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Review

# Critical review of current and emerging quantification methods for the development of influenza vaccine candidates

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ABSTRACT

Significant improvements in production and purification have been achieved since the first approved influenza vaccines were administered 75 years ago. Global surveillance and fast response have limited the impact of the last pandemic in 2009. In case of another pandemic, vaccines can be generated within three weeks with certain platforms. However, our Achilles heel is at the quantification level. Production of reagents for the quantification of new vaccines using the SRID, the main method formally approved by regulatory bodies, requires two to three months. The impact of such delays can be tragic for vulnerable populations. Therefore, efforts have been directed toward developing alternative quantification methods, which are sensitive, accurate, easy to implement and independent of the availability of specific reagents. The use of newly-developed antibodies against a conserved region of hemagglutinin (HA), a surface protein of influenza, holds great promises as they are able to recognize multiple subtypes of influenza; these new antibodies could be used in immunoassays such as ELISA and slot-blot analysis. HA concentration can also be determined using reversed-phase high performance liquid chromatography (RP-HPLC), which obviates the need for antibodies but still requires a reference standard. The number of viral particles can be evaluated using ion-exchange HPLC and techniques based on flow cytometry principles, but non-viral vesicles have to be taken into account with cellular production platforms. As new production systems are optimized, new quantification methods that are adapted to the type of vaccine produced are required. The nature of these new-generation vaccines might dictate which quantification method to use. In all cases, an alternative method will have to be validated against the current SRID assay. A consensus among the scientific community would have to be reached so that the adoption of new quantification methods would be harmonized between international laboratories.

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## 1. Introduction

Human influenza viruses cause seasonal flu each year throughout the world. The virus can be fatal in the elderly, young children and immunocompromised population, causing up to 500,000 deaths according to the World Health Organization (WHO) [1]. Prevention of the disease is achieved via the use of vaccines, which typically contain a combination of two influenza A strains, and one or two influenza B strains. Influenza B is mostly found in human [2] and is not likely to cause pandemics, as opposed to influenza A which is found in large animal reservoirs such as aquatic birds, poultry and domestic swine [3] and presents therefore a higher probability of mutating and becoming infectious to humans. Hence, new vaccines are required every year due to antigen shifts and drifts of two glycoproteins expressed at the surface of the virus, hemagglutinin (HA) and neuraminidase (NA). By nomenclature, these two proteins will define the subtype of the virus with 18 types of HA (H1–H18) and 11 types of NA (N1–N11). Viruses can further be classified into strains based on the host of origin, geographical location and year of isolation. Over the period 2000–2011, antigenic drift resulted in 22 strain changes [4]. Certain strains have been circulating in the population for years and even decades. Therefore, immunity has been built up through direct exposure to the virus, and/or vaccination. However, very little immunity if any is present in the general population when a new strain appears, causing pandemics like the one observed in 2009 with H1N1 A/California/04/2009. In the last one hundred years, we have witnessed four pandemics. Fifty million people died in 1918–1919

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during the most deadly influenza pandemic recorded [5]. Given the potential impact of the virus, the fast production of new and efficient vaccines is critical.

Fertilized chicken eggs have been used since the 1940s for vaccine production [6]. Eggs are incubated for several days after inoculation with the seed virus to allow the virus to replicate. The allantoic fluid is then harvested, and the virus is inactivated and purified. However, millions of eggs from biosecure flocks are required since approximately only one dose of vaccine can be obtained per egg. Most of the manufacturing steps are automated but necessitate large operating facilities to meet cost-effectively the current demand for seasonal vaccination campaigns. To address these issues, several new technologies and platforms have been developed or are being evaluated in order to replace or complement egg-based vaccines. For instance, vaccine production in cell culture using bioreactors allows fast production in large quantities. A number of mammalian cell lines such as PER.C6 cells (human derived embryonic retinal cells) [7], HEK293 cells (Human embryonic kidney cells) [8], Vero (monkey kidney cells) [9], MDCK (canine kidney cells) [10,11], and EB66 (duck derived embryonic stem cell) [12] are being evaluated for the manufacture of influenza vaccines, and were recently reviewed in detail [13]. Notably, the FDA approved two new vaccines in 2012 and 2013 respectively: Flucelvax (Novartis) is generated in MDCK cells [14] while Flublok (Protein Science corporation), the first approved vaccine made of recombinant protein, is produced in insect cells [15]. Plants are also emerging as a promising production platform for the generation of subunit vaccines and viral-like particles [16–18]. When using tobacco plants, a new lot of vaccine can be produced within as little as three weeks [19].

Regardless of the production system, the release of a new vaccine is significantly slowed down by delays in the production of reagents required for quantification using the single radial immunodiffusion (SRID) assay. This technique is the main method formally approved by the WHO and national regulatory bodies for the quantification of influenza. Strain-specific antibodies used for detection are produced by WHO Essential Regulatory Laboratories within two to three months. As a result, new lots of vaccines that can be manufactured within three weeks, have to be retained for several months until SRID reagents are made available. New methods for viral quantification are therefore needed to accelerate vaccine release. Several different strategies are being proposed and can be grouped into three main categories: methods based on the use of antibodies, techniques that physically count the number of particles or quantify HA without antibodies, and assays to evaluate infectivity.

Here, we review the limitations of well-established methods, as well as the challenges of emerging methods that have been developed for the quantitation of influenza vaccine candidates. The focus is on methods that are easily implemented in the laboratory and require minimal investments and expertise.

## 2. Antibody-dependant methods

### 2.1. Conventional methods

The SRID assay has been used extensively for the quantification of influenza vaccines since 1978 [20] and is considered a potency assay because it measures the biologically active form of HA (i.e. in its trimeric form). In this assay, an agarose gel containing polyclonal anti-HA antibodies is punched with 4 mm wells. The wells are loaded with dilutions of a standard of known concentration, alongside samples that are solubilized with detergent. Precipitation rings due to antibody–antigen complexes are detected with a coomassie blue stain, and their diameter is proportional to the concentration of HA (Fig. 1).

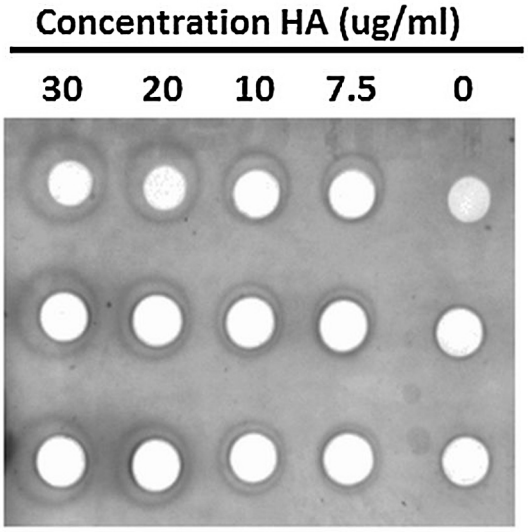


**Fig. 1.** Example of a typical standard curve generated in the SRID assay. Recombinant HA from H1N1/Puerto Rico/8/1934 (Protein Sciences) was treated with 1% Zwittergent 3–14 and 1 μg/ml trypsin-TPCK, and loaded at different concentrations. Sheep polyclonal anti-HA serum (NIBSC, 03/242) was used.

However, the SRID assay presents a few limitations. It is highly variable and time-consuming, it does not measure monomeric or oligomeric HA and it was originally developed for egg-based vaccines. The suitability of the SRID assay to quantify vaccines produced in other platforms such as cell cultures is questionable, since reagents used to quantify egg-based vaccines cannot be used for cell-based vaccines especially with unsplit viruses [21]. Furthermore, the SRID assay requires the use of strain-specific anti-HA1 polyclonal antibodies. HA is composed of two subunits: HA1 (also called globular region or head) and HA2 (also called stem region). HA1 is highly variable while HA2 is conserved among subtypes and strains. The SRID assay requires antibodies that are directed against the exposed epitopes present on HA1. It also necessitates the use of polyclonal antibodies because multiple contact points between the antigen and antibodies are required to form the precipitation rings. In short, the SRID assay has made its proof over time and allows the quantification of different subtypes in trivalent vaccines through the use of subtype-specific antibodies and standard antigens. However, the production of these reagents takes about three months. The assay is also fraud with high variability depending on the operator due to the analysis method employed; in many instances, the diameter of the precipitation rings is manually measured using a magnifying eye piece and a ruler.

### 2.2. Universal (pan-HA) antibodies

Traditionally, antibodies are produced against the HA1 globular head of HA. This region is specific for each strain and mostly exposed. The stem region of HA on the other hand, is proximal to the viral membrane and is therefore partially hidden. Functionally, the stem region undergoes significant conformational changes once taken up by cells in order to ensure the fusion of the viral membrane to cellular membrane, which eventually leads to viral replication. This step is critical and explains why the stem region of HA is highly conserved among the influenza strains [22,23]. As a result, antibodies against conserved regions of HA2 have been developed with the goal of generating antibodies with broader specificity. The first antibodies generated were against either Influenza A group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16) or influenza A group 2 (H3, H4, H7, H10, H14, H15). More recent research programs have led to

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