Chapter 9

Development of Recombinant Measles Virus-Based Vaccines

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Abstract

This chapter describes the development of recombinant measles virus (MV)-based vaccines starting from plasmid DNA. Live-attenuated measles vaccines are very efficient and safe. Since the availability of a reverse genetic system to manipulate MV genomes and to generate respective recombinant viruses, a considerable number of recombinant viruses has been generated that present antigens of foreign pathogens during MV replication. Thereby, robust humoral and cellular immune responses can be induced, which have shown protective capacity in a substantial number of experiments.

For this purpose, the foreign antigen-encoding genes are cloned into additional transcription units of plasmid based full-length MV vaccine strain genomes, which in turn are used to rescue recombinant MV by providing both full-length viral RNA genomes respective anti-genomes together with all protein components of the viral ribonucleoprotein complex after transient transfection of the so-called rescue cells. Infectious centers form among these transfected cells, which allow clonal isolation of single recombinant viruses that are subsequently amplified, characterized in vitro, and then evaluated for their immunogenicity in appropriate preclinical animal models.

Key words Recombinant measles virus, Rescue of *Morbillivirus*, Vaccine platform, *Paramyxoviridae*

1 Introduction

1.1 Background

Vaccines are the most effective way to prevent infectious disease in terms of safety and cost–benefit ratio. However, at present, the development of vaccines to the point of licensing for human use takes decades and sometimes has proven hardly possible as exemplified by the HIV pandemic. To minimize the time for vaccine development and to be safe, it is necessary to develop strategies that allow for the immediate initiation of standardized vaccine development, leading to successful and safe candidate vaccines in a minimal amount of time. One strategy is to use well-known, already authorized vaccines with exceptional safety and efficacy records as a platform to present critical antigens of the pathogen which is the focus of vaccine development. Thereby, efficacious immune responses are triggered in immunized animals and patients

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not only against the vaccine vector, but also against the additionally present extra antigen. One of these potential vaccine platforms currently under development are recombinant vaccine strain-derived measles viruses.

1.2 Advantages of Measles Virus-Based Vaccines

Unmodified live-attenuated measles virus (MV) vaccine strains are efficient replicating vaccines. Besides revealing an excellent safety record, both humoral and cellular immune responses are elicited, which are responsible for long-lasting protection [1–3]. Therefore, the WHO targets eradication of measles by using these vaccines [4]. The vaccine's manufacturing process is extremely well established [5] and millions of doses can be generated quite easily and quickly. Generation of recombinant MV from DNA via reverse genetics became feasible [5] and allows for the robust expression of different antigens, as outlined below, during replication of the modified recombinant MV vaccine viruses.

Thereby, generally robust immune responses against vector and foreign antigens are induced after vaccination of transgenic MV-susceptible IFNAR$^{-/-}$-CD46Ge mice [6], nonhuman primates, or human patients (*see* Table 1) indicating the high efficiency of the system. Interestingly, pre-vaccinated animals with protective immunity against measles were still amendable to vaccination with the recombinant MV, since significant immune responses against the foreign antigen(s) are still induced [7, 8]. Also in human probands, levels of antibodies against chikungunya virus (CHIKV) Env antigens did not display a negative correlation with preexisting antibody levels against the MV vector backbone [9].

1.3 Potential Applications (i.e., Potential Deliverable Antigens/Disease Targets)

A plethora of different antigens of various viruses and of one bacterium have been cloned into recombinant MV genomes, and the respective recombinant viruses have already been rescued. These projects are summarized in the following table (Table 1).

As depicted, mainly structural proteins, especially viral envelope glycoproteins have been expressed as antigens by the yet generated recombinant MV-derived vaccines, simply due to the expectation that especially humoral immunity against these antigens may result in neutralizing antibodies with protective capacity. However, the vector system proved also capable to induce robust cellular immune responses. Moreover, also secreted, cytoplasmic, or membrane bound markers, such as different luciferases, GFP, lacZ [13], or cellular proteins such as carcinoembryonic antigen (CEA) [41] can be expressed by recombinant MV as demonstrated during its characterization and application as a potential oncolytic virus. Even membrane pore proteins such as the sodium iodide symporter (NIS) [42] and up to three different transgene cassettes with a size potentially exceeding 5 kb [13] have been successfully expressed by recombinant MV, demonstrating the general versatility of this vector system.

Table 1
Recombinant vaccines derived from measles virus

Pathogen	Antigen	Position[a]	Immunity[b]	Protection[c]	Reference
HBV	sHBsAg	P, H, L	ELISA, nAbs	n.t. (MV)	[10–12]
SIVmac	Env (+ Gag)	P	ELISA	n.t.	[13, 14]
MuV	HN, F	P	n.t.	n.t.	[14, 15]
WNV	E	P	nAbs, ELISpot	Yes	[16, 17]
HIV-1	Env	P	nAbs, ELISA, IFNγ-ICS	n.t.	[7, 18–24]
DENV	E, M; EDIII	P	ELISA, Cytokines	n.t.	[25–28]
SARS-CoV	S, N	P	ELISA, nAbs, ELISpot	Yes	[24, 29]
HPV	L1	P	ELISA, nAbs	n.t.	[30, 31]
HCV	C, E1, E2; E1/Ft, E2/Ft	P	ELISA, nAbs	n.t.	[32, 33]
Helicobacter pylori	NAP	N	ELISA, ELISpot	n.t.	[34]
RSV	F, G, M2-1, NP	N, P	ELISpot	Yes	[15, 35–37]
EBV	gB350	N, P	ELISpot	n.t.	[37]
MERS-CoV	S	H	ELISA, nAbs, ELISpot	Yes	[38]
NiV	G	N	ELISA	Yes	[39]
CHIKV	C-E3-E2-6k-E1	P	ELISA, nAbs, ELISpot	Yes	[8, 9]
JEV	prM-E	P	ELISA, nAbs	n.t.	[40]
FluV	HA	P	n.t.	n.t.	[15]

[a]Relative genomic position of the ATU; N indicates first position in the genom, P and H indicate position of the ATU directly following P and H gene cassettes, respectively
[b]Triggered antigen-specific immune responses after immunization determined by measuring total antibodies (ELISA), neutralizing antibodies (nAbs), or reactive T cells determined by ELISpot or intracellular cytokine staining (ICS)
[c]Protective capacity of vaccine-induced immune responses after challenge of the appropriate animal model determined by reduction of pathogen load or attenuation of etiopathology

The targeted viruses span a couple of genera and families. Also the developmental stage of the different vaccines is quite diverse, ranging from the demonstration of successful antigen expression by the recombinant MV, spanning the demonstration of humoral or cellular immunogenicity against the encoded antigen up to demonstration of the vaccine's protective efficacy in appropriate pre-clinical animal models. Of note, recombinant MV encoding the glycoprotein antigens of Chikungunya Virus, MV-CHIKV, has already been tested in a clinical phase I study in human volunteers [9]. After demonstrating efficacy in appropriate mouse and primate animal models [8], this recombinant vaccine delivered proof of

principle for safety and immunogenicity in human patients, irrespective of preformed anti-measles immunity [9].

Thus, the route for clinical development of such recombinant, MV-derived vaccines is open. On the one hand, these recombinant vaccines may be valuable to be used during primary measles vaccination to immunize children simultaneously against measles and a secondary pathogen of concern for the respective pediatric population without the need to vaccinate these children with two different vaccines. As an example, MV expressing hepatitis B virus small antigen (HBsAG) may be used in regions with high HBV prevalence to protect children early on from this potentially chronic infection ("buy one, get one free" strategy).

On the other hand, recombinant MV is one of the potential vaccine platforms that can be used for the (fast track) development of vaccines against emerging pathogens, for which fast availability of a vaccine may be critical. In this respect, our MV-derived vaccine against the corona virus responsible for the Middle East Respiratory Syndrome (MERS-CoV) expressing the MERS-CoV glycoprotein S [38] has been among the first and most progressed vaccine candidates, which were evaluated by the Saudi Arabia Ministry of Health [43] and the WHO [44].

2 Materials

2.1 Plasmid DNA and cDNA

1. Full-length MV genome plasmids such as pBR-MV_{vac2}-GFP(H) [10] or p(+)PolII-MV_{vac2}-ATU(P) [38] (*see* **Note 1**).
2. The MV genomes encoded on these plasmids contain the so-called additional transcription units (ATUs). To facilitate insertion of foreign ORFs, usually single-cutter restriction endonuclease recognition sites are placed between the start and stop signals of the ATU, in the above mentioned examples 5´ *MluI* and 3´ *AatII* (*see* **Note 2**).
3. T7-promoter driven expression plasmids for MV proteins being components of the viral RNP complex (i.e., nucleocapsid protein N, phosphoprotein P, viral RNA-dependent RNA-Polymerase L), such as pEMC-Na, pEMC-Pa, or pEMC-La [5, 45], respectively.
4. PolII-Promoter driven expression plasmids for MV RNP complex proteins, such as pCA-MV-N, pCA-MV-L, and pCA-MV-P [46].
5. cDNA of the foreign antigen to be expressed by the recombinant vaccine. The ORF has to be flanked 5´ by *MluI* and 3´ by *AatII* restriction sites (or alternative sites being part of the ATU) such that the genome length of the putative recombinant vaccines obeys to the "rule-of-six" [47] (*see* **Note 3**).

2.2 PCR Components

1. Expand High-Fidelity PCR System (Roche) or standard PCR protocol.
2. Primers to generate antigen cDNA to be cloned into an ATU of MV genomes.
3. Sense primers have to encompass the 5′ restriction site being part of the ATU directly followed by the START-ATG codon and the approx. 20–25 following nucleotides of the antigen ORF.
4. Antisense primers have to encompass the 3′ restriction site being part of the ATU followed by a STOP codon and the approx. 20–25 preceding nucleotides of the antigen ORF. It may be necessary to include extra nucleotides between restriction site and STOP codon to obey the rule-of-six (*see* **Note 3**).

2.3 Enzymatic Restriction Reaction Components

1. Restriction endonucleases and respective buffers (e.g., purchased by New England Biolabs (NEB)).
2. Nuclease-free H_2O.
3. DNA to be digested.

2.4 Ligation Reaction Components

1. Standard ligation kit such as Rapid Ligation Kit (Roche).
2. Purified DNA fragments with compatible ends after restriction digest.

2.5 Bacteria Culture Components

1. Bacteria: *E. coli*, e.g., Top10 F′ (F′{*lac*I^q Tn10 (Tet^R)}, mcrA, Δ(*mrr-hsd*RMS-*mcr*BC), Φ 80 *lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *ara*D139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L (Str^R), *end*A1, *nup*G).
2. Luria–Bertani (LB) medium: Bacto tryptone 1% (v/w), yeast extract 0.5% (w/v), NaCl 1% (w/v), pH 7.0.
3. S.O.C. medium (Invitrogen): tryptone 2% (w/v), yeast extract 0.5% (w/v), NaCl 10 mM, KCl 2.5 mM, $MgCl_2$ 10 mM, $MgSO_4$ 10 mM, glucose 20 mM.
4. DNA isolation kits suited for DNA isolation from small (5 mL) or medium sized (200 mL) culture volumes.

2.6 Plasmid Transfection Components

1. $CaPO_4$-transfection: commercial Kit, e.g., ProFection Mammalian Transfection System.
2. 5 mL polystyrene roundbottom tube.
3. Lipofection: Lipofectamine® 2000 Transfection Reagent (Invitrogen).

2.7 Eukaryotic Cell Culture Components

1. Vero cells (African green monkey kidney): ATCC CCL-81.
2. 293T cells (human embryonal kidney): ATCC CRL-3216.
3. 293-3-46 (transgenic HEK cells) [5].
4. Geneticin (G418) solution, 100 mg/mL.

5. Cell are cultivated in DMEM supplemented with 10% fetal bovine serum and 2 mM L-Gln with additional 1.2 mg/mL of geneticin, when used for rescue as described in [5].
6. All cells were cultured at 37 °C in a humidified atmosphere containing 6% CO_2 for no longer than 6 months after thawing of the original stock.

2.8 Western Blot

1. RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% (w/v) NP-40, 0.5% (w/v) sodium-desoxycholat, 0.1% (w/v) sodium dodecylsulfate (SDS), pH 8.0) supplemented with Protease Inhibitor Cocktail Complete (Roche Diagnostics, Mannheim, Germany).
2. Antibody/serum recognizing MV protein, e.g., nucleocapsid protein N.
3. Antibody/serum recognizing foreign antigen.
4. Standard SDS-PAGE and Western blot equipment and material.

2.9 Animal Experiments

1. IFNAR$^{-/-}$-CD46Ge: Mice deficient in (*knockout* insertion) α/β-interferon receptor (IFNAR) receptor gene, expressing human CD46 gene with human-like tissue specificity for human CD46 locus, derived from C57Bl/6 [6].
2. 30 G needles, one for each vaccine to be injected.
3. 2 mL syringes with fine scale, one for each vaccine to be injected.

3 Methods

3.1 Cloning of Full-Length Measles Virus Genomes Encoding Foreign Antigens

1. Generation of gene segments encoding the desired foreign antigen of the target pathogen may be generated by gene synthesis. The (codon-optimized) ORF encompassing START and STOP codons is flanked by the respective restriction sites, which allow direct cloning of the gene segment into the ATU of a full-length MV genome plasmid. It may be necessary to include a specific number of extra nucleotides between STOP codon and 3´restriction site to obey the rule-of-six (*see* **Note 3**).

   Alternatively, the desired ORF is amplified on the basis of (plasmid) cDNA or cDNA directly isolated (eventually after reverse transcription) from the target pathogens' genomes using (RT-)PCR. For this purpose, specific primers have to be designed as outlined above, depending on the exact structure of the ATU and the ORF to be amplified.
2. The resulting cDNA segments as well as the MV genome plasmids are then treated by the respective restriction endonucleases using the following standard reaction mix:

1–10 μg DNA.

5–10 U per restriction enzyme.

5 μl 10× NEB buffer (depending on the enzyme used).

5 μl 10× BSA (if required by the applied enzyme).

Fill up to 50 μl with nuclease-free H_2O.

DNA digestions proceeds at the temperature required by the respective enzymes to allow thorough digestion of the DNA, optimally overnight.

3. The desired segments are purified after restriction digestion by standard agarose gel electrophoresis (*see* **Note 4**). The purified genome-containing plasmid backbone and the antigen-ORF containing insert are ligated using standard ligation conditions with at least tenfold molar excess of the insert:

   50 ng vector DNA.

   150 ng insert DNA.

   2 μl 5× DNA dilution buffer.

   Fill up to 10 μl with nuclease-free water and mix.

   10 μl 2× T4 DNA ligation buffer.

   1 μl (5 U) T4 DNA ligase.

   Incubate for 10–15 min at room temperature.

4. Ligated DNA should be directly transformed into chemically competent *E. coli* strains such as Top10F′ being able to amplify large plasmids and cultured on LB-agar plates containing the selection antibiotic at the appropriate temperature (*see* **Note 1**).
5. After overnight or 48 h of cultivation, single bacterial colonies can be picked into 5 mL LB medium, each, containing the respective selection antibiotic.
6. After culture at the appropriate temperature (*see* **Note 1**) employing permanent shaking, a 500 μl probe of each sample is drawn the next morning or 48 h after picking the colonies, and stored for few hours at 4 °C during isolation of plasmid DNA using standard procedures for DNA preparation from small culture volumes.
7. The isolated DNA is analyzed by treatment with appropriate restriction enzymes (e.g., *HindIII* for Edmonston B-derived MV).
8. Probes of clones displaying the expected DNA pattern after agarose gel electrophoresis are used to inoculate 200 mL LB medium, each containing the respective selection antibiotic. 24 or 48 h after inoculation (the bacteria optimally being still in the logarithmic growth phase), bacteria are pelleted and

plasmid DNA is isolated using standard procedures for DNA isolation from medium sized bacterial cultures.

9. After checking the integrity and identity of the isolated plasmid DNA by enzymatic restriction nuclease treatment and agarose gel electrophoresis, the genome plasmids now containing the modified MV genomes are ready for the rescue of recombinant viruses.

3.2 Introduction to Rescue of Recombinant Measles Viruses

At least three protocols for the rescue of recombinant measles virus are available [5, 45, 46] (*see* **Note 5**), which differ mainly in the cell lines, plasmids, as well as the transfection protocol used for the transfection of recombinant MV DNA into the rescue cell line.

Carry out all procedures aseptically under laminar flow and at room temperature unless otherwise specified. Eukaryotic cells are generally cultured at 37 °C, 5% CO_2, and 95% humidity in an incubator.

3.3 Rescue by Transfection of the 293-3-46 Helper Cell Line [5]

1. Seed 8×10^5 293-3-46 cells/well in 2 mL complete DMEM + 1.2 mg/mL G418 into six-well plates and incubate overnight (*see* **Note 6**).
2. The next day, cells should be approximately 80% confluent bevor starting with transfection and the medium of the cells was replaced by medium without G418 approx. 3 h before transfection.
3. Transfection of 293-3-46 helper cells (calcium phosphate transfection): The ProFection Mammalian Transfection System is used for transfection. In detail, 5 µg of MV genome plasmid and 25 ng pEMC.La are added to 25 µl $CaCl_2$ [2 M] in a 1.5 mL reaction tube. The reaction volume is filled up to 200 µl with nuclease-free H_2O. 200 µl 2× HBS buffer are filled into a 5 mL polystyrene round bottom tube and strongly vortexed, while the DNA containing solution is carefully added dropwise to the 2× HBS buffer. The transfection mix is then incubated for 30 min at RT, added dropwise to the medium of the cells, and cells with transfection mix are incubated for 24 h. After incubation, a heat shock of the transfected 293-3-46 cells is done for 3 h at 42 °C in a water bath (*see* **Note 7**).
4. In parallel, overlay cell cultures are prepared by seeding 8×10^5 Vero cells/10 cm cell culture dish in 10 mL complete DMEM. After the heat shock, 293-3-46 cells are cultivated for further 48 h, before 293-3-46 cells are overlaid onto prepared overlay cells. During the incubation period examine for syncytia formation, daily (*see* **Note 8**).

3.4 Transfection Protocol Using a PolII Polymerase-Based Rescue System [46]

1. Seed 8 × 10^5 293T cells/well in 2 mL complete DMEM into six-well plates and incubate overnight (*see* **Note 6**).
2. The next day, cells should be approx. 80% confluent bevor starting transfection.
3. Transfection: 4 μg of the respective MV genomic cDNA plasmid are mixed with 0.4 μg pCA-MV-N, 0.4 μg pCA-MV-L, and 0.1 μg pCA-MV-P (helper-plasmids); add 250 μl Opti-MEM to this mixture (*see* **Note 9**). In parallel mix 12.5 μl Lipofectamine 2000 and fill up to 250 μl with Opti-MEM. Incubate both mixtures for 5 min. Mix both solutions and gently mix by vortexing at level 4. Incubate for 20 min to build lipid–DNA complexes. During the incubation period change medium on the prepared six-well plates to 1.5 mL Opti-MEM. After incubation add the total of the lipid–DNA complex solution (500 μl) dropwise to the six-well. Incubate for 4 h. Then, change medium again to complete DMEM and incubate for 2 days.
4. Seed 3 × 10^6 Vero cells/10 cm cell culture dish in 10 mL complete DMEM and incubate for 4 h. Completely suspend transfected 293T cells by pipetting up and down and spread 1 mL of the solution evenly onto the prepared 10 cm cell culture dish (overlay; *see* isolation of single MV clones). Incubate for 3 days. During the incubation period examine for syncytia formation, daily (*see* **Note 8**).

3.5 Transfection Protocol Using T7 Polymerase Based Rescue System and a T7 Pol Expressing Vaccinia Virus [45]

1. Seed 5 × 10^5 Vero cells/well in 2 mL complete DMEM into six-well plates and incubate overnight (*see* **Note 6**).
2. The next day, cells should be approx. 80% confluent bevor starting transfection.
3. Infection: Cells are infected with a T7-encoding vaccinia virus such as MVA-T7 at an MOI of 1–5 (*see* **Note 10**). 45 min after infection, the medium is replaced and cells are transfected.
4. Transfection: 1.5 μg of the respective MV genomic cDNA plasmid are mixed with 1.5 μg pEMC-Na, 1.5 μg pEMC-Pa, and 0.5 μg EMC.La (helper plasmids) and transfected using a commercially available lipofection method as described above in the protocol using the PolII-based rescue system. Transfected cells are cultured and examined for syncytia formation, daily (*see* **Note 8**).

3.6 Isolation of Single Infectious MV Clones

1. If you find syncytia, pick them as outlined below (*see* Subheading 3.6, **step 4**).
2. If no syncytia are visible on day 4 post overlay, split the overlay culture by passaging the culture 1:4. For that purpose, seed 8 × 10^5 Vero cells/10 cm cell culture dish in 10 mL complete DMEM and incubate for 4 h. Aspirate the medium from the 10 cm cell culture dish of the overlay and wash once with 5 mL

PBS. Detach the cells by incubating with 1.5 mL trypsin–EDTA for 5 min. After complete detaching (check by microscope) stop trypsin incubation by adding 2.5 mL complete DMEM and suspend the cells by pipetting up and down. Seed 25% of the cell suspension to the prepared 10 cm cell culture dish. The remaining 75% of the cell suspension are transferred into a 15 mL tube and snap-frozen in liquid nitrogen for 5 min. The frozen cell suspension is thawed at 37 °C in a water bath and the resulting cell debris is pelleted by centrifugation at 3000 × *g* for 5 min at 4 °C. Transfer the supernatant to the 10 cm cell culture dish dropwise and incubate overnight (*see* **Note 11**).

3. Again check for syncytia formation (*see* **Note 8**). If there is no syncytia formation wait for one additional day and if there is still no syncytia formation, repeat the rescue.
4. If there is syncytia formation, seed one six-well plate/virus with 3×10^5 Vero cells/well in 2 mL complete DMEM per well and incubate for 4 h. Identify six different syncytia (*see* **Note 8**) and mark them by surrounding the syncytia at the bottom side of the 10 cm cell culture dish. Harvest one syncytium by scratching the marked area with a 200 µl pipette tip and aspirating in parallel 60 µl. Transfer the aspirated 60 µl to one of the prepared six wells. Repeat the procedure to obtain six different virus clones (one six-well plate/virus).
5. Incubate the six-well plate and check regularly for the level of infection (at least twice a day). If a well is near complete infection, aspirate 1 mL medium and detach the infected cells by scratching with a cell scraper. Transfer the remaining 1 mL including the virus to a 15 mL tube and snap-freeze in liquid nitrogen for 5 min (*see* **Note 12**). Thaw the frozen virus solution at 37 °C in a water bath (*see* **Note 13**) and pellet the remaining cell debris by centrifugation at 3000 × *g* for 5 min at 4 °C. Aliquot the so-called "passage 0" (P0) virus supernatant at 300 µl each and store at −80 °C.
6. For infection of virus in passage 1 (P1), seed 5×10^6 Vero cells/15 cm cell culture dish in 20 mL complete DMEM and incubate for 4 h. Thaw one of the P0 virus aliquots at room temperature and distribute the virus suspension across the 15 cm cell culture dish. Slew the 15 cm cell culture dish immediately for a few seconds (*see* **Note 14**). Incubate the 15 cm cell culture dish and check regularly for the level of infection (*see* **Note 15**).
7. When the 15 cm cell culture dish is completely infected, aspirate the medium completely and add 1 mL Opti-MEM. Detach the infected cells by scratching with a cell scraper. Transfer the medium including all cell debris by a 1 mL pipette (*see* **Note 16**)

to a 15 mL falcon and snap-freeze in liquid nitrogen for 5 min (*see* **Note 12**). Thaw the frozen virus solution at 37 °C in a water bath (*see* **Note 13**) and pellet the remaining cell debris by centrifugation at 3000 × *g* for 5 min at 4 °C. Distribute the P0 virus supernatant into 300 µl aliquots and store at −80 °C. To obtain the virus titer measured as 50% tissue culture infective dose ($TCID_{50}$), perform an endpoint dilution assay with one aliquot according to Spearman–Karber [48].

3.7 Amplification of Recombinant Measles Viruses (See Note 17)

1. For generation of P1 virus stocks 5 × 10^6 Vero are seeded in quadruplicates into 15 cm dishes and cultivated for around 4 h at 37 °C.
2. The cells were infected with 300 µl of P0 stock. Vero cells infected with MV vaccine strains are cultivated at 32 °C.
3. When almost all cells are infected (*see* **Notes 8** and **15**), the P1 culture is harvested by freezing and thawing of cells. The medium of the infected cells is completely removed (*see* **Note 18**) and 1 mL Opti-MEM is added to the infected cells. The cells were carefully detached using a cell scraper and the cell suspension is transferred into a 15 mL tube, which is snap-frozen in liquid nitrogen (*see* **Note 12**). The suspension is then thawed at 37 °C (*see* **Note 13**) and subsequently centrifuged at approx. 3000 × *g*, 5 min, 4 °C. The supernatant is aliquoted into 300 µl aliquots in cryotubes and stored at −80 °C (*see* **Note 19**). The P1 virus stocks are titrated on Vero cells according to Kaerber and Spaerman [48].
4. To generate P2 and subsequent MV cultures (*see* **Note 20**), 5 × 10^6 Vero cells are seeded in quadruplicates in 15 cm dishes and cultivated for around 4 h at 37 °C. Then, the cells are infected with a multiplicity of infection (MOI) of 0.03 of the titrated parental virus stock and cultivated at 32 °C. When almost all cells are infected, the P2 culture is harvested as described above and titrated on Vero cells [48].

3.8 Characterization of Recombinant Measles Virus-Based Vaccines by Western Blot Analysis

1. For the preparation of cell lysates 3 × 10^5 Vero cells/well in 2 mL complete DMEM per well are incubated for around 4 h.
2. The cells are infected with an MOI of 0.03 of the respective virus.
3. When almost all cells are infected, medium is removed and the cells are washed with 1 mL PBS at 4 °C for 5 min (*see* **Note 21**).
4. The cells are then incubated with 500 µl RIPA buffer for 10 min on ice (*see* **Note 22**).
5. The cell suspension is transferred into precooled 1.5 mL reaction tubes and centrifuged at 17,000 × *g* for 15 min at 4 °C to remove the cell debris.

6. The protein containing supernatant is transferred into a fresh pre-cooled 1.5 mL reaction tube and stored at −80 °C. Frozen cell lysates are thawed on ice for further Western blot applications according to standard conditions using antibodies recognizing an MV protein, e.g., the nucleocapsid protein, to standardize for infection, and another antibody recognizing the foreign antigen to allow assessment of proper antigen expression by the recombinant vaccine.

3.9 Vaccine Characterization by Determining Viral Growth Kinetics in Vitro

1. For the generation of growth curves, 1×10^5 Vero are seeded per well of a 12-well plate. After 24 h, the cells are infected with the virus of interest with an MOI of 0.03 in six replicates.
2. Released virus in the supernatant and cell-associated virus is sampled 24, 48, 72, 96, 120, and 144 h post infection.
3. To harvest the supernatant, 500 µl medium are removed in duplicates and transferred into pre-cooled 1.5 mL reaction tubes and quick-frozen.
4. To harvest the cell-associated virus 1 mL Opti-MEM is added to one well of infected cells, which are detached from the well using a cell scraper and transferred into a pre-cooled 1.5 mL reaction tube and quick-frozen.
5. The cell suspension is thawed at 37 °C (*see* **Note 13**) and subsequently centrifuged at 855 × *g*, 5 min, and 4 °C. The virus containing supernatant was also stored 2× 500 µl aliquots at −80 °C.
6. Both the released virus and the cell-associated virus are titrated on Vero cells in 8 two-fold dilutions with two replicates per time point and virus.

3.10 In Vivo Characterization of Recombinant Vaccine Viruses

1. IFNAR$^{-/-}$-CD46Ge mice (*see* **Note 23**) aged 6–12 weeks receive 1×10^5 $TCID_{50}$ MV diluted in Opti-MEM in a final volume of 200 µl.
2. Mice are injected i.p. into the left, caudal abdomen using a 30 G needle.
3. After 4 weeks, mice receive a booster immunization of 1×10^5 $TCID_{50}$ MV diluted in Opti-MEM to a final volume of 200 µl, which are injected as before (*see* **Note 24**).
4. Four to 7 days after booster immunization, splenozytes are harvested from immunized mice to assess abundance of antigen- or vector-specific T cells by ELISpot or intracellular cytokine staining after stimulation with respective antigens or peptides, either following standard protocols or using commercially available kits according to the manufacturer's instructions.

5. Before immunization ("pre-bleed"), directly before the booster vaccination ("post-prime") or 21 days after booster vaccination ("post-boost"), 200 µl blood are taken from each mouse, and sera are separated to assess abundance of vector- or target-specific antibodies by titrating neutralizing titers or total antibody titers by ELISA.
6. If the IFNAR$^{-/-}$-CD46Ge mouse strain is susceptible to the pathogen to which the vaccine is directed against, vaccinated mice can be challenged subsequent to immunization. The challenge relies on an established infection protocol of (unvaccinated) animals, which results in symptomatic infections or even death, thereby allowing to test the protective capacity of the vaccine. Parameters such as course of disease or pathogen load in specific organs are assed (*see* **Note 25**).

4 Notes

1. Both examples pBR-MV$_{vac2}$-GFP(H) and p(+)PolII-MV$_{vac2}$-ATU(P) contain, as all genome plasmids for the rescue of recombinant MV, a full-length MV vaccine strain genome. pBR-MV$_{vac2}$-GFP(H) is derived from the plasmid backbone pBR322 (low copy) and expresses the viral RNA antigenome under the control of a T7-promoter, whereas p(+)PolII-MV$_{vac2}$-ATU(P) has a pBluescript (high copy) backbone and is PolII-driven. Plasmids containing full-length MV genomes tend to be a bit delicate to handling procedures. Therefore, plasmids with a high-copy plasmid backbone should be amplified in *E. coli* at 30 °C, whereas low-copy plasmids can be amplified at 37 °C. In addition, it pays to directly pick clones or isolate plasmids from growing cultures instead of storing liquid bacteria cultures or colony plates with MV genome-containing plasmids at 4 °C for more than few hours (≪ overnight!). As an alternative, bacteria pellets can be stored frozen at −20 °C before plasmid isolation.
2. Each MV gene cassette is flanked by conserved genetic elements representing start and stop signals for the viral polymerase complex. By duplicating these highly conserved intergenic sequences, a new transcription unit can be inserted in virtually any position of the virus genome, allowing insertion and expression of foreign genes such as antigen ORFs. The relative genomic position of the ATU allows regulation of the inserted gene's expression due to the transcriptional gradient found in *Mononegavirales*. The further upstream the ATU cassette is located, the higher the amount of mRNA being transcribed in infected cells and the higher protein expression. However, if the encoded gene product interferes with MV replication, too

high expression levels of the encoded antigen may be detrimental.

3. The number of nucleotides in MV genomes can be exactly divided by 6, presumably due to one N protein binding to six nucleotides. Also recombinant genomes have to obey this rule; otherwise no recombinant virus can be rescued. Therefore, it has to be considered that the length of the inserted gene segment can be divided with the same rest by 6 as the length of the segment removed from the genome during cloning, if gene cassettes are inserted into genome plasmids. Thereby, one makes sure that the length of the resulting full-length genome is multiple of 6, again.
4. Due to the size of the genome-containing plasmid (approx. 20 kDa), voltage during agarose gel electrophoresis should be restricted to max. 70 V.
5. While T7-based rescue of MV, especially using the helper cell line 293-3-46, is the standard rescue system guaranteeing precise start and stop of the transcribed anti-genomic viral RNA due to the precise start of T7-driven transcription and stop due to specific termination signals in conjunction with the ribozyme flanking the viral genome sequences [5], efficiency of the rescue is quite variable and depends considerably on the status of the rescue cells. Moreover, syncytia formation using 293-3-46 cells after overlay is not too efficient [5], further limiting rescue efficiency especially of virus variants with limited fusion activity (unpublished own observation). Vaccinia Virus driven rescue allows usage of cell lines other than 293, which may be better suited for propagation of one specific MV variant, but depends on the quality of the used plasmids. PolII-driven virus rescue is very efficient and usually results in recombinant MV with precise genomes, but the lower precision of start and stop of Poll II transcription may allow completion of virus from genomes not being in line with the rule of six [46].
6. Prepare one more six-well plate than required for the rescue of the different MV. This additional well is used as transfection control.
7. Seal six-well plate properly to avoid contamination of the cells.
8. If the recombinant MV will additionally express GFP or other fluorescent marker proteins, check for GFP-expression with the help of a fluorescence microscope to identify syncytia. If no easy marker protein for easy evaluation is encoded, check for syncytia formation via light microscope in phase contrast.
9. For transfection control, use 4 μg of for example EGFP expression plasmids such as pEGFP-N1 without any MV cDNA or helper-plasmids and transfect as described for MV cDNA.

10. If using another Vaccinia Virus than MVA for T7 Pol expression, one has to make sure that the rescued recombinant MV can be separated from (co-) replicating Vaccinia Virus, which may be a tedious process due to the laborious methods needed to physically separate the different virus populations.
11. **Step 2** seems to be slightly more efficient in our hands, but it includes one additional freeze–thaw cycle.
12. After quick-freezing in liquid nitrogen, the tube containing the virus suspension can be stored at −80 °C for several days.
13. Check thawing of the virus regularly, the virus is heat sensitive and incubating the virus at 37 °C will lead to significantly reduced virus titers.
14. It is essential to slew the cell culture dish immediately as well as properly to avoid erratic infection of Vero cells, which will lead to lower virus titers.
15. The growth rate is depending on the virus strain and might be modulated by the additional genetic information. Therefore, check the virus growth regularly to develop a feeling for the growth rate.
16. Scratch the cells to on edge of the cell culture dish and hold the plate inclined while aspirating the medium including all cell debris.
17. While MV vaccine strains authorized to be used as human vaccines are usually regarded as safe and accordingly being handled under BSL-1 conditions, recombinant viruses—even those bearing identical vaccine strain genomes—may be handled under BSL-1 or BSL-2 conditions depending on the locally responsible national or regional regulatory authorities. Under very rare circumstances, single recombinant MV may be placed into BSL-3, depending on the nature of the expressed foreign antigen.
18. Try to remove the medium completely while following **Note 16**. This will increase virus titers.
19. The number of aliquots can be modified, depending on freezer-space and experimental plans.
20. For passages >P5 it is sufficient to infect only one 15 cm dish and store around five aliquots, because those viruses are not used for experiments, normally.
21. Slew the plate gently a few times during incubation.
22. Slew the plate vigorously a few times during incubation.
23. Experimental mouse work has to be carried out in compliance with the regulations of the respective animal protection law.
24. Mice are sacrificed or used for further studies dependent on the respective experimental schedule.

25. Efficacy of the recombinant vaccines can be most directly assessed using a challenge experiment directly revealing protection by abolishing or attenuating the etiopathology. In absence of an appropriate challenge, quantitative abundance of antigen-specific (neutralizing) antibodies or T cell reactivity can indicate the protective efficacy of the recombinant vaccine.

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