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Methods Mol Biol. Author manuscript; available in PMC 2015 January 01.

Published in final edited form as:

Methods Mol Biol. 2014 ; 1144: 305–327. doi:10.1007/978-1-4939-0428-0_21.

The Murine Intravaginal HSV-2 Challenge Model for Investigation of DNA Vaccines

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Abstract

DNA vaccines have been licensed in veterinary medicine and have promise for humans. This format is relatively immunogenic in mice and guinea pigs, the two principle HSV-2 animal models, permitting rapid assessment of vectors, antigens, adjuvants, and delivery systems. Limitations include the relatively poor immunogenicity of naked DNA in humans and the profound differences in HSV-2 pathogenesis between host species. Herein, we detail lessons learned over the last few years investigating candidate DNA vaccines in the progesterone-primed female mouse vaginal model of HSV-2 infection as a guide to investigators in the field.

Keywords

Herpes simplex virus; Animal model; DNA vaccine; Antibody; Polymerase chain reaction; Latency; Dorsal root ganglia

1 Introduction

There is no licensed vaccine for herpes simplex virus type 1 (HSV-1) or herpes simplex virus type 2 (HSV-2). Inbred mice do not recapitulate some of the features of HSV infections in humans. For example, murine infections tend to be either fatal at high inoculum or fail to establish themselves at low inoculum, while human primary infections are rarely fatal. The infectious inoculum dose is not known in humans. Additionally, humans have spontaneous reactivations leading to shedding of infectious virus at epithelial surfaces [1, 2], while mice at best have rare molecular evidence of reactivation, even when stressed or immune suppressed, and never reactivate all the way to shedding of infectious virus [3, 4]. Nonetheless, both species exhibit the establishment of HSV latency in dorsal root ganglia (DRG) that innervate sites of epithelial inoculation, and both show worsening of disease course with immune suppression. The animal efficacy phase of the preclinical study of a candidate HSV vaccine typically sequences murine immunogenicity and then protection studies and then progresses to guinea pig and first-in-human stages [5].

Unmanipulated female mice have a 4-day estrus cycle and are refractory to vaginal HSV-2 inoculation. Exogenous progesterone pre-treatment alters vaginal physiology [6], HSV receptor levels [7], and perhaps immune mechanisms [8] to render animals more susceptible. Animal age [6] and strain [9] are other critical variables for HSV susceptibility for some viral strains and routes of inoculation. Balb/c mice tend to be more susceptible to virus challenge [9], and in general are easier to handle. However, C57BL/6 have a thoroughly

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characterized CD8 T-cell response [10], HSV TCR transgenics [11], and many genetic variants such that they may be preferable for some experiments.

1.1 DNA Vaccines and Vaccination

At the time of writing there is no DNA vaccine licensed for use in humans. Despite this, DNA vaccines continue to be attractive. Advantages are the ability to produce broad immune responses including CD4, CD8, and antibody [12], storage and shipping stability, ease of production, predictable cost, the potential for in vivo post-translational modification of vaccine product, and a long duration of antigen presentation. Sequences can be modified to reflect microbial strain changes, to target the MHC class I pathway through the addition of ubiquitin or other motifs, to optimize codon utilization, or to include immunostimulatory CpG motifs. DNA vaccines can also be delivered by diverse routes and in concert with adjuvants or enhancers aimed at increasing the uptake or protein expression or creating an immunogenic microenvironment [13, 14].

In mice, vaccine route can be varied to mimic potential human delivery routes or to target different immune pathways. With intramuscular (IM) injections of DNA vaccine, antigen processing and immune priming likely occur in the draining lymph nodes. In contrast, antigen-presenting cells (APC) in the immediate area are thought to play a key role in the immune response to DNA vaccines delivered to the dermis [2, 15]. Additionally, vaccination in the skin has been reported to be up to tenfold more potent than the IM route [2] and therefore to have the potential for dose-sparing.

DNA vaccines can be prepared in-house or manufactured by third party contractors. In general these vaccines consist of: (1) bacterial plasmid backbone with an antibiotic resistance gene and bacterial origin of replication, (2) a strong eukaryotic promoter, and (3) DNA coded vaccine insert. Our group's experience with the pVAX1 vector, manufactured by Invitrogen, is detailed herein. This nonproprietary molecule is commercially available, but other vectors with similar features can also be used. pVAX1 is specifically designed for DNA vaccine development with a CMV promoter, multiple cloning site, and kanamycin resistance for selection in *E. coli.* It is recognized that DNA vaccines for clinical trial administration to humans typically lack antibiotic resistance markers. A strategic decision is required as to whether to move directly to one of these proprietary vectors for preclinical testing [16]. When producing vaccine in-house, make enough vaccine to complete your studies. With vaccine doses as high as 100 μg each, a 100 animal study with two doses/animal could easily require over 20 mg of vaccine. Outsourcing can be attractive but requires additional decisions concerning Good Manufacturing Process (GMP) specifications and costs. Special efforts must be made to monitor the purity and identity of DNA vaccines. We recommend resequencing the final vaccine construct and checking for expression of the *bona fide* protein as outlined below. In situations where we have not had a mAb, we have used polyclonal human immune sera or human CD8 T-cell clones specific for the HSV-2 gene of interest [12]. *E. coli* strains typically used to manufacture plasmid are derivatives of the “safe” lab strain K-12 but still have an altered endotoxin. This TLR4 agonist that could have an unrecognized adjuvant effect and level should be carefully monitored.

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There are several design considerations for DNA vaccines. HSV sequences are GC rich and some coding regions have repeat elements; these features can lead to cloning difficulties or instability. Codon optimization is important in some viral systems and has been reported for HSV-2 [17, 18]. Intellectual property, institutional review board (ethics), and cost considerations may favor synthesis of the gene of interest or routine PCR cloning to obtain the initial HSV-2 inserts.

1.2 Virus and Virus Challenge

Several challenge strains of HSV-2 are in use. A nearly complete genome is available for the virulent strain 186 (GenBank JX112656.1). The prototype strain HG52 has mutations rendering it less virulent [19] and is therefore disfavored. Some researchers are using low-passage, near wild-type strains in animal HSV-2 research and finding them more challenging to obtain protection. While we have not yet applied this to DNA vaccines, this is a quite rational reality check [20]. Sequence matching between vaccine and challenge strain is important. In our work, we sequenced strain 186 and wild-type HSV-2 tegument genes *UL46* and *UL47*, found consistent differences with HG52, and matched DNA vaccines to the wild-type consensus and the challenge strain [12]. We use a replication-competent, attenuated HSV-2 strain delivered vaginally as positive control vaccine. We selected a strain based on availability [21] and a track record of near-sterilizing, durable immunity that appears to depend on CD4 T-cells [22]. Diverse attenuated strains are available [23]. These are also helpful for studying immune responses in infection versus vaccination and comparing the two contexts. Live attenuated vaccines set a high bar and help to differentiate simple survival from higher levels of protection. One disadvantage of the TK-minus positive control is the establishment of DRG latency, making this vaccine endpoint problematic.

1.3 Murine Challenge Endpoints

1.3.1 Survival—When HSV-2 strain 186 is introduced vaginally to progesterone-treated mice and infection establishes itself as indicated by local lesions and viral replication, death usually occurs by day 6–8. There is a very narrow window near the 50 % lethal dose (LD_{50}) within which some animals seroconvert but survive. With specific criteria for euthanasia, including hind limb paralysis, ataxic gait, immobility, or dehydration, survival is a relatively objective and unambiguous endpoint. Very occasionally, challenged mice treated with negative control vaccines have survived to 21 days. To determine infection, we compare pre-infection and day 21 serum from survivor mice in a simple ELISA using whole UV-inactivated HSV-2 as a coating antigen and survivor animal serum at 1:100 and 1:300 dilution as detailed elsewhere [12]. A threefold increase in OD_{450} is consistent with infection. Mice that fail to mount specific antibody responses could have either had sterilizing immunity or been improperly inoculated. Attention to detail in progesterone treatment, vaginal inoculation, and the titration and storage of challenge virus are important to minimize this ambiguous situation. To study anamnestic immunity that is primed by vaccine and boosted by infection in mouse groups not expected to survive, we will sometimes include additional mice for sacrifice on day 6 after challenge to permit both immune boosting and survival endpoints.

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1.3.2 Clinical Score—Many HSV-2 murine challenge models use a 0–4 scoring scale. Scores of 3 or 4, as detailed below, trigger humane euthanasia. Mice typically show few symptoms through day 5, but their condition can deteriorate rapidly thereafter and twice daily monitoring is appropriate. Scores provide a continuous variable of overall efficacy to distinguish otherwise similar vaccines.

1.3.3 Vaginal HSV-2 DNA Copy Number Assessed by Quantitative PCR—
Measure of microbial titer is a regular part of preclinical vaccine studies and many groups have used plaque assays to titer vaginal swabs and other tissues after HSV-2 challenge. We prefer PCR based on cost and the local availability of a sensitive and accurate assay [24]. The main value is ranking of test vaccines that lead to 100 % survival. Many HSV-2 vaccines are based on glycoprotein D (gD, encoded by gene *US6*), known for several years to lead to complete mouse protection when administered as a DNA vaccine [25]. The failure, to date, of gD2 vaccines in humans is another story altogether [26, 27]. A measure of vaginal replication such as PCR can distinguish vaccines leading to survival only from vaccines that decrease early mucosal replication. Day 1 (24 h) vaginal HSV-2 DNA copy number is correlated with survival [2] and DRG HSV-2 DNA load at latent time points in survivor mice [28]. The useful TK-minus positive control typically leads to near-sterilizing immunity, especially by day 5 [17].

1.3.4 Specific Immunity—Antibody and T-cell assays are typically used to confirm immunogenicity and the delivery of the desired antigen. It is less clear that we have a good correlate of efficacy for mouse survival, and as no vaccine has consistently worked in humans, targeting of such assays is still uncertain. Pure antigen is best to detect specific antibodies, and given the multiple protein targets available within HSV-2 and the proprietary nature of some candidate protein antigens [26], this may be difficult to obtain. We have substituted commercially available gD1 for the desired gD2 in some work [2, 13] and used relatively crude HSV-2 protein made by eukaryotic host cell transient transfection for tegument proteins [12]. T-Cell responses mediated by CD4 and CD8 T-cells are widely sought after and can be detected by several methods. One can consult the literature for commonly used HSV-2 proteins such as gD2, recalling that epitopes are mouse H-2 haplotype specific. To test other HSV-2 proteins, preliminary vaccine-only (no challenge) experiments are done in which immune splenocytes are tested for interferon-γ responses to overlapping peptide sets covering the HSV-2 protein(s) of interest. The phenotype (CD4 versus CD8) of responding cells are established in depletion studies as detailed [12]. It is helpful to independently establish that the same T-cell epitopes are also recognized by immune splenocytes in the context of actual HSV-2 infection. The attenuated TK-minus HSV-2 strain is used for this purpose [12]. We specifically note that splenocytes harvested from noninfected mice sometimes show high background in IFN-γ ELISPOT. This problem has occurred in temporal waves in our animal facility and while likely related to inflammation or infection, veterinary care staff can have difficulty identifying a discrete problem that can be fixed. Detailed antibody and T-cell assay methods are not addressed herein but myriad primary or methods sources are available.

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1.3.5 HSV-2 Latency in DRG

Controversy exists as to whether HSV-2 vaccines for human use should be sterilizing, preventing local infection and the establishment of DRG latency, or merely ameliorate disease [5]. HSV-2 does establish latency in mice, and careful dissection followed by explant culture or PCR can detect and measure this variable. While explant culture proves infectious virus, we prefer PCR for the reasons outlined above. Animal models support the contention that DRG HSV-2 load is related to reactivation [29]. There is a learning curve in establishing the dissection method, throughput is limited even for skilled operators, and the TK-minus positive control virus leads to positive DRG PCR such that this endpoint is not useful for this vaccine unless a strain-specific PCR is designed. We generally measure a number of HSV-2 genomes present and a number of mouse genomes present using GAPDH as a diploid housekeeping gene. Results are expressed as HSV-2 DNA copy number per million mouse cells [13]. Regarding timing, most investigators wait between 60 and 100 days after inoculation, although valuable immunology research has been done in latently infected DRG at 30 days [30].

2 Materials

Materials and reagents comparable to the standards in our lab can be used at the discretion of the specific lab.

2.1 Mice

Female Balb/c or C57BL/6 mice at sexual maturity, age 5–6 weeks (*see* **Note 1**).

2.2 Vaccines and Vaccine Delivery

Vaccine composition and route will be tailored to specific research. We include as gold standard positive control a replication-competent HSV-2 strain attenuated through deletion of part of gene *UL23* encoding thymidine kinase (TK). This TK-minus virus requires specific institutional approval. Though it is less virulent than wild-type HSV-2, TK-minus strains are acyclovir resistant, leading to occupational health concerns (*see* **Note 2**).

1. Positive control TK-minus HSV-2 strain at a titer of 10^8 pfu/ml or higher.
2. Positive control amino acids 1–340 glycoprotein D (gene *US6*) of HSV-2 cloned into commercially available pVAX1 (Invitrogen). This is an alternative positive control.
3. Negative control pVAX1 plasmid.
4. Researcher-selected and -sourced test vaccine(s) with or without adjuvants, excipients, stabilizers, preservatives, etc.
5. Appropriate negative controls for test vaccines, typically containing the same buffers, adjuvants, etc. but no active compound. Note that TLR agonists delivered locally can protect the vagina [31]. Innate immunity-stimulating adjuvants, if used, should therefore be incorporated into controls.
6. TK-minus positive control virus: Seed stocks were obtained from Dr. Greg Milligan at the University of Texas Medical Branch, Galveston, Texas and

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originally published by Stanberry et al. [21]. Stocks should be regrown in *Mycoplasma* negative Vero or similar cells, tittered by standard plaque assay, and stored in single-use aliquots at −80 °C. We confirmed deletion within *UL23* by PCR comparing virulent strain 186 and TK-minus using PCR primers at the 5' and 3' ends of the coding region followed by agarose gel electrophoresis and sequencing. Strain 186 lead to a product of approximately 1.1 kb, while product from the TK-minus strain was considerably shorter, reflecting internal deletion.

7. pVAX1-gD2 positive control vaccine: please see our publication for details [2]. Briefly, gD2 amino acids 1–340 were cloned by PCR from a random clinical HSV-2 isolate into pVAX1 (Invitrogen). Similar results have been obtained by gene synthesis. pVAX1 expresses the insert under the control of a CMV promoter. Plasmid was harvested from small-scale *E. coli* cultures under kanamycin selection and sequenced to prove identity. Seed was provided to a commercial DNA manufacturer for near-GMP material for pVAX1 and PVAX1-gD2 at 1 mg/ml with defined endotoxin levels. With regard to amount, three IM injections of 10 μg per mouse at 21-day intervals lead to solid protection. Plan ahead and make a single large batch for the positive control group. The gD2 construct is predicted not to localize to cell membranes due to deletion of the C-terminal transmembrane domain within amino acids 341–393 [32]. To check expression of gD2 we used flow cytometry [2]. Briefly, vaccine from the manufacturer was transfected into Cos-7 cells (obtained from ATCC) with Fugene 6 (Roche) per the package insert. After 48 h cells were permeabilized with Cytoperm/Cytofix (Pharmingen) per the manufacturer and stained for flow cytometry using as first antibody, mouse anti-gD mAb 2C10 (Santa Cruz Technologies), and as secondary antibody allophycocyanin-labeled goat anti-mouse IgG (Biolegend). The result was that pVAX-1 control-transfected Cos-7 were negative for specific fluorescence, while pVAX-1-gD2-transfected cells were 20–40 % positive. Vaccine stocks were stored at −20 °C until use.

8. pVAX1 empty vector control: prepare identically to pVAX1-gD2.

9. Test vaccines: prepare investigator-specific DNA vaccines, optimally in a manner similar to that of a positive control DNA vaccine such as the one discussed above. Regarding amount, plan for in the range of 10–100 μg per mouse per vaccination and 2–3 vaccinations per mouse. Single large batches are therefore preferable. We have found that “Endo-Free” Midi- or Maxi-prep kits (Qiagen) are adequate if one is not using a commercial vendor. In addition to sequencing the final vaccine, it is preferable to verify expression of the *bona fide* HSV-2 protein. The use of a specific mAb as outlined for gD2 can be pursued if such a reagent is available. In our work with DNA vaccines encoding HSV-2 tegument proteins, we used both humoral and cellular human immunology probes. VM92 cells were transfected with the candidate DNA vaccines and supernatants collected and plated onto ELISA plates, and probed with pooled human serum obtained either from HSV-2-infected persons or HSV-1/HSV-2 dually seronegative persons. Specific binding of only the immune sera was observed for each HSV-2 protein tested [12]. For CD8 T-cell tests, Cos-7 cells were co-transfected with both the test vaccine and the relevant

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HLA class I heavy chain cDNA and co-incubated with CD8 T-cell clones specific for the HSV-2 protein under study, as detailed in a previous methodology paper [33]. The expected result is that only cells transfected with both the HLA and HSV-2 protein trigger specific interferon-γ release [12].

2.3 Intramuscular (IM) Vaccine Delivery to the Rectus Femoris Quadriceps Muscle

1. 2″ × 2″ gauze sponges (Fisher).
2. Vaccine and diluent such as PBS (40 g NaCl, 1 g KCl, 5.7 g Na_2HPO_4, 1 g KH_2PO_4, add H_2O to a final volume of 5,000 ml, adjust pH to 7.4, autoclave, store at 4 °C).
3. 29 gauge, ½″, 0.3 cc insulin syringes (Becton Dickinson) (*see* **Note 3**).

2.4 Intradermal (ID) Vaccine Delivery to the Pinna (Ear)

1. Anesthetic: Ketamine/xylazine. Ketamine is obtained from the clinical pharmacy at 100 mg/ml and xylazine at 20 mg/ml. Please note that xylazine stocks also come at 100 mg/ml and care is required to check each bottle. Both are stored at room temperature. A pre-mix is made and stored at room temperature for up to 28 days. Mix 0.65 ml (100 mg/ml) ketamine with 0.22 ml (20 mg/ml) xylazine and 9.13 ml sterile saline. The final solution contains 6.5 mg/ml ketamine and 0.44 mg/ml xylazine and is dosed at 20 μl/g of body weight (*see* **Note 4**).
2. Needle and syringe 25 gauge 5/8′ safety-lok™ syringes (Becton Dickinson) for anesthesia.
3. Optional: Blu-tack™ (manufactured by Bostik) (*see* **Note 5**).
4. Vaccine and diluent such as PBS.
5. 30 gauge, ½′, 0.3 cc ultra-fine insulin syringe (Becton Dickinson); one per ear to be injected. We recommend this specific product for this application.

2.5 Virus Challenge

Virus culture/preparation is not detailed herein. Researchers should be competent in virus handling and growth at BSL-2 levels. We use *Mycoplasma* negative stocks of Vero cells originally obtained from the American Type Culture Collection (ATCC) to grow virus. Obtain institutional approvals for each strain used including the TK-minus strain if appropriate. Store stocks in small ~100 μl aliquots at −80 °C in screw-cap, O-ring style tubes.

1. Biosafety cabinet certified to BSL-2.
2. Medroxyprogesterone 150 mg/ml. This is obtained from the clinical pharmacy as Depo-provera™. Amount needed is 2 mg/animal. This is also required prior to TK-minus virus immunization used for positive vaccine control.
3. 1 ml sterile syringes 25 gauge 5/8′ safety-lok™ syringes (Becton Dickinson) for medroxyprogesterone injection, one per animal.

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4. HSV-2 strain 186 or other virulent strain with titer of 10^8 pfu/ml or higher (*see* **Note 6**).
5. Normal mouse serum prepared from naïve animals 0.1 % solution in PBS (*see* **Note 7**).
6. Sterile 1.7 ml DNAse/RNAse-free microfuge polypropylene conical-bottom tubes for virus dilution.
7. Ketamine/xylazine anesthetic pre-mix (*see* Subheading 2.4, **item 1**).
8. Sterile syringes for anesthetic (*see* Subheading 2.4, **item 2**).
9. Calcium alginate swab (Fisher), one per animal.
10. 2–20 μl range adjustable pipette and sterile nuclease-free filter pipette tips.
11. 10 % bleach in water.

2.6 Challenge Study Endpoints

2.6.1 Survival

1. Record keeping materials in animal facility.
2. Excel spread sheet with two columns for each day (morning and evening) and one row per animal.
3. Institutionally and facility approved method for getting paper data out of animal room, for example plastic bags with disinfectant spray to outside of bag.

2.6.2 Clinical Score

1. Record keeping materials in animal facility.
2. Excel spread sheet with two columns for each day (morning and evening) and one row per animal.
3. Reference sheet/card with disease score criteria for disease score 0–4.

2.6.3 Vaginal Swab for HSV-2 DNA Copy Number via PCR

1. Digestion buffer: 100 mM KCl, 10 mM Tris–HCl pH 8.0, 25 mM EDTA, 0.5 % Nonidet P-40; store at room temperature prior to use. Digestion buffer can be made as a 5× solution. For 5× solution: 3.7 g KCl, 50 ml 1 M Tris–HCl pH 8.0, 250 ml 0.5 M EDTA pH 8.0, 50 ml Igepal CA-630, add deionized molecular biology grade water to a final volume of 1,000 ml. Dilute 1:5 to make working solution. Note that Ipegal CA-630 (Sigma Aldrich) is chemically indistinguishable from Nonidet P-40, which is no longer commercially available.
2. 2 ml Polypropylene sterile O-ring tubes (Sarstedt) with 1 ml PCR digestion buffer, one tube per animal per day. Pre-label tube with animal ID number and day.
3. Sterile mini-tip urethral swabs one per animal per day (Copan).
4. Small sharp scissors.

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2.6.4 Blood Collection for Serologic End Points (See Note 8)

1. Ketamine/xylazine anesthetic (*see* Subheading 2.4, **items 1** and **2**).
2. Glass Natelson blood capillary collection tubes (Fisher), non-sterile.
3. 2′ × 2′ gauze sponges (Fisher).
4. Blood collection: "Microtainer™" non-sterile serum separator tubes (Becton Dickinson) or sterile Eppendorf tubes if downstream cell culture-based assays such as neutralization assays that require sterility are anticipated.
5. Optional: Artificial tears ointment sterile ophthalmic petrolatum and mineral oil lubricant (NDC).

2.6.5 DRG Dissection

1. Dissecting scope such as SMZ-800 Zoom stereo (Nikon).
2. Light source such as NI-30 single gooseneck illuminator (Nikon).
3. Low quality dissection scissors.
4. Low quality dissection forceps.
5. Acceptable surface on which to perform dissections such as disposable dissecting board or dense sturdy styrofoam covered with Kimtech science benchtop protector (Fisher); one piece large enough to cover the dissection work surface for each animal.
6. 20 gauge syringe needles.
7. Student Vannas spring scissors for laminectomy (Fine Science Tools#91500-09). We specifically recommend this item.
8. Two of each high quality forceps (Fine Science Tools, #11271-30 and #11272-30). We specifically recommend these items.
9. Vannas spring scissors for DRG excision and lower spine laminectomy (Fine Science Tools #15012-12). We specifically recommend this item.
10. Sterile O-ring cryovials (2 ml, Sarstedt), one per animal, pre-label.
11. Digestion buffer 150 μl/animal, 10 mM Tris-HCl, 25 mM EDTA, 10 mM KCl, 1 % Igepal C-630.

3 Methods (*See* Note 9)

3.1 Mouse Restraint

1. Place the mouse on a surface it can grasp.
2. Gently pull on mouse tail and maintain light pressure, mouse will try to pull away.
3. Grasp the scruff of the neck with other thumb and forefinger.
4. Maintain hold of tail.

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5. A mouse can be restrained with one hand large/flexible enough to hold the tail against the pad of the hand with the little finger while scruffing the neck with thumb and forefinger. This causes hand fatigue over time.

3.2 Mouse Husbandry

1. Ensure that institutional approval is in place.
2. Follow facility/institutional requirements concerning work with HSV-2. In the USA, HSV-2 is a biosafety level 2 (BSL-2) agent. Nomenclature may vary internationally. Comply with housing and procedure room standards at all steps after mice are vaccinated with attenuated HSV-2 positive control, if used, or challenged with live HSV-2.
3. Order mice through your commercial vendor, or source mice according to your study design. Adjust age at purchase to allow 1-week acclimatization for animals before starting study (*see* **Note 10**).

3.3 IM Vaccine Delivery

1. This requires two persons and is frequently done the same day as blood collection (*see* **Note 11**).
2. The first person restrains the mouse using standard neck scruff/tail method.
3. The hind leg to be injected must also be held fully extended by person 1. The leg should be held extended in a natural direction, neither straight back nor straight out perpendicular to the spine. Leaving the leg slightly loose makes it easier to pinch and locate the quadriceps muscle.
4. Person 1 holds the mouse so that its ventral side faces person 2.
5. Person 2 wipes the anterior of the mouse thigh with 70 % ethanol applied with the 2×2 gauze. Be careful to avoid genital/rectal areas as this will agitate the animal.
6. Person 2 gently squeezes the quadriceps femoris muscle group with thumb and forefinger.
7. Person 2 inserts needle and injects while maintaining gentle pressure on muscle with thumb and forefinger. The muscle should feel like a large round grain of rice. On second and later injections, it will feel and possibly look larger. As you push the syringe plunger you should feel the muscle swell and stay swollen. If you do not feel this, you have missed. Practice with dye if allowed by your institution including dissection of leg to locate the injection point to gain skill at IM injections. Use a sharp/new needle at least every four injections. We typically inject bilaterally 50 μl per side per injection; thus, we use a new needle every two mice.
8. Remove needle and discard in appropriate sharps container.

3.4 ID Vaccine Delivery to the Ear Pinna

1. Anesthetize mouse using ketamine/xylazine mouse mix (*see* **Note 12**).

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2. Place a small amount of Blu-tack on fore- or middle finger.
3. Place mouse prone (face down) in front of you.
4. Using thumb, gently press the inner, ventral of the mouse ear against the Blu-tack so that pinna is as flat and planar as possible with no wrinkles or folds.
5. With the bevel of the syringe-mounted 30 gauge needle facing up very carefully push the needle tip into the dermis. Catch the skin and then slide gently until the beveled tip and 1 mm of the shaft of the needle are completely covered by skin. The needle tip should still be visible through the very thin skin even though it is intradermal (*see* **Note 13**).
6. Gently and slowly push on the syringe plunger to form a small bleb of vaccine in the pinna. The maximum volume is 10 μl.
7. Slowly without shaking remove the syringe from the ear.
8. Gently remove ear from Blu-tack and finger to prevent vaccine from being pushed or squeezed out.
9. Discard sharp in appropriate sharps container.
10. Use a new sharp needle for each ear.

3.5 Virus Challenge

3.5.1 Administration of Medroxyprogesterone

1. Six days prior to wild-type virus challenge or administration of TK-minus vaccine virus, mice will be treated with 2 mg/animal of medroxyprogesterone.
2. Dilute medroxyprogesterone to 20 mg/ml in sterile PBS on the day of administration.
3. Administer 100 μl (2 mg) subcutaneously. Holding the awake mouse by the loose skin on the back of the neck with the thumb and index/forefinger, insert the needle into the subcutaneous space between the back of the mouse's head and your fingers. Inject 100 μl of 20 mg/ml medroxyprogesterone solution (*see* **Note 14**).

3.5.2 Establish the 50 % Lethal Dose (LD_{50})—It is necessary to establish the LD_{50} for each specific viral challenge strain, virus batch, mouse strain, and mouse chronologic age prior to carrying out experiments with vaccines (*see* **Note 15**).

3.5.3 Live Virus Challenge (See Note 16)—Set up your work area as to eliminate unwanted spread of virus and to maintain workflow.

1. Dilute virus in PBS/0.1 % naïve mouse serum so the desired inoculum is in 10 μl (*see* **Note 17**).
2. Chose the desired challenge dose(s) based on the scientific goals of the study. We typically challenge at 50–100 × LD_{50}. To differentiate between moderately and highly active vaccines, some studies may use a dose range including lower or higher challenges. In our hands, effective DNA vaccines can provide 100 %

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protection at up to 500 × LD_{50} [17]. Some investigators use a two-dimensional matrix in which both vaccine dose and HSV-2 challenge dose are independently varied to rank vaccine candidate efficacy.

3. Anesthetize mice. We prefer ketamine/xylazine as isoflurane will not keep mice motionless long enough to ensure vaginal residence of the inoculum.
4. Avoid handling mice for 5 min after they have been inoculated to minimize the amount of inoculum that exits the vagina.
5. Scruff anesthetized mice such that spine is straight and stretched to full length without hunching, holding the tail with the little finger.
6. Remove mucus from the vaginal introitus. Using a calcium alginate swab, clean the vagina. Thick, even gelatinous mucus is normal. Often gently rotating the swab a few times can wind up the mucus to ease removal.
7. Draw 10 μl of diluted virus into a filter-tip 2–20 μl pipette tip.
8. Gently insert pipette tip into mouse vagina.
9. Slowly push pipette plunger. If you see inoculum spilling out, readjust pipette tip or your restraint of the mouse. A fully stretched out mouse optimizes retention of the inoculum.
10. Place pipette tip in bleach gently pipette up and down.
11. Discard pipette tip in sharps container.
12. Gently place mouse supine (face up) in cage to sleep for 5–15 min.
13. Observe that all mice are awake prior to leaving area.

3.6 Challenge Study Endpoints

3.6.1 Survival

1. Check mice according to your protocol and animal care and use standards. We are generally required to check mice twice per day for 21 days.
2. It is best for consistency if one researcher completes all animal evaluations for pre-morbid conditions requiring euthanasia.
3. Humanely euthanize animals showing premorbid grade 3 or 4 changes (*see* Subheading 3.6.2 and **Note 18**).

3.6.2 Clinical Score

1. It is best for consistency if one researcher completes all animal evaluations.
2. Gently grab mouse by tail taking care not to startle. Lift by the tail so that the anal/ genital region is visible and note of any abnormal appearance.
3. Asses fur, posture, gait, and hydration level.
4. Assign a score based on appearance of animal and disease scoring criteria.

5. Disease score:

   0—Normal.

   1—Perianal/genital erythema.

   2—Perianal swelling and erythema. May have slightly ataxic gait and/or slightly ruffled coat. The normal mouse coat is glossy and shiny.

   3—Purulent lesions, partial or complete paralysis in one or both hind limbs, visible weight loss or dehydration, very poor grooming, perianal urine staining due to loss of bladder control.

   4—Immobile, complete hind limb paralysis, severe dehydration, little or no grooming.

3.6.3 Vaginal HSV-2 DNA Measurement by PCR (See Note 19)

1. Aliquot 1 ml digestion buffer to each 2 ml sterile, nuclease-free O-ring cryovial tube.
2. Restrain the non-anesthetized mouse using the one hand method (*see* Subheading 3.1). Turn mouse supine so that the head is away from you and the tail is towards you. Two persons, one holding and one taking the sample, enables less operator hand fatigue but is overall more challenging.
3. Gently insert sterile swab into mouse vagina and rotate swab 360° within 2 s. The main difficulty is inadequate restraint or a hunched mouse posture. Inflammation should not be limiting on days 1–5. The entire cotton tip of the swab should be inserted.
4. Place swab in designated tube with digestion buffer.
5. Use scissors to trim plastic handle of swab, leaving the swab tip behind in the tube. Trim enough that you can be sure to close the tube. Scissors should be sharp and tubes in a secure holder to avoid recoil and spilling after swab snipping.
6. Secure cap onto tube.
7. Store samples at −20 °C until they are ready for processing for PCR.

3.6.4 Blood Collection (See Note 20)

1. Obtain institutional approval for all bleeds including schedule. Ensure operators are trained.
2. Anesthetize animals using ketamine/xylazine mouse mix or isoflurane. Do not anesthetize too many animals with mouse mix or some may awaken too early. Generally ten animals anesthetized at a time will sleep long enough for a less skilled operator to bleed them all.
3. Place mouse on its side, on a clean, cushioned, and thermal neutral or warmed surface so all four legs point towards you. As you will alternate left and right eye with serial blood collections, mice laying on their right flank for the initial

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collection will be lying on their left flank for the next collection. We generally limit the total number of retro-orbital bleeds to four in the life of the mouse, two from each side, with an interval of at least 2 weeks between bleeds from any one eye.

4. The head should be oriented toward your dominant side and the hand you will use to collect blood. Keep mice warm; if slow to awaken, rewarm. Some twitching is normal during anesthesia but they should not withdraw to noxious forepaw stimulation, it is often associated with lighter anesthesia or with an early waking state.
5. Using your thumb and forefinger to gently pull the skin above and below the eye away from the eye. The forefinger is above the eye and the thumb below.
6. The eye should be slightly protruding from the socket.
7. Gently and decisively push blood capillary collection tube behind the eye between the upper eyelid and the top of the eyeball. The tip of the capillary tube is puncturing the capillary bed behind the eye.
8. Hold capillary tube in place until the blood flows up to the point where you have predetermined your collection volume.
9. Using 2″ × 2″ gauze, gently close the eye and hold pressure for several seconds.
10. Place capillary tube into blood collection tube. Using your trimmed transfer pipette, apply pressure to push the blood into the separator tube (non-sterile) or sterile microcentrifuge tube. Work quickly before the blood clots in the capillary tube.
11. Apply ophthalmologic ointment to the eye (optional).
12. Allow blood to clot in the secondary collection tube at least 60 min at room temperature.
13. Spin samples at approximately 9,300 RCF or about 10,000 rpm on a small benchtop microcentrifuge for 10 min. Use a pipette to transfer serum to a storage tube.

3.6.5 Lumbosacral DRG Dissection (See Note 21)

1. Euthanize mice using CO_2 overdose or another approved non-physical method (*see* **Note 22**).
2. Spray carcass completely with 70 % ethanol to constrain hair and particulates.
3. Completely remove skin. Make a small incision in the skin of mouse flank using common dissecting scissors. Gently grasp the skin on either side of the incision, pull towards the head and tail until the skin is mostly removed.
4. Use common dissecting scissors to trim away skin (*see* **Note 23**).
5. Remove all thoracic and abdominal viscera using common forceps and scissors.
6. Trim away the most ventral portion of the rib cage using common scissors (*see* **Note 24**).

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7. Label mice so you can track animal identity. Store in an airtight container at 4 °C to prevent drying of carcasses.
8. Pin mouse down on dissection surface ventral side down. Pin hind legs out from body fully extended at about a 45° angle from spine in the caudal direction. 20 Gauge syringe needles work well. Pins directly through the middle of the footpads will hold the best.
9. Pin the front legs. Gently pull on them so the entire spine is stretched out.
10. Using the student Vannas scissors carefully cut through the muscle on either side of the spine down the length of the spine (*see* **Note 25**).
11. Carefully fillet away the muscle from the dorsal bony spinal column.
12. Place pre-labeled sample tube in dry ice and remove cap (no buffer).
13. Locate the first or second thoracic vertebrae finding the first or second most rostral sets of ribs.
14. Using the student Vannas scissors make a cut into the spine, perpendicular to the length of the spine. This cut should be a half cross section. You are cutting only through the dorsal part of the spinal column. The ventral half of the bony spinal column should be left intact (*see* **Note 26**).
15. Alternating between sides, cut at about the dorsal/ventral margin down the spine towards the tail (*see* **Note 27**).
16. As you cut the spine away you can excise the DRG or you can wait until you complete the laminectomy to excise them all at once.
17. Gently grasp the nerve fiber on either side of the DRG using either of the fine science tools forceps. Using the finer Vannas spring scissors trim the nerve fiber away from either side best you can (*see* **Note 28**).
18. Carefully place the DRG into the labeled O-ring vial that is resting in dry ice. The moist tissue should stick to the very cold tube wall.
19. Remove all DRG that are relevant to your study design. We typically aim for 10–14 DRG from each side from the lumbosacral region.
20. Cap tube and spin at high speed in a microcentrifuge to get all DRG to the bottom of the sample tube.
21. Add 150 μl of PCR digestion buffer.
22. Store at −20 °C until delivery to the PCR lab.

4 Notes

1. Purchase females from commercial vendors or breed HLA or other transgenics [34] at your facility. Animals born the same day are more expensive than animals within an age range. We usually tolerate a small range and buy animals at sexual maturity at 5–6 weeks. Adjust age at purchase to allow 1 week acclimatization prior to

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vaccination, and so that the age at HSV-2 challenge corresponds to that age at which the LD_{50} was established. Strain is important. Balb/c tend to be more susceptible to HSV. We use this strain to set a higher bar for survival and allow lower viral inoculums. CD4 and CD8 epitopes in selected HSV-2 proteins have been identified in some strains but not others, thus influencing strain choice depending on the ORF under study. Balb/c are behaviorally easier to manipulate than are C57BL/6 in the non-anesthetized state. C57BL/6 have a thoroughly characterized CD8 T-cell response [10], HSV TCR transgenics, and many genetic variants such that they may be preferable for some experiments. The number of mice per experimental group is a critical parameter set in consultation with a biostatistician based on preliminary data from the user's lab and/or the literature, the expected effect size, and the desired degree of certainty concerning the results. At our institution, ethical committee approval includes group size. Small groups may lead to failure to detect differences, while large groups lead to unnecessary animal suffering.

2. A variety of replication-competent attenuated or replication-incompetent HSV-2 strains can be used in the place of TK-minus if available to the investigator. We also include a DNA vaccine positive control containing partial-length gene *US6*, encoding glycoprotein D of HSV-2, detailed herein. Acyclovir in drinking water at 1 mg/ml is an alternative positive control. Acyclovir requires daily drug dilution and water bottle changes for 21 days. Obtain acyclovir for intravenous injection from a clinical pharmacy at 50 mg/ml, and hold concentrated drug at room temperature. Each day, dilute to 1 mg/ml in sterile water and refill water bottles. The cost is low. There is less protection of the DRG than with effective vaccines. Occasional late deaths have been noted after day 21 for acyclovir.

3. Typically 50 μl is the maximum dose that can be administered to the quadriceps, or 100 μl/mouse if injected bilaterally. If multiple injections with a single syringe are permitted we do not recommend more than four per needle, as the needles dull quickly. When using this method, fill syringes to 200 μl.

4. The amount needed is 300–350 μl per mouse per anesthesia depending on weight and age. We estimate weight visually. We typically use 300 μl for younger Balb/c mice for the first and second administration, but by the age of challenge, typically 10–12 weeks, we use 350–400 μl. Follow institutional guidelines concerning controlled substance licensing, storage, disposal, and frequency of administration. Animals may develop tolerance if used too frequently. In our experience, C57BL/6 have less development of tolerance and we use 300 μl/dose and 350 μl at challenge. In general, if at the last administration prior to challenge seems at all inadequate, increase the dose for challenge. Do not exceed 400 μl of drug/mouse. Overdose is possible and generally lethal. Ketamine will not inhibit the hind leg withdrawal reflex. Use forepaw withdrawal to noxious stimulation to test the depth of anesthesia. We typically draw 600–800 μl per syringe/needle and reuse for two mice total per syringe/needle.

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5. The optional nature of this product is partly a safety issue. The alternative to using Blu-tack™ is to use only a gloved finger.
6. Stocks may lose titer at −80 °C at the rate of about 0.3 $\log_{10}$/year and should be periodically re-titered. LD_{50} will need to be preestablished by titration in mice of the same strain and target age in prior experiments. While stocks are being grown and titrated, observe microscopically for large syncytia formation. Scattered syncytia are normal for strain 186 but very large syncytia may indicate mutations that can influence virulence.
7. This is used at 0.1 % in PBS to dilute virus prior to inoculation.
8. We use commercially available truncated glycoprotein D of HSV-1 as test antigen when we use test vaccines that contain gD2 as a protein or DNA construct. gD1 and gD2 have highly similar amino acid sequences. ELISA details are published [2]. For other test vaccines, investigator-specific ELISA antigens will be required. Neutralizing antibody titers are also frequently performed, especially if the test vaccines contain the glycoproteins typically associated with HSV neutralizing antibodies: gB, gD, and/or gH/gL. Neutralizing assays are not detailed herein.
9. The outline of animal procedures is not a substitute for adequate hands-on training. Follow all institutional requirements regarding training for specific procedures, such as retrobulbar bleeds, subcutaneous, intramuscular, intradermal, and intraperitoneal injections, use of anesthetics and restraint and euthanasia techniques. Follow institutional guidelines and preferences regarding housing, restraint, blood sampling, anesthesia, and euthanasia. Training in vaginal inoculation, vaginal swabs, and DRG dissection may not be available from your institution and practice animals, if allowed, may be reasonable.
10. Consider ordering a few extra animals for critical studies as some may die due to shipping stress. Stressed animals may impact on study outcomes. Observe each mouse for inflammation or irregular health before beginning study. Young Balb/c can be very skittish, jump from great heights, and run away. Therefore operate with extreme care.
11. It is best to do IM injection before retro-bulbar blood collection requiring anesthesia, or when animals are awake enough to crawl around after blood collection. Animals with quadriceps muscle tone are much easier for IM injection as one can find the muscle and also palpate the expected small swelling after successful injection.
12. Isoflurane will not work because mice need to be under anesthesia for at least a couple minutes. It is best to have two persons with one giving anesthesia and the second giving vaccine.
13. It is very helpful to pay attention to the reflectivity of the needle. Depending on operator's vision the only difference you may see between a needle lying flat on mouse ear and a needle that is properly inserted intradermal is that the inserted needle will have a “matte” appearance in comparison.

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14. This is difficult for one person with skittish C57BL/6 mice. One person can restrain and another inject. An alternate injection site of loose skin on the rump can be used. If there is significant visible leakage of drug, repeat the procedure with estimation of the amount of leakage and record the extra injection. We prefer to use a single needle/syringe for every two animals. Preparing multiple syringes ahead of time does not work well because the drug is formulated as a suspension that settles out very rapidly. The master concentrate and diluted drug vials should be swirled prior to each use. Two operators are therefore preferable.

15. In brief, mice are obtained or aged in the facility to reach the proper age and then inoculated with virulent HSV-2 vaginally as described in Subheading 3.5.3, after medroxyprogesterone pre-treatment. Typically we range from 10 to 10,000 pfu/mouse in ½ log_{10} increments and test 8–10 animals per dose. We have found that the LD_{50} is typically between 100 and 1,000 pfu/mouse dependent on virus strain and batch and mouse strain. LD_{50} values in our hands are typically moderately (three to fivefold) higher for C57BL/6 than for Balb/c; that is, Balb/c are moderately more susceptible. Specific institutional approval is required at our institution prior to carrying out LD_{50}-finding studies. We use the same endpoints for humane euthanasia for LD_{50}-finding studies that are used in vaccine studies.

16. This is much easier with two operators. One person can anesthetize, clean the vaginal area and/or bleed, while the second person performs the inoculation. Diluted virus, bleach, and a sharps container should be accessible to the person doing inoculations. Space for at least one mouse cage should also be available within reach. Pre-tear the paper off the back of the individual paper pouches of the cleaning swabs so that the ends are accessible.

17. Transport virus to animal room on dry ice and thaw gently at room temperature. Then place leftover concentrated virus on wet ice. Dilute by two- to tenfold dilutions using a fresh pipette tip each time and gentle vortexing. Dilute the virus in a PBS 0.1 % normal mouse serum. Diluted virus can be used over 1–2 h held at room temperature. We dilute enough virus for about 40 mice. If the experiment is larger, we go back to the concentrated stock held on wet ice and prepare a second working tube. Wear personal protective equipment.

18. Operators should become certified in and comfortable with one or more modes or humane euthanasia following institutional guidelines and preferences. We use CO_2 overdose followed in some instances by cervical dislocation.

19. Perform this procedure on the days that are appropriate for your study. We generally study days 1, 3, and 5 after inoculation. This allows differentiation of vaccines that allow survival but still permit brisk local replication from vaccines that provide sterilizing or near-sterilizing local protection in addition to survival.

20. General instructions are given here for retro-orbital bleed. Operators should be trained and certified at their local facility. The maximum volume we obtain by this method is 1 % of body weight every 2 weeks. For a 20 g mouse this is 200 μl. The amount needed per antigen for ELISA is generally on the order to 10 μl depending

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on starting dilution and number of Ig types tested. It is always a good idea to use artificial tears when using ketamine anesthesia as mouse eyes remain open and will dry out. This increases the risk of eye bleed complications. It is very important to place pressure on eye both to stop bleeding and prevent blood from building up behind the eye. This increases the risk of eye loss or scaring.

21. Detailed photomicrographs and a protocol are available [35]. Perform this procedure on a limited number of mice per day and increase as experience builds. There is a steep learning curve, especially for those unaccustomed to microscope work. This work requires sitting still for long periods of time. Make the workstation as comfortable as possible, optimizing chair height, bench height, microscope eye piece angle, and distance from edge of bench to work area. Breaks, stretching, and eye exercise (distant focus) reduce fatigue. While HSV-2 is not thought to disseminate or to cause latent or lytic infection outside of the DRG in mice at late time points, the entire dissection should be performed carefully using personal protective equipment. The initial dissection of skin and viscera removal should be performed in a BSL-2 biosafety cabinet.

22. Do not use cervical dislocation or a guillotine. Nonphysical methods are preferred to maintain the spinal integrity. This is especially important for the spinal cord dissection. The pinning of the carcass to the dissection board will be simpler and the intact spine makes the laminectomy much easier.

23. When you remove the skin from the legs by pulling, be especially careful, particularly with the front legs. It is possible to pull too hard and disrupt the integrity of the joints. Gently grasp the leg near the shoulder or hip and using your other hand pull the skin down the leg.

24. Removal of the ventral portion of the rib cage allows the carcass to lie flat during dissection. It is helpful to leave most of the rib cage intact however as the ribs can be used as reference points to locate specific DRG levels.

25. At this point it is important to be mindful of not cutting through any bone. The idea of this step is to fillet the muscle away from the spine making it more accessible for a clean dissection. Especially for beginners, the more muscle that is removed from the dorsal and lateral sides of the spine the easier the laminectomy will be to perform.

26. A laminectomy is much easier to perform when the ventral half of the spine is intact. An intact ventral portion of the spine stabilizes the carcass during dissection.

27. Depending on comfort with this procedure and eyesight this step may be done without a microscope. When performing several of these dissections, rest breaks are advisable. It may be helpful to visualize cutting the entire dorsal half of the bony spinal canal off, leaving behind the intact spinal cord, DRG, and nerves cradled in the ventral half of the spinal column. If you imagine that the spinous processes (the most dorsal vertebral processes) are in the 12 o'clock position, the DRG are located at about 4 and 8 o'clock on either side of the spine. At the lumbosacral transition, the spine curves ventral and the hips can get in the way of

your scissors. Go slowly and carefully, from this point on in the caudal direction it is easy to lose DRG as they are smaller and the dissection trickier. After passing this little transition the finer Vannas spring scissors are better suited for the laminectomy. This portion of the laminectomy is easiest performed with the microscope.

28. The nerve fibers can be fairly elastic. If the fiber wraps up around the DRG it may be difficult to untangle the DRG from the fiber. Take care to avoid tangling nerve fibers. This can be slightly exacerbated by the fact that even the fine forceps may not provide a solid grip on the nerve tissue.

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