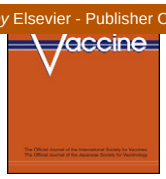
Vaccine 29 (2011) 5031–5039

Vaccine

journal homepage: www.elsevier.com/locate/vaccine



Towards ambient temperature-stable vaccines: The identification of thermally stabilizing liquid formulations for measles virus using an innovative high-throughput infectivity assay

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ARTICLE INFO

Article history:
Received 28 October 2010
Received in revised form 18 April 2011
Accepted 20 April 2011
Available online 25 May 2011

Keywords:
Measles
Thermostability
Formulation
High-throughput
Cold-chain
Gates Foundation Grand Challenges in Global Health

ABSTRACT

As a result of thermal instability, some live attenuated viral (LAV) vaccines lose substantial potency from the time of manufacture to the point of administration. Developing regions lacking extensive, reliable refrigeration ("cold-chain") infrastructure are particularly vulnerable to vaccine failure, which in turn increases the burden of disease. Development of a robust, infectivity-based high throughput screening process for identifying thermostable vaccine formulations offers significant promise for vaccine development across a wide variety of LAV products. Here we describe a system that incorporates thermal stability screening into formulation design using heat labile measles virus as a prototype. The screening of >11,000 unique formulations resulted in the identification of liquid formulations with marked improvement over those used in commercial monovalent measles vaccines, with <1.0 log loss of activity after incubation for 8 h at 40 °C. The approach was shown to be transferable to a second unrelated virus, and therefore offers significant promise towards the optimization of formulation for LAV vaccine products.

1. Introduction

It is estimated that 50% of lyophilized vaccines are discarded annually [1], and temperature instability is an appreciable contributing factor in this wastage. The majority of vaccines, particularly live attenuated viral (LAV) vaccines against measles and polio [2,3], require careful temperature regulation from the point of manufacture through administration to preserve their stability and therefore efficacy [4,5], i.e. the cold chain. Although this challenge is largely solved in developed markets, in much of the developing world, where ambient temperatures can exceed 40 °C, the cold-chain infrastructure is incomplete or unreliable. Failures in the cold chain have contributed to local outbreaks and the resurgence of disease in the developing world [6–14].

The development of thermostable vaccines would dramatically improve access to effective vaccines to the global populations most in need and represents a major step to realizing the full benefit of vaccines in preventing infectious diseases and saving lives worldwide [15–18]. However, the development of thermally stable vaccine formations presents significant technical challenges and is of little commercial interest to major pharmaceutical companies focused on the developed world. As a result, preparation of "Vaccines That Do Not Require Refrigeration" was identified as one of the 14 Grand Challenges in Global Health put forth by the Bill & Melinda Gates Foundation [19].

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0264-410X
doi:10.1016/j.vaccine.2011.04.079

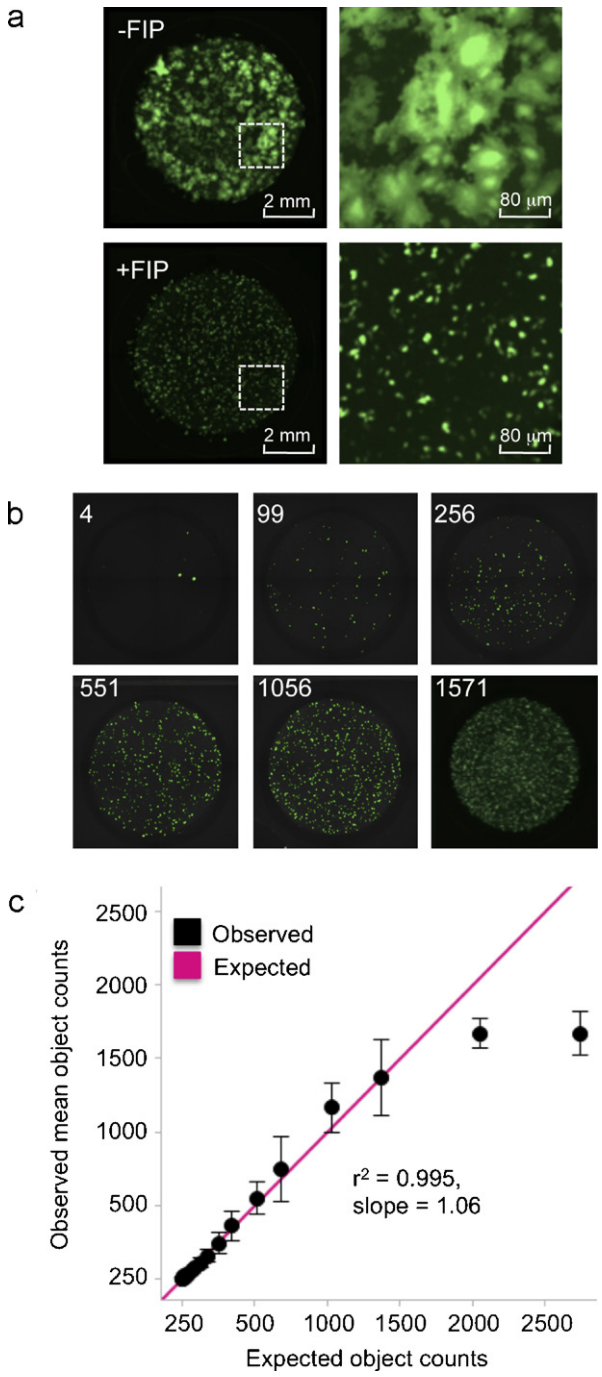

Fig. 1. Infectivity-based image analysis. (a) A Vero cell monolayer infected with recombinant measles virus expressing enhanced green fluorescent protein (MVeGFP) in a single well of a 96-well plate with (+) and without (−) FIP addition at 1 h post-inoculation. Absence of FIP results in cell–cell fusion (syncytia) preventing quantitation of discrete, infected cells. (b) Representative images from a single 2-fold virus dilution series, showing the number of fluorescent objects (individual MVeGFP-infected cells, +FIP) counted by a custom image analysis algorithm. (c) The response of the assay is linear up to ~1600 object counts (r^2 = 0.995), a dynamic range of ~2.2 log units. In order to normalize virus counts for each experiment, a single control formulation was included on every plate. Through *a priori* statistical power analysis, 16 control replicates per 96-well plate and 8 test replicates per experiment were considered sufficient to achieve the desired power of 0.9 and specificity of 0.8 at a standardized effect size of 1.0. In practice, 10 replicates of each formulation

Measles LAV is an ideal candidate for reformulation. Despite the existence of a safe and effective vaccine, the World Health Organization reports 25–30 million cases of measles each year and measles remains a leading cause of vaccine-preventable death among children under 5 years old. Recent reinvigorated efforts across a broad spectrum of approaches have helped reduce measles deaths worldwide from 750,000 in 2000, but there were still an estimated 197,000 fatalities in 2007 [20]. Interruption of endemic transmission of measles virus (MV) requires that >95% of the population be immune [21], highlighting the need for complete, effective vaccination coverage in communities.

MV is inherently labile, losing 50% potency after 1 h at 22–25 °C and almost 100% after 1 h at 37 °C [22]. Reducing the moisture content in the vaccine, most commonly through lyophilization [17], or alternatively through spray drying [23], can lead to dramatic improvements in the stability of the vaccine during storage and distribution; however, reconstitution prior to vaccination is still required. Even successful commercial LAVs such as Attenuvax® (Merck) lose 1 log of potency after 8 h at 37 °C in the reconstituted (liquid) form (internal data). Although single dose vials are used in developed countries, multi-dose vials are ubiquitous in the developing world due to cost considerations. In practice, a vial may be reconstituted and kept so throughout the course of a full clinic day, without adequate cooling and without adherence to the WHO guidelines around diluent temperature, storage temperature and time, and discard [24]. Thus, improvement in the stability of liquid (reconstituted) measles vaccine at ambient temperatures could deliver significant value in the developing world.

Herein we describe the development of a high throughput (HT) screening platform capable of simultaneously evaluating the thermostability performance for hundreds of MV formulations. The HT approach is ideal for complex vaccine formulations because of the intensive and time-consuming nature of traditional formulation development and the enormity of the possible formulation space. Using this HT process, we identified multiple formulations capable of maintaining the potency of the vaccine in the liquid state at 40 °C for at least 8 h. These formulations may offer increased thermal stability for a monovalent measles vaccine when compared to currently marketed products, and in some cases also offer a cost benefit and eliminate the need for animal-derived components.

2. Materials and methods

2.1. Commercial vaccines

The measles vaccine Attenuvax® (Merck) contains phosphate buffer, sodium chloride, stabilizers (sorbitol, sucrose, hydrolyzed gelatin, and human albumin), and residual culture medium [25]. The measles vaccine M-VAC™ (Serum Institute of India) includes tricine, amino acids (alanine, arginine, histidine), and stabilizers (lactalbumin hydrolysate, hydrolyzed gelatin) [26].

2.2. Measles virus growth and harvest

Measles virus encoding enhanced green fluorescent protein [27] (MVeGFP) was grown by infecting a 50% confluent monolayer of Vero cells (CCL-81, ATCC) in 100 mm cell culture plates (Corning) at a 0.015 multiplicity of infection in OptiMEM (GIBCO). After 1-h incubation at 37 °C/5% CO_2, OptiMEM containing 2% fetal bovine serum (FBS, GIBCO) was added to the inoculated cells. Cells were further incubated at 37 °C/5% CO_2 until 90–100% of cells exhibited cytopathic effect. To harvest virus, infected cells were scraped from plates, and excess growth medium was removed following low speed centrifugation (300 × *g*). Cell pellets were resuspended in 2 ml of OptiMEM, freeze-thawed, and centrifuged. Resulting

supernatant containing virus was titered using the assay described in Section 2.3, aliquoted, and stored at −80 °C. To expand stocks of Moraten and Edmonston-Zagreb viruses from Attenuvax® and M-VAC™ vaccines (respectively), lyophilized vaccines were reconstituted, serially diluted into serum-free DMEM (GIBCO), and added to Vero (Moraten) or MRC-5 (Edmonston-Zagreb) cells (CCL-171, ATCC) and then processed as described for MVeGFP.

2.3. Fluorescent infectivity assay

Vero cells were seeded at 2×10^4 cells/well in DMEM containing 5% FBS on 96-well ViewPlates (Perkin Elmer). Following a 1 h room temperature incubation [28], cell plates were incubated overnight at 37 °C/5% CO_2. Virus was diluted 1:9 into formulation and thermally challenged. After further diluting 1:3 into OptiMEM, samples were added to cells (25 μL) and centrifuged at low speed ($311 \times g$) for 10 min. Assay plates were incubated at 37 °C/5% CO_2 for 80 min to allow viral adsorption to cells. Fusion inhibitory protein (FIP, Z-D-Phe-Phe-Gly-OH, Bachem), dissolved in DMSO and diluted to a final concentration of 155 μM in OptiMEM containing 2% FBS/1% penicillin–streptomycin (GIBCO), was then added to wells (75 μL) to prevent syncytia formation and secondary infection. After 30 h at 37 °C/5% CO_2, cells were fixed with 4% paraformaldehyde (EMS). Images were captured with a Cellomics VTi Arrayscan using a FITC filter and 2.5× objective lens (Fig. 1). Infectious units ('IU') denote the titer of virus determined from the fluorescence-based assay as opposed to plaque-forming unit (pfu) titer measured by plaque assay.

2.4. High throughput (HT) experimentation

The complete HT formulation procedure will be described elsewhere (Development of an integrated high throughput system for identifying formulations of live virus vaccines with greater thermostability: application to the monovalent measles vaccine; manuscript in preparation). In brief, in-house Design of Experiment software created screening protocols. After 1.11× unary stock formulation creation (to account for 1:9 dilution with measles stock), a high-throughput combinatorial liquid dispenser created complex formulations in 96-well deepwell plates (Matrix). A Hamilton Starlet transferred formulations to 96-well assay plates (Corning). Virus was diluted to 8×10^6 IU ml^{-1} in OptiMEM and added into the BioCube (Protedyne). The remainder of the process is described in Fig. 2 and the fluorescent infectivity assay. For each formulation in HT experiments, $n = 4$–10.

2.5. Automated image analysis

Automated image analysis was performed on each well of 96-well assay plates using a custom image analysis algorithm developed using the Matlab image processing toolbox environment (version R2006b, MathWorks).

2.6. Excipients in validation experiments

L-Asparagine anhydrous, sodium D-gluconate, glycine, sodium sulfate anhydrous, L-serine, D-(+)-trehalose dihydrate, L-valine, and tricine were obtained from Sigma–Aldrich. Sodium citrate dihydrate and sucrose were obtained from JT Baker. GELITA SOL-KKA (porcine) gelatin was obtained from Gelita USA.

2.7. MVeGFP validation assay

The validation assay was conducted in a similar manner to the method described above with the following modifications. To ensure that Moraten virus from Attenuvax® did not affect MVeGFP infection, Attenuvax® was exposed to visible light (at room temperature) in order to photoinactivate [29] the vaccine-strain virus. MVeGFP was then diluted (∼1:200) into Attenuvax® while the remaining formulations were prepared as previously described. At each timepoint, $n = 24$/formulation.

2.8. Moraten measles virus fluorescence assay

The Moraten assay was performed as described for the MVeGFP validation assay with the following modifications. Following thermal challenge, reconstituted Attenuvax® was diluted 1:5 into OptiMEM. For non-Attenuvax® formulations, Moraten virus was diluted to 8×10^5 IU ml^{-1} in OptiMEM prior to addition to formulation. After fixation, cells were permeabilized with 1% Triton-X for 5 min at room temperature and incubated with 1:500 antibody to measles nucleoprotein (MAB8906F, Millipore) for 30 min at 37 °C prior to imaging. At each timepoint, $n = 12$/formulation.

2.9. Plaque assays

Serial dilutions of formulated virus was added to 50% confluent Vero cells in 6-well plates (Corning). After 4 h at 37 °C/5% CO_2, cells were overlaid with DMEM containing 1% methylcellulose/2%FBS and further incubated for 5 days. Cells were fixed with 1% crystal violet in methanol and plaques manually counted under a light microscope. Titer was calculated by multiplying average plaque count (from duplicate wells) by dilution factor. For thermal challenge, vaccines were reconstituted per manufacturers' instructions. At each timepoint, $n = 2$/formulation.

2.10. Adenovirus transferability assay

Adenovirus (Ad-CMV-eGFP; Vector Biolabs) assays were conducted using similar methods described for MVeGFP except there was neither a centrifugation step nor FIP added post-inoculation. Cells were fixed and analyzed after 72 h of infection. At each timepoint, $n = 24$.

3. Results

3.1. Bioassay development and optimization

Live measles vaccine potency directly correlates with infectivity [16]. WHO requirements describe two alternative ways of determining the potency of live measles vaccine: measurement of plaque forming units (PFU) or tissue culture infective doses ($TCID_{50}$). Both assays are time intensive, highly variable, and limited in throughput as they require expert visual analysis. Thus, a novel, quantitative cell-based *in vitro* measles infectivity assay (Fig. 1) for quantifying the infectivity of MV in standard 96-well microtiter plates was developed. The fluorescence-based assay uses a recombinant Edmonston-derived laboratory-adapted MV expressing enhanced green fluorescent protein (MVeGFP) [27] and is quantitated using automated image analysis. The assay has a wide dynamic range (≥2.0 orders of magnitude), low variability (Relative Standard Deviations, RSDs ≤30%, as measured through the thousands of control formulations across the screening campaign), and short duration (<4 days).

Two additional measures not typically used during measles infection were implemented to optimize this assay for the HT screening process. First, fusion inhibitory protein (FIP) was used to prevent cell-to-cell spread and therefore secondary infections, and thereby increase the dynamic range of the assay. In a typical MV infection, neighboring cells fuse to form multinucleated syncytia, which markedly vary in size, shape, brightness and sharpness.

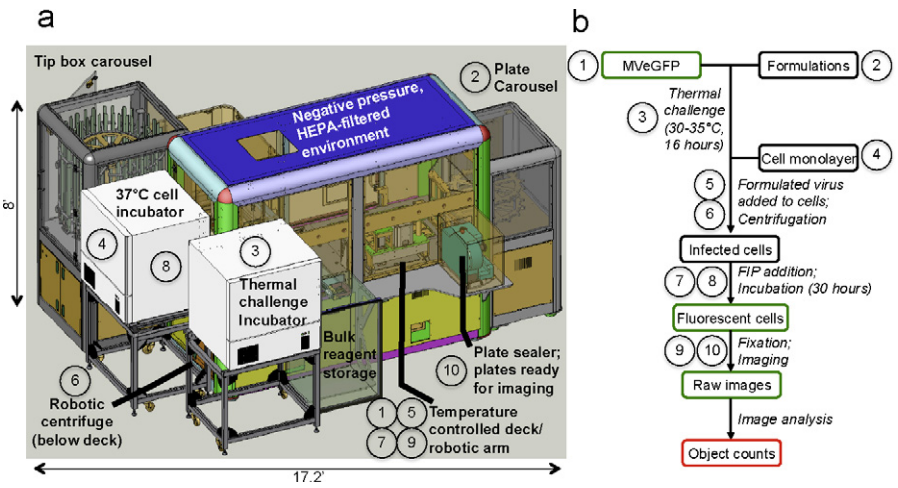

Fig. 2. High-throughput (HT) assay for the determination of formulation-dependent thermal inactivation of MV. Formulations were created from individual excipient stock solutions dispensed to 96-well microtiter plates according to the experimental design using automated liquid handling instruments. All further operations were conducted on a custom-built, fully integrated robotic BioCube system (Protedyne, Inc; Windsor, CT). Within the automated system (a), the experimental process (b) begins when the (1) MVeGFP is combined with (2) unique formulations. (3) Formulated virus is thermally challenged at an elevated temperature, diluted 1:3 into OptiMEM, and added to (4) Vero cell monolayers in (5) 96-well plates. (6) Plates are centrifuged and briefly incubated to facilitate primary infection and (7) fusion inhibitory protein (FIP) is added to prevent secondary infection. (8) After 30 h of incubation at 37 °C, (9) cells are fixed with paraformaldehyde and stored in PBS. (10) Plates are manually removed from the BioCube and imaged on a Cellomics Arrayscan. The resulting images are analyzed using a custom algorithm (Supplementary Algorithm online). The greater the thermal protection conferred by a formulation to the virus, the higher the cell infectivity, and thus the higher the fluorescent "object count.".

Physical overlaps between syncytia create an upper limit on dynamic range, and their non-uniform appearance makes accurate quantification challenging, especially when using automated image analysis. FIP prevents syncytia formation through an unknown molecular mechanism [30]. When FIP is added shortly after the initial infection, fluorescent infectious centers remain discrete, single objects of uniform size and shape (Fig. 1a), each representing a single cell infected by MVeGFP. Second, the relatively low titer of MV in typical cell culture (~10^6 plaque-forming units) plus the additional reduction of virus concentration as a result of its dilution into formulation places limits on the upper bound of detection. In order to address these challenges, we introduced a "spinoculation" step. Centrifugation of inoculated cell monolayers at low speed has been shown to enhance the detection of viable virus (e.g. for HIV [31]), presumably by bringing infectious particles into close contact with the cells, thereby facilitating infection. Addition of FIP to the viral inoculum prior to centrifugation completely eliminated infection, suggesting that the molecular mechanism of viral entry is not affected (results not shown). Spinoculation, however, causes an apparent increase in viral titer of approximately 0.5 log10 increasing the upper end of the range (Fig. 1b). This apparent increase in titer reduces consumption of virus during HT screening and allows for greater dilution of virus stock into formulation.

FIP and spinoculation increase the dynamic range of the assay approximately 2.5-fold from 1.8 logs (~5 to ~300 object counts, data not shown) to ~2.2 logs (~10 to ~1500 object counts) and generate reproducible, robust data (RSDs of 5–15%) (Fig. 1c), thus offering significant advantages over traditional plaque or $TCID_{50}$ assays.

3.2. Assay automation

In order to achieve the desired throughput (>10^4 formulations), we developed an integrated system (Fig. 2a), combining software (including design of experiment, sample tracking, data visualization, and analysis), hardware (liquid dispensing, plate handling, and fluorescence imaging), and experimental workflow (Fig. 2b) (Development of an integrated high throughput system for identifying formulations of live virus vaccines with greater thermostability: application to the monovalent measles vaccine; manuscript in preparation). A combination of in-house designed, custom modified, and off-the-shelf hardware and software were used.

The impact of intra- and inter-plate systematic variability typical of cell-based assays in microtiter plate formats [32] was reduced through careful experimental design choices and data normalization using on-plate controls. The solutions implemented to overcome these challenges will be discussed in greater detail separately (Maximizing the value of cell-based high throughput screening data through experimental design and data normalization; manuscript in preparation).

In HT small molecule screening it is common practice to evaluate the performance of the assay based on the negative and positive controls (Z') [33] and the proportion of hits found (i.e. hit rate). In thermal stability screening of virus formulations, neither a true negative control (no infectivity) nor a true positive control is informative. In theory, it is possible to benchmark formulation performance against either a commercial vaccine or the pre-thermal challenge viral titer for each assay. However, this proved impossible in practice due to the limited availability of monovalent vaccine and the impracticality of processing non-thermally challenged control plates simultaneously with thermally challenged samples. In practice, the primary goal of identifying formulations capable of thermally stabilizing the virus was readily achieved through simple rank ordering of formulation performance, followed by validation of 'high performing' hits using manual assays such as plaque assays.

3.3. Formulation screening strategy

A formalized screening strategy to guide experimental design was applied. A list of >200 excipients including buffers, stabilizers, solubilizers, preservatives, and tonicifiers compiled from marketed parenteral formulations, the FDA 'Generally Regarded As Safe' (GRAS) list, and the literature was narrowed based on considerations of safety, cost, manufacturing, and ethical issues. Ultimately, 98 unique excipients were screened (Supplementary Table Online).

The fully combinatorial formulation space represented by 98 excipients is many orders of magnitude larger (1×10^9 unique formulations with just 6 excipients each) than is tractable, even for HT screening (~10^4). Therefore, a 'linear' strategy was devised in

Table 1
High throughput screening stages.

Stage	Excipient classes	Formulation order	Variables	Control formulation	Unique formulations
1	Buffers	1	Excipient (17) Concentration (5) pH (7)	Mimic of Attenuvax®	218
II	Buffers, stabilizers	2	Excipient (70) Concentration (3)	Sodium citrate pH 7.4, 50 mM	3134
III	Buffers, stabilizers	4	Excipient (50)	Sodium citrate pH 6.0, 50 mM	2740
IV	Buffers, stabilizers, antioxidants, chelating agents	5–8	Excipient (18)	2.9% (w/v) porcine gelatin in sodium citrate pH 6.0, 50 mM	3200
V	Buffers, stabilizers, antioxidants, chelating agents	4	Excipient (22) Concentration (3)	2.9% (w/v) porcine gelatin, asparagine 50 mM, and trehalose 100 mM in sodium citrate pH 6.0, 50 mM	1280
–	Various	5–8	Excipient (62) Concentration (3)	As per screening stage	1251
Total					11,823

which formulations of increasing complexity were evaluated in five 'Stages,' each comprised of multiple individual screens (Table 1). The best performing formulations (highest object counts) were identified from each screen and taken forward as the basis of the design of the more complex formulation space to be evaluated in the next stage.

A linear strategy inherently risks missing any dramatic synergistic effects between excipients that are never tested in combination (having been eliminated from consideration during earlier steps) and the true maxima in concentration space (which is only explored coarsely). To reduce these risks, 4 additional screens aimed to cover both a broader sampling of the overall formulation space ('shotgun' screens) or to finely explore concentration effects of promising formulations ('targeted' screens) were interspersed in the process.

3.4. HT screening results

A total of 11,823 unique formulations (as defined by combination of excipients, excipient concentrations, and pH) were screened in 35 HT screens comprising 5 stages of linear screening and additional non-linear screens (Table 1, full and summarized datasets in Supplementary Data Online). Intra-assay variability was typically in the range of 10–25% RSDs normalized across control formulations, and all assays reported had RSDs below 30%. The highest performing formulations (based on rank ordered normalized object counts) were selected at each stage as the basis of the design of the subsequent stage. Pairwise comparisons of formulation performance quoted are significant at the $p < 0.05$ level by standard t-test, with 4–10 replicates per formulation. A small number of datapoints attributed automation error were removed from the calculations. In general, as the complexity of the formulations increased with progression through the stages, the performance of the top formulations from each stage increased. Increases in performance were incremental or additive at best, and no truly synergistic effects ($AB \gg A + B$) were observed.

Stage I was designed to broadly assess the effect of buffers on viral stability (29 variables, 218 unique formulations). Citrate pH 7.4, citrate pH 6.0, potassium phosphate pH 7.4, and histidine pH 7.4 were identified as the highest performing buffers. In Stage II, they were combined with stabilizers (73 variables, 3134 unique formulations). Formulations containing gelatin, valine, citrate, and trehalose were typically high performing, and citrate pH 6.0 was generally the best performing buffer background. In Stage III (50 variables, 2740 unique formulations), higher order combinations of the same excipients used in Stage II yielded increased performance.

A non-linear screen examined the effects of varying the concentrations in two high-performing quaternary formulations identified in Stage III (Fig. 3a). In both cases, the highest performing concentration combinations had approximately 30% higher counts than the single combination tested in the Stage III screen, suggesting that simultaneous co-optimization of concentrations can significantly, but probably not radically, improve formulation performance. In a second non-linear screen, additional excipients from several new classes (including antioxidants, chelating agents, and surfactants) were tested (Fig. 3b). High performers included sodium gluconate and xylitol, which were then included in the design of Phase IV. Both positive (e.g. sodium gluconate) and negative (Tween 20 and Tween 80) concentration effects were observed. At higher concentrations, Tween likely shifts from behaving like a stabilizer to becoming a detergent, causing disruption of the virion lipid envelope. Likewise, non-polar amino acids were better performers than other classes of amino acids, but the reasons for this are unclear.

In Stage IV (18 variables, 3200 unique formulations), higher order formulations (5–8 excipients) including promising buffer/stabilizer combinations were combined with antioxidants and chelating agents. The same excipients continued to perform well, including citrate pH 6.0, gelatin, trehalose, and valine. Finally, in Stage V (25 variables, 1280 unique formulations), a limited concentration optimization of 22 high performing formulations showed that for most excipients stability decreased as concentrations increased. Interestingly, ionic components including, $MgSO_4$ and $MgCl_2$ [34], have been shown to affect the stability of the MV. Both xylitol and sodium gluconate have been shown to bind to Ca^{2+} [35], suggesting one potential mechanism for the stabilization effect.

Fig. 3c graphically depicts the linear screening strategy by focusing on the progression of formulations tested through all five stages that led to a single high-performing final candidate formulation, starting with citrate 50 mM (pH 7.4) in Stage I and building incrementally to a partially concentration optimized formulation of citrate 50 mM (pH 6.0), gelatin, trehalose, sucrose, asparagine, and glycine (Formulation C in Table 2) in Stage V.

3.5. Validation and transferability

In order to confirm "hits" identified during HT screening, a suite of validation assays were applied following completion of each screening stage (the final validated formulations are described in Table 2). In the HT assay, the viral inoculum added to cells contains

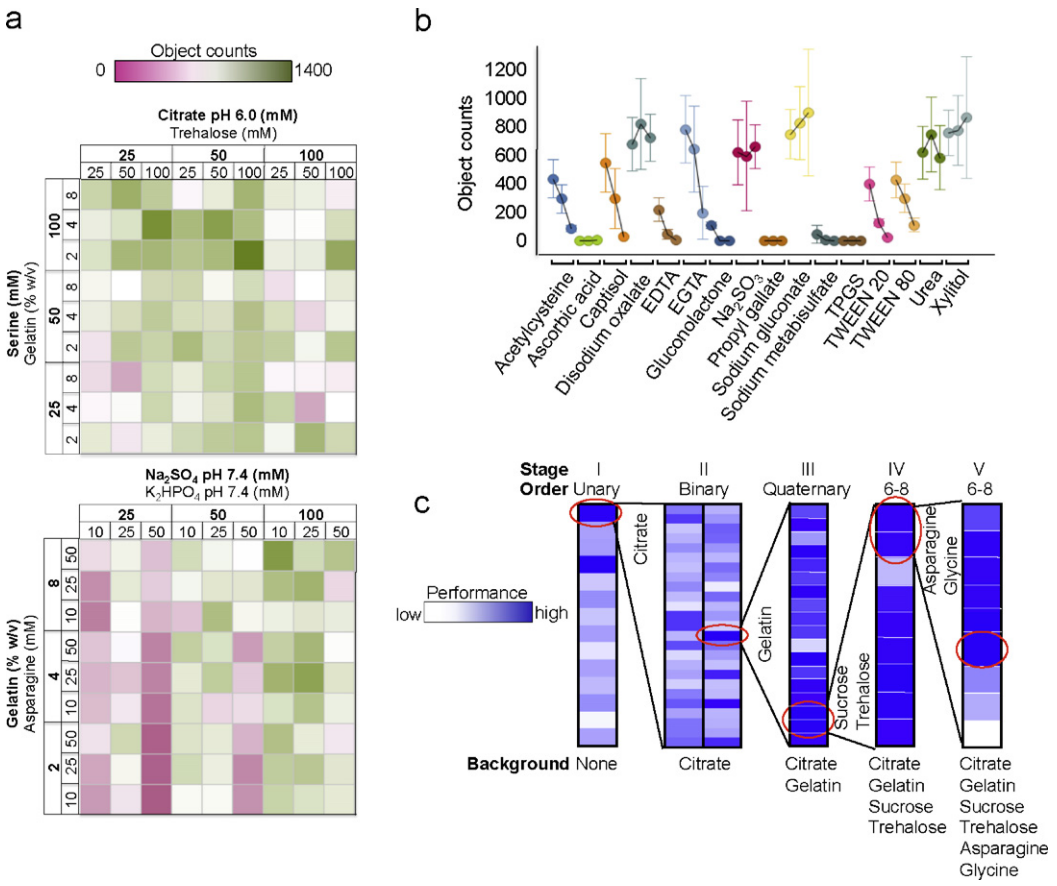

Fig. 3. Representative HT screening results. (a) Heat maps showing performance range (top) during excipient concentration optimization of 2 quaternary formulations: sodium citrate pH 6.0 (25, 50, 100 mM), trehalose (25, 50, 100 mM), serine (25, 50, 100 mM), gelatin (2, 4, 8%, w/v) (middle); sodium sulfate pH 7.4 (25, 50, 100 mM), potassium phosphate pH 7.4 (10, 25, 50 mM), gelatin (2, 4, 8%, w/v), asparagine (10, 25, 50 mM) (bottom). (b) Concentration-dependent effects on thermal stability. Against a background of citrate pH 6.0 (50 mM) and gelatin (4%, w/v), 16 excipients were screened at three concentrations (w/v). For each excipient, concentration range is shown with respect to the *x*-axis (lowest concentration on the left, highest concentration on the right). Excipients were tested at 0.1%, 0.2%, and 0.4%, w/v, with the following exceptions: captisol (2, 4, 8%, w/v); disodium oxalate, propyl gallate, TPGS (0.05, 0.10, 0.20%, w/v); gluconolactone, sodium gluconate (0.5, 1.0, 2.0%, w/v); Tween 20, Tween 80 (0.01, 0.15, 0.02%, v/v); xylitol (1, 2, 4%, v/v). (c) Thermal stability performance of 100 related formulations (0.85% of the total) illustrating the formulation design progression through the five stages of HT screening (numerical range of performance varies by stage). In Stage I, citrate was among the best performing unary formulations and thus was included in Stage II. In binary combination with certain excipients, including gelatin, citrate continued to perform well and was continued in Stage III, etc. The majority of formulations screened in Stage IV resulted in <1.0 log loss of virus after 35 °C thermal challenge for 16 h.

Table 2
Validated formulations following Stage IV screening.

Formulation	Stage	Order	Formulation recipe
A	II	2	Sodium citrate 50 mM pH 6.0, porcine gelatin 4%
B	III	4	Sodium citrate 50 mM pH 7.4, porcine gelatin 2.9%, trehalose 100 mM, valine 50 mM pH 7.4
C	IV	6	Sodium citrate 50 mM pH 6.0, porcine gelatin 2.9%, trehalose 100 mM, sucrose 100 mM, asparagine 50 mM pH 7.4, glycine 100 mM pH 7.4
D	IV	6	Sodium citrate 50 mM pH 6.0, trehalose 100 mM, asparagine 50 mM pH 7.4, glycine 100 mM pH 7.4, serine 100 mM pH 7.4, sodium gluconate 100 mM
E	IV	7	Sodium citrate 50 mM pH 6.0, porcine gelatin 2.9%, sorbitol 100 mM, asparagine 50 mM pH 7.4, praline 100 mM pH 7.4, serine 100 mM pH 7.4, sodium gluconate 100 mM
F	IV	7	Potassium phosphate (dibasic) 10 mM pH 7.4, porcine gelatin 2.9%, sodium sulfate 100 mM, sucrose 100 mM pH 7.4, glycine 100 mM pH 7.4, valine 50 mM pH 7.4, sodium gluconate 100 mM
G	IV	8	Potassium phosphate (dibasic) 10 mM pH 7.4, porcine gelatin 2.9%, trehalose 100 mM, sodium sulfate 100 mM, asparagine 50 mM pH 7.4, glycine 100 mM pH 7.4, proline 100 mM pH 7.4, sodium sulfite 100 mM
H	IV	8	Tricine 50 mM pH 7.4, porcine gelatin 2.9%, trehalose 100 mM, sodium gluconate 100 mM, glycine 100 mM pH 7.4, serine 100 mM pH 7.4, sucrose 100 mM, sodium sulfate 100 mM

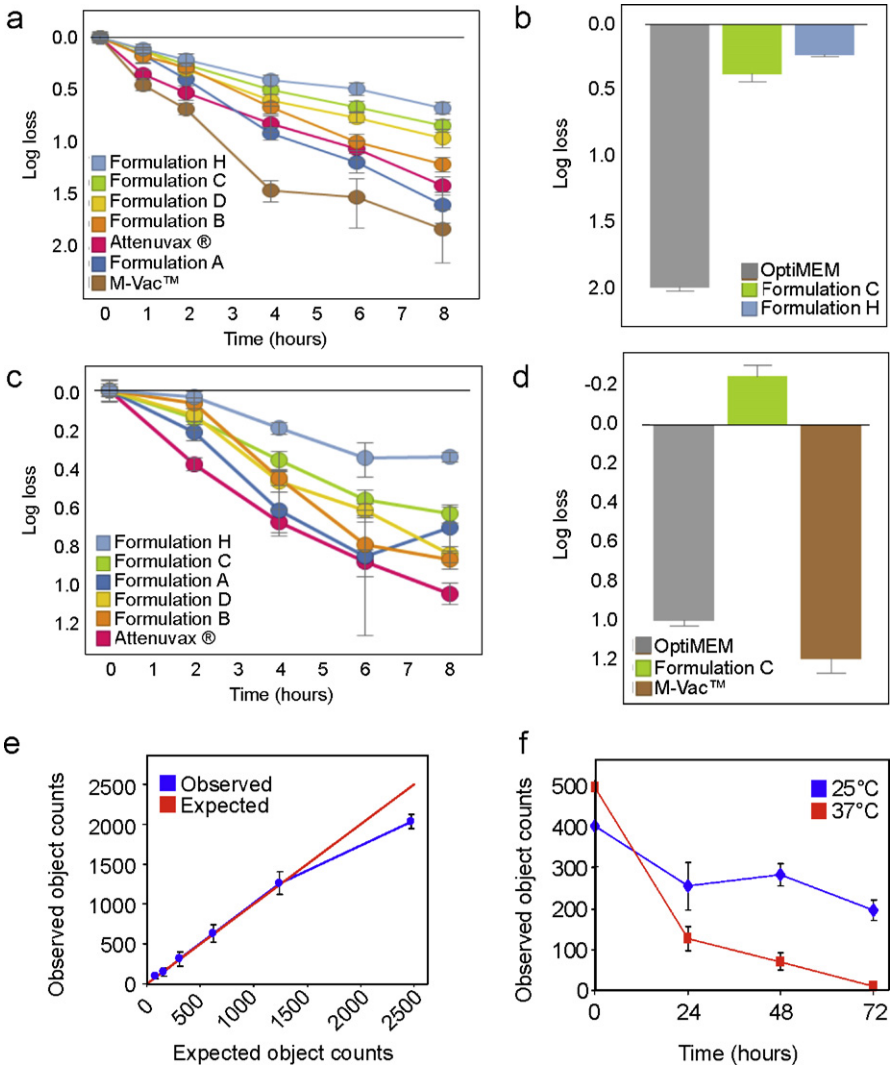

Fig. 4. Validation and transferability of the HT process. (a) Accelerated degradation of MVeGFP for up to 8 h at 40 °C in marketed monovalent measles vaccine (Attenuvax®, M-Vac™) or custom formulations (described in Table 2). Experiment and analysis conducted in a similar manner to HT screening. (b) Plaque assay showing MVeGFP degradation after 4 h at 40 °C in a subset of formulations. (c) Accelerated degradation of Moraten MV for 8 h at 40 °C. Experiment conducted as described in (a) except that following fixation, cells were permeabilized and incubated with measles-specific, FITC-conjugated antibody. (d) Plaque assay showing Edmonston-Zagreb MV degradation after 4 h at 40 °C. (e) Transferability of screening methods to non-measles virus shown with adenovirus expressing eGFP (Ad-eGFP). Ad-eGFP was added to cell monolayers using a two-fold dilution scheme. Observed object counts (from the same custom algorithm used for MVeGFP quantitation) were in close agreement with the expected counts. (f) Ad-eGFP was formulated in basic cell medium, OptiMEM, and thermally challenged for 24, 48, or 72 h at 25 °C or 37 °C. Time- and temperature-dependent inactivation was seen, but on different time-scales than for MVeGFP, indicating a difference in intrinsic thermal stability of the viruses.

residual, diluted formulation from thermal challenge which could render cells more permissive to infection, and therefore cause an artificial increase in object counts independent from thermal stabilization of virus. All of the high-performing formulations were confirmed to be not acting through this trivial mechanism (data not shown). In accelerated degradation studies over 8 h at 40 °C, formulations based on citrate and tricine demonstrated superior stabilizing effects (Fig. 4a) relative to those in a potassium phosphate background (data not shown). It is possible that sodium citrate has a slight deaggregating effect on virus (thereby giving rise to an apparent increase in viral titer) as opposed to a strictly protective effect, as suggested from studies with rotavirus vaccine [36]. It is also notable that tricine is the buffer background of M-VAC™ vaccine [26]. After 8 h at 40 °C, MVeGFP formulated in formulations C and H suffered <1.0 log loss while the commercial measles vaccines, Attenuvax® and M-VAC™, decreased by 1.4 logs (1.35–1.53) and 1.9 logs (1.67–2.19), respectively. Assessment of the formulations by the traditional plaque assay closely correlated with the results of the MVeGFP accelerated degradation assay (Fig. 4b). Overall, the rank order of formulation stability is identical for both methods, supporting the validity of the HT screening strategy.

MVeGFP was used as a surrogate for the HT screens because fluorescence is an easily quantifiable endpoint. The most promising formulations were validated using the same non-recombinant measles strains used in commercial vaccines, Edmonston-Zagreb (EZ, used in M-VAC™ from Serum Institute of India) and Moraten (used in Attenuvax® from Merck). Attenuvax and formulated Moraten were thermally challenged at 40 °C for up to 8 h, and infection was quantified following Cellomics data acquisition using the existing MVeGFP algorithm via an immunofluorescence assay utilizing a FITC-conjugated anti-measles antibody (Fig. 4c). Attenuvax loses 1.0 log (90% counts) of activity after 8 h while formulations A and C only experience a ~0.6 log loss. The tricine-based formulation H exhibited the greatest thermostability, losing only 0.35 log, similar to the results seen with MVeGFP. Interestingly, MVeGFP appears to be less thermally stable than Moraten in the other common formulations.

Finally, the most promising formulations were combined with EZ vaccine strain virus, challenged at 40 °C for 4 h, and titered using a plaque assay (Fig. 4d). Non-challenged, formulated virus was used as a control to calculate log loss and the plaque assay data again supports the HT screening data. The lead candidate formulations are highly stabilizing with no significant loss in activity, whereas the commercial M-VAC™ vaccine suffers >1 log loss. These infectivity data suggest that the two vaccine strains, Moraten and EZ, have differential inherent thermal stability (e.g. formulation C in Fig. 4c vs. d) as has been suggested previously [37,38] which may result in slightly different behaviors in the same formulation. It is also important to note that while vaccine-strain virus has been used to validate candidate formulations, manufacturing conditions for the commercial vaccines may affect viral stability. For example, it has been reported that the level of cytopathic effect during viral harvest can affect the thermal stability of virus [37].

As proof of concept of broad transferability of the formulation stability screening platform to non-related viruses, the screening process was applied to adenovirus expressing eGFP (Ad-eGFP). A linear response to increasing viral titer was seen with RSDs of 10–20% (Fig. 4e) showing that the assay has similar performance characteristics using either measles or adenovirus. However, Ad-eGFP is inherently more thermally stable than MVeGFP, losing all infectivity after 72 h at 37 °C (Fig. 4f) compared to just a few hours at 37 °C for MVeGFP. The difference in thermal stability may be attributed to the presence (measles) or absence (adenovirus) of a viral envelope as the enveloped viruses are noted for greater temperature sensitivity than non-enveloped viruses [39].

4. Discussion

Maintenance of vaccine efficacy in the absence of a cold chain has the potential to extend immunity against deadly diseases into the world's poorest communities and thereby save tens of thousands of lives each year. Although alternative approaches for MV stabilization are being explored [26,40], the reformulation of existing LAVs is a promising approach towards eliminating the need for refrigeration during their storage, distribution, and use while not requiring major modifications to the existing manufacturing process. This screening platform allows for reformulation of existing vaccines and could also be integrated into the formulation design process in the developmental stage of new vaccines. Although in the present work, the screening process was applied towards increasing LAV resistance to higher temperatures, an analogous process could be applied for addressing sensitivity to cold or freezing, or towards optimization against performance metrics other than infectivity.

As a proof-of-concept, we applied the screening platform to MV, and several formulations were validated with vaccine strain virus that suffer <1.0 log loss after 8 h at 40 °C in the liquid state. This is a significant gain in thermal stability relative to two representative commercial vaccines (Attenuvax® and M-VAC™) and would allow the reconstituted multi-dose vials of vaccine to be used for a full working day in a health clinic without access to refrigeration.

This dataset represents the most comprehensive information to date on the thermal stability of MV in liquid formulation, and therefore may be of broad interest to the MV and vaccine development communities. We acknowledge that thermal stability in the reconstituted (liquid) state must be paired with stability in the lyophilized state. The HT screening platform described here has been extended to address the more technically challenging problem of evaluating diverse lyophilized formulations, and we will report those results separately (High throughput screening of lyophilization conditions: application to the monovalent measles vaccine; manuscript in preparation). Also, the underlying biophysical effect of excipients on virus has not been explored during this project; however, this topic is being rigorously pursued by other groups [41].

In order for a reformulation to be implemented, the change must be attractive for the vaccine producer. We recognize that a firmly entrenched manufacturing process is a high barrier to adoption. Furthermore, the levels of thermostability required for refrigeration-free storage and distribution have not been a high priority for pharmaceutical companies targeting developed world markets where the cold chain is ubiquitous and reliable. However, improved thermal stability promises a reduction in manufacturing and distribution costs through elimination of vaccine wastage and refrigeration infrastructure. Because many of the formulations identified do not contain animal-derived products such as human albumin or porcine gelatin, there are additional advantages in the areas of cost of goods, regulatory concerns, and ethical/religious considerations. As an alternative approach to complete reformulation, a new diluent may be used for reconstituting existing lyophilized vaccines. For example, M-VAC™ vaccine reconstituted with a simple, inexpensive diluent (50 mM sodium citrate dihydrate pH 7.4) showed 0.5 log loss after 4 h at 40 °C (data not shown) as compared to 2.5 log loss when reconstituted with water for injection.

5. Conclusion

The development of a robust, infectivity-based screening process for identifying thermostable vaccine formulations offers remarkable promise for vaccine development and reformulation of both heat-sensitive (e.g. varicella, rotavirus, and OPV vaccines) and cold-sensitive (*H. influenzae* type b, pneumococcal polysaccharide, hepatitis vaccines) [42] vaccine products.

Acknowledgements

This work was funded by the Foundation for the National Institutes of Health through the Bill & Melinda Gates Foundation Grand Challenges in Global Health initiative. Dr. R. Dhere at the Serum Institute of India provided the M-VAC™ vaccine. P. Balaji, K. Briasco, E. Cash, K. Chmielewski, T. Dowie, A. Gandhi, R. Gyory, S. Hong, D. Klein, C. Lee, K. Marks, J. Matamoros, D. Pristin, B. Pullman, I. Risenberg, K. Sebes, A. Tebbe, and L. Yin provided technical assistance. In particular, we are grateful to C. Burke, D. Carucci, J. Carpenter, J. Dingerdissen, R. Dobbelaer, M. Gottlieb, J. van Hoof, D. Lans, R. Middaugh, P. Molino, T. Monath, V. Truong, D. Volkin, and S. Weiner for their project guidance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.04.079.

References

[1] http://www.who.int/en/ [accessed September 20, 2010].
[2] Black FL. Growth and stability of measles virus. Virology 1959;7:184–92.
[3] Newman JF, Tirrel S, Ullman C, Piatti PG, Brown F. Stabilizing oral polio vaccine at high ambient temperatures. Vaccine 1995;13:1431–5.
[4] Cheriyan E. Monitoring the vaccine cold chain. Arch Dis Child 1993;69:600–1.
[5] Cheyne J. Vaccine delivery management. Rev Infect Dis 1989;11:S617–22.
[6] Lerman SJ, Gold E. Measles in children previously vaccinated against measles. JAMA 1971;216:1311–4.
[7] Markowitz LE, Preblud ST, Fine PEM, Orenstein WA. Duration of live measles vaccine-induced immunity. Pediatr Infect Dis 1990;9:101–10.
[8] Burgess MA, McIntyre PB. Vaccines and the cold chain: is it too hot or too cold? Med J Austr 1999;171:83–4.
[9] Gazmararian JA, Oster NV, Green DC, Schussler L, Howell K, Davis J, et al. Vaccine storage practices in primary care physician offices. Am J Prev Med 2002;23:246–53.

[10] Liddle JLM, Harris MF. How general practitioners store vaccines. A survey in south-western Sydney. Med J Austr 1995;162:366–8.
[11] Nelson CM, Wibisono H, Purwanto H, Mansyur I, Moniaga V, Widjaya A. Hepatitis B vaccine freezing in the Indonesian cold chain: evidence and solutions. Bull World Health Organ 2004;82:99–105.
[12] Omilabu SA, Oyefolu AO, Ojo OO, Audu RA. Potency status and efficacy of measles vaccine administered in Nigeria: a case study of three EPI centres in Lagos, Nigeria. Afr J Med Med Sci 1999;28:209–12.
[13] Carrasco R, Dinstrans R, Montaldo I, Medina E, Reyes M, Vergara I, et al. The cold chain and the expanded program on immunization in Chile: an evaluation exercise. Bull Pan Am Health Organ 1982;16:261–71.
[14] Haddad S, Bicaba A, Feletto M, Taminy E, Kabore M, Ouedraogo B, et al. System-level determinants of immunization coverage disparities among health districts in Burkina Faso: a multiple case study. BMC Int Health Hum Rights 2009;9:S15.
[15] Zaffran M. Vaccine transport and storage: environmental challenges. Dev Biol Stand 1996;87:9–17.
[16] Galazka A, Milstien J, Zaffran M. Thermostability of vaccines. WHO Vaccine Immune News; 1998.
[17] Burke CJ, Hsu TA, Volkin DB. Formulations, stability, and delivery of live attenuated vaccines for human use. Crit Rev Ther Drug Carrier Syst 1999;16:1–83.
[18] Peetermans J. Factors affecting the stability of viral vaccines. Dev Biol Stand 1996;87:97–101.
[19] http://www.grandchallenges.org [accessed September 20, 2010].
[20] Dabbagh A, Gacic-Dobo M, Featherstone D, Strebel P, Okwo-Bele JM, Hoekstra E, et al. Progress in global measles control and mortality reduction, 2000–2007. JAMA 2009;301:1430–1.
[21] Griffin DE, Pan CH, Moss WJ. Measles vaccines. Front Biosci 2008;13:1352–70.
[22] Melnick JL. Thermostability of poliovirus and measles vaccines. Dev Biol Stand 1996;87:155–60.
[23] Ohtake S, Martin RA, Yee L, Chen D, Kristensen DD, Lechuga-Ballesteros D, et al. Heat-stable measles vaccine produced by spray drying. Vaccine 2010;28:1275–84.
[24] WHO. Temperature sensitivity of vaccines. WHO Publications; 2006, http://www.who.int/vaccines-documents/DocsPDF06/847.pdf.
[25] Merck. Package Insert for the Attenuvax(R) mono-valent measles vaccine; 2006 [cited February 21, 2011]; available from: http://www.merck.com/product/usa/pi_circulars/a/attenuvax/attenuvax_pi.pdf.
[26] Burger JL, Cape SP, Braun CS, McAdams DH, Best JA, Bhagwat P, et al. Stabilizing formulations for inhalable powders of live-attenuated measles vaccine. J Aerosol Med Pulm Drug Deliv 2008;21:25–34.
[27] Duprex WP, McQuaid S, Hangartner L, Billeter MA, Rima BK. Observation of measles virus cell-to-cell spread in astrocytoma cells by using a green fluorescent protein-expressing recombinant virus. J Virol 1999;73:9568–75.
[28] Lundholt BK, Scudder KM, Pagliaro LA. Simple technique for reducing edge effect in cell based assays. J Biomol Screen 2003;8:566–70.
[29] Cutchins EC, Dayhuff TR. Photoinactivation of Measles virus. Virology 1962;17:420–5.
[30] Plemper RK, Compans RW. Mutations in the putative HR-C region of the measles virus F2 glycoprotein modulate syncytium formation. J Virol 2003;77:4181–90.
[31] O'Doherty U, Swiggard WJ, Malim MH. Human Immunodeficiency virus type 1 spinoculation enhances infection through virus binding. J Virol 2000;74:10074–80.
[32] Shaneyfelt ME, Burke AD, Graff JW, Jutlia MA, Hardy ME. Natural products that reduce rotavirus infectivity identified by a cell-based moderate-throughput screening assay. Viol J 2006:3.
[33] Zhang JH, Chung TD, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen 1999;4:67–73.
[34] Wallis C, Melnick JL, Rapp F. Different effects of $MgCl_2$ and $MgSO_4$ on the thermostability of viruses. Virology 1965;26:694–9.
[35] Hamalainen M, Knuuttila M, Svanberg M, Kosinken T. Comparison of the effect of gluconate, lactose, and xylitol on bone recalcification in calcium deficient rats. Bone 2009;11:429–38.
[36] Peterson SE, Wang S, Ranheim T, Einterz-Owen K. Citrate-mediated disaggregation of rotavirus particles in RotaTeq vaccine. Antiviral Res 2006;69:107–15.
[37] Klamm H, Pollex G, Henning U. Thermal inactivation of different measles virus strains. Acta Virol 1991;35:200–2.
[38] Dilraj A, Cutts FT, Bennett JV, Fernandez de Castro J, Cohen B, Coovadia HM. Persistence of measles antibody two years after revaccination by aerosol or subcutaneous routes. Pediatr Infect Dis J 2000;19:1211–3.
[39] Wallis C, Melnick JL. Stabilization of enveloped viruses by dimethyl sulfoxide. J Virol 1968;2:953–4.
[40] Lin WH, Griffin DE, Rota PA, Papania MJ, Cape SP, Bennett DJ, et al. Successful respiratory immunization with dry powder live-attenuated measles virus vaccine in rhesus macaques. PNAS 2011;108:2987–92.
[41] Kissmann J, Ausar SF, Randolph A, Braun C, Cape SP, Sievers RE, et al. Stabilization of measles virus for vaccine formulation. Hum Vaccine 2008;4:350–9.
[42] Vaccine handling and storage.Pickering LK, Baker CJ, Kimberlin DW, Long SS, editors. Report of the committee on infectious diseases. 28th ed. Elk Grove Village, IL: American Academy of Pediatrics; 2009. p. 13–7.