total of 2.9 mg of virus for influenza (0.415×10^{-15} g/virion), 41 mg of vaccinia (6×10^{-15} g/virion), 0.64 mg of polio (0.092×10^{-15} g/virion), or 2.8 mg for a virus whose mass is the average of 52 different viruses (0.4×10^{-15}g/virion). The mass of a "word dose" for live polio would fit under one fingernail.

The mass of live virus needed to immunize the entire US population is ~3.1 mcg for a small parvovirus while for a large Rhabdovirus ~842 mcg would be required.

Since virion masses range from ~ 0.009 fg for a small Parvovirus to 1.7 fg for a large Rabdovirus, the viral mass per dose would range from 0.9 to 1,700 fg, or between $1/10{,}000^{th}$ to 1/63 of the mass one human red cell.

This is why mosquitos are so effective even though they are designed by evolution to withdraw blood rather than to inject infected saliva.

In stark contrast to the above, it would require ~4.7 kilos of virus to immunize the entire US current population with 15 mcg doses of a killed vaccine.

The ratio of the mass of virus require for a killed versus a live vaccine is, for a small Parvovirus, a factor of ~1.5 billion, and for a large Rabdovirus is ~5.5 million. These are noticeable differences.

These calculations are theoretical in the sense that not all physical virions are infectious. However, the numbers are useful for order of magnitude estimations of central vaccine problems.

This all suggests that the major objective should be to develop live vaccines as rapidly as possible.

In the chaos of an advancing pandemic, and given modern mass communication, every medical, research, production, and testing detail will be available to all on the evening news. it is essential for the public to see some visible action. And that action should be to attempt to vaccinate at least some of the health care workers immediately at risk. This can only be done with a killed vaccine.

We have described how this may be done, and how purity can be measured. Unusual side effects that would be worse than infection are difficult to imagine. While many objections may be raised, in the actual event, and given the long history of success with impure vaccines, we must conclude that whoever actually has the facilities and the skill to do it, should make and inject a high purity radiation-killed vaccine as soon as possible .

It is essential to know as rapidly as possible whether a killed vaccine can be made and whether it provides protective immunity. It is also essential to know where, in infected humans and animals, exploitable masses of virus are to be found. Using immunological assays based on isolated killed virions it is feasible to determine whether a new vaccine produces an immune response, i.e., whether a killed vaccine works.

The mass of killed immunogen per current vaccine dose is often given in arbitrary units not readily convertible to mass; however, in some cases, mass is given in micrograms. These generally range from 5-50 micrograms per dose. Taking 15 micrograms per dose as effective, and assuming that the dose was all virus, the number of virions per dose would range from ~16.6 x 10^{12} for small parvoviruses to ~8.8 x 10^{9} for rabdoviruses.

To immunize the entire US current population with 15 mcg doses of a killed vaccine would require ~ 4.7 kilos of killed virus.

The ratios of the mass of virus require for a killed versus a live vaccine is, for a small Parvovirus, a factor of ~1.5 billion, and for a large Rabdovirus is ~5.5 million.

Thus the logical strategy for virodefense, is to begin with individual-doses of killed vaccines because these can be quickly made, and then to be ready to rapidly scaled up to hundred or thousand dose levels.

This is hopefully to be followed by a smooth transitioned to a live vaccine as soon as one can be developed. However this may not occur rapidly, in which case an effort should be made to make and distribute as much killed vaccine as possible.

Using reverse genetics seed stocks for a new influenza vaccine can be made in as little as one to two weeks (89). All other types of vaccines, including subunit, DNA, and

peptide vaccines should be made in parallel in case of failure of the main strategy, even though these may not, in the end, prove necessary.

We have started with rapid vaccine production because if this cannot be done or does not work, only drastic public health efforts offer any immediate hope. However, if rapid vaccination is on the horizon, then rapid initial viral screening for discovery becomes essential. In fact, mass screening for viruses should, it is proposed, be the initial major activity of an operational Virome Project Laboratory.

5.3 Estimates of Doses Available in Vertebrate Sources

It appears that viral loads in infected patients or animals peak sharply with fever, and subsides rapidly (in most cases) as antibodies begin to appear. If viruses are to be efficiently harvested from infected sources, then one must know when and where they are present, and in the largest numbers.

Unfortunately, there have been few systematic studies on viral loads in different tissues infected with different viral pathogens, and at different times after infection. The data, which were originally obtained by infectivity measurements, are now generally gotten using PCR-based methods. These can only be used to detect and measure known viruses for which we have specific reagents. Hence the importance of physical counting methods that can be universally applied, as described in this document.

Note that PCR-based methods actually measure genome copy number and ***not*** the number of intact virions that could be potentially isolated to make killed vaccines.

At the start, it is important to ferret out as much information as we can in order to estimate the probability that rapid small-scale vaccination can actually be done.

Conventional egg grown influenza vaccines contain ~15 mcg of each of three different virus strains per dose, or ~45 mcg total. The yield per egg is a maximum of ~50 mcg so on the average one egg is required for 1-2 doses. The chorioallantoic volume is ~ 10 ml giving ~ 5 mcg/ ml. Since an influenza virion mass is ~0.415 x 10^{-15} g, the yield is ~ 1.2 x 10^{-10} grams of virus/ml. This provides a rough indication of the total virion mass we need to find in any source worth exploiting for rapid vaccine development.

Additional available fragmentary data includes the following. SARS postmortem human lung tissue can contain up to 10^9 viral copies/g (90), potentially yielding ~ 44 doses of 15 mcg each of a killed virus vaccine from a kilogram of tissue.

In human gastric cancer the mass equivalent of 1.3 killed vaccine doses of JCV per gram has been found (91). The viral loads in renal allograft tissue from patients with BK virus nephropathy can be very high, and one pair of infected kidneys may contain sufficient virus for 250 killed vaccine doses (92).

The EBV viral load in a nasal NK/T-cell lymphoma was 186 mcg/g, equal to 12 killed vaccine doses per gram (93).

In studies on a turkey coronavirus, some samples of infected intestine were reported to contain almost the equivalent of 160 doses per gram. (94). The spleen viral load in naturally occurring hanta virus infections has been reported to be as high as 0.56 x 10^{11} genome copies per mg of tissues, giving 62 killed vaccine doses per gram (95).

Pigeons naturally infected with a circovirus the Bursa of Fabricius had an estimated 189,000 KVV doses per gram of tissue, spleen contained an estimated 120,000 doses/gram, and one liter of serum would contain 120,000 doses, assuming, in all cases,

the highest titer found (96). In foot-and-mouth disease virus infected bovine tongue which was found to have 1.5 x 10^{11} per gram(97). This equals 21 ug/gm or ~1 killed virus vaccine dose per gram.

On of the highest viral loads recorded occurred in 20 day old Peking ducks on day six after being injected subcutaneously with duck virus enteritis (DVE) , and was 10^{13} virions per gram(98). Assuming a vial mass of 2.3 x 10^{-15}, this is 2.3 mg/gram of tissue, and equals 1,500 doses per gram. Assuming body weight of 160 grams, and that kidneys are 1% of body mass, this would equal ~2,400 15 mcg doses per animal.

Urine is a potential and largely overlooked source of infectious viruses. Viuria has been reported in measles, cytomegalovirus, coxsackie, vaccinia, rubella, adenoviruses, mumps, classical swine fever, hanta virus infections and West Nile virus. The time course of excretion has not been studied in detail to our knowledge for any of these viruses.

Polyomavirus and BK virus-induced nephropathy have emerged as an important cause of renal graft dysfunction. In BK virus associated nephropathy the urine concentration may reach 10^{11} virions per ml (99). BK virus has an approximate mass of = .035 x 10^{-15} g, and one vaccine dose would contain ~4.3 x 10^{11} virions which would be contained in less than 5 ml of urine.

In interstitial cystitis the urinary viral load of polyoma virus was over 10^{8} copies per ml in urine excreted over a 10 month period, giving the equivalent of one vaccine dose every three days (100).

This indicates that, given an average daily urinary volume of 1,500 ml, the ease with which urine samples may be acquired, and the existence, as described previously, of centrifuges capable of recovering viral loads from ~100 liters per day, the preparation of vaccines from urine should be the considered at the start of any epidemic and especially a pandemic. Urine has the advantage of being readily available in quantity, and filtration methods are also available for rapidly isolating viruses from it.

This all suggests, but does not prove, a high probability that sufficient viral mass for at least a few doses of a killed vaccine can be found in the blood or urine of infected febrile patients, and in autopsy material.

Variolation was the first example of what could now be called vaccination for immunizing large populations against a viral disease. In the procedure a mass of semi-liquid material was removed from the pustules of active smallpox cases and then inserted under a flap of skin, usually on the forearm, of a recipient who then had a mild case of the disease, recovered, and was then immune for life. The case mortality rate for Variola major was around 30%, but was only ~3% for those variolated. However, those undergoing variolation could readily infect others with full-blown disease, proving that unattenuated virus was present in those variolated. Thus variolating a small group of people could start a full-blown epidemic in the surrounding population. For this reason, the first response to this procedure was to ban it, and the second was to accept it, but to quarantine those being variolated.

In variolation the immunogen is believed to have contained live virus, non-infectious but otherwise complete particles, empty capsids, capsids in various stages of either assembly or digestion, peptides of viral origin, viral nucleic acids, some possibly intact virus-containing human cells, and unknown ingredients that could act as adjuvants.

It appears that this heterogeneous mass stimulates the immune system slightly ahead of massive infection.

The actual mass of virus in tissues of individuals infected with small pox is difficult to estimate, but given the pustule mass indicated in Figure 67, and its distribution all over the body, it would be expected to quite high.

Figure 67. Arm of Child Infected with Smallpox.

Visual indication of the mass of virus-infected material potentially available

There is little precise data on the viral load in tissues infected with rabies virus partly because genetic variations between slightly different strains makes PCR analyses inconsistent.

To produce immunity against a fully infectious pathogen, Pasture, Roux and colleagues first developed a rabies vaccine in 1885. It was actually a series of vaccines made from infected rabbit spinal chord dried for increasing periods of time.

In the first four following decades other methods of vaccine production were used, all employing virus infected nerve tissue as the antigen source. These employed the same theory of serial injection of increasingly virulent rabies-infected tissue.

In the 1900s Fermi (101, 102) and Semple [84] introduced chemically inactivated brain tissue vaccines and used the same vaccine for all injections. This made it possible to both test the safety and potency on larger batches, and to distribute the vaccine in vials for individual use. Reactions to basic myelin proteins were minimized by using newborn mice to grow the virus because myelinization is incomplete at birth.

In 1973 the World Health Organization recommended that these vaccines be discontinued because some batches contained residual, live fixed rabies virus.

The lesson from this work is the one stressed here, which is that in many cases it may be possible to make radiation-killed vaccines directly from infected human tissues with only minor side effects.

It must be concluded that the first priority in a viral pandemic is to find out for certain that a virus is indeed present, where and when it is present and to identify it. One has no advance notice of what the virus might be like.

From this history of vaccine development and present knowledge, it is concluded that there is a reasonable probability that in a lethal pandemic there will be tissues available that have a high enough viral load to allow vaccines to immunize health care workers immediately at risk provided that the required isolation systems are available.

While a reversion to ideas from the era of Pasteur may not be initially appealing, simple approaches that work are to be preferred to exquisite methods that come too late. Very simple radiation or chemically inactivated serum or tissue extracts must be considered *in extremis*.

5.4 General Strategy and Process for Killed Vaccine Development

The general strategy is to provide a direct path from clinical samples and patients to an injectable killed vaccine, while simultaneously acquiring sequence data to make a live one. Visible progress in doing this is essential to the maintenance of public trust and order. Note that no animal testing is envisioned as an absolute requirement before the initial human vaccination. The vaccine strategy is based on the postulates first, that no fully inactivated pure virus vaccine is toxic in itself, and second, that human serum subjected to effective radiation sterilization contains no active pathogens, and is safe to administer to humans

Hence it is safe to transfuse human viremic serum, inactivated with high doses of ionizing radiation, into any and all human recipient anywhere on the globe. This, then, can not only be the first step but also the final fall back position in case a live vaccine is not made in time. Note that one need not be concerned that the serum is free of other viruses because these would also be killed by radiation.

All of the detection, separation and purification methods are focused on providing first, sufficient viral mass to begin killed virus immunization immediately and to provide a cohort of vaccinated individuals to begin convalescent serum collection.

Eggs and mice have been traditional sources of crude vaccines, but they require considerable advance notice for large scale production. With the availability of the K-II (CC40) system it becomes feasible to isolate viruses from kilograms of tissue, which are more readily obtained from larger animals or human sources.

The pig has long been thought to be the best available model for the production of organs that might be transplanted into man, however they are normally too large and difficult to handle for research purposes. Therefore, over fifty different strains of so-called miniature swine have been developed largely with the aim of developing strains whose organs can be transplanted into man, and some have been genetically modified to reduce immuno-rejection.

Normal farm breeds grow from a birth weight of 0.5 – 1 kg to a weight of >100 kg in four months. The Hanford strain reaches the body weight of an adult human in a year, while smaller breeds weight <30 kg at the same age.

In the same period miniature pigs can grow from ~0.25 kg to 7-20 kg, depending on the strain. Laboratory methods and systems for using miniature swine are now available in many different laboratories throughout the world. Maintaining even a hundred miniature pigs is much less expensive than maintaining a large tissue culture

facility in a constant state of readiness with many different cell types.

Obviously, swine flu virus can be grown in such animals, and cascaded centrifuge systems can be designed around the K-II (CC40) to process virus-rich tissues automatically. Large homogenizers can prepare 5-10% tissue homogenates that are then run through one or more prefilters to remove connective tissue and other large particles, then through low speed continuous-flow centrifuges, of which several commercial types and available, and then through the K-II (CC40) in up to 100-liter batches, representing 5 to 10 kg of tissue.

Organ weights for the Hormel-Hanford strain of miniature swine at 196 days are: Liver 590 g, kidney 70.7 g, spleen 50.6 g, brain 98, and pancreas 39.7. This means that between ~10 – 50 animals could be processed in one run, depending on the organ used. It is difficult to estimate the scale of animal production to reach commercial requirement until virus yield is known. This points up the importance of a full-scale operating facility to discover, for example, whether pig fetuses might have a very wide range of virus susceptibilities, and reach a high viral load per gram with many known human pathogens reach in eggs.

Maximizing the yield in virus production is a new science, and an essential one for virodefense. If yield can be increased by one or two orders of magnitude by suppression of humoral responses for example, the problem of making large quantities of killed vaccines would be greatly advanced. While much is known about how viruses themselves down regulate immune responses (103), there is at present little information on how to up-regulate virus production in intact animals.

Cyclosporine A and tacrolimus inhibit virus production in vitro (104), but immunosuppression increases viruria in vivo (91), Only experimental studies on the effects of various drugs and cytokines on viral loads in various organs and body fluids of experimental animals will solve the problem of maximizing vaccine production in animals.

5.5 Live Vaccine Development with Testing and Production in Humans

A variety of methods have been described for genetically altering a virus make it function as a live vaccine. These include adding genetic material which diminishes pathogenicity, removing sections coding for virulence, or recoding the genome with synonymous codons so that the same protein gene products are formed, but on a different schedule. The latter is a blunt, direct, simple, rapid and reproducible method, but not necessarily optimal method for making a live vaccine candidate (105-107)). With extended research on a wide variety of viruses, it will doubtless be optimized. Using reverse genetics it has been suggested that vaccine seed viruses for live influenza vaccines could be prepared in a few weeks (89).

This means that live vaccines can be prepared within days or weeks after a virus is sequenced. A major advantage of this approach is that a very large number of change are made to genome, making reversion to pathological state unlikely, although research has shown that this process occurs more rapidly than originally thought (108).

Different live vaccine viruses will have different sensitivities to drying, and in all cases will be sensitive to the support medium, temperature, and humidity. It is hoped that by the time a killed vaccine has been administered, and is found to produce virus-specific antibodies, a live vaccine candidate may be available.

Immunized individuals can then be inoculated with a corresponding live vaccine to first see if it has some unexpected toxicity, and second, whether there is a booster effect. Alternatively, live vaccine could be given to unimmunized individuals, in the hope that these could be rescued by passive immunization with convalescent antisera if the vaccine is ineffective, assuming that such sera are available.

The exact procedures will have to be improvised on the spot. And would depend on extent and immediacy of the threat, on previous experience with viruses similar to the one causing the pandemic, and on whether any individuals are available that have survived infection.

As described previously, live vaccines generally contain ~1,000 active virions, which is an almost vanishingly small mass, invisible to the naked eye. In underdeveloped areas of the world this fact could be useful. For example, the 10^3 virions could easily be dried on a needle, and then be sent through the mail for immediate use. Preliminary studies suggest that partially hydrolyzed polyvinylpyrollidone can stabilize phage for several weeks on a needle tip.

If a group of individuals from each interested country can be identified as free of known viruses, these can be given a live vaccine, and could return to their countries of origin where they could be bled locally. It is obvious that a live virus grows in the vaccine recipient, and the question is when does the titer reach a maximum and what is that maximum.

Since only 10^3 live virions are needed per dose, 1 ml of a vacinee having 10^6 virions/ml would supply the vaccine for 1000 individuals. (This approach may also work for urine). While some viral pathogens have been reported not to occur in blood, it may be that this information is incorrect, and that a low viremia may actually occur transiently shortly after all infections. These questions deserve careful examination.

If a mixture of live vaccine candidates is injected, it is evident that natural selection will favor those growing most rapidly. While many different live vaccine versions will doubtless be made, the ones that grow the fastest and will still eliciting immunity, will be the best ones for human vaccine production.

If humans cannot be vaccine producers (donors), then it is essential to have available a variety of animals to test as producers, and facilities for producing the live vaccine from them.

6.0 Conclusions and Discussion

In all large scale, complex, urgently time dependent science-based projects, it is essential to have as complete a plan at the outset at the outset, especially when there is a unique definable product to be made. And no amount of progress substitutes for the product. In this project the product is priceless before a pandemic, and almost worthless after.

All true defense projects have something analogous to a proving ground.

It is concluded that the systems and technologies required to both justify and actually organize an integrated Virome Laboratory now exist, that it can be in initial operation within six months, and can reach the target throughput time of eight week at the end of one year, given the will and resources. The procedures for doing this are encompassed in present DOD and HHS legislation under the term, “other transactions”.

It is proposed that the first task should be to construct and test a microbanding system capable of isolating, detecting, and sequencing a few thousand virions. With this target the intervening steps can be defined and fall in a logical order.

The second task, the preparation of a radiation-killed vaccine, has been shown to follow directly from the application of high-resolution two-dimensional zonal centrifugation monitored by biophysical virus counting.

The third task is the synthesis and testing of many different versions of an effective live vaccine, with virus selection in human or animal tests. The aim is a strain that is effective and that also produces a sufficient viremia to allow vaccine production in human vaccinees.

By systematically and routinely going through these processes with animal and human viral pathogens, the risks associated with the production and testing procedures can be evaluated and minimized.

The entire procedure can only be optimized and made predictable on the basis of long-term operation and experience.

The only truly crash efforts in science-based defense were those of the Manhattan Project and a brief period when it was realized that gas centrifugation might change the face of nuclear weapons production. This document derives from these efforts. The zonal centrifuge was conceived while working post WWII in the Y-12 Electromagnetic plant which is shown in Figure 68. The scale of the K-25 Gaseous Diffusion Plant in indicated in Figure 69, and the Building eventually dedicated to the Zonal Centrifuge Program is shown in Fig. 70. A gas centrifuge cascade at K-25 is shown in Figure 71.

Figure 68. Y-12 Electromagnetic Plant

Original work on zonal centrifuges was done in the Biology Division which by chance was located in the red brick building in center right. This is the size of a facility required to develop one separation technology on a crash basis.

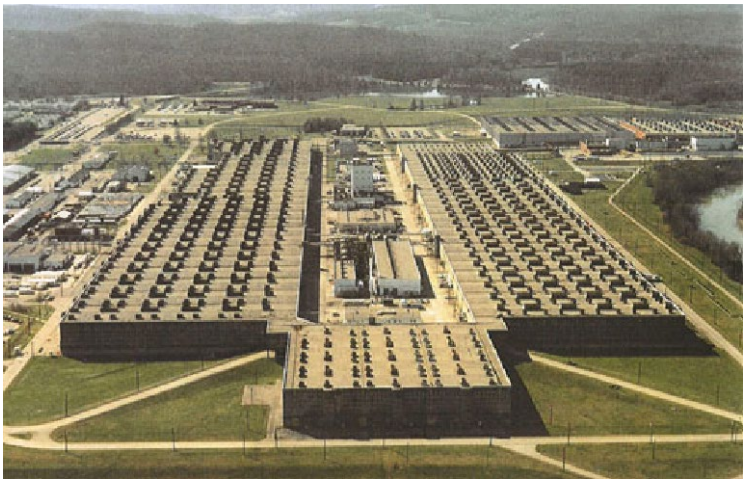


Figure 69. K-25. The Oak Ridge Gaseous Diffusion Plant

K-25 cost $512 million to build, or $6.5 billion in 2010 dollars. The mile-long, U-shaped plant covered forty-four acres, was four stories high and up to 400 feet wide. Engineers developed special coatings for the hundreds of miles of pipes and equipment to withstand the corrosive uranium hexafluoride gas that would pass through the plant's 3,000 repetitive diffusion stages (together making up a cascade).

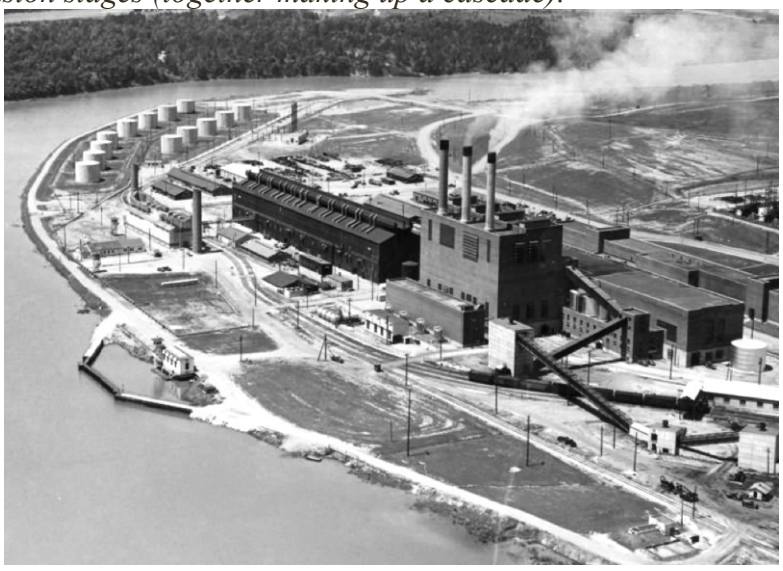


Figure 70. Building K-703

The majority of the work described here was done in the center building which was converted to virus work.

Figure 71. Gas Centrifuge Cascade at K-25

Figure 71 shows a 42-foot-long centrifuge cascade developed and constructed at K-25.

No trace of the original zonal centrifuge project remains at Oak Ridge. Gas centrifugation for uranium enrichment was privatized in the early 1960s, and then abandoned in favor of the AVLIS process which was in turn abandoned in favor of a new American Gas Centrifuge program with no interest in liquid ultracentrifuges.

Note on Non-Virological Applications of Physical Bioparticle Separations.

The original objective of the work described was to isolate putative human oncoviruses. The technology is equally applicable to bacteria, yeasts and mold including, but not limited to those which may cause human disease.

Evidence is now growing which suggests that Alzheimers disease and amyotrophic lateral sclerosis may have a mycological origin (109, 110). With the technology described here the organisms involved may be isolated while still living.

The majority of bacterial species on earth are unculturable by current methods (111). There exists an ocean of diversity that is not available. This accounts for the collapse of basic antibiotic discovery in about 1987 and the present " discovery void".

Absence of methods for bacterial pathogen culture also means that it is difficult to understand the differences between a fully pathogenic strain and an attenuated one. Treponema pallidum thus far cannot be kept in continuous culture *in vitro*. Fortunately it can be readily isolated from infected rabbit tissue by zonal centrifugation (112). The laboratory system described here therefore has wide uses in the field of infectious diseases.

References

1. Anderson NG, Cline GB, Harris WW, Green JG. Isolation of virus particles from large fluid volumes. . Berg G, editor. New York: Interscience Publishers; 1967.
2. Anderson NG. Evolutionary significance of virus infection. Nature. 1970 1970;227(265):1346-7.
3. Schuler A, Fitzgerald J, Inglesby T, O'Toole T. Executive positions of influence in biodefense: The Bio-Plum Book. Biosecuity and Bioterrorism 2004 2:328-341.: Biosecuity and Bioterrorism; 2004. p. 328-41.
4. Russell PK, Gronvall GK. U.S. Medical countermeasure development since 2001: a long way yet to go. Biosecur Bioterror. 2012 Mar;10(1):66-76. eng.
5. Northrop JH. The resistance of living organisms to digestion by pepsin or trypsin. J Gen Physiol. 1926;9:497-502.
6. Anderson NG. Studies on isolated cell components. VI. The effects of nucleases and proteases on rat liver nuclei. Exptl Cell Res 1953;5:361-74.
7. Allander T, Emerson SU, Engle RE, Purcell RH, Bukh J. A virus discovery method incorporating DNase treatment and its application to the identification of two bovine parvovirus species. Proc Natl Acad Sci U S A. 2001 Sep 25;98(20):11609-14. PubMed PMID: 11562506. eng.
8. Toplin I. Purification of the Moloney and Rauscher murine leukemia viruses by use of zonal ultracentrifuge systems. Appl Microbiol. 1967:582-9. PubMed PMID: 6035050. eng.
9. Toplin I, Mizell M, Sottong P, Monroe J. Zonal centrifuge applied to the purification of Herpesvirus in the Lucke frog kidney tumor. Appl Microbiol. 1971;21:132-9. PubMed PMID: 5540668. eng.

10. Toplin I, Sottong P. Large-volume purification of tumor viruses by use of zonal centrifuges. Appl Microbiol. 1972;23:1010-4. eng.
11. Wen K, Ortmann AC, Suttle CA. Accurate estimation of viral abundance by epifluorescence microscopy. Applied and environmental microbiology. 2004 l;70:3862-7. PubMed PMID: 15240256. eng.
12. Brakke MK. Density gradient centrifugation: A new separation technique. J Am Chem Soc. 1951;73:1847-8.
13. Anderson NG, Harris, W W, Barber, A.A., Rankin, Jr., C.T., and Candler, E.L. . Separation of sub cellular components and viruses by combined rate- and isopycnic-zonal centrifugation. Natl Cancer Inst Monograph No 211966. p. 253-84.
14. Anderson NG, Waters DA, Nunley CE, Cline GB. Two new simple zonal centrifuges rotors: B-XIV and B-XV. Fed Proc. 1966;25:421.
15. Anderson NG. The zonal ultracentrifuge. A new instrument for fractionating mixtures of particles. J Phys Chem. 1962;66:1984-9.
16. Anderson NG, Barringer HP, Babelay EF, Fisher WD. The B-IV zonal ultracentrifuge. Life Sci. 1964;3:667-71.
17. Anderson NG, Barringer HP, Babelay EF, Fisher WD. The B-Iv Zonal Ultracentrifuge. Life sciences. 1964 Jul;3:667-71. PubMed PMID: 14203962. eng.
18. Anderson NG, Barringer HP, Babelay EF, Nunley CE, Bartkus MJ, Fisher WD, et al. The design and operation of the B-IV zonal centrifuge system. Natl Cancer Inst Monogr. 1966;21:137-64.
19. Anderson NG, Nunley CE, Rankin CTJ. Analytical techniques for cell fractions. XV. Rotor B-XXIX--a new high-resolution zonal centrifuge rotor for virus isolation and cell fractionation. Anal Biochem. 1969;31:255-71.
20. Anderson NG, Price CA, Fisher WD, Canning RE, Burger CL. Analytical Techniques for Cell Fractions. IV. Reorienting Gradient Rotors for Zonal Centrifugation. Anal Biochem. 1964;7:1-9. PubMed PMID: 14106917. eng.
21. Anderson NG, Rankin CTJ, Brown DH, Nunley CE, Hsu HW. Analytical techniques for cell fractions. XI. Rotor B-XXIII--a zonal centrifuge rotor for center or edge unloading. Anal Biochem. 1968;26:415-9.
22. Anderson NG, Waters DA, Fisher WD, Cline GB, Nunley CE, Elrod LH, et al. Analytical techniques for cell fractions. V. Characteristics of the B-XIV and B-XV zonal centrifuge rotors. Anal Biochem. 1967;21:235-52.
23. Anderson NG, Waters DA, Nunley CE, Gibson RF, Schilling RM, Denny EC, et al. K-series centrifuges. I. Development of the K-II continuous-sample-flow-with-banding centrifuge system for vaccine purification. Anal Biochem. 1969;32:460-94.
24. Anderson NG, Gerin JL, Anderson NL. Global screening for human viral pathogens. Emerging infectious diseases. 2003;9:768-74. PubMed PMID: 12890315. eng.
25. Breitbart M, Felts B, Kelley S, Mahaffy JM, Nulton J, Salamon P, et al. Diversity and population structure of a near-shore marine-sediment viral community. Proc Bioll Sci. 2004;271:565-74. PubMed PMID: 15156913. eng.
26. Edwards RA, Rohwer F. Viral metagenomics. Nature reviews. 2005 Jun;3(6):504-10. PubMed PMID: 15886693. eng.
27. Wommack KE, Bench SR, Bhavsar J, Mead D, Hanson T. Isolation independent methods of characterizing phage communities 2: characterizing a metagenome. Methods in molecular biology (Clifton, NJ. 2009;502:279-89. PubMed PMID: 19082562. eng.

28. Bench SR, Hanson TE, Williamson KE, Ghosh D, Radosovich M, Wang K, et al. Metagenomic characterization of Chesapeake Bay virioplankton. Applied and environmental microbiology. 2007 Dec;73(23):7629-41. PubMed PMID: 17921274. eng.
29. Angly F, Rodriguez-Brito B, Bangor D, McNairnie P, Breitbart M, Salamon P, et al. PHACCS, an online tool for estimating the structure and diversity of uncultured viral communities using metagenomic information. BMC bioinformatics. 2005;6:41. PubMed PMID: 15743531. eng.
30. Wommack KE, Bhavsar J, Ravel J. Metagenomics: read length matters. Applied and environmental microbiology. 2008;74:1453-63. PubMed PMID: 18192407. eng.
31. Gerin JL, Anderson NG. Purification of influenza virus in the K-II zonal centrifuge. Nature. 1969;221:1255-6.
32. Reimer CB, Baker RS, Van Frank RM, Newlin TE, Cline GB, Anderson NG. Purification of large quantities of influenza virus by density gradient centrifugation. J Virol. 1967;1:1207-16.
33. Hilleman MR, McAleer WJ, Buynak EB, McLean AA. Quality and safety of human hepatitis B vaccine. Developments in biological standardization. 1983;54:3-12. eng.
34. Peck FB, Jr. Purified influenza virus vaccine. A study of viral reactivity and antigenicity. JAMA. 1968;206:2277-82. eng.
35. Breillatt JP, Brantley JN, Mazzone HM, Martignoni ME, Franklin JE, Anderson NG. Mass purification of nucleopolyhedrosis virus inclusion bodies in the K-series centrifuge. Appl Microbiol. 1972;23:923-30. eng.
36. Breillatt JP, Martignoni ME, Mazzone HM. Host-specific virus isolation for large-scale insect destruction. ORNL-4419. ORNL-NSIC Rep. 1969:8-9. PubMed PMID: 5372835.
37. Martignoni M, Breillatt JP, Anderson NG. Mass purification of polyhedral inclusion bodied by isopycnic banding in zonal rotors. J Invert Path. 1968;23:507-10.
38. Hilleman MR, McAleer WJ, Buynak EB, McLean AA. The preparation and safety of hepatitis B vaccine. The Journal of infection. 1983;7 Suppl 1:3-8. PubMed PMID: 6674367. eng.
39. Anderson NG, Anderson NL, Taylor J, Goodman J. Large-scale oligonucleotide synthesizers. I. Basic principles and system design. Appl Biochem Biotechnol. 1995 1995;54(1-3):19-42.
40. Cho N, Barringer HP, Amburgey JW, Cline GB, Anderson NG, McCauley LL, et al. Problems in biocontainment. Natl Cancer Inst Monogr. 1966 1966;21:485-502.
41. Brussaard CP. Enumeration of bacteriophages using flow cytometry. Methods in molecular biology (Clifton, NJ. 2009;501:97-111. PubMed PMID: 19066815. eng.
42. Eid J, Fehr A, Gray J, Luong K, Lyle J, Otto G, et al. Real-time DNA sequencing from single polymerase molecules. Science. 2009 Jan 2;323(5910):133-8. PubMed PMID: 19023044. eng.
43. Pichot C. Surface-functionalized latexes for biotechnological applications. Current Opinion in Colloid & Interface Science. 2004;9(3-4):213-21.
44. Mullbacher A, Ada GL, Hla RT. Gamma-irradiated influenza A virus can prime for a cross-reactive and cross-protective immune response against influenza A viruses. Immunology and cell biology. 1988;66 (Pt 2):153-7.
45. Furuya Y, Chan J, Regner M, Lobigs M, Koskinen A, Kok T, et al. Cytotoxic T cells are the predominant players providing cross-protective immunity induced by {gamma}-

irradiated influenza A viruses. J Virol. 2010 May;84(9):4212-21. PubMed PMID: 20164231. Pubmed Central PMCID: 2863765.
46. Furuya Y, Regner M, Lobigs M, Koskinen A, Mullbacher A, Alsharifi M. Effect of inactivation method on the cross-protective immunity induced by whole 'killed' influenza A viruses and commercial vaccine preparations. The Journal of general virology. 2010;91(Pt 6):1450-60.
47. Alsharifi M, Mullbacher A. The gamma-irradiated influenza vaccine and the prospect of producing safe vaccines in general. Immunology and cell biology. 2010 Feb;88(2):103-4.
48. Pollard E. Inactivation of viruses for vaccine purposes using ionizing radiation and heat. The Yale journal of biology and medicine. 1957;29:436-43. PubMed PMID: 13423369. eng.
49. Traub FB, Friedemann U, Brasch A, Huber W. High intensity electrons as a tool for preparation of vaccines. I. Preparation of rabies vaccine. J Immunol. 1951;67:379-84. PubMed PMID: 14898073. eng.
50. Wiktor TJ, Aaslestad HG, Kaplan MM. Immunogenicity of rabies virus inactivated by -propiolactone, acetylethyleneimine, and ionizing irradiation. Appl Microbiol. 1972;23(5):914-8.
51. Gruber J. Immunogenicity of Purified Venezuelan Equine Encephalitis Virus Inactivated by Ionizing Radiation. Infection and immunity. 1971;3:574-9. PubMed PMID: 16558020. Eng.
52. Reitman M, Tribble HR. Inactivation of Venezuelan Equine Encephalomyelitis Virus by gamma-Radiation. Appl Microbiol. 1967;15:1456-9. PubMed PMID: 16349765. Eng.
53. Henner WD, Grunberg SM, Haseltine WA. Sites and structure of gamma radiation-induced DNA strand breaks. The Journal of biological chemistry. 1982;257:11750-4. PubMed PMID: 7118909. eng.
54. Pomares P, Velez F, inventors; Technology Application Services, assignee. Method and apparatus for sterilizing and /or embalming corpses and specimens. USA1993 June 8, 1993.
55. Hennes KP, Suttle CA, Chan AM. Fluorescently Labeled Virus Probes Show that Natural Virus Populations Can Control the Structure of Marine Microbial Communities. Applied and environmental microbiology. 1995;61:3623-7. PubMed PMID: 16535146. Eng.
56. Suttle CA. Viruses in the sea. Nature. 2005;437:356-61. eng.
57. Suttle CA, Chan AM, Cottrell MT. Use of Ultrafiltration To Isolate Viruses from Seawater Which Are Pathogens of Marine Phytoplankton. Applied and environmental microbiology. 1991;57:721-6. PubMed PMID: 16348439. Eng.
58. Fout GS, Medappa KC, Mapoles JE, Rueckert RR. Radiochemical determination of polyamines in poliovirus and human rhinovirus 14. The Journal of biological chemistry. 1984;259:3639-43. PubMed PMID: 6323434. eng.
59. Arthanari H, Basu S, Kawano TL, Bolton PH. Fluorescent dyes specific for quadruplex DNA. Nucleic acids research. 1998 Aug 15;26(16):3724-8. PubMed PMID: 9685488. eng.
60. Yao ZP, Demirev PA, Fenselau C. Mass spectrometry-based proteolytic mapping for rapid virus identification. Analytical chemistry. 2002;74(11):2529-34. PubMed PMID: 12069233. eng.

61. Blouin AG, Greenwood DR, Chavan RR, Pearson MN, Clover GR, MacDiarmid RM, et al. A generic method to identify plant viruses by high-resolution tandem mass spectrometry of their coat proteins. Journal of virological methods. 2009;163:49-56. eng.
62. Go EP, Wikoff WR, Shen Z, O'Maille G, Morita H, Conrads TP, et al. Mass spectrometry reveals specific and global molecular transformations during viral infection. Journal of proteome research. 2006;5:2405-16. PubMed PMID: 16944953. eng.
63. Jackson GW, McNichols RJ, Fox GE, Willson RC. Toward universal flavivirus identification by mass cataloging. J Mol Diagn. 2008;10:135-41. PubMed PMID: 18258926. eng.
64. Shaw ML, Stone KL, Colangelo CM, Gulcicek EE, Palese P. Cellular proteins in influenza virus particles. PLoS pathogens. 2008;4:e1000085. PubMed PMID: 18535660. eng.
65. Thomas JJ, Bakhtiar R, Siuzdak G. Mass spectrometry in viral proteomics. Accounts of chemical research. 2000 Mar;33(3):179-87. PubMed PMID: 10727207. eng.
66. Zeng R, Ruan HQ, Jiang XS, Zhou H, Shi L, Zhang L, et al. Proteomic analysis of SARS associated coronavirus using two-dimensional liquid chromatography mass spectrometry and one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by mass spectroemtric analysis. Journal of proteome research. 2004 May-Jun;3(3):549-55. PubMed PMID: 15253436. eng.
67. Curry A, Appleton H, Dowsett B. Application of transmission electron microscopy to the clinical study of viral and bacterial infections: present and future. Micron. 2006;37:91-106. PubMed PMID: 16361103. eng.
68. Roingeard P. Viral detection by electron microscopy: past, present and future. Biology of the cell / under the auspices of the European Cell Biology Organization. 2008;100:491-501. PubMed PMID: 18627353. eng.
69. Singh HK, Madden V, Shen YJ, Thompson BD, Nickeleit V. Negative-staining electron microscopy of the urine for the detection of polyomavirus infections. Ultrastructural pathology. 2006;30:329-38. PubMed PMID: 17090512. eng.
70. McFerran JB, Clarke JK, Curran WL. The application of negative contrast electron microscopy to routine veterinary virus diagnosis. Research in veterinary science. 1971;12:253-7. PubMed PMID: 4325008. eng.
71. Woolcock PR, Shivaprasad HL. Electron microscopic identification of viruses associated with poult enteritis in turkeys grown in California 1993-2003. Avian diseases. 2008;52(2):209-13.
72. Bron C, Gremillet P, Launay D, Jourlin M, Gautschi HP, Bachi T, et al. Three-dimensional electron microscopy of entire cells. Journal of microscopy. 1990;157:115-26. PubMed PMID: 2299660. eng.
73. McEwen BF, Renken C, Marko M, Mannella C. Chapter 6: Principles and practice in electron tomography. Methods in cell biology. 2008;89:129-68. PubMed PMID: 19118675. eng.
74. Sharp D. Particulate component of plasma from fowls with avian erythro-myeloblastic leucosis. J Immunol. 1960a;84:507-13.
75. Sharp DG, Bracken EC. Quantitation and morphology of equine abortion virus in hamsters. Virology. 1960b;10:419-31.
76. Mathews J, Buthala DA. Centrifugal sedimentation of virus particles for electron microscopic counting. J Virol. 1970;5:598-603. PubMed PMID: 5419912. eng.

77. Muller G. [Electron microscopical particle counting in virology. I. Centrifuge tubes for the direct sedimentation of viruses on grids]. Archiv fur die gesamte Virusforschung. 1969;27:339-51. PubMed PMID: 5369726. Elektronenmikroskopische Partikelzahlung in der Virologie. I. Zentrifugierrohrchen zur direkten Sedimentation von Viren auf Netztrager. ger.
78. Anderson NL, Jackson A, Smith D, Hardie D, Borchers C, Pearson TW. SISCAPA peptide enrichment on magnetic beads using an in-line bead trap device. Mol Cell Proteomics. 2009 May;8(5):995-1005. PubMed PMID: 19196707. eng.
79. Shi L, Sanyal G, Ni A, Luo Z, Doshna S, Wang B, et al. Stabilization of human papillomavirus virus-like particles by non-ionic surfactants. Journal of pharmaceutical sciences. 2005 Jul;94(7):1538-51. PubMed PMID: 15929070. eng.
80. Kuhr M, S B, U R, Wolff D. Coatings on plastics with the PICVD technology. Thin Solid Films. 2003;442:107-16.
81. Diamandis E. Next Generation Sequencers. Clinical Chemistry. 2009.
82. Harris TD, Buzby PR, Babcock H, Beer E, Bowers J, Braslavsky I, et al. Single-molecule DNA sequencing of a viral genome. Science. 2008;320:106-9. PubMed PMID: 18388294. eng.
83. Quan PL, Briese T, Palacios G, Ian Lipkin W. Rapid sequence-based diagnosis of viral infection. Antiviral research. 2008;79:1-5. PubMed PMID: 18367256. eng.
84. Branton D, Deamer DW, Marziali A, Bayley H, Benner SA, Butler T, et al. The potential and challenges of nanopore sequencing. Nature biotechnology. 2008;26:1146-53. PubMed PMID: 18846088. eng.
85. Anderson NG, Breillatt JP. Isopycnometric serology: a new technique based on buoyant density changes in latex beads. Nat New Biol. 1971 1971;231(21):112-4.
86. Rogers PH, Michel E, Bauer CA, Vanderet S, Hansen D, Roberts BK, et al. Selective, controllable, and reversible aggregation of polystyrene latex microspheres via DNA hybridization. Langmuir. 2005 Jun 7;21(12):5562-9. PubMed PMID: 15924490. eng.
87. Becquart P, Wauquier N, Mahlakoiv T, Nkoghe D, Padilla C, Souris M, et al. High prevalence of both humoral and cellular immunity to Zaire ebolavirus among rural populations in Gabon. PLoS ONE. 2010;5(2):e9126. PubMed PMID: 0161740.
88. Hasleton PS. The internal surface area of the adult human lung. Journal of anatomy. 1972 Sep;112(Pt 3):391-400. PubMed PMID: 4564685. Pubmed Central PMCID: 1271180.
89. Palese P. Making better influenza virus vaccines? Emerging infectious diseases. 2006;12:61-5. PubMed PMID: 16494719. eng.
90. Mazzulli T, Farcas GA, Poutanen SM, Willey BM, Low DE, Butany J, et al. Severe acute respiratory syndrome-associated coronavirus in lung tissue. Emerging infectious diseases. 2004;10:20-4. PubMed PMID: 15078592. eng.
91. Lu MC, Yu CL, Yin WY, Tung CH, Huang KY, Liu SQ, et al. Increased prevalence of polyomavirus BK viruria that correlates with thrombocytopenia in patients with systemic lupus erythematosus on intensive immunosuppressive therapy. Autoimmunity. 2009;42:216-23. PubMed PMID: 19301203. eng.
92. Randhawa PS, Vats A, Zygmunt D, Swalsky P, Scantlebury V, Shapiro R, et al. Quantitation of viral DNA in renal allograft tissue from patients with BK virus nephropathy. Transplantation. 2002;74:485-8. PubMed PMID: 12352906. eng.

93. Hsieh PP, Tung CL, Chan AB, Liao JB, Wang JS, Tseng HH, et al. EBV viral load in tumor tissue is an important prognostic indicator for nasal NK/T-cell lymphoma. Am J Clin Pathol. 2007 Oct;128:579-84. PubMed PMID: 17875508. eng.
94. Chen YN, Wu CC, Bryan T, Hooper T, Schrader D, Lin TL. Specific real-time reverse transcription-polymerase chain reaction for detection and quantitation of turkey coronavirus RNA in tissues and feces from turkeys infected with turkey coronavirus. Journal of virological methods. 2010;163:452-8. PubMed PMID: 19917315. eng.
95. Korva M, Duh D, Saksida A, Trilar T, Avsic-Zupanc T. The hantaviral load in tissues of naturally infected rodents. Microbes and infection / Institut Pasteur. 2009;11:344-51. PubMed PMID: 19397875. eng.
96. Duchatel JP, Todd D, Willeman C, Losson B. Quantification of pigeon circovirus in serum, blood, semen and different tissues of naturally infected pigeons using a real-time polymerase chain reaction. Avian Pathol. 2009;38:143-8. PubMed PMID: 19322713. eng.
97. Zhang Z, Alexandersen S. Quantitative analysis of foot-and-mouth disease virus RNA loads in bovine tissues: implications for the site of viral persistence. J Gen Virol. 2004;85:2567-75. PubMed PMID: 15302950. eng.
98. Qi X, Yang X, Cheng A, Wang M, Zhu D, Jia R. Quantitative analysis of virulent duck enteritis virus loads in experimentally infected ducklings. Avian diseases. 2008 Jun;52(2):338-44. PubMed PMID: 18646467. eng.
99. Tong CY, Hilton R, MacMahon EM, Brown L, Pantelidis P, Chrystie IL, et al. Monitoring the progress of BK virus associated nephropathy in renal transplant recipients. Nephrol Dial Transplant. 2004;19:2598-605. PubMed PMID: 15292464. eng.
100. Eisen DP, Fraser IR, Sung LM, Finlay M, Bowden S, O'Connell H. Decreased viral load and symptoms of polyomavirus-associated chronic interstitial cystitis after intravesical cidofovir treatment. Clin Infect Dis. 2009;48:e86-8. PubMed PMID: 19292661. eng.
101. Semple D. The preparation of a safe and efficient rabies vaccine. . Government Printing Office of India; 1911.
102. Fermi C. Immunizierung der Muriden mi tollwut und normaler Nervensubstanz gegen die nachfolgende sukutane infection von Strassenvirus. Hyg Infektionskr. 1908 1908;58:221.
103. Gack MU, Albrecht RA, Urano T, Inn KS, Huang IC, Carnero E, et al. Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. Cell host & microbe. 2009;5:439-49. PubMed PMID: 19454348. eng.
104. Mortola E, Endo Y, Ohno K, Watari T, Tsujimoto H, Hasegawa A. The Use of Two Immunosuppressive Drugs, Cyclosporin A and Tacrolimus, to Inhibit Virus Replication and Apoptosis in Cells Infected with Feline Immunodeficiency Virus. Vet Res Comm. 2004;22:553-63.
105. Coleman JR, Papamichail D, Skiena S, Futcher B, Wimmer E, Mueller S. Virus attenuation by genome-scale changes in codon pair bias. Science. 2008 Jun 27;320(5884):1784-7. Pubmed Central PMCID: 1.
106. Mueller S, Coleman JR, Papamichail D, Ward CB, Nimnual A, Futcher B, et al. Live attenuated influenza virus vaccines by computer-aided rational design. Nature biotechnology. 2010 Jul;28(7):723-6. PubMed PMID: 20543832. Pubmed Central PMCID: 2902615.
107. Mueller S, Coleman JR, Papamichail D, Ward CB, Nimnual A, Futcher B, et al. Live attenuated influenza virus vaccines by computer-aided rational design. Nature biotechnology. 2011 Jul;28(7):723-6. PubMed PMID: 20543832. eng.

108. Bull JJ, Molineux IJ, Wilke CO. Slow fitness recovery in a codon-modified viral genome. Molecular biology and evolution. 2012 Apr 24. PubMed PMID: 22532576. Eng.
109. Pisa D, Alonso R, Rabano A, Rodal I, Carrasco L. Different Brain Regions are Infected with Fungi in Alzheimer's Disease. Scientific reports. 2015;5:15015. PubMed PMID: 26468932. Pubmed Central PMCID: 4606562.
110. Alonso R, Pisa D, Marina AI, Morato E, Alberto Rábano A, Rodal I, et al. Evidence for Fungal Infection in Cerebrospinal Fluid and Brain Tissue from Patients with Amyotrophic LateralSclerosis. Int J Biological Sciences. 2015;11(5):546-58.
111. Stewart EJ. Growing Unculturable Bcteria. JBact 2012;194(16):4151-66.
112. Thomas ML, Clark JW, Jr., Cline GB, Anderson NG, Russell H. Separation of Treponema pallidum from tissue substances by continuous-flow zonal centrifugation. Appl Microbiol. 1972 Apr;23(4):714-20. PubMed PMID: 4622979. Pubmed Central PMCID: 380424.