Reducing Variability of High-Throughput Herpes Simplex Virus Neutralization Assays by Utilizing an Assay-Ready Cell Line and Overlay Techniques

James Perry, Amy Baccari, Johanna K. Kaufmann, Jin Yuan, Debbie Long, Jessica Flechtner, Lisa McNeil

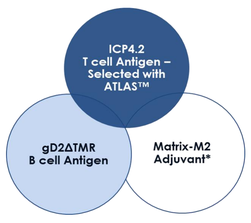
Genocea Biosciences Inc., Cambridge, MA 02140

Abstract

GEN-003 is a candidate immunotherapy for the treatment of genital herpes which induced humoral and cell-mediated immune responses in Phase 1 and 2 clinical trials. Accurate and rapid measurement of functional neutralizing antibodies (NAb) is important to assess GEN-003 immunogenicity. Here, an Assay-Ready Vero cell line and cell overlay techniques were evaluated for their ability to decrease variability and advance throughput of a cell-based colorimetric HSV neutralization assay. Assay-Ready cell lines provide reduced risk of contamination, abolish cell quality drift, and facilitate continuous use of identical low passage material throughout a clinical trial. The cell overlay technique has the potential to shorten the neutralization assay from 42 to 24 hours, an improvement over the gold-standard four-day plaque assay. The consistency of Assay-Ready cell growth, viability and NAb titers were compared to cultured Vero cells by examining reporter gene expression of the cells post-infection with recombinant HSV-2 engineered to express β-galactosidase. After thawing, Assay-Ready cells had greater than 94% viability, consistent with observations in cultured Vero cells. Equivalent expression of β-galactosidase was detected 18 hours post-infection by both cultured and Assay-Ready Vero cells. The NAb titers of six pooled serum samples (with low, medium and high titers) tested in 20 replicates over five days resulted in <15% CV for Assay-Ready cells compared to <20% with cultured Vero cells. Sera from thirty GEN-003 clinical trial participants were tested in the neutralization assay using Assay-Ready cells and cultured Vero cells. The fold change of NAb titers post-immunization over baseline strongly correlated between both cell types (R^2 = 0.945). However, initial experiments examining cell overlay techniques increased variability of NAb titers up to 52% CV. While continued efforts to refine the neutralization assay are in progress, Assay-Ready cells exhibit a clear improvement to this neutralization assay as GEN-003 enters Phase 3 clinical trials.

Introduction

- Genital herpes, which is characterized by recurrent painful ulcers, is primarily caused by HSV-2 and affects more than 500 million people worldwide[1].
- HSV-2 infection increases the risk of HIV-1 transmission[2] and causes severe disease in infants and in immunocompromised individuals[3].
- Prior attempts to develop prophylactic and therapeutic HSV-2 vaccines have failed.
- The effective control of primary and recurrent HSV-2 disease is likely to require T and B cell immunity[4,5].
- GEN-003 is a candidate subunit vaccine comprised of two viral antigens, ICP4.2 and gD2ΔTMR, and the adjuvant Matrix-M2 (Novavax, Gaithersburg, MD)[6,7].



- GEN-003 will begin Phase 3 clinical trials soon, and accurate assessment of the vaccine's immunogenicity remains a top priority. Here, we describe efforts to streamline the method and reduce variability of a Colorimetric HSV Neutralization Assay by implementing the use of an Assay-Ready cell line and cell overlay techniques.

Materials & Methods

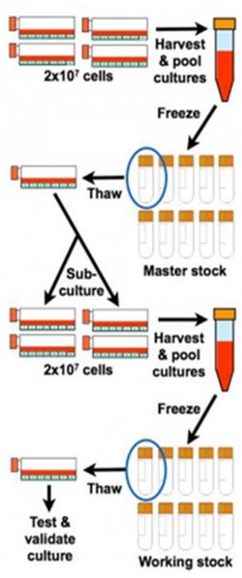
Assay-Ready Vero Cells. Vero cells were licensed from the ATCC by acCELLerate GmbH (Hamburg, Germany), which expanded the cells and passaged them for seven rounds prior to cryopreservation[8]. These Assay-Ready Vero cells underwent strict quality control comparable with current GMP guidelines. Ten percent of each batch was tested for post-thaw viability, debris/cell ratio, aggregation, sterility (bacteria, yeast, fungi, and mycoplasma), proliferative capacity, and morphology prior to shipment. A master stock of vials[9] was prepared strictly for Genocea's usage, and working aliquots were provided at a concentration of 1x10^7 cells/vial.


large scale cell production


automatic dispensing into vials


controlled rate cryopreservation

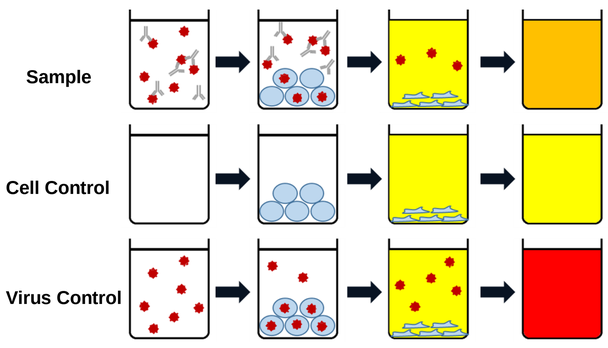

aliquots of assay ready FROZEN INSTANT CELLS

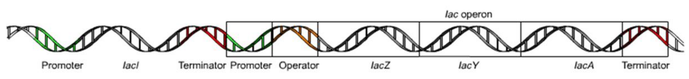


Assay-Ready Vero Post-Thaw Assessment. Each vial was thawed according to the manufacturer's suggested guidelines in a 37°C water bath for approximately 3 - 4 minutes. The cells were centrifuged at 180g for 4 minutes prior to staining with Trypan Blue and counting on a hemocytometer. Assay-Ready Vero cells were evaluated for quality and uniformity by assessing –

- Post-thaw viability
- Cell count consistency
- Proliferative rate in culture

Colorimetric HSV Neutralization Assay. The colorimetric HSV neutralization assay was performed as previously described[10]. A four parameter logistic regression model was used for the determination of 50% NAb titers in Softmax Pro software version 6.2.1.



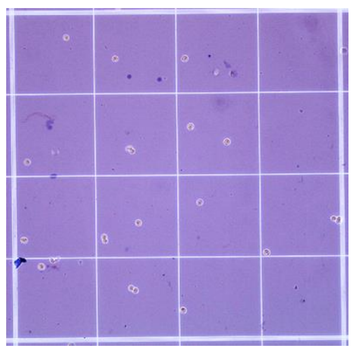


Recombinant HSV-1 (KOS/tk12) engineered to contain the *lacZ* gene under control of the ICP4 promoter encoding β-galactosidase, and recombinant HSV-2 (333) which contains the *lacZ* gene inserted between UL3 and UL4 were used for each experiment[11]. Each virus was produced and provided by the lab of Patricia Spear at Northwestern University. NAb titers were defined as the reciprocal of the serum dilution which produced a 50% reduction in mean OD_{562} of the virus control wells. Evaluation of Assay-Ready Vero cells examined performance in comparison to traditional Culture Vero cells by assessing–

- Confluency in 48 & 96-well plates
- Recombinant HSV dilutions
- Infection duration
- Reporter gene expression
- Dynamic range of OD_{562} values
- 50% NAb titer variability
- Clinical titer fold-change pre & post immunization

Culture and Assay-Ready Vero Cell Overlay. Here we also examine the feasibility of using a cell overlay technique to shorten the duration of the assay. By omitting overnight incubation of Culture and Assay-Ready Vero cells in 96-well plates, and instead overlaying them into wells containing the incubated mixture of serum and virus, the Overlay technique could shorten the duration of the assay from 3 days to 24 hours. This would also increase the number of days experimental setup is feasible throughout a five day work week.

High Viability and Consistent Cell Counts from Vial to Vial for Assay-Ready Vero Cells



Assay-Ready Vials (n = 15)

% Viability	Cell Count % CV
> 94 %	4 %

Figure1. %Viability and %CV of Assay-Ready Cell Counts. Fifteen vials of Assay-Ready Vero cells were diluted 1:20 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1mM sodium pyruvate, 2mM L-glutamine, 100U/mL penicillin and 100ug/mL streptomycin. A 1:4 dilution of the cell mixture in Trypan Blue was used for determining post-thaw viability and cell counts via hemocytometer. Viability was calculated by dividing total viable cells by the total cell count in the four outer regions of the hemocytometer.

Assay-Ready Vero Cells Proliferate More Slowly Than Culture Vero Cells

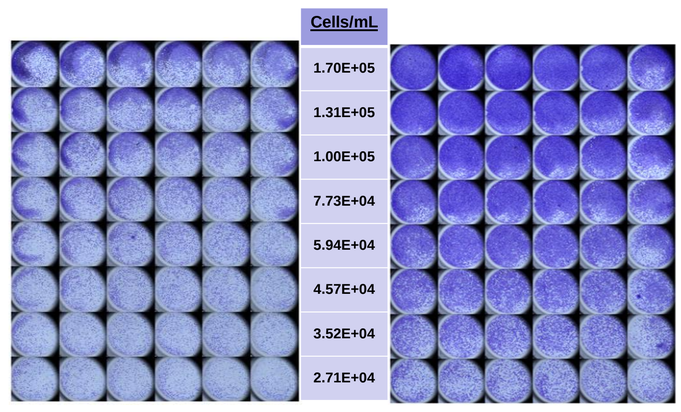


Figure 2. Culture and Assay-Ready Vero Titration in 48-well Plates. Culture and Assay-Ready Vero cells were titrated in 48-well plates, and stained with 0.4% Crystal Violet in 95% ethanol for comparison of cell confluency after 18 hours of incubation at 37°C and 5% atmospheric CO_2. Both cell lines contain six replicates for each dilution which ranged in concentration from 2.71E+04 – 1.70E+05 cells/mL. Each row shown above is representative of a 30% increase in plating concentration and 15-100% confluency as determined by the operator.

Reporter Gene Expression and Dynamic Range of OD_{562} values are equivalent in Assay-Ready and Culture Vero Cells

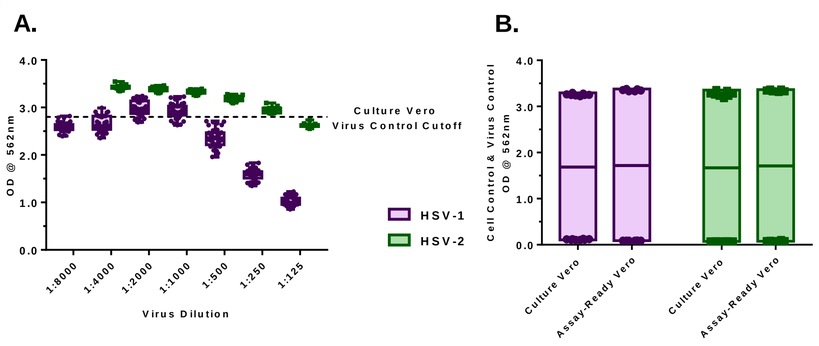


Figure 3. HSV-1 & HSV-2 Titrations on Confluent Assay-Ready Vero Cells. (A) To assess reporter gene expression of Assay-Ready Vero cells infected with recombinant HSV-1 and HSV-2, the titration above was run in 96-well plates with 36 replicates for each virus dilution. A confluent monolayer of Assay-Ready cells was infected for 18 (HSV-2) or 20 hours (HSV-1) before development of the plates. The box and whiskers plot displays OD_{562} for all replicates, alongside the virus control cutoff for infected Culture Vero Cells (shown by the black dotted line). **(B)** Assay-Ready and Culture Vero cells were infected with recombinant HSV in 96-well plates and assessed for their minimum and maximum OD_{562} values after the addition of substrate. Infected cells, or the Virus Control, show the maximum response while uninfected cells, or Cell Control, result in the minimum OD_{652} values shown above. Both cell lines have an equivalent range of minimum and maximum values, or Dynamic Range, when infected with HSV-1 or HSV-2 in the assay. The OD_{562} of each replicate (n = 16) and the mean value is plotted above.

Assay-Ready Vero Cells yield lower titers but reduced variability compared with Culture cells

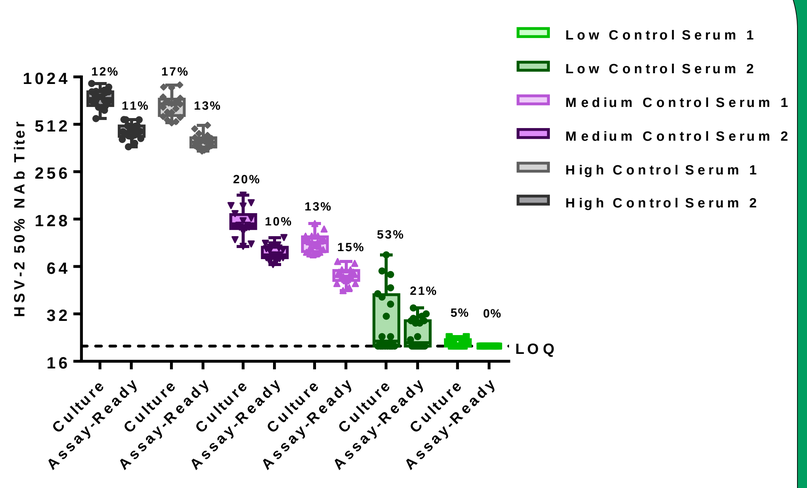


Figure 4. HSV-2 NAb Titers in Low, Medium, and High Titer Serum Controls. The box and whisker plot above displays mean, minimum, and maximum HSV-2 NAb titers of six control serum samples (with low, medium and high titers). Each control serum sample was tested in 20 replicates over five days, using both Assay-Ready and Culture Vero cells. The black dotted line represents the limit of quantification (LOQ = 20) for the assay, and % CV for each control serum sample is shown above its respective plot. %CV was calculated by dividing the standard deviation of all titers by the mean value.

Overlay Method Measures Higher HSV-2 Titers in Culture Vero Cells Compared to the Traditional Method

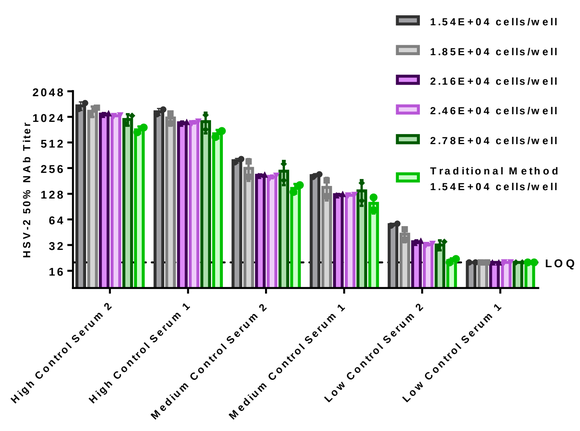


Figure 5. HSV-2 NAb Titers in Culture Vero Cells Using the Overlay Method. Five concentrations of Culture Vero cells were run in the Colorimetric HSV Neutralization Assay using the Overlay method. Six control serum samples with low, medium, and high titers were tested for a direct comparison of HSV-2 NAb titers produced using the Traditional and Overlay methods. Mean NAb titer for two replicates of each concentration is plotted above alongside the LOQ (20) of the assay.

Titers Measured using Traditional and Overlay Methods are Highly Correlated

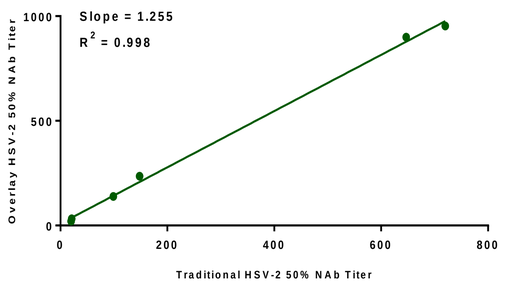


Figure 6. HSV-2 NAb Titer Correlation for Traditional and Overlay Methods. Low, medium, and high titer control sera were assessed using the Overlay method and Culture Vero cells. Overlay NAb titers were compared to titers produced using the Traditional method for the same control sera. The correlation of mean HSV-2 NAb titers produced by the both methods is plotted above for Overlay concentration of 2.78E+04 cells/well.

Titers Measured Using Overlay Method with Assay-Ready Cells are Highly Variable

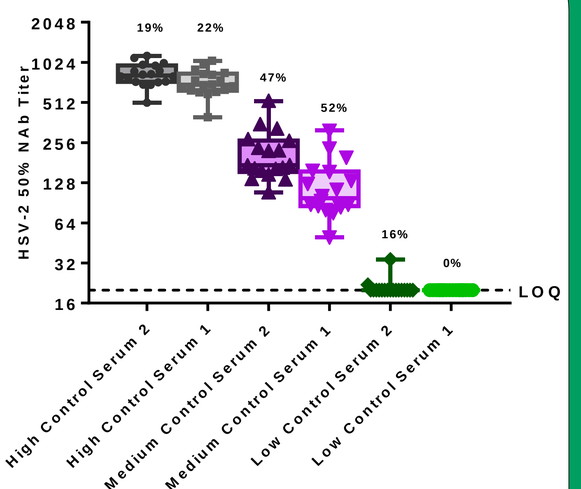


Figure 7. HSV-2 NAb Titers in Assay-Ready Vero Cells Using the Overlay Method. Low, medium, and high titer control sera were assessed using the Overlay method and Assay-Ready Vero cells. A box and whisker plot of HSV-2 NAb titers (n = 18) is show above alongside the % CV for each sample. % CV was higher than values using the Traditional method and Assay-Ready or Culture Vero cells alone.

Despite Absolute Differences in Titer, Assay-Ready and Culture Vero Cells Yield Significant Correlations in Fold Change Measurements

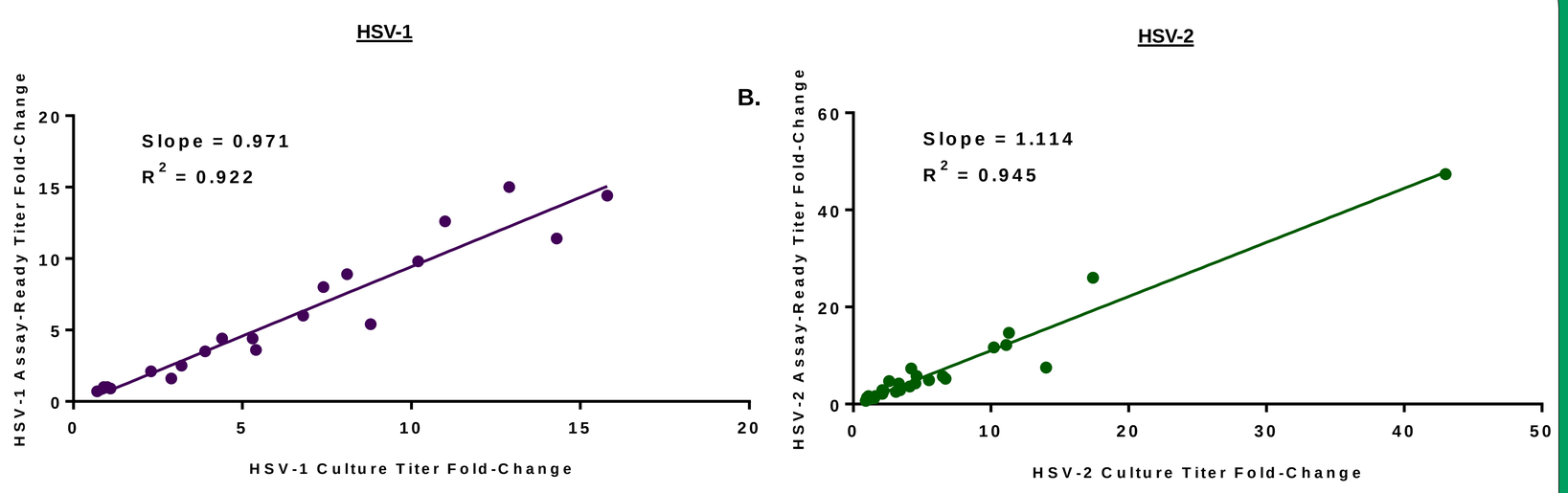


Figure 8. HSV-1 & HSV-2 Correlations of Post-Immunization Fold-Change using Assay-Ready and Culture Vero Cells. Thirty subject serum sets, pre and post-immunization with GEN-003, were run in the Colorimetric HSV-1 **(A)** & HSV-2 **(B)** Neutralization Assay using Assay-Ready and Culture Vero cells. Subjects were vaccinated with placebo, antigen or GEN-003, and had a range of titers when run previously using Culture Vero cells and the traditional method. Assay-Ready Vero cells measure lower titers on average when compared to the titers of Culture Vero cells, but all subjects maintained comparable fold-change measurements (Slopes = 0.971 & 1.114) in both cell lines and virus types. GEN-003 NAb responder threshold was determined by a natural history study and established as a change in NAb titer ≥ two-fold over baseline. All subjects maintained their original responder status to vaccination when using Assay-Ready Vero cells for the determination of post-immunization titer fold-change.

Conclusions

- High post-thaw viability and reproducible cell counts are reflective of the quality and consistency afforded by Assay-Ready cell technology.
- The thaw and use format abolishes weekly cell culture and can facilitate experimental setup outside of standard cell culture schedules.
- Adoption of this cell line facilitates continuous use of low passage material and eliminates concerns of cell quality drift throughout clinical and potency testing.
- The Overlay method using Assay-Ready Vero cells increased titer variability when compared to the values generated using the Traditional method, further evaluation of this technique will be investigated in the future.
- Comparable determination of change in titer by the Assay-Ready Vero cell line suggests accurate assessments of humoral immunogenicity for the candidate immunotherapy, GEN-003.
- Genocea will use Assay-Ready Vero cells for Phase 3 neutralizing antibody testing to reduce titer variability, cost, and the workload of the assay.

References

1. Wald et al., *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis.* 2007; **36**
2. Corey et al., *J AIDS* 2004; **35**:435-445.
3. Corey and Handsfield., *JAMA* 2000; **283**:791-794.
4. Straus et al., *Lancet* 1994; **343**:1460-1463.
5. Belshe et al., *N Engl J Med* 2012; **366**:34-43.
6. Skoberne et al., *J Virol* 2013; **87**:3930–3942
7. Long et al., *Virology* 2014; **464–465**:296–311.
8. "Assay Ready frozen instant cells" accellerate.me.
9. "Creating a certified working cell bank for important cell lines in your research program." http://www.bionique.com/
10. Baccari, A. et al., *Vaccine* **34**(33);3901-06. 2016 Jun 15.
11. "lac operon" https://en.wikipedia.org/wiki/Lac_operon.

Acknowledgements

The authors wish to thank the laboratory of Patricia Spear for providing the recombinant virus strains, and the research participants and study personnel who made these experiments possible. This work was funded by Genocea Biosciences Inc.