

EPIDEMIC
ALERT &
RESPONSE

WHO guidance on development of influenza vaccine reference viruses by reverse genetics



Department of Communicable Disease
Surveillance and Response
Global Influenza Programme

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1. Introduction

The World Health Organization (WHO) reviews the world influenza epidemiological situation and virological data twice annually and, if necessary, recommends new influenza vaccine strains. Following the WHO recommendations, it is usually necessary to develop vaccine reference viruses which have the antigenic properties of the recommended strain, but with improved growth properties for more efficient vaccine production. For more than twenty years, high growth reassortant influenza viruses have been prepared by infecting an embryonated hen's egg with the wild type recommended strain and A/PR/8/34, a laboratory adapted virus that grows to high titre in eggs. The goal has been to generate reassortants that contain six gene segments from A/PR/8/34 virus plus the haemagglutinin (HA) and neuraminidase (NA) gene segments of the newly-recommended wild type strain (6:2 reassortants). However, since the traditional approach is empirical, more than two gene segments may derive from the wild type strain with the remainder from PR8, generating for example 5:3 or 4:4 or other reassortants, and these have been effective high growth strains. Generally, the gene constellation of high growth reassortants is not determined.

In recent years, plasmid-based reverse genetics has been applied to the rational development of influenza virus high growth reassortants (1–7). In each case, 6:2 reassortants were created in which the HA and NA segments were derived from a variety of wild type viruses including H1N1, H3N2, H5N1 and H5N3 human and avian viruses with the remaining six segments from A/PR/8/34. Generally these have grown as well as the equivalent high growth reassortants derived empirically. The reverse genetics process involves the use of mammalian cells and, during development of the technology, 293T cells, 293T/MDCK cell mixtures and Vero cells have been used. However, the requirement for a mammalian cell in the reverse genetics process imposes specific regulatory requirements when the rescued virus is intended for use in the development of human vaccine.

Reference viruses are not subject to the comprehensive regulatory requirements that apply to vaccines as they are not part of vaccine manufacture *per se*. However, as they are often used for the development of human vaccine seed viruses, appropriate procedures should be followed in their preparation to ensure an adequate level of quality and safety. Moreover the derivation of the reassortant virus should be approved by the National Regulatory Authority. The guidance provided below is to ensure clarity and worldwide consistency in the procedures used to develop and test human influenza reference viruses produced by reverse genetics, and has been developed after consultation with WHO Collaborating Centres for Reference and Research on Influenza, regulatory agencies and vaccine manufacturers.

2. Materials used in reverse genetics

2.1 Wild type influenza virus

Traditional reassortant viruses are derived only from wild type viruses isolated and grown in hens' eggs, or in a validated clean cell culture system. All other systems may harbour unknown contaminants that could adversely affect the quality of the reassortant virus. However, in deriving a reassortant by reverse genetics, any source of influenza virus genetic material is acceptable, as any potential contaminant of the virus isolation system, or present in the clinical sample, will be eliminated during the molecular cloning step. Thus, the originating wild type virus for a reverse genetics based reassortant may be derived from eggs, non-validated mammalian cell culture, or directly from clinical material.

The wild-type virus should be handled under appropriate conditions for an infectious agent (see especially, section 5). It should also be characterized by a WHO Collaborating Centre for Influenza as antigenically suitable for vaccine virus development.

2.2 Cells

Mammalian cells may harbour microbiological contaminants that could adversely affect the quality of a reference virus and subsequent vaccine seed. Consequently, cells for use in deriving vaccine reference viruses by reverse genetics should be validated to be free of contaminants, as determined by the national control authority. Cells approved for human vaccine production should be used.

The cells should be based on a cell bank system. Cells used for the reverse genetics process should be passaged minimally from the cell bank and, for cells specifically validated for vaccine production, within the maximum number of passages permitted for vaccine production. Culture media and other materials used for cell propagation should comply with the guidance given below in section 2.3.

Reassortant viruses, derived in laboratories where approved cells have not been used, are not recommended for use in the development of vaccine seed viruses.

Initial demonstrations of the use of plasmid-based reverse genetics for the derivation of reassortants made use of 293T or 293T/MDCK cells (1–4). While optimal for reverse genetics, 293T cells are not validated for the preparation of human vaccine and so viruses derived on them should not be used for this purpose. Of the alternative cell systems available, the most suitable appears to be Vero cells, banks of which have been approved for the production of human vaccines. Vero cells have been shown to be amenable to the reverse genetics process (5–8) and reference viruses specifically for use in human vaccine development have been prepared using validated Vero cells (6, 7). Development of other cell lines that may be appropriate for use in reverse genetics and in human vaccine production is being pursued.

Additional guidance related to considerations for tissue cultures and materials used to support tissue cultures for vaccine production can be found in the WHO Expert Committee on Biological Standardization, 47th report, Requirements for the use of animal cells as in vitro substrates for the production of biologicals (9).

2.3 Reagents

All reagents used for the reverse genetics derivation of a reference virus should be of good quality. This includes materials used for cell propagation, bacterial growth media, reagents used for plasmid DNA purification and reagents used during cell transfection and virus rescue. All products of animal origin (especially bovine sourced material) used in the preparation of a reference virus should be from an acceptable source and comply with WHO recommendations on Transmissible Spongiform Encephalopathies (10). All steps should be carefully scrutinized for the origin of their component parts. Typical reagents that are, or may contain, materials of animal origin are bovine serum used in cell media, porcine trypsin used in cell propagation, bacterial growth media, RNase used during plasmid DNA purification and bovine serum albumin used during virus recovery. Animal products used for cell propagation (serum and trypsin) should preferably be irradiated or otherwise treated to inactivate potential viral contaminants. The use of animal-free components should be explored.

3. Laboratory facilities and procedures

Reverse genetics procedures should be carried out in a microbiological safety cabinet within a laboratory area which is either separate from other influenza virus activities or which can be disinfected before work is initiated. After transfection of cells with plasmid DNA, the cells must be considered as infectious and, depending on the virus being rescued, should be kept at an appropriate level of biological containment. If a national competent authority determines a reverse genetics derived influenza virus to be a genetically modified organism, there should be compliance with appropriate requirements.

Cells for use in reverse genetics should be cultivated in a clean laboratory designated for cell culture and separate from work with biological agents.

Wherever possible, procedures should be conducted according to standard operating procedures. All activities associated with the derivation and characterization of a vaccine reference virus, e.g. cloning of the HA and NA genome segments, plasmid DNA preparation, cell culture, reverse genetics, virus propagation and all analytical tests should be recorded in laboratory notebooks. Records should be kept of all batch numbers for materials used. Laboratory records should include documentation that no other influenza viruses or their genetic material or other plasmids are handled at the same time as the rescue work in order to avoid cross contamination.

4. Characterization of the reference virus

The antigenic character of the reference virus should be assessed and shown to be identical to that of the wild type virus from which the HA and the NA segments were obtained. The nucleotide sequence of the HA and NA genes of the reference virus should be determined and should be compared with the sequence of the respective clones and of the genes from the original wild-type virus. Any differences should be noted. An assessment of the level of residual plasmid in the reference virus should be made using e.g. PCR technology.

- The virus titre should be determined in the appropriate substrate.
- The reference virus should be tested for bacterial and/or fungal contamination using normal procedures¹.
- A protocol should be prepared, summarizing virus development and documenting the items listed above in sections 2–4.
- The virus should also be assessed by a WHO Collaborating Centre for Influenza to confirm that antigenic and genetic characteristics are suitable for use as a vaccine reference virus.

5. Development of a reference virus from highly pathogenic influenza viruses

Work with highly pathogenic influenza viruses should take place at a high level of biological containment (e.g. BSL3+ or 4 as advised by WHO and national safety authorities).

Reverse genetics and recombinant DNA mutagenesis technology should be used to eliminate the HA pathogenicity motif (multibasic amino acids at the HA cleavage site) in order to produce a virus with properties suitable for large-scale vaccine manufacture.

The tests performed to demonstrate that a virus derived by reverse genetics is no longer pathogenic have been described by WHO (*11*) and include a chick embryo lethality test, a chicken pathogenicity test, a ferret pathogenicity test and sequencing.

All the above considerations on source materials, derivation and characterization (sections 2–4) also apply to a reference virus derived from a highly pathogenic influenza virus.

¹ See <http://www.who.int/biologicals/publications/trs/areas/vaccines/sterility/en/>

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