
Preface

Vaccine research and development is advancing at an unprecedented pace, with an increasing emphasis on rational design based upon a fundamental understanding of the underlying molecular mechanisms. The aim of this volume is to provide a selection of contemporary protocols that will be useful to both novice and advanced practitioner alike. The variety of procedures required to design, develop, produce, and assess a vaccine is immense and covers aspects of chemistry, biochemistry, molecular biology, cell biology, and immunology. No single volume can hope to cover these topics exclusively. Rather, here we attempt to provide a methods sourcebook focusing on hands-on practical advice. Complementary and background information may be found in other volumes in the *Methods in Molecular Medicine* series. Of particular interest are volumes on *Dendritic Cell Protocols*, *Interleukin Protocols*, *Vaccine Adjuvants*, and *DNA Vaccines*.

Since the publication of the first edition of *Vaccine Protocols* there have been major advances, particularly in the areas of bacterial genomics, antigen-specific T-cell quantification, genetic manipulation of vaccine vectors, the harnessing of natural molecules concerned with the regulation of immune responses, and the burgeoning field of DNA vaccinology. Hence, the extensive revision of this edition with new chapters on live viral vaccine vectors, attenuated bacterial vectors, immunomodulators, MHC-peptide tetrameric complexes, and the identification of vaccine candidates by genomic analysis. Additionally, chapters from the first edition have been updated to accommodate state-of-the-art methods in vaccinology. We have maintained the overall structure of the book to comprise essentially three basic chapter types. First, those describing in detail the development and production of vaccines using specific techniques, including genetic manipulation of viruses or bacteria to produce live attenuated vaccines, vaccine vectors, or inactivated toxins and the production of synthetic peptides and conjugate vaccines. Second, chapters describing more general techniques for vaccine formulation and delivery and assessment of immune responses. Finally, a few chapters review the critical areas of scale-up to manufacture, vaccine quality assurance, and clinical trials in order to prime the reader with the relevant background information. Together with a general overview of vaccines, the present volume on *Vaccine Protocols* should provide valuable and relevant practical information to all those seeking to produce improved or novel vaccines.

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Temperature-Sensitive Mutant Vaccines

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1. Introduction

Many live virus vaccines derived by empirical routes exhibit temperature-sensitive (ts) phenotypes. The live virus vaccines that have been outstandingly successful in controlling poliomyelitis are the prime example of this phenomenon. The three live attenuated strains developed by Albert Sabin were derived from wild-type isolates by rapid sequential passage at high multiplicity of infection (MOI) in monkey tissue *in vitro* and *in vivo*, a regimen that yielded variants of reduced neurovirulence. Concomitantly, the three vaccine strains developed ts characteristics, a phenotype that correlated well with loss of neurovirulence. The reproductive capacity at supraoptimal (40°C) temperature, the *rct* phenotype, proved to be a useful property for monitoring the genetic stability of the attenuated virus during propagation, vaccine production, and replication in vaccinees. Nucleotide sequencing of the genome of the poliovirus type 3 attenuated virus and its neurovirulent wild-type progenitor (the Leon strain), revealed that only ten nucleotide changes, producing three amino acid substitutions, differentiated the attenuated derivative from its virulent parent despite its lengthy propagation in cultured cells. One of the three coding changes, a serine-to-phenylalanine substitution at position 2034 in the region encoding VP3, conferred the ts phenotype. A combination of nucleotide sequencing of virus recovered from a vaccine-associated case of paralysis and assay in primates of the neurovirulence of recombinant viruses prepared from infectious cDNA established that two of the ten mutations in the type three vaccine strain were associated with the loss of neurovirulence. The mutation conferring temperature-sensitivity was one of these mutations (*1*).

Since all three independently modified poliovirus vaccines exhibit temperature-sensitivity, it is likely that ts mutations in general may be attenuating by diminishing reproductive potential without appreciable loss of immunogenicity. However, it is not clear why temperature-sensitivity *per se* should adversely affect replication of poliovirus in the central nervous system (CNS), while allowing replication to proceed nor-

mally in the gut. Since the restrictive temperature in the case of the poliovirus vaccines is above normal *in vivo* temperature, it is possible that a mild febrile response following initial infection is sufficient to limit the amount of virus leaving the gut epithelium and to reduce the likelihood of access of the virus to the CNS.

The potential of ts mutants as live virus vaccines is more obvious in the case of infections by the respiratory route, since the mean temperature of the nares and the upper respiratory tract is likely to be significantly lower than that of the lower respiratory tract. Thus, virus replication can proceed unrestricted in the nasopharyngeal epithelium, inducing both local and systemic immune responses, and causing minimal discomfort to the host. Temperature-sensitive mutants employed in this way must have sufficient genetic stability to ensure that the host organism can mount an effective immune response before virus penetrates into the lower respiratory tract. Experimental ts mutant vaccines have been developed for a number of human respiratory viruses, and these have shown promise in experimental animals and in volunteer trials (2). None has been approved for clinical use because of uncertainties regarding their genetic stability and concern about their semi-empirical mode of development. However, ts mutant vaccines for respiratory diseases are gaining favor again as a result of the increasing ease with which the genomes of viruses can be manipulated in a controlled manner by recombinant DNA technology. The over-riding advantage of live virus vaccines is their inherent property of auto-amplification and their ability to induce a balanced immune response (2).

Although the genomes of single-stranded and double-stranded DNA viruses and those of positive-stranded RNA viruses are amenable to manipulation by standard recombinant DNA methodology, until recently the introduction of specific mutations by reverse genetics was not possible in the case of negative-stranded RNA viruses. However, appropriate methodology has been developed now for genetic engineering of the genomes of both segmented genome negative-stranded RNA viruses (3) and non-segmented negative-stranded RNA viruses (4). The existence of an infectious DNA copy of the wild-type viral genome is a prerequisite for implementation of reverse genetics. The current strategy requires also the prior existence of an empirically derived attenuated (usually ts) vaccine virus, which can be utilized as a model for the identification of the genetic determinants of virulence. Recently, the first successful incorporation of a foreign gene into the genome of the double-stranded RNA virus, reovirus type 3, has been reported (5). It remains to be seen whether this approach can be generalized into a method of reverse genetics applicable to other mammalian double-stranded RNA viruses.

The first part of this chapter describes three approaches that have been used successfully to produce experimental ts attenuated viruses, and the second part provides an example of a reverse genetics approach for insertion of attenuating mutations into a viral genome, which can serve as a model.

1.1. Classical Empirical Approach

Three approaches have been used successfully to produce experimental ts attenuated viruses. The first approach involves continuous passage of a virulent virus at

gradually reduced temperatures to produce a strain that is no longer able to replicate efficiently at supraoptimal or physiological temperatures. Cold-adapted (ca) viruses with potential as vaccines have been derived for influenza virus and several paramyxoviruses. The rationale behind this approach is that spontaneous ts mutants will accumulate and gradually diminish the pathogenic potential of the virus. The advantage of this procedure is that the process is progressive. It can be monitored continuously and terminated when the appropriate degree of attenuation has been obtained. The accumulation of spontaneous mutations is likely to achieve better genetic stability because the final phenotype is the product of many small incremental changes rather than a few major changes, which may be subject to reversion at high frequency. Such a ca virus may exhibit a reduced capacity to multiply at normal body temperature in addition to its extended low-temperature range. However, this is not always observed, and virus may become attenuated without becoming temperature-adapted. Such attenuated viruses are described as cold-passaged (cp) virus, or (cp/ts), where they exhibit a ts phenotype (6–8).

The second approach is sequential passage of virus in the presence of a mutagen in order to accelerate the accumulation of mutants. This method could be combined with passage at low temperature to enhance the selection of ts mutants, but it has been used mainly at normal incubation temperatures to optimize virus replication (important in the case of base analog mutagens) and to maximize the yield of mutants. A Rift Valley fever virus vaccine has been derived in this manner using 5-fluorouracil (5-FU) as the mutagen (9).

The third approach is direct isolation of single spontaneous or induced ts mutations. Individually, such mutants are generally too unstable genetically to be suitable as vaccines. Greater stability can be achieved by isolating several ts mutants. The isolation of multiple ts mutants is achieved by isolation of single ts mutants sequentially at progressively reduced restrictive temperatures, as described below.

The protocols outlined in the following sections have been used in the generation of an experimental human respiratory syncytial virus vaccine (10,11), but they are generally applicable with minor modification. Human respiratory syncytial virus (order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Pneumovirus*) has a narrow host range, grows to low titer, and is intolerant of extremes of heat and pH. Thus, modification of the protocols listed here for use with other viruses usually entail no more than a relaxation of some of the specific restrictions and a change of cell substrate.

2. Materials

2.1. General Virology and Mutagenesis

1. A class II laminar flow safety cabinet, located in a dedicated laboratory with restricted access.
2. Disposable gloves, gowns, and face masks.
3. A minimum of two CO₂-gassed incubators.
4. A circulating water bath able to maintain temperature within $\pm 0.2^{\circ}\text{C}$.
5. A refrigerated bench centrifuge.

6. A UV-microscope.
7. A liquid nitrogen storage cylinder.
8. Tissue-culture-grade sterile disposable plastic ware: 150 cm², 75 cm² and 25 cm² flasks with vented caps; 50-mm Petri dishes; 6-well cluster plates, 96-well flat-bottomed plates; 1-mL, 5-mL, and 10-mL disposable pipets; plugged narrow-bore Pasteur pipets; screw-capped freezer vials.
9. Glasgow Minimum Essential Eagle's Medium (GMEM) or equivalent, supplemented with 200 mM glutamine, antibiotics (100 ug/mL streptomycin and 100 U/mL penicillin). Used with 10% mycoplasma-free fetal calf serum (FCS) for cell propagation, with 1% FCS for maintenance of infected cell cultures and as a diluent.
10. Cell freezing medium; GMEM 70%, glycerol 10%, FCS 20%.
11. Versene (EDTA) and versene/trypsin solutions for cell transfer; used at a concentration of 0.5 g porcine trypsin plus 0.2 g EDTA per L.
12. Neutral red stain.
13. Agar or agarose.
14. DAPI stain (*see Note 5*).
15. MRC-5 human diploid embryonic lung cells (ATCC No. CCL 171).

2.2. Reverse Genetics

1. An infectious full-length cDNA clone of the wild-type viral genome.
2. A vaccinia virus recombinant expressing the T7 RNA polymerase, or, when available, susceptible mammalian cells stably expressing the T7 RNA polymerase.
3. Support plasmids expressing the essential proteins for replication and encapsidation of the viral genome.
4. Susceptible cells able to support virus replication and encapsidation, and expression of the vaccinia virus T7 RNA polymerase recombinant and the support plasmids.
5. Standard reagents and equipment for recombinant DNA manipulation.
6. Specific primers for polymerase chain reaction (PCR) synthesis.
7. Thermal cycler for PCR synthesis (*see Note 6*).

3. Methods

3.1. General Methodology

All experimental operations should be conducted according to appropriate safety regulations/guidelines in a class III facility in which all equipment and reagents are dedicated solely to vaccine development. There should be no exchange of materials or reagents with other laboratories during the course of the project. The virus isolates, media, and cell cultures should be stored in dedicated refrigerators, preferably housed within the containment area. Gowns, gloves, and perhaps special footwear should be worn at all times. Staff used on the project should not be assigned during the same working day to other tasks that could bring them into contact with other viruses and cells. Visitors should be kept from the working area.

3.1.1. The Cell Substrate

Use a cell substrate approved for vaccine development and production from the outset. A diploid cell line is mandatory in the case of vaccines destined for ultimate use in humans. The MRC-5 cell line is appropriate for most purposes. MRC-5 cells

grow slowly and achieve confluence at low density; their useful life may extend up to about 40 passages. Beyond this time, there is a progressive retardation of growth rate.

1. Establish an adequate cell bank at the outset from a low-passage seed to ensure an adequate supply of cells for the entire enterprise. MRC-5 cells can be propagated in growth medium consisting of Eagle's medium with non-essential amino acids (Glasgow formulation), supplemented with antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) and FCS. The concentration of foetal calf serum is the critical factor (*see Note 1*). FCS is preferred because of its content of growth factors and the absence of inhibitory substances and antibodies present in adult animal sera (*see Note 2*). Newborn or other animal sera may be suitable for some purposes (*see Note 3*). Heat-treat sera for cell culture (30 min at 56°C) before use to destroy complement and other nonspecific inhibitory substances.
2. Propagate MRC-5 cells as monolayer cultures by seeding 1×10^6 cells into a large (150 cm²) tissue culture-grade plastic flask with vented lid. Incubate in a 5% CO₂-gassed incubator at 35–37°C. Confluence should be achieved within 4–5 d, providing a yield of 5–10 $\times 10^6$ cells.
3. Harvest cells by removing the incubation medium and washing the monolayer with 20 mL 0.2% (v/v) versene solution. Incubate in the presence of 20 mL versene without calcium and magnesium for 5–10 min at room temperature, then wash with 20 mL trypsin/versene solution followed by incubation in the presence of 2.5 mL trypsin/versene at 37°C until the cells detach. If the cells do not detach within 5 min, decant and add fresh trypsin/versene.
4. For continued propagation of cells, seed large (150 cm²) flasks at a density of 2.5×10^6 /mL. Use small (25 cm²) flasks for virus production and 50-mm-diameter plastic Petri dishes for virus infectivity assay, both seeded at 1×10^6 per mL. Seed multi-well plates with 2.5×10^6 cells distributed proportionately.
5. Establish a cell bank. To do this amplify low-passage MRC-5 cells by 1:1 splits, prepare a cell suspension containing 2.0×10^6 cell per mL in a storage medium consisting of Eagle's growth medium containing 10% glycerol and 20% FCS (*see Note 4*). Distribute aliquots of 1.5 mL into 1.8-mL screw-capped freezer vials and freeze the vials slowly (at approx 1°C per min) from room temperature to –70°C. Transfer the vials to the vapor phase of a liquid nitrogen storage cylinder.
6. Screen cell cultures for the presence of extraneous agents. Check visually and by subculture for the presence of fungi and yeasts. Inoculate samples of fluids into broth culture for the detection of bacteria. Use DAPI-staining of cells for detection of mycoplasma (*see Note 5*). More detailed treatments of this topic can be found in reviews by Knight (*12*) and Gwaltney et al. (*13*).

3.1.2. Temperature Control

The accurate control of temperature of incubation is essential. The greatest control can be achieved by total immersion of cultures in a circulating water bath maintaining temperature with an accuracy of at least $\pm 0.2^\circ\text{C}$. Screw-capped glass medicinal flat bottles are generally superior to plastic flasks for this purpose. Petri dishes can be placed in plastic containers, which are gassed and sealed before submergence.

However, it is possible to obtain satisfactory temperature control using many commercial cell culture fan-assisted incubators, provided there is adequate attention to humidification, preheating of reagents and containers, and control on access.

3.1.3. Virus Source

It is important to initiate vaccine development with a recent virus isolate that has relevance to current epidemiological circumstances. The virus should originate from material isolated from a patient or a diseased animal, and be inoculated directly into the vaccine-approved cell substrate (*see Note 7*). It is preferable to inoculate the clinical specimen into cell culture, or into antibiotic-containing transport medium with subsequent inoculation into cell culture in the laboratory. After inoculation, the transport medium should be held at low temperature, unfrozen. Delay between sampling and isolation into cell culture should be kept to a minimum.

1. Establish a genetically homogeneous stock by propagation from single plaques. To do this, isolate virus from individual plaques appearing on cell monolayers infected at limiting dilution. The time of incubation and the temperature will depend on the particular virus. For respiratory syncytial virus, incubate at 37°C under a 0.9% (v/v) agar (or agarose, because the growth of some viruses is inhibited by impurities in commercial agar) overlay for 5–7 d. Visualize the plaques by addition of a 0.05% (v/v) neutral red-containing agar overlay at d 4 or 5 (*see Note 8*). Pick plaques into 1 mL growth medium from monolayers with single plaques whenever possible, by inserting a sterile Pasteur pipet through the agar overlay and removing an agar plug and attached cells. Resuspend the sample and after dilution plate immediately onto fresh monolayers, without intervening freeze-thawing. Many viruses are sensitive to freeze-thawing, and in the case of respiratory syncytial virus, a preferential loss of ts virus was observed (*14*).
2. Repeat the cycle of infection and plaque picking at least three times; exceptionally, in the case of an avian pneumovirus (*Turkey rhinotracheitis virus*), it was necessary to carry out ten sequential re-isolations before homogeneous stocks of two distinct plaque morphology mutants were obtained from a mixed parental stock (*15*).
3. Amplify the final plaque isolate by sequential passage in small (25 cm²), medium (75 cm²), and large (150 cm²) flask cultures to achieve the final volume of virus-containing supernatant required. Pool the fluids from the final passage in large flask cultures and clarify by low-speed centrifugation in a refrigerated bench centrifuge. Distribute into freezer vials as 1-mL aliquots and store frozen in the vapor phase of a liquid nitrogen storage cylinder.
4. Test several randomly selected aliquots of this stock virus for the presence of fungal, mycoplasmal, and bacterial contaminants by inoculation into appropriate detection media. Freedom from adventitious cytopathic viral agents can be verified by prolonged incubation of MRC-5 cells inoculated with the triple-cloned stock virus rendered non-infectious by exposure to specific neutralizing antiserum. The absence of known viral pathogens can be ensured by inoculation of appropriate susceptible cells and screening by immunofluorescence using specific monoclonal antibodies (MAbs). An updated consideration of likely pathogens is given by Gwaltney et al. (*13*).

3.1.4. Mutagenization

Decide whether to attempt isolation of spontaneously occurring mutants or to employ mutagens to induce mutations and thus enhance the frequency of recovery of mutants (*16*). RNA viruses have high mutation rates because viral RNA-dependent RNA polymerases lack proofreading capability. As a consequence, ts mutants can be isolated without mutagen treatment in the case of viruses that achieve moderate to

high progeny virus titers during growth in cultured cells. In the case of viruses that do not grow to high titer, such as respiratory syncytial virus, it may be essential to use mutagens. Even in the case of a virus such as Rift Valley fever virus, which does grow to moderate titers, it may be expedient to use mutagens to accelerate modification of the virus during propagation *in vitro* (see **Subheading 3.5.**). Mutagenization is obligatory in the case of DNA-containing viruses.

1. Choose an appropriate mutagen. Chemical mutagens are more controllable than ionizing or non-ionizing radiation, and are preferred. In the case of easily purified viruses that grow to high titer, it is feasible to carry out the mutagen treatment *in vitro* by exposing purified virus or nucleic acid to the mutagenic agent for varying periods of time. The mutagen is usually added to virus-infected cells to induce mutations, predominantly by causing mis-incorporation of nucleotides during replication. 5-bromodeoxyuridine (BUDR) and 5-fluorouracil (5-FU) are the first choice for DNA-containing viruses and for RNA-containing viruses, respectively (see **Note 9**).
2. Determine the concentration of mutagen required to enhance the yield of mutants. To do this, inoculate replicate susceptible cell monolayers with the plaque-purified virus at a ratio of 1 pfu per cell. After adsorption, remove the virus inoculum by three changes of medium, and then add a range of concentrations of a base analog mutagen (e.g., 5-FU or 5-BUDR) made up in normal incubation medium. In the case of the mutagenization of RNA-containing viruses with 5-FU, final concentrations in the range of 10–500 µg per mL are appropriate. At these concentrations, the viability of MRC-5 cells is not seriously affected for several days.
3. Then incubate the mutagen-exposed infected cell cultures at a suboptimal temperature (30–33°C) until cytopathic effect is maximal in cultures containing no mutagen (see **Note 10**). Harvest the supernatant fluids and clarify by low-speed centrifugation.
4. Remove the mutagen by dialysis for 18–24 h against changes of normal incubation medium (with antibiotics, but without serum).
5. Determine the reduction in virus yield by assay of residual infectivity at both *permissive* and *restrictive* temperatures (see **Subheading 3.2.**). Identify the minimum mutagen concentration at which there is a measurable increase in the difference between the infectivity titers at permissive and restrictive temperatures of incubation (see **Subheading 3.2.**). Use this mutagen concentration as the initial treatment, and subsequently adjust in response to the yields of mutants obtained. The protocol described is designed to limit the frequency of isolation of multiple mutations so that the majority of the ts mutants isolated are the consequence of single base changes (see **Note 11**).

3.2. Isolation of TS Mutants Following Mutagen Treatment

To avoid the re-isolation of mutant virus originating from the same mutational event, several mutagenic treatments of wild-type virus should be carried out independently with only one ts mutant isolated from each treatment. The isolation of ts mutants of respiratory syncytial virus by three methods is described here as examples. As a preliminary step to all three methods, first establish appropriate permissive and restrictive temperatures as follows. Assay the wild-type unmutagenized virus for plaque-forming ability on monolayers of susceptible cells incubated at a range of temperatures between 30°C and 42°C. The *permissive temperature* should be the lowest temperature of incubation at which the virus yield does not depart significantly from

the mean, and the *restrictive temperature* should be the highest incubation temperature at which the titer does not differ by more than 1 log₁₀ unit from the mean.

3.2.1 Screening After Pre-Amplification

1. Plate out the mutagen-treated virus at limiting dilution on MRC-5 monolayers in 50-mm plastic Petri dishes, or six-well cluster plates. Aspirate off the inoculum and add an agar-containing overlay. Incubate the infected monolayers at the predetermined permissive temperature until macroscopic plaques are clearly visible. Add a second agar overlay containing neutral red stain, but no serum, and allow it to solidify. Incubate at the permissive temperature for an additional 24–48 h, avoiding exposure to light.
2. Choose monolayers with single or a few well-dispersed plaques for plaque picking (*see Note 12*). Plaque picking is accomplished by inserting a narrow bore (~1-mm diameter) sterile cotton wool-plugged Pasteur pipet into the agar above the plaque. Withdraw an agar plug and expel it directly onto a fresh cell monolayer or into a storage vial containing a small volume of growth medium. It is not necessary to scrape the monolayer; sufficient infectivity will be present in cells attached either to the base of the agar plug, or as virus that has diffused into the agar.
3. The screening of the plaque-picked isolates for temperature-sensitivity can be carried out either after preliminary amplification to provide a reference stock, or directly by inoculation onto replicate monolayers that are then incubated at the permissive and restrictive temperatures. The pre-amplification of plaque-picked virus is generally a more efficient and reliable procedure, although time-consuming and more expensive in terms of consumables. Inoculate each plaque isolate directly onto a monolayer of susceptible cells in a small (25 cm²) screw-capped flask. Adsorb for 1 h at permissive temperature; then add 3 mL of maintenance medium without removal of the inoculum. Incubate at the permissive temperature until the cytopathic effect is extensive. The advantage of this procedure is that screening and the isolation of clones can be carried out at different times.
4. To screen the amplified isolates for temperature-sensitivity, inoculate two sets of monolayers of susceptible cells in 96-well flat-bottomed cluster plates and adsorb at room temperature for 30 min. Then add maintenance medium and incubate one set of plates at permissive temperature and one set at restrictive temperature until the cytopathic effect is extensive in control wells simultaneously infected with wild-type virus (*see Note 13*). Those isolates that fail to produce cytopathic effect on monolayers incubated at restrictive temperature are putative ts mutants.
5. To ensure the absence of any carry-over of wild-type virus, initiate second and third cycles of cloning of these putative ts mutants by plating out and re-isolation of single plaques.
6. Verify and quantify temperature-sensitivity by determining plaque counts for the triple-cloned virus at restrictive and permissive temperatures. Those mutants that differ by less than three log₁₀ units are unlikely to be useful, and should be discarded unless none better can be isolated.

3.2.2. Screening Without Amplification

An alternative procedure, which is more economical in time and consumables, is screening without pre-amplification. This follows the procedure outlined in the previous section, with the difference that fluid from the storage vial containing the agar plug is inoculated directly onto duplicate assay plates for incubation at the two temperatures. This procedure is the preferred one, in which the yield of infectious virus from a single plaque is appreciable (>1000 pfu). In the case of viruses for which the

yield may be low (<100 pfu), this procedure is prone to error and the number of false-positives can be high. Putative ts mutants, however, must undergo the same triple cloning and verification procedure to provide a substrate for the next round of mutagenesis.

3.2.3. Screening by Replica Plating

An accelerated version of the preceding method is to bypass the isolation step by inoculating the agar plug in equal proportions directly into two corresponding wells in duplicate 96-well blocks. One block is then incubated at permissive temperature, and the other block at restrictive temperature. Since no virus has been stored, the block incubated at permissive temperature serves as the repository of the isolates as well as the assay control block. When a potential mutant is identified, virus is recovered from the corresponding well of the block incubated at permissive temperature, and subjected to triple cloning as previously described. This method is fast and economical, but vulnerable to losses by fortuitous contamination, since a single contaminated well entails loss of the remaining 92 isolates.

Sectored plates can substitute for multi-well plates. These are prepared as follows.

1. Allow 2.5 mL of agar overlay to solidify in 50-mm Petri dishes containing confluent monolayers of MRC-5 cells. Mark the plates into sectors on the underside with indelible ink.
2. Inoculate picked plaques onto duplicate sectored plates. Insert the Pasteur pipet through the agar overlay and expel half of the plug onto one plate. Inoculate the other half of the plug similarly onto the corresponding sector of a duplicate plate.
3. After a period of 30 min at permissive temperature in a humidified incubator for adsorption, add a second agar-containing overlay of 2.5 mL to seal the plates. Incubate one of each pair of plates at permissive temperature, and the other at restrictive temperature.
4. Absence of cytopathic effect at the restrictive temperature indicates a putative ts mutant. Produce an amplified stock by propagation of virus from the control plate incubated at permissive temperature.

This procedure is rapid and less vulnerable to losses by contamination than the multi-well plate method. It has the advantage that plaque morphology differences can be screened simultaneously with temperature-sensitivity.

3.3. Establishment of Mutant Stocks and Determination of Mutant Phenotype

The protocols outlined in **Subheading** include verification of the temperature-sensitivity of putative mutants and the growth of triple-cloned stocks.

It is also necessary to undertake some preliminary characterization of the phenotype of the first harvest of mutants in order to select a suitable mutant to form the parental virus for the next round of mutagenesis. Properties of relevance are a matter for intuitive decision for each particular infectious agent, and no general guidance can be given here; detailing of methodology is inappropriate (*see ref. 17* for methods of phenotyping for a respiratory syncytial virus).

In the case of respiratory syncytial virus, it was found that the mutants that showed most promise in trials in adult volunteers were those with an RNA-positive phenotype and thus able to support viral protein (antigen) synthesis at the restrictive temperature.

In general, mutants with RNA-negative phenotypes, although showing dramatic reduction in pathogenicity, did not induce good immune responses in adult volunteers (10,11).

3.4. Vaccine Development by Sequential Selection of Multiple TS Mutants

The following protocol is generalized, with specific details for respiratory syncytial virus inserted for illustration (10,11).

3.4.1. Isolation of the Primary Series of (Putative Single) TS Mutants

1. Establish permissive and restrictive temperatures and prepare a triple-plaque purified stock of the parental virulent virus as described in **Subheadings 3.1.3.** and **3.1.4.**, respectively. In the case of respiratory syncytial virus, the initial restrictive temperature was 39°C, and a permissive temperature of 31°C was used throughout.
2. Carry out mutagen treatment as described in **Subheading 3.1.4.** Respiratory syncytial virus was mutagenized by infection of MRC-5 cells at a multiplicity of approx 1, followed by incubation in maintenance medium (Eagle's medium plus glutamine, antibiotics, and 1% (v/v) FCS) supplemented with 100 µg/mL 5-FU (see **Note 14**).
3. Plate out at limiting dilution samples of the supernatant fluids of several independently mutagenized cultures.
4. Carry out plaque picking and screening for temperature-sensitivity as described in **Subheading 3.2.** It was observed in the case of respiratory syncytial virus that yields of ts mutants were improved by avoiding freeze-thawing.
5. Determine the phenotypes of at least five mutants originating from independently mutagenized cultures as described in **Subheading 3.3.** In the case of respiratory syncytial virus, the single mutant with the greatest temperature restriction at 39°C (e.g., the ratio of plaques at 39°C/plaques at 31°C), in conjunction with an RNA-positive phenotype, was identified as the most suitable parental virus for the next round of mutagenesis. A stock of this mutant, designated *tsIA*, was prepared by triple-cloning and amplification as described in **Subheading 3.1.3.**

3.4.2. Isolation of a Secondary Series of (Putative Double) TS Mutants

1. Mutagenize the primary series ts mutants, *tsIA* in the case of respiratory syncytial virus, as outlined in **Subheading 3.1.4.** For the second round of mutagenesis, choose a mutagen with a different chemical specificity from that used in the first round of mutagenesis. The mutagen used in isolation of the candidate respiratory syncytial virus vaccine double-mutant *tsIB* was an acridine derivative, known as ICR 340 (10).
2. Incorporate the mutagen into standard maintenance medium at an appropriate concentration (10 µg/mL ICR 340 for respiratory syncytial virus) and incubate the mutant-infected cells in its presence until cytopathic effect (CPE) is far advanced in control unmutagenized cultures. Again avoid freeze-thawing (see **Note 15**).
3. Plate out the mutagenized virus at limiting dilution and pick plaques from plates incubated at permissive temperature as before (see **Subheading 3.3.**). In the case of viruses of moderate to good stability, growing to reasonable titer, the mutagen should be removed by dialysis against maintenance medium before inoculation and plaque picking.
4. Pick isolated plaques, or preferably plaques from single-plaque plates, and screen for temperature-sensitivity. In the case of respiratory syncytial virus, screening for temperature-sensitivity was carried out at a restrictive temperature arbitrarily chosen as one degree below that used for selection of the first mutant, in this case, 38°C.

5. Determine the phenotypes of at least five mutants from independently mutagenised cultures as described before in **Subheading 3.3**. In the case of respiratory syncytial virus, the single mutant with the greatest temperature restriction at 38°C, in conjunction with an RNA-positive phenotype at that restrictive temperature, was identified as the most suitable parental virus for the next round of mutagenesis. A stock of this mutant, designated *ts1B*, was prepared by triple-plaque purification and amplification as described in **Subheading 3.1.3**.

3.4.3. Isolation of a Tertiary Series of (Putative Triple) TS Mutants

1. Mutagenize the secondary *ts* mutant, designated mutant *ts1B* in the case of respiratory syncytial virus, as outlined in **Subheading 3.1.3**. Choose a mutagen of a different specificity from that used in the preceding mutagenesis. However, in the case of the respiratory syncytial virus project, 5-FU was used again because experience has shown that this mutagen is the most reliable and consistent for RNA viruses (**16**).
2. Plate out independently mutagenized lots of the second series mutant (mutant *ts1B*) at limiting dilution and incubate the plates at permissive temperature.
3. Pick well-dispersed plaques, preferable plaques from single-plaque plates, and screen for temperature-sensitivity at a third restrictive temperature. In the case of respiratory syncytial virus, this was arbitrarily chosen as 1°C below (37°C) that used for selection of the second series mutant. The single mutant with the greatest temperature restriction at 37°C, in conjunction with an RNA-positive phenotype at that restrictive temperature, was selected as the third series (putative triple) mutant, which represented the endpoint of the project.
4. Produce a stock of this mutant, designated *ts1C* in the case of respiratory syncytial virus, by three sequential re-isolations from single plaques and amplification (*see Subheading 3.1.3*.) for subsequent evaluation as a candidate vaccine (**10,11,18**).

3.5. Modification of Virus by Continuous Mutagenization

A successful protocol for continuous mutagenization has been described for Rift Valley fever virus (Family *Bunyaviridae*, Genus *Bunyavirus*) by Caplen et al. (**9**). The following is a brief outline of their method, modified to permit accumulation of *ts* mutations.

1. Infect monolayers of MRC-5 cells at a multiplicity of 0.1 pfu/cell. Remove unadsorbed virus by several washes with growth medium and finally replace with growth medium containing 10% (v/v) FCS and 200 µg/mL 5-FU (*see Note 16*). Incubate the infected cultures for 3–4 h at 33°C in a 5% CO₂-gassed incubator. Remove unadsorbed or desorbed virus by discarding the culture fluid and replacing it with fresh medium containing the same concentration of 5-FU.
2. Harvest the culture supernatants after a further 48–72 h incubation at 33°C.
3. Titrate the yield of infectious virus on MRC-5 cell monolayers by incubation for 4 d at 33°C under a 0.5% agarose overlay containing a reduced concentration (4%) of FCS before application of a second 0.5% agarose overlay containing 0.01% neutral red stain.
4. After incubation for a further 18–24 h, pick two large well-separated plaques and inoculate onto fresh MRC-5 cell monolayers in screw-capped flasks.
5. Harvest the fluid from these infected cultures after 48–72 h incubation at 33°C, and clarify by low-speed centrifugation. Store the clarified supernatant at –70°C.
6. Assay these yields for plaque formation at a predetermined restrictive (38–40°C) and permissive (31–33°C) temperature.

7. Initiate the next round of infection and mutagenesis using the virus that exhibits the greatest differential between the assays at the permissive and restrictive temperatures.
8. Continue this procedure as long as there is evidence of an increase in temperature-sensitivity without decline in yield at the permissive temperature (*see Note 17*).

A control series of plaque isolations, propagated under identical conditions but in the absence of mutagen, is required to properly evaluate the progress of mutagenization and the accumulation of ts virus.

3.6. Modification by Passage at Low Temperature

There are many routes to achieving modification of virus by passage at low temperature. Since these are determined to a large extent by the host range and biological properties of specific viruses, it is not appropriate to present a protocol here.

The reader is referred to the literature on the derivation of ca experimental influenza A virus (Family *Orthomyxoviridae*) vaccines. Here, there is the added dimension of utilization of subunit reassortment. A ca virus has been used successfully as a donor of attenuating genes for the construction of other attenuated influenza A viruses (*19*).

3.7. Evaluation of Vaccine Potential of Modified Virus

The evaluation of the vaccine potential of any virus modified by the procedures described here, or by any other methodology, is a problem of great complexity, and is beyond the scope of this chapter. Each virus poses a different set of challenges, and specific solutions must be sought without regard to precedent.

The normal path to authenticating the merits of a candidate human vaccine is through evaluation in whatever animal models are available, through volunteer trials in sero-positive individuals, ultimately to trials in sero-negative individuals (*2,6,20,21*).

3.8. Reverse Genetic Approaches

3.8.1. Overall Strategy

The ability to produce infectious virus from cDNA provides an alternate route for production of precisely characterized attenuated viruses. The evaluation of empirically derived candidate vaccines identifies the sites of attenuating mutations, and such mutations can be introduced by reverse genetics into other related viruses that may possess more appropriate antigenic characteristics, or to enhance the genetic stability of existing candidate vaccine strains (*22,23*).

Paradoxically, the technique of reverse genetics has been exploited to greatest extent in the case of the negative-strand RNA viruses, long the most inaccessible to controlled genetic modification by site-specific mutagenesis. In the case of negative-strand RNA viruses, the genomic and the anti-genomic RNA are not infectious, and the minimal replicative unit is the nucleocapsid, which contains the nucleocapsid (N) protein tightly bound to the RNA, together with the phosphoprotein (P) and the polymerase (L) protein, which are less tightly bound. The basic strategy, developed first for rabies virus (*4*), involves providing genes encoding the essential nucleocapsid components, N, P, and L, in cells together with a cDNA producing the anti-genome.

This strategy depends on intracellular synthesis of T7 RNA polymerase, which is most conveniently supplied by a vaccinia virus recombinant, to direct the synthesis of mRNAs encoding the components of the viral nucleocapsid complex and a copy of the cloned genome (usually as the +ve sense anti-genome) terminated with a ribozyme sequence to achieve precise termination of the transcript. The intracellularly synthesized nucleocapsid proteins (N, P, and L, supplied by support plasmids) mediate the replication of both the +ve sense antigenomic RNA and transcribe -ve sense genomic RNA, which also results in the production of the remaining virus structural proteins and packaging of functional nucleocapsids. The infectious progeny virus is separated from the vaccinia-T7 recombinant and propagated for analysis of phenotype. This general procedure is applicable to all negative-strand RNA viruses, varied only by the inclusion of any additional essential nucleocapsid components when applicable (25).

Since all of these operations involve nucleotide sequence-specific components and primers and standard recombinant DNA methodology, a generalized protocol is inappropriate. Instead, two protocols for critical stages common to all strategies are presented in detail.

3.8.2. Site-Directed Mutagenesis

Site-directed mutagenesis is performed using standard recombinant DNA technology involving oligonucleotide-directed mutagenesis of an infectious cDNA clone of the virus genome. This is achieved by a polymerase chain reaction (PCR), in which one of the primers encodes the desired mutation as a mismatch to the wild-type sequence. If the mutation is close to a naturally occurring restriction enzyme cleavage site, the replacement of the wild-type sequence with the mutation is straightforward. If there is no appropriate cleavage site, then the PCR fragment containing the mutation can be joined to other PCR fragments that extend the sequence to a usable cleavage site, either by overlapping PCR or by direct ligation (*see Note 16*). Overlapping PCR utilizes two sets of primers, in which one of each set includes complementary sequences at their 5'-termini, and two separate PCR reactions are performed, followed by ligation of the products (**refs. 22,23, and 24** provide examples of how these protocols are integrated into the complete procedure, including the substitution of commercial kits in place of the following protocols).

3.8.2.1. SITE-SPECIFIC MUTAGENESIS BY OVERLAPPING PCR

1. Overlapping PCR utilizes two sets of primers in which one of each set includes complementary sequences at their 5'-termini.
2. Two separate PCR reactions are performed using cDNA as the template. The following is a reliable procedure, which uses Gibco Taq polymerase and the buffers supplied by the manufacturer.
3. PCR reaction mix (on ice):
 - a. 10 μ L 10X buffer.
 - b. 3 μ L $MgCl_2$.
 - c. 2 μ L detergent (1% w-1) (optional).
 - d. 2 μ L 10 mM dNTP mix (*see Note 18*).
 - e. 1 μ L primers (~0.4 μ g).

- f. 4 μL cDNA.
 - g. 79 μL sterile distilled water.
 - h. 0.4 μL Taq polymerase.
4. The amounts of primer or cDNA employed may be different, in which case the amount of sterile distilled water should be adjusted so that the final volume remains 100 μL . The 10X buffer, MgCl_2 , and detergent are supplied ready-made with the Taq enzyme (*see Note 19*).
 5. Use the appropriate program on the PCR thermal cycler. Use 1-min extension times for each kilobase to be amplified.
 6. To check if your PCR has worked, run a sample on an agarose gel with an appropriate ladder for calibration. Different concentrations of agarose are required to resolve different size fragments: 2% gel for fragments <500 bp; 1% gel for fragments 500 bp–3K; 0.7% gel for fragments >3K.
 7. The products can be purified by agarose gel electrophoresis to remove unincorporated primers. Alternatively, cut out the PCR product from the agarose gel using a clean scalpel blade.
 8. Use a Qiagen gel extraction kit to purify the PCR product.
 9. PCR is performed a second time using only the outside set of primers. PCR reactions should be carried out using thin-walled tubes.
 10. The product of this reaction is then inserted into the plasmid carrying the cDNA copy of the viral genome using appropriate restriction enzyme sites.

3.8.2.2. PCR LIGATION

1. Two PCR reactions are performed using cDNA as the template.
2. Purify the PCR products by agarose gel electrophoresis.
3. Add 1 mM ATP reaction buffer (*see Subheading 3.8.2.1.*) to each PCR product. Raise the temperature to 70°C and then add 50 U of T4 polynucleotide kinase. Incubate at 37°C in a 50 μL vol for 30 min.
4. Mix 5 μL of each phosphorylated primer (adjusted to approx equimolar proportions) with 400 U of T4 DNA ligase, and leave for 15 min at room temperature.
5. Use 1 μL of this ligation reaction as the template for a PCR with appropriate external primers and insert the product into a plasmid carrying the virus genome copy using convenient restriction sites.

3.8.3. Transfection

The second critical stage is the recovery of the mutagenized virus from the infectious cDNA clone. A reliable method using Lipofectace (Gibco-BRL) is described here.

1. In a 12-well tissue-culture-plate seed cells (in this example, Hep-2 cells) per well in GMEM supplemented with fetal bovine serum (FBS) at a final concentration of 10%. Incubate at 37°C for 18–24 h (*see Note 20*). The goal is to obtain 50–75% confluent monolayers. (NB: Since transfection efficiency is sensitive to culture confluence, it is important to maintain a standard seeding protocol from experiment to experiment *see Note 21*).
2. Prepare the following solutions in separate polypropylene tubes: Solution A: For each transfection, dilute 1 μg plasmid DNA in 50 μL serum-free Optimem (Gibco-BRL). Solution B: For each transfection, dilute 4 μL Lipofectace (Gibco-BRL) reagent in 50 μL serum-free Optimem (Gibco-BRL).

3. Combine the two solutions, mix gently by pipetting up and down and incubate at room temperature for 15 min. The solution may appear cloudy; however, this will not affect the transfection.
4. Aspirate medium from the cells.
5. For each transfection, add 400 μL serum-free Optimem (Gibco-BRL) to each tube containing the lipid-DNA complexes. Mix gently and overlay the diluted complex onto the cells.
6. Incubate the cells for 5 h at 37°C.
7. Add 500 μL GMEM (10% FBS) without removing the transfection mixture. If toxicity is a problem, remove the transfection mixture and replace with normal medium. Replace medium with fresh medium at 18–24 h following the start of transfection.
8. Assay cell extracts for virus activity 48–72 h after the start of transfection.

4. Notes

1. Normally a concentration of 10% (v/v) is adequate, but sera of different origin may vary in suitability, and each batch should be tested before use. For most viruses optimal yields are obtained with optimally growing cells. Assay of virus concentration by plaque formation is best achieved at concentrations of FCS, which maintain viability but restrict growth (0.5–1%).
2. FCS originating from the United Kingdom and other European countries should not be used for vaccine development and production because of the risk of contamination by the bovine spongiform encephalopathy (BSE) prion.
3. For example, adult sheep serum has good growth-stimulating properties and is a good substitute if the presence of antibody is not a concern. However, the serum must originate from scrapie-free animals.
4. Care should be taken to remove any residual trypsin by sedimentation of the cells from the transfer medium prior to resuspension in storage medium.
5. Seed sterile degreased cover slips in 30-mm sterile Petri dishes with approx 10^5 cells per dish. Grow to subconfluence and fix in methanol. Treat with a solution of 1 $\mu\text{g}/\text{mL}$ DAPI (4,6 diamidino-2-phenylindole) for 15 min at 37°C. Remove stain and wash in methanol. Examine by fluorescence microscopy. Uncontaminated cells have brightly fluorescing nuclei but unstained cytoplasm, whereas mycoplasmal-contaminated cells show cytoplasmic and surface fluorescence.
6. In all cases of PCR-mediated mutagenesis, the reaction should be performed with proof-reading polymerases and a low cycle number (<25) to limit misincorporation. (If PCR machine is not of the heated lid variety, put a drop of mineral oil on top of the mix to prevent evaporation during the PCR.)
7. It is important before initiating the process of vaccine development to verify by whatever means available that the isolate retains the characteristic pathogenic properties of the wild-type virus.
8. Neutral red has a photodynamic inactivating effect on virus. Consequently, the overlaid monolayers and any solutions containing neutral red should not be exposed to light more than is necessary.
9. 5-fluorouridine or 5-azacytidine can be used as alternative mutagens for RNA viruses, and nitrous acid, hydroxylamine, ethyl methane sulphonate, ethyl ethane sulphonate, N-methyl-N-nitro-N-nitrosoguanidine, and proflavine and other acridines are alternative mutagens for DNA and RNA viruses (16).

10. In the case of respiratory syncytial virus, it was observed that a concentration of 50 μg per mL 5-FU or above inhibited cytopathic effect and reduced virus yield.
11. A concentration of mutagen which reduced the yield of progeny virus from respiratory syncytial virus-infected MRC-5 cells at permissive temperature to approx 1% of normal was found to give yields of ts mutants at frequencies in the range 1–5%.
12. Plaque picking is intended to recover progeny virus originating from a single infectious particle. In practice, this cannot be achieved reliably when there is more than one plaque on the monolayer, because there is always a fluid layer between the cell monolayer and the overlying agar allowing lateral diffusion of released virus. Nonetheless, this is not a serious concern with highly cell-associated viruses and plaques can be picked from plates with several plaques. It should be remembered, however, that virus-infected cells can extend beyond the boundary of the visible lytic plaque.
13. Each cluster plate should contain two uninoculated cell control wells and two wild-type virus infected wells. Thus, 92 isolates can be screened on each pair of multi-well plates.
14. 5-FU (Sigma, London) can be made up as a stock solution of 1 mg/mL in sterile distilled water, filtered through a 0.22- μm filter, and stored frozen as aliquots at -20°C .
15. If an interruption is unavoidable, even thermosensitive viruses such as respiratory syncytial virus can be stored at an accurately maintained $+4^{\circ}\text{C}$ for a few days without serious loss of infectivity.
16. The concentration of 5-FU used by Caplen et al. (9) was that required to reduce the virus yield 10^2 – 10^3 -fold.
17. Modification of the pathogenetic properties of Rift Valley fever virus required 16–18 cycles of mutagenesis.
18. 10 mM dNTP mix is prepared as follows: 5 μL 100 mM dATP; 5 μL 100 mM dTTP; 5 μL 100 mM dGTP; 5 μL 100 mM dCTP; 30 μL H_2O . Make sure to use dNTPs and not ddNTPs. Label the dNTP stock and store it at -20°C .
19. When setting up PCR reactions, use the PCR-grade Gilson micropipets and barrier filter tips to avoid contamination. Always include a negative control.
20. For 6-well plates or 35-mm dishes, the amount of DNA and lipofectace should be multiplied by 2.5.
21. For transfection of other cell lines using Lipofectace or Lipofectamine, the amount of DNA and lipid used for transfection should be determined separately. By keeping the amount of DNA constant (2 μg is a good starting point), different amounts of Lipofectace or Lipofectamine should be used (e.g., 1, 2, 4, 6, 8, and 10 μL of each in the first instance).

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