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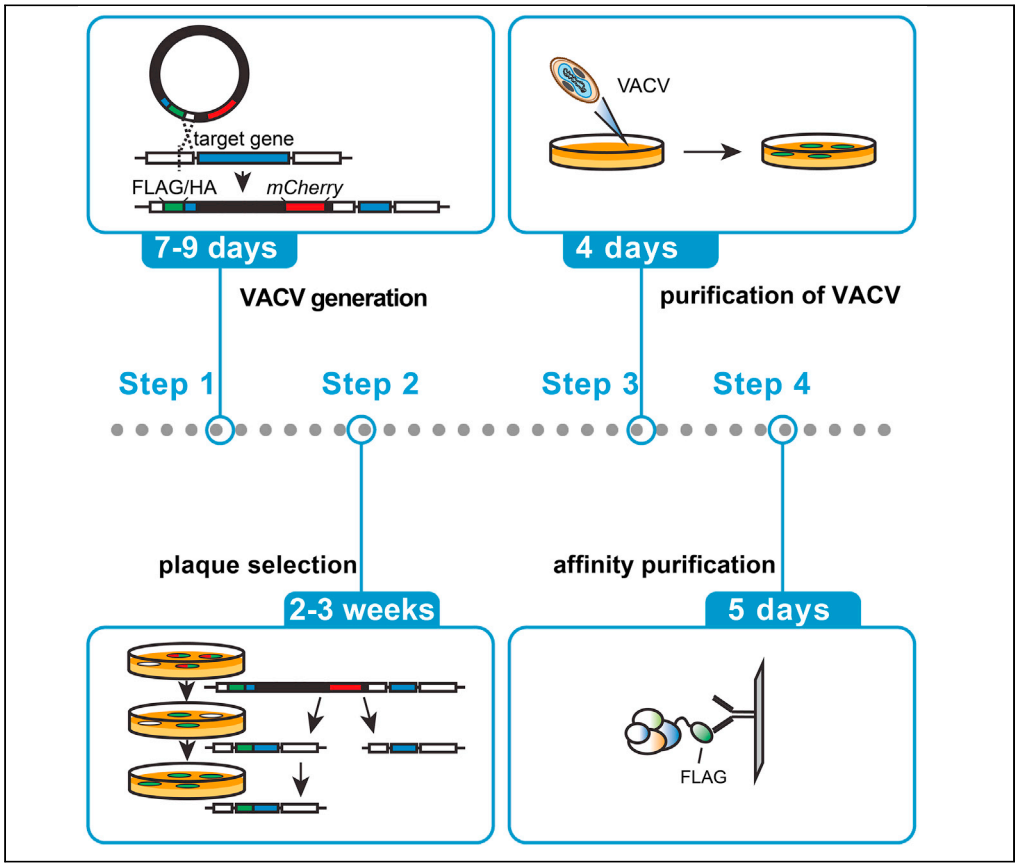
A generic protocol for the affinity-purification of native macromolecular complexes from poxvirus-infected cells



The functional and structural characterization of macromolecular complexes requires protocols for their native isolation. Here, we describe a protocol for this task based on the recombinant poxvirus Vaccinia expressing tagged proteins of interest in infected cells. Tagged proteins and their interactors can then be isolated via affinity chromatography. The procedure is illustrated for the Vaccinia virus encoded multi-subunit RNA polymerase. Our protocol also allows the expression and isolation of heterologous proteins and hence is suitable for a broader application.

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Highlights

Generation of endogenously tagged Vaccinia virus (VACV) strains

Generation of VACV strains expressing heterologous proteins

Protocol for the affinity purification of native macromolecular complexes

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A generic protocol for the affinity-purification of native macromolecular complexes from poxvirus-infected cells

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SUMMARY

The functional and structural characterization of macromolecular complexes requires protocols for their native isolation. Here, we describe a protocol for this task based on the recombinant poxvirus Vaccinia expressing tagged proteins of interest in infected cells. Tagged proteins and their interactors can then be isolated via affinity chromatography. The procedure is illustrated for the Vaccinia virus encoded multi-subunit RNA polymerase. Our protocol also allows the expression and isolation of heterologous proteins and hence is suitable for a broader application.
For complete details on the use and execution of this profile, please refer to Grimm et al. (2019).

BEFORE YOU BEGIN

In this protocol, we describe the purification of Vaccinia virus RNA polymerase (vRNAP) complexes as an example for the isolation of native and enzymatically active protein assemblies. We use FLAG and HA affinity tags for purification, but other tags may likewise work in this work scheme. This generic procedure can also be adopted for the purification of other Vaccinia virus (VACV) -encoded proteins, heterologous factors and their interacting partners.

The protocol starts with the generation of the recombinant VACV expressing either endogenous (i.e., viral) or heterologous FLAG/HA-tagged proteins. In general, recombinant VACV are generated inside infected cells by the homologous recombination between replicating virus DNA and transfected plasmid DNA containing the desired insert (Ball 1987; Falkner and Moss, 1990; Marzook et al., 2014). To direct the insertion of the foreign DNA into the desired genomic position, viral sequences (referred to as "flanks") are included in the transfected plasmid. Because only a small percentage of the progeny viruses are recombinants (usually 10^{-4} to 10^{-3}), isolation of recombinant viruses is facilitated by the incorporation of genetic markers in the transfected plasmid DNA. Once the desired recombinant VACV is generated, purification of tag-containing complexes can conveniently be performed from infected cultivated cells.

Before you begin, it is important to:

1. Check whether you have all necessary reagents, cell lines and viral strains listed in the key resource and materials and equipment tables.
2. Handle genetically engineered VACV strains and/or VACV clinical isolates in certified or governmentally registered biosafety level 2 laboratories.

KEY RESOURCE TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
GLV-1h439	(Grimm et al., 2019) available from the authors upon request.	N/A
Western reserve strain (wild-type)	ATCC	VR-119
vRB12	(Blasco, R., Moss, B. 1995)	N/A
Chemicals, peptides and recombinant proteins		
3× FLAG-peptides (lyophilized powder)	Sigma-Aldrich	Cat#F4799-25MG
Agarose Low melting point – analytical grade	Promega	Cat#V2111
Anti-FLAG M2 Affinity Gel	Sigma-Aldrich	Cat#A2220-25ML
Ammonium peroxydisulphate (APS)	Carl Roth	Cat#9592.1
Bis-(2-hydroxyethyl) imino-tris-(hydroxymethyl)-methane (Bis-Tris)	Carl Roth	Cat#9140.8
ClaI restriction enzyme	Thermo Fisher Scientific	Cat#ER0141
Carboxymethylcellulose sodium (CMC)	Sigma-Aldrich	Cat#4888
Crystal Violet	Merck	Cat#6158
1,4-Dithiothreitol (DTT)	Carl Roth	Cat#6908.2
Dulbecco's Modified Eagle Medium (DMEM 1×)	Gibco	Cat#41965-062
Dulbecco's Phosphate Buffered Saline (PBS)	Gibco	Cat#14190-169
Fetal bovine serum (FBS)	Gibco	Cat#10270106
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Carl Roth	Cat# HN78.3
Hypoxanthine ≥99.0% (HXT)	Sigma-Aldrich	Cat#H9377-25G
Magnesiumchlorid ($MgCl_2$)	VWR-Merck	Cat#1.05833.1000
Minimum Essential Media (MEM)	Gibco	Cat#11935046
Mycophenolic acid ≥98% (MPA)	Sigma-Aldrich	Cat#M5255-50MG
Natriumchlorid ≥99,5% p.a (NaCl).	Carl Roth	Cat#3957.5
Nonidet P 40 (NP-40)	Sigma-Aldrich	Cat#74385
Opti-MEM Reduced Serum Medium	Gibco	Cat#31985047
Penicillin Streptomycin (P/S; 10,000 U/mL)	Gibco	Cat#15140122
Phenol/Chloroform/Isoamyl alcohol 25:24:1	Carl Roth	Cat# X985.2
Protease inhibitor cocktail (tablets)	Merck	Cat#11697498001
Proteinase K	Merck	Cat#1.24568
Rotiphorese Gel 30%	Carl Roth	Cat#3029.1
Rotiphorese Gel 40%	Carl Roth	Cat#3030.1
SalI restriction enzyme	Thermo Fisher Scientific	Cat#ER0642
Sodium Acetate (NaOAc)	Carl Roth	Cat#6773.1
Sodium dodecyl ulphate (SDS)	Carl Roth	Cat#CN30.3
Sucrose	Carl Roth	Cat#4621.1
Tetramethylethylenediamine (TEMED)	Carl Roth	Cat#8142.1
TransIT-X2 System	Mirus	Cat#MIR6000
Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl)	Carl Roth	Cat#9090.3
Trypsin EDTA (0.25%), Phenol red	Gibco	Cat#25200056
Xanthine ≥99% (XT)	Sigma-Aldrich	Cat#X7375-25G
Critical commercial assays		
In-fusion Snap Assembly Master mix (10 rnx)	Takara Bio	Cat#638947
Deposited data		
Core vRNAP structure	(Grimm et al., 2019; Hillen et al., 2019)	PDB: 6RIC
Complete vRNAP structure	(Grimm et al., 2019; Hillen et al., 2019)	PDB: 6RFL
Vaccinia virus Complete genome	NCBI Reference Sequence: NC_006998.1	https://www.ncbi.nlm.nih.gov/nuccore/NC_006998.1
Experimental models: Cell lines		
BS-C-1	ATCC	ATCC CCL-26
CV-1	ATCC	ATCC CCL-70

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
HeLaS3	ATCC	ATCC CCL-2.2
HEK293T	ATCC	ATCC CRL-3216
Recombinant DNA		
pRB21	(Blasco, R., Moss, B. 1995)	N/A
TDS vector	(Marzook et al., 2014)	N/A

MATERIALS AND EQUIPMENT

Reagents

DMEM with 10% or 2% FBS		
Reagents	Final concentration	Final volume
Dulbecco's Modified Eagle Medium	1×	445 mL/485 mL
FBS	10 % / 2 %	50 mL/10 mL
P/S (Penicillin/Streptomycin)	1 %	5 mL
milliQ H_2O	-	to 500 mL
Store at 4°C for 2 weeks		

4% (stacking gel)- 12% (running gel) Bis-Tris denaturing PAGE		
Reagents	Final concentration	Final volume
Rotiphorese Gel 30%	4 / 12 %	2 mL/8 mL
1.25 M Bis-Tris (pH 6.65)	358 mM	5.7 mL
10% APS	1:100	0.2 mL
TEMED	1:1000	0.02 mL
milliQ H_2O	-	to 20 mL
Prepare fresh before use, storage not recommended		

10%–30% or 24%–40% sucrose density gradient solutions, 36% sucrose cushion		
Reagents	Final concentration	Final volume
10× gradient buffer	1×	10 mL
Sucrose	10% / 24% / 30% / 36% / 40% (w/v)	10 gr / 24 gr / 30 gr / 36 gr / 40 gr
milliQ H_2O	–	to 100 mL
Prepare fresh before use, storage not recommended (filter sterilize the solution using a 0.22 μm filter)		

6× SDS-PAGE loading dye		
Reagents	Final concentration	Final volume
1 M Tris-HCl pH 6.8	318 mM	3.185 mL
SDS	315 mM	0.9 gr
1 M DTT	590 mM	5.9 mL
Glycerol	23.5 % (w/v)	2.35 gr
Bromphenol blue	0.06 % (w/v)	0.006 gr
milliQ H_2O	–	to 10 mL
Store at −20°C for 3 months		

10× Gradient Buffer		
Reagents	Final concentration	Final volume
5 M NaCl	1.5 M	3.6 mL
1 M HEPES-NaOH (pH 7.5)	0.5 M	6 mL

(*Continued on next page*)

Continued		
Reagents	Final concentration	Final volume
1 M MgCl2	15 mM	0.180 mL
1 M DTT	10 mM (add before use)	0.120 mL
milliQ H_2O	–	to 12 mL
Prepare fresh before use, storage not recommended (filter sterilize the solution using a 0.22 μm filter)		

Crystal Violet Staining Solution		
Reagents	Final concentration	Final volume
Crystal Violet	1% (w/v)	5 gr
EtOH	20% (v/v)	100 mL
milliQ H_2O	–	to 500 mL
Store at 25°C in the dark for 6 months		

Elution Buffer		
Reagents	Final concentration	Final volume
5 M NaCl	150 mM	6 mL
1 M HEPES-NaOH (pH 7.5)	50 mM	10 mL
1 M MgCl2	1.5 mM	0.3 mL
1 M DTT	1 mM (add before use)	0.2 mL
milliQ H_2O	–	to 200 mL
Prepare fresh before use, storage not recommended (filter sterilize the solution using a 0.22 μm filter)		

Lysis Buffer		
Reagents	Final concentration	Final volume
5 M NaCl	150 mM	6 mL
1 M HEPES-NaOH (pH 7.5)	50 mM	10 mL
1 M MgCl2	1.5 mM	0.3 mL
10% NP-40	0.5 %	10 mL
1 M DTT	1 mM (add before use)	0.2 mL
milliQ H_2O	–	to 200 mL
Protease inhibitor cocktail (tablets)	1/1000 (v/v) (add before use)	
Prepare fresh before use, storage not recommended (filter sterilize the solution using a 0.22 μm filter)		

Selection Inhibitors solutions	
Reagents	Final concentration
Hypoxanthine	10 mg/mL in 0.1 M NaOH
Mycophenolic acid	10 mg/mL in 0.1 M NaOH
Xanthine	10 mg/mL in 0.1 M NaOH
Prepare fresh before use, store for 1 week at 4°C (filter sterilize the solution using a 0.22 μm filter)	

10× TEN Buffer		
Reagents	Final concentration	Final volume
1 M Tris pH 7.5	100 mM	5 mL
0.5 M EDTA pH 8	10 mM	1 mL
5 M NaCl	1 M	10 mL
milliQ H_2O	–	to 50 mL
Store at 4°C for 2 weeks (filter sterilize the solution using a 0.22 μm filter)		

Wash Buffer		
Reagents	Final concentration	Final volume
5 M NaCl	150 mM	6 mL
1 M HEPES-NaOH (pH 7.5)	50 mM	10 mL
1 M MgCl2	1.5 mM	0.3 mL
10% NP-40	0.1 %	2 mL
1 M DTT	1 mM (add before use)	0.2 mL
milliQ H_2O	–	to 200 mL
Prepare fresh before use, storage not recommended (filter sterilize the solution using a 0.22 μm filter)		

STEP-BY-STEP METHOD DETAILS

Generation of recombinant VACV expressing FLAG/HA-tagged VACV proteins from the endogenous locus

Timing: 2 days for step 1

Timing: 1 h for step 2

Timing: 3–4 days for step 3

Timing: 2–3 days for step 4

Timing: 2–3 weeks for step 5

Below, we summarize and explain the steps required to generate a recombinant VACV expressing FLAG/HA-tagged VACV proteins from its endogenous genomic locus. The protocol has been adopted from the transient dominant selection (TDS) method, which has been initially described by Falkner and Moss (1990) and has been further developed by Marzook et al. (2014). In our modified protocol, recombinant VACV are identified by a combination of metabolic and fluorescent selection.

Overview of the required steps:

First: Determination of the virus titer. Prior to any infection experiment, it is important to perform a plaque assay to determine the viral infectivity of your virus stock. 10-fold dilutions of a virus stock are prepared, these aliquots are used to infect cell monolayers that are then covered with agar or cellulose. After 2 days of incubation, the viral plaques are stained and can be counted to calculate the virus titer.

Second: Identification of the tag insertion site. The VACV genome is highly compact with overlapping open reading frames (ORFs) and only small intergenic regions. Consequently, promoter sequences are often localized in the ORF of a neighboring gene. Moreover, ORFs are present on both strands of the genome and transcription occurs in both directions (Czarnecki and Traktman, 2017). In order to generate a virus that expresses a FLAG/HA fusion protein from the endogenous locus, an insertion site should be selected without interfering with the expression of the neighboring ORFs. Consequently, whether the VACV protein of interest can be expressed as an N- or C-terminal FLAG/HA fusion protein is dependent on the genome localization of the specific ORF. For example, *E9L* can only be tagged at the C-terminus, while *J3R* only at the N-terminus.

For the isolation of vRNAP complexes, the FLAG/HA- tag was inserted at the 3′ of the *A24R* ORF (encoding for the VACV RNA polymerase subunit Rpo132). (Grimm et al., 2019, 2021).

Third: Generation of the transfer plasmid. The transient dominant selection (TDS) vector contains *mCherry*, as well as the *Escherichia coli* guanine phosphoribosyl transferase (*gpt*) gene, as

phenotypic markers to allow the identification and selection of recombinant viruses (Marzook et al., 2014). In this step, the FLAG/HA sequences and the viral sequences (flanks 1 and 2) that direct the insertion of the FLAG/HA tag are cloned into the TDS vector.

Fourth: Transfection of the TDS vector. The generated TDS vector is transfected into VACV infected cells to allow homologous recombination to occur between flank 1 or flank 2 of the vector and the respective sequence in the VACV DNA. This single crossover event results in the integration of the full-length plasmid into the viral genome (Figure 4).

Fifth: Isolation of recombinant VACV. The recombinant VACV generated in step four are specifically isolated by consecutive rounds of plaque purification. The first rounds are performed in the presence of specific virus growth inhibitors that allow growth of recombinant viruses only. These recombinant viruses have incorporated the TDS vector and therefore express the *gpt* gene, which confers resistance to the growth inhibitors. After the enrichment of recombinant VACV, plaque purification is performed in absence of growth inhibitors. Because of the presence of direct repeats in this "intermediate" virus, a second crossover event occurs when selection is removed resulting in the formation of either wildtype virus or recombinant virus containing the FLAG/HA sequence in the targeted place in the genome. Loss of the selection markers (*gpt* and *mCherry* genes) can easily be observed by fluorescence microscopy.

1. Determination of the VACV stock titer (First)
   Before starting any infection, it is advisable to determine the titer (plaque-forming virus particles per mL, Pfu) of your VACV stock. This titer will be further used to calculate the amount of virus required to infect a certain number of cells.
   Commonly used strains of VACV, such as Western reserve, forms plaques when grown in a cell monolayer under a cellulose or agarose liquid overlay. Below, we describe how to determine the titer of a virus stock:
   a. Seed 0.25×10^6 of CV-1 cells (ATCC # CCL-70) per well in 1 mL of DMEM medium with 10% FBS (see materials and equipment table) in a 12-well plate.

   Note: CV-1 cells were used for the determination of the VACV stock titer, because they are rapidly propagating and viral plaques are easily detectable on a CV-1 cell monolayer. Other cell types, such as BSC-1 can also be used.
   Incubate cells at 37°C in 5% CO_2 until they reach a confluency of 80% (approximately 24 h (h)).

   b. Thaw a virus aliquot and sonicate for 1 min in a water bath sonicator at 4°C for at least three cycles with a 1 min pause on ice in between cycles. Prepare 10-fold serial dilutions from 10^{-1} to 10^{-8} in 1 mL of DMEM medium with 2% FBS (see materials and equipment table).

   Note: DMEM medium with 2% FBS increases the efficiency of cell infection and cell survival. However, infection experiments can also be performed in DMEM medium with 10% FBS.

   c. Remove medium from the 12-well plate.
   d. Add 300 µL of viral suspension per well and incubate for 1 h at 37°C in 5% CO_2.
   e. Add 500 µL of 2.5% (w/v) CMC medium on top of each well and incubate plate for 48 h at 37°C in 5% CO_2.
   f. Remove medium, add 200 µL of crystal violet solution (0.5% w/v of crystal violet in 20% v/v methanol solution) per well and incubate at 25°C for 2 h.
   g. Remove crystal violet solution and wash with milli-Q water. Dry plate and count plaques.
   h. Calculate virus titer in Pfu per mL as indicated below:

Pfu/mL = # of plaques counted × 1/µL of lysate × 1000 µL/ 1 mL × 10^{+x} (dilution)

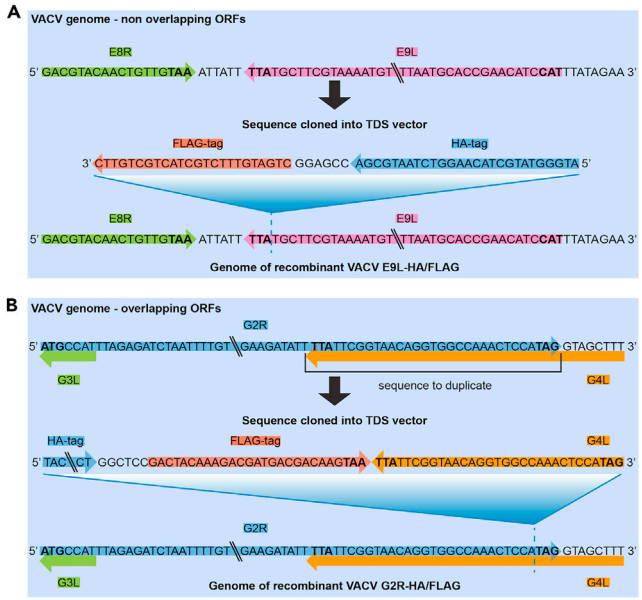

Figure 1. Schematic representation for insertion of FLAG/HA tag into different sites of the VACV genome

(A) HA/FLAG C-terminal tagging of E9L (gene encoding the DNA polymerase). The E9L sequence does not overlap with the adjacent E8R gene.

(B) HA/FLAG C-terminal tagging of G2R (gene encoding the late transcription factor G2). The G4L overlapping sequence is partially duplicated inside the TDS vector in order to prevent disruption of the G4L endogenous sequence. The transcription directions of the genes are indicated by arrows. ORF (Open Reading Frames).

2. Identification of tag-insertion sites (Second)

Whether your gene of interest can be expressed as an N- or C-terminal FLAG/HA fusion proteins depends on the specific genomic location and on its neighboring ORFs. To identify potential tag insertion sites that avoid the disruption of neighboring ORFs and/or promoter elements follow the steps summarized below:

a. Use public databases, such as NCBI (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/) for genome accession of the Western Reserve VACV strain, # AY243312 or any other VACV strain you may want to use.
b. Identify the transcription directions of the ORF to be tagged and its neighboring ORFs. The direction of transcription is contained in the ORFs name: "L" indicates transcription from left to right (e.g., *E3L*) whereas "R" indicates transcription from right to left (e.g., *A24R*).
c. Localize the promoter. In general, promoters are in close proximity to the start codon and can be identified by consensus sequences and/or have been mapped by reporter assays or next generation sequencing techniques (Davison and Moss, 1989a, 1989b; Yang et al., 2010).
d. To minimize the possibility of disrupting promoter elements or interfering with the expression of neighboring ORFs, consider the following for the selection of an insertion site:
    i. If possible, choose an intergenic (=noncoding) region where the neighboring gene has its 3′ end (see Figure 1A).
    ii. If tagging must be performed at a site where two ORFs overlap, duplication of the overlapping sequences can ensure that expression of a neighboring gene to the gene of interest is not affected. An example is illustrated in Figure 1B.

3. Generation of the FLAG/HA TDS vector (Third)

In this section, we describe the generation of the VACV TDS vector that will be used for tagging of the VACV ORF of interest (Falkner and Moss, 1990; Marzook et al., 2014). The TDS vector contains

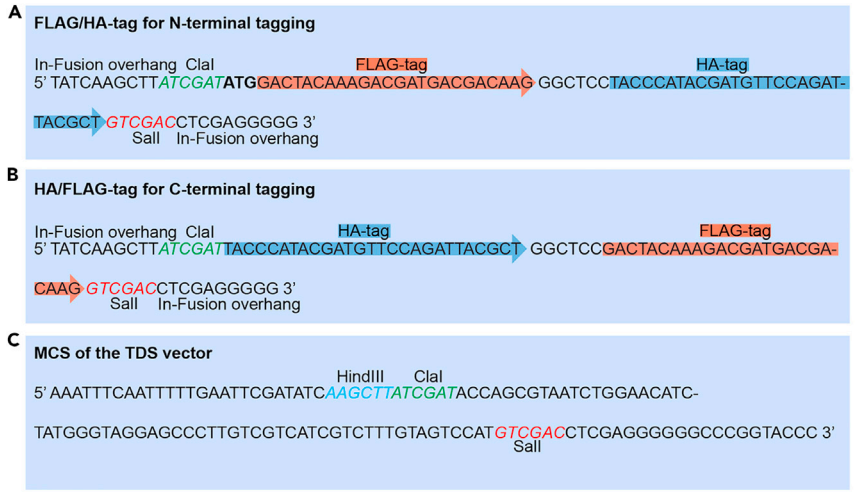

Figure 2. Sequences of FLAG/HA-tag oligonucleotides and of the TDS vector multiple cloning site (MCS)

(A and B) oligonucleotide sequences used to tag proteins with FLAG/HA at the N-terminus (A) or at the C-terminus (B). The In-Fusion HD Cloning Kit was used for the cloning procedure, In-Fusion overhangs and restriction sites are indicated.

(C) The MCS sequence of the TDS vector. FLAG-tag (orange), HA-tag (blue), ClaI restriction site (green), SalI restriction site (red), HindIII restriction site (light blue).

unique Cla I, HindIII and Sal I restriction sites, an ampicillin resistance gene and the mCherry as well as the *gpt* genes as phenotypic markers to allow the identification and selection of recombinant viruses (Marzook et al., 2014). Expression of *gpt* and mCherry are driven by the VACV synthetic early/late promoter (Cordeiro et al., 2009).

In the first part (step a-b), the FLAG/HA tag for either C-terminal or N-terminal tagging is inserted into the vector. In the second part (step c-f), the regions upstream (flank 1) and downstream (flank 2) of the selected tag insertion site, are cloned into the same TDS vector. In the final vector, the N- or C-terminal FLAG/HA tag sequence is sandwiched in between flanks 1 and 2.

Cloning and primer designing are performed using the In-Fusion HD Cloning Kit according to the manufacturer's protocol (Takara) but alternative cloning methods can also be used.

a. Design oligonucleotides containing the FLAG and HA sequences either with a preceding ATG (for N-terminal tagging) or a terminal stop codon (for C-terminal tagging). The FLAG and HA sequences are separated by a small linker (GGCTCC). Oligonucleotides with these features and suitable for In-Fusion HD Cloning are displayed in Figures 2A and 2B.
b. Digest the empty TDS vector with ClaI and SalI and perform an infusion reaction with either the N- or C-terminal FLAG/HA oligonucleotides. The multiple cloning site of the TDS vector is shown in Figure 2C.
c. PCR-amplify the sequences upstream (flank 1) and downstream (flank 2) of the desired FLAG/HA insertion site using VACV genomic DNA as template. Each flank should be approximately 500 nucleotides in length (Figure 3A). A significant drop in recombination frequency is observed with regions of homology of less than 200 nucleotides (Yao and Evans, 2001).
d. Digest the TDS vector generated in 2. with ClaI and perform an infusion reaction with the flank 1 PCR product.
e. Digest the plasmid generated in 4. with SalI and perform an infusion reaction with the flank 2 PCR product.
f. Verify the generated plasmid by sequencing using T7 promoter specific primer (5′ TTCGAT ATCAAGCTTATCGAT 3′). Examples of N- and C-terminal FLAG/HA TDS vectors are shown in Figure 3B.

4. Infection and transfection procedures (Fourth)

In this step, cells are infected with wild-type virus and subsequently transfected with the TDS

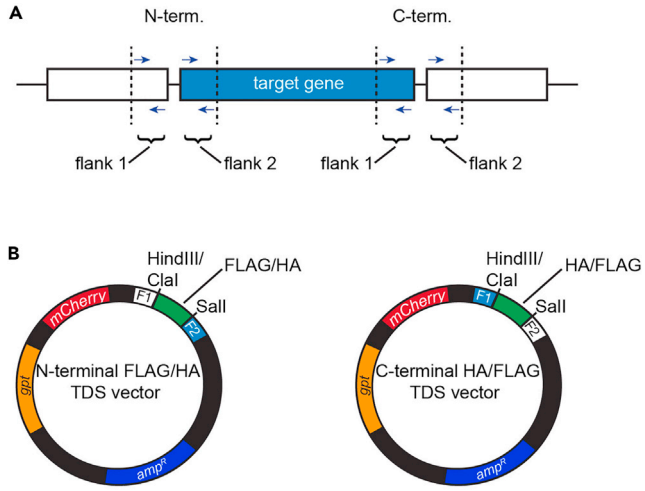

Figure 3. Schematic representations of the flanking regions of the target gene and the Transient Dominant Selection (TDS) recombinant vector

(A) Representation of the viral gene of interest (blue) and neighboring genes (white). The homology arms, flank1 and flank 2 are indicated. The total length of each flank should be approximately 500 nt. Primer positions used to amplify each flank are displayed as arrows.

(B) Schematic of the TDS vector used for N- or C-terminal FLAG/HA tagging. The *gpt* and *mCherry* genes for selection of recombinant VACV are depicted in yellow and red, respectively. The flank 1 and flank 2 homology arms are illustrated flanking the FLAG/HA tag (green). HindIII, ClaI and SalI are unique restriction sites present in the TDS vector.

vector that has been generated in the previous step. Homologous recombination occurs either via flank 1 or flank 2 and results in the integration of the entire plasmid into the VACV genome including the *gpt* and mCherry selection genes (Figure 4, step 1).

a. 24–48 h before transfection, seed 1×10^6 of HEK293T cells (ATCC # CRL-3216) per well in 2 mL of DMEM medium with 10% FBS in a 6-well plate. Grow cells at 37°C until they reach 60%–80% confluency in a humidified incubator equilibrated with 5% CO_2.

Note: HEK293T cells were employed for the infection and transfection procedures due to their high transfection efficiency and permissibility to VACV infection. However, other cell lines can be also used for the same purpose.

b. Prepare virus inoculum (1 mL/well) by diluting crude wild-type virus stock to a 0.05–0.1 Multiplicity of Infection (MOI) in DMEM medium with 2% FBS.
   MOI is the ratio between the number of viral particles and number of host cells in infection media; e.g., MOI of 1 means that for one host cell there is one viral particle.
   Calculate the amount of virus necessary to infect one well of a 6-well plate with an MOI (multiplicity of infection) of 0.05 with the formula shown below:
   mL of virus per well = Total cells/mL × (Volume of cell re-suspension) mL × MOI (0.05) / Pfu/mL
c. Remove medium from cells, replace with 1 mL of virus inoculum and incubate in 5% CO_2 at 37°C for 2 h.
d. About 30 min before the end of the incubation time, prepare the transfection reaction. Per reaction, dilute 3–4 ug of vector in 0.5 mL of Opti-MEM medium. Add 7.5 μL of transfection reagent, such as TransIT-X2 System (Mirus), and incubate 20 min at 25°C according to the manufacturer's protocol (https://www.mirusbio.com/assets/protocols/ml061-transit-x2-dynamic-delivery-system-user-protocol.pdf).
e. Aspirate virus inoculum and replace with 1 mL of DMEM medium with 10% FBS. Add transfection mixture drop-by-drop and incubate at 37°C for 24–48 h.
f. Harvest cells with a disposable cell scraper and collect the whole cell suspension in a sterile tube.

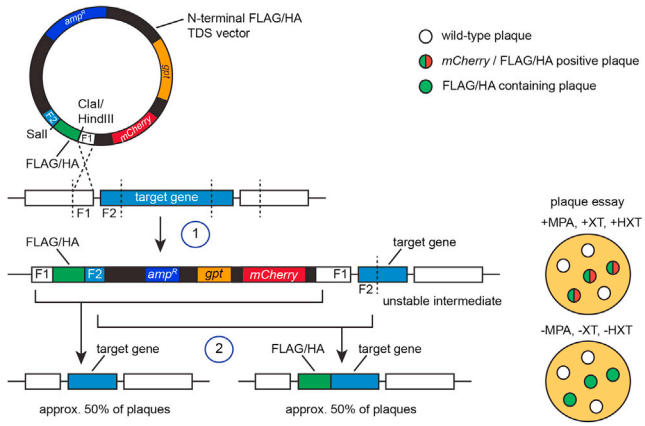

Figure 4. Schematic outline of the homologous recombination events with the TDS method

The first step is a single crossover event that results in the integration of the full-length plasmid into the VACV genome including the selection marker *gpt*, allowing virus growth in the presence of mycophenolic acid (MPA) (here, recombination via flank 1 (F1) is shown). Because of the presence of direct repeats, this intermediate is unstable and can only be maintained and isolated in the presence of MPA. The positive plaques (red and green plaques) also contain *mCherry* (red), that allows visualization by fluorescence microscopy. In step 2, MPA is removed from the medium. A second recombination event results either in loss of the integrated plasmid resulting in wild type virus (via F1 recombination) (white plaques) or in FLAG/HA containing virus (green plaques) (via F2 recombination) (step 2).

Note: To harvest cells, trypsin can also be used instead of cell scraper. However, we do not recommend trypsin usage, because prolonged trypsinization may affect the integrity of the purified complex (see step 43).

g. Virus progenies are released from infected cells by three consecutive freeze-/thaw cycles. For this, the cell suspension is first snap-frozen in liquid nitrogen, then incubated in a water bath at 37°C until complete thawing.
h. Store the virus-containing cell suspension at −80°C. The viral suspension can be stored for a couple months at −80°C without losing infectivity.

Note: Transfection with Calcium Phosphate, Lipofectamine or other transfection reagents are also compatible with the procedure described here (see step 4, d).

⚠ CRITICAL: Cell lines used for this protocol should be tested for mycoplasma contamination before usage as it affects the transfection efficiency and also virus infectivity.

5. Isolation of recombinant VACV (Fifth)

In this step, recombinant VACV expressing the FLAG/HA tagged viral protein of interest will be isolated. The cell lysate from step 4, h (see previous section "infection and transfection procedures") contains a mix of parental viruses and viruses that have an integration of the entire TDS vector. Due to the presence of duplicate sequences (flanks 1 and 2), the viruses carrying the TDS vector are highly unstable and are only maintained by their resistance to MPA conferred by *gpt*. In order to isolate the recombinant viruses, 3 to 4 rounds of plaque purification are performed in the presence of mycophenolic acid (MPA), xanthine (XT) and hypoxanthine (HXT). MPA blocks the synthesis of purine nucleotides, but viruses expressing *gpt* can initiate an alternative pathway for purine synthesis using XT and HXT as precursors (Boyle and Coupar, 1988). Recombinant viruses expressing *gpt* are able to form plaques under MPA selection (Lorenzo at al., 2004). mCherry further simplifies the identification of recombinant plaques by fluorescence microscopy (Figure 4, step 1).

Subsequently, the next rounds of plaque purification are performed in the absence of selective reagents (without MPA, XT and HXT), which results in a second homologous recombination event (Figure 4, step 2). Depending on the position of the recombination event with respect to the insertion

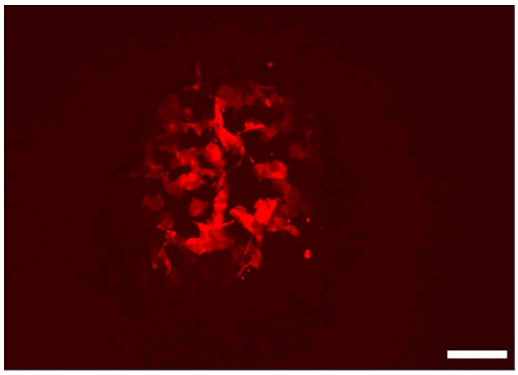

Figure 5. Representative mCherry-positive VACV plaque

Immunofluorescent microscopy picture. BSC-1 cells were infected with VACV. VACV mCherry-positive plaque is visualized in red. Scale bar 200 μm.

(either via flank 1 or via flank 2), either the desired recombinant virus or the parental virus is retained (Figure 4, step 2).

The experimental steps for VACV selection are as follows:

a. Seed 2×10^5 of BSC-1 cells (ATCC #CCL-26) per well in 2 mL of DMEM medium with 10% FBS in a 6-well plate. Incubate cells at 37°C in 5% CO_2 for 24 h.

Note: Other cell lines giving detectable-size plaques may be employed for the purpose of recombinant plaque selection, such as BSC-1.

b. 16–24 h prior to infection, incubate BSC-1 cells with 2 mL of selection growth medium containing 1/400 volumes of 10 mg/mL MPA, 1/40 volumes of 10 mg/mL XT and 1/670 volumes of 10 mg/mL HXT.

c. Thaw the cell suspension containing virus from step 4, g (see infection and transfection procedures above) and sonicate for 1 min in a water bath sonicator (output maximum, duty cycle constant) at 4°C for at least three cycles with a 1 min pause on ice in between cycles.

Note: The sonication settings are optimized for HeLa S3, CV-1 and BSC-1 cell lines.

d. Prepare 10-fold serial dilutions of the cell suspension in DMEM medium with 2% FBS.

e. Remove media from BSC-1 cells (see step 5, b) and add 1 mL of the cell suspension per well (use 10^{-1}or 10^{-3} dilutions).

f. Incubate cells for 1–2 h at 37°C.

g. Remove inoculum and replace with 1 mL of a 1:1 mixture of 2% low melting point agarose and 2× MEM medium, containing MPA, XT and HXT (see step 5, b).

h. Incubate infected cells at 37°C for 48 h to allow for formation of VACV plaques. (Figure 5)

i. Use a 200 μL pipet tip to pick single, mCherry-positive, clearly separated plaques and transfer into fresh tubes containing 0.5 mL of DMEM medium with 2% FBS.

j. Freeze – thaw each tube and sonicate as described in step 5, c.

k. To further purify the isolated viral positive plaques from eventual contamination of wild-type virus, repeat steps 5, a to 5, k at least three times.

l. Perform two or three rounds of plaque purification without selection: Repeat steps 5, a to 5, k without MPA, XT and HXT using the last selected mCherry-positive plaques as starting material.

m. Pick mCherry negative plaques.

Generation of recombinant VACV expressing heterologous FLAG/HA-tagged proteins

Timing: 3–4 days for step 6

Timing: 2–3 days for step 7

Timing: 2–3 weeks for step 8

In addition to generating viruses that express FLAG/HA-tagged VACV proteins, heterologous FLAG/HA fusion proteins can also be incorporated into the viral genome. The described method uses the recombinant VACV strain (vRB21), where the viral *F13L* ORF is replaced with the *gpt* gene (Blasco and Moss, 1991). Loss of *F13L* causes a severe defect in virus spread, resulting in the formation of very small plaques only after prolonged incubation times (Blasco and Moss 1991). By using the VACV transfer plasmid pRB21, the *F13L* ORF of vRB21 can be restored and a foreign gene can be simultaneously introduced (Blasco and Moss 1995). In this plasmid, the *F13L* coding sequence is flanked by VACV DNA that directs homologous recombination into the natural *F13L* locus, placing the foreign gene (under the control of a VACV promoter) in an intergenic region immediately downstream of the *F13L* stop codon. The recombinant viruses are identified and isolated based on their ability to form normal sized plaques after a standard 2 days incubation (Blasco and Moss, 1995). Below, we summarized the steps required to generate recombinant VACV expressing a foreign FLAG/HA tagged protein.

First: Generation of the pRB21 plasmid. Cloning of the FLAG/HA-tagged gene of interest into the pRB21 plasmid (Blasco and Moss, 1995). This plasmid contains the *F13L* coding sequence flanked by VACV sequences that direct homologous recombination into the endogenous *F13L* locus and simultaneously place the desired FLAG/HA-tagged protein (under the early/late VACV promoter) immediately downstream of the *F13L* stop codon.

Second: Transfection of the pRB21 plasmid generated in step 1 into vRB12 infected cells. The insertion of the *F13L* ORF along with the FLAG/HA-tagged gene of interest occurs by homologous recombination via both flanks, resulting in the replacement of the *gpt* gene with *F13L* and the gene of interest.

Third: Identification and isolation of recombinant viruses based on their ability to form plaques.

6. Generation of the pRB21 transfer vector (First)
   In this section, we describe the procedure to clone the FLAG/HA-tagged protein of interest into pRB21. Specifically, pRB21 contains the VACV *F13L* ORF, as well as an ampicillin resistance gene and the multiple cloning site (Blasco and Moss, 1995). The multiple cloning site is preceded by the synthetic VACV early/late promoter (Chakrabarti et al., 1997) and terminated with a VACV early transcription termination signal (Yuen and Moss, 1987). The whole cassette (*F13L* ORF and the MCS including promoter and termination signals) is framed with VACV sequences preceding and following the endogenous *F13L* ORF site in the viral genome (Figure 6).
   a. Digest the pRB21 vector with the desired restriction enzyme(s) present in the MCS (Figure 6A).
   b. Design oligonucleotides to amplify the entire gene of interest with an N- or C-terminal FLAG/HA tag for cloning into the pRB21 vector.
   c. Amplify and clone the desired insert into the pRB21 vector. Verify the generated plasmid by sequencing using the pRB21 sequencing primer (5′- GTGAGCTCACATTCAAAGAG-3′).

7. Infection and transfection procedures (Second)

To allow homologous recombination between the generated pRB21 vector and the viral genome, cells are infected with vRB12 (Blasco and Moss, 1991) and subsequently transfected with the generated pRB21 vector construct. After transfection, homologous recombination results in the incorporation of the *F13L* ORF and the recombinant ORF of interest into the *F13L* site in the vRB12 genome (Figure 6B).

The steps are identical to steps 4, a – 4, h described in the "infection and transfection procedures" of the previous section on tagging endogenous VACV proteins. Note that in step 4, b the vRB12 virus is used for infection instead of wild-type virus.

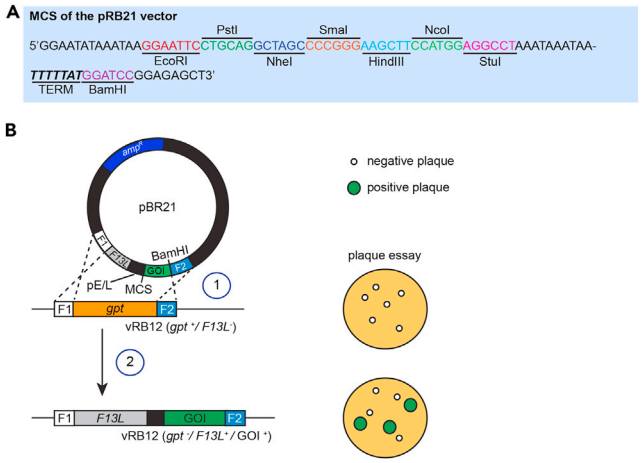

Figure 6. Generation of recombinant VACV expressing heterologous proteins

(A) Schematic of the multiple cloning site (MCS) of the pRB21 vector. TERM, early transcription termination signal.

(B) The insertion of the "Gene Of Interest" (GOI) (green) is a result of a double crossover event between the vRB12 genome and the pRB21 vector involving both flanks (step 1). The morphology of the plaques of the vRB12 strain is smaller in comparison to the wild-type strain (small white circles). After recombination, the GOI together with the entire *F13L* gene are inserted into the *F13L* locus (step 2). The plaques of the recombinants have normal size (big green circles). pE/L, early/late promoter.

⚠ CRITICAL: To amplify the vRB21 strain an MOI of 1 Pfu/cell or more is recommended. Virus titer can be estimated by counting the number of plaques at 5th day post-infection.

8. Isolation of recombinant VACV (Third)

After transfection, recombinant viruses are identified by their ability to form large plaques within 2–3 days (Figure 6B).

The steps are identical to steps 5, a – 5, m in the "isolation of recombinant VACV" of the previous section on tagging endogenous VACV proteins, but without the use of selection reagents in the medium. Instead, large plaques are selected and amplified (see step 5, i, 5, k and 5, l of the "isolation of recombinant VACV" of the previous section).

Purification of VACV genomic DNA

Timing: 2 days

Once recombinant VACV plaques have been identified, the correct insertion of the FLAG/HA tag or the *F13L* gene along with foreign FLAG/HA-tagged protein of interest are confirmed by sequencing

9. Seed 2×10^5 BSC-1 cells per well in 2 mL of DMEM medium with 10% FBS in a 6-well plate and incubate at 37°C in 5% CO_2 for 24 h.
10. Sonicate recombinant virus (1.5×10^7 Pfu) and infect BSC-1 cells with 150 μL of crude viral extract per well (see step 5, j in the previous section).
11. 48 h post-infection, aspirate medium and scrape cells in 500 μL PBS buffer. Transfer 400 μL of the suspension into a new tube and add 50 μL of 10× TEN buffer (see materials and equipment table), 50 μL of 10% SDS and 5 μL of 20 mg/mL of Proteinase K. Incubate at 56°C for at least 2 h.
12. Add 600 μL of Phenol/Chloroform/Isoamyl alcohol 25:24:1, vortex briefly and centrifuge at 16,000×*g* in a tabletop centrifuge for 10 min at 4°C.

13. Collect the aqueous phase (upper phase) and add an equal volume of Chloroform. Vortex briefly and centrifuge at 16,000×*g* for 10 min at 4°C.
14. Transfer the aqueous phase into a new tube and add 1/10 v/v of 3M NaOAc (pH 5.2) and 3 volumes of 99.8% EtOH.
15. Incubate at least 30 min at −20°C and centrifuge at 16,000×*g* for 20 min at 4°C.
16. Remove supernatant and wash pellet with 1 mL of 80% EtOH and centrifuge samples at 5,600×*g* for 15 min. Remove supernatant and dry DNA pellet for 30 min and re-suspend in milliQ H_2O (approx. 30 μL). The expected concentration is between 0.3 and 0.5 μg.
17. Sequence DNA from 10 different plaques.

For the TDS vector system, use primers that were employed for the cloning of the viral flanking regions into the respective TDS vector.

For the *F13L* system, use primers specific for the *gpt* and *F13L* genes. Recombinant virus plaques containing the desired gene of interest should be PCR positive for *F13L* but negative for *gpt*.

F13L primers:

F: 5′-TATGGATTTTAGATCCGATCA-3′

R: 5′- TACAAGCTGTTTATCTATACTA-3′

gpt primers:

F: 5′- TTGCGAGATATGTTTGAGAAT-3′

R: 5′- CAGTTGCCGCCACGGTA-3′

⚠ CRITICAL: Phenol/Chloroform/Isoamyl alcohol extraction has to be performed under a fume hood.

Purification of VACV

Timing: 4 days

Once recombinant VACV have been identified, they are amplified for future complex purifications, such as for the purification of vRNAP complexes as described in the next section.

HeLa S3 cells are used routinely for large-scale propagation of VACV from positive plaques but other cell types such as CV-1 may also be used.

18. Seed 5–7×10^6 HeLa S3 cells per 145 cm^2 dish in 20 mL of DMEM medium with 10% FBS. For large-scale virus production, approximately 50×145 cm^2 dishes are infected and incubated at 37°C in 5% CO_2 until cells reach 90%–100% confluency (approx. 72 h).

Note: By infecting 50×145 cm^2 dishes, usually 3–5×10^9 Pfu of purified VACV are obtained. However, virus titer can differ in between preparations.

19. Before infection, remove medium from dishes and add 9 mL of DMEM medium with 2% FBS.
20. Thaw the crude virus stock (10^8 Pfu/mL) and sonicate for 1 min in a water bath sonicator at 4°C for at least three cycles with a 1 min pause on ice in between cycles. Add 200 μL of crude virus stock to each plate. Incubate cells at 37°C until the cytopathic effect (e.g., once cell lysis starts, cells round up and get notably bigger) becomes visible (approximately 2 days post infection).

21. Remove medium and harvest cells with a cell scraper. Pellet cells in a 50 mL Falcon tube at 1,800×*g* for 5 min at 4°C, remove supernatant and re-suspend pellet in 35 mL of 10 mM Tris-HCl (pH 8.8).

Note: The re-suspended pellet can be stored at −80°C if not immediately used and thawed in a water bath at 37°C when proceeding to step 22.

22. Homogenize cell suspension with 40 strokes in a glass dounce homogenizer with a tight pestle. Monitor the degree of the cell homogenization with a light microscope. Homogenization is good, when 70%–80% of cells have been lysed, the remaining portion will be visible as intact cells.
23. Centrifuge cells for 5 min at 4°C and 1,800×*g* to remove cellular debris. Transfer supernatant to a new Falcon tube.
24. To collect the remaining viral particles still adhering to cell debris, re-suspend the cell pellet in 3–5 mL of 10 mM Tris-HCl (pH 8.8) and centrifuge cells 5 min at 4°C and 1,800×*g*.
25. Pool supernatants from steps 23 and 24 and split in 3–5×5 mL aliquots. Sonicate for 1 min in a water bath sonicator at 4°C for at least three cycles with a 1 min pause on ice in between cycles.
26. Layer the suspension on top of a 36% sucrose cushion, use 17 mL of sucrose solution per tube (Ultra-Clear Tubes, 1× $3^{1/2}$ in., 25 × 89 mm, Beckman Coulter # 344058). Centrifuge at 26,000×*g* for 2 h at 4°C. If you use a Beckman Coulter Optima L-80 XP Ultracentrifuge, use Rotor TST 28.38 and set the Acceleration and Deceleration to Maximum. If you use other centrifuges, adjust settings accordingly.
27. Remove supernatant and re-suspend pellet in 1 mM Tris-HCl 8.8. At this stage virus is pure enough for further experiments.
28. It might be necessary to determine virus titer in order to fine-tune your subsequent infection/purification protocols. An easy and reliable way to determine the titer is by plaque assay (see "determination of vacv titer" in the "before you begin" section).
29. For normal applications, the virus purification described above is sufficient. However, some applications require highly pure virus stocks. These can be prepared by combining the sucrose cushion purification (see above) with a second purification step using a sucrose gradient.
30. Lay 1 mL of sucrose cushion purified virus on top of a 24%–40% sucrose gradient and centrifuge for 40 min at 26,000×*g* at 4°C (use Beckman Coulter Optima L-80 XP Ultracentrifuge; Rotor TST 28.38; Acceleration and Deceleration – Maximum or equivalent).

Note: To prepare a 24%–40% sucrose gradient, manually lay 6.8 mL each of 40%, 36%, 32%, 28% and 24% of sucrose solutions on top of each over in a centrifuge tube (see step 30) and let it seat for 12–18 h at 4°C before usage.

31. The virus is visible as a milky band approximately in the middle of the centrifuge tube. Aspirate sucrose above the band and carefully collect the virus-containing fraction with a sterile pipet.
32. Place the collected virus in a sterile centrifuge tube and bring volume up to 30 mL with 1 mM Tris-HCl (pH 8.8).
33. Centrifuge at 32,900×*g* for 1 h at 4°C (Beckman Coulter Optima L-80 XP Ultracentrifuge; Rotor TST 28.38; Acceleration and Deceleration - Maximum), then aspirate and discard supernatant.
34. Resuspend the pellet in 1 mL of 1 mM Tris-HCl (pH 8.8) and sonicate for 3 cycles at 4°C.
35. Split sonicated virus into smaller aliquots and store at −80°C

Purification of native VACV RNA polymerase complexes

Timing: 5 days

The procedure preceding the vRNAP complexes isolation is outlined in Figure 7.

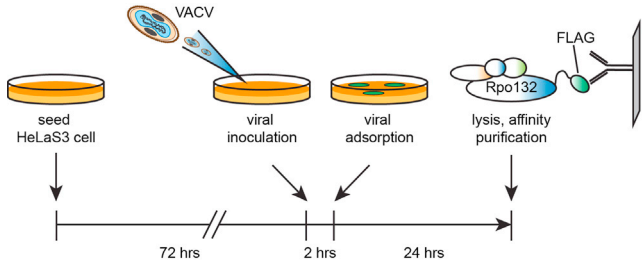

Figure 7. Schematic representation of the time-line preceding the native vRNAP complexes purification

Seed HeLa S3 cells on 145 cm^2 dishes and when they reach 90%–100% confluency (approximately 72 h), infect cells with recombinant VACV expressing Rpo132-HA/FLAG fusion protein. After two h of viral adsorption, remove virus inoculum and 24 h later start with the vRNAP complexes purification.

36. Seed 5–7×10^6 HeLa S3 cells in 50×145 cm^2 dishes (use cells with low passage number) in DMEM medium with 10% FBS and incubate for 72 h at 37°C in 5% CO_2.
37. Once HeLa S3 cells reach 90%–100% confluency, take one dish out of the initial 50 dishes, harvest cells by trypsinization and count number of the cells per plate by using a cell counting chamber.

Note: We do not recommend using lower cell confluency, because the yield of the purified complex will be lower. However, it is possible to infect cells at lower confluency, such as 60%–70%.

38. Calculate the amount virus (GLV-1h439 virus encoding Rpo132-HA/FLAG) necessary to infect one dish with an MOI of 1.2 with the formula shown in the "infection and transfection procedures" section.

Note: Virus stocks prepared according to the protocol described here have a titer of approx. 10^9 Pfu/mL but exact values may differ.

39. Remove medium and add 9 mL of DMEM medium with 2% FBS.
40. Thaw an aliquot of the virus stock and sonicate before use.
41. Dilute the sonicated virus stock in DMEM medium with 2% FBS and add 1 mL of the diluted virus stock to each dish. Incubate at 37°C for 2 h.
42. Replace inoculum with 20 mL of DMEM medium with 10% FBS, and incubate cells for 24–40 h at 37°C in 5% CO_2.

Note: 24–40 h post infection, cells are still attached to the plate and Rpo132-HA/FLAG is well expressed.

43. Remove medium from infected cells and collect cells with a cell scraper. Pool cell suspensions in a 50 mL falcon tube and centrifuge at 1,800×*g* for 5 min at 4°C.

Note: Cell pellets may be stored at −80°C for up to 1 month.

44. Equilibrate FLAG-agarose beads with lysis buffer (see materials and equipment table). Centrifuge beads at 2,054×*g*, for 2 min at 4°C and remove buffer from the resin. Wash beads twice with 10 bed volumes of Wash buffer (see materials and equipment table).

Note: The amount of the FLAG-agarose beads needs to be optimized according to the level of the expression of the target protein. Rpo132-HA/FLAG is a highly-expressed protein during virus replication. In this case, approximately 2 mL of FLAG-agarose affinity gel (= 1 mL of beads volume + 1 mL of storage buffer) was used for 50 dishes of HeLa S3.

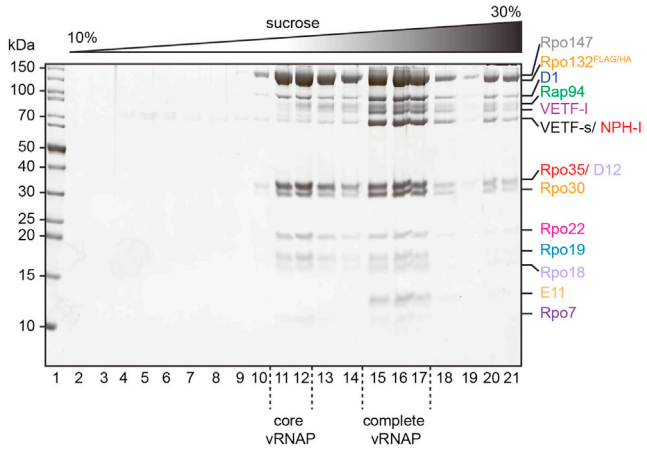

Figure 8. Isolation and characterization of native vRNAP complexes

(A) Purification of Rpo132-HA/FLAG-containing complexes from HeLa S3 infected cells with GLV-1h493 using anti-FLAG affinity column. Rpo132-HA/FLAG eluate was separated on a 10%–30% sucrose density gradient. Fractions were analyzed by Bis-Tris denaturing PAGE and visualized by silver staining. Core vRNAP complexes are mainly present in fraction 11 and 12, while complete vRNAP complexes sediment in fraction 15, 16, and 17.

45. Add Protease inhibitor cocktail to the lysis buffer (see materials and equipment table). Re-suspend cell pellet in 30 mL of lysis buffer. Incubate sample on ice for 20 min and clear the lysate from cellular debris by centrifugation at 15,000×*g* for 20 min at 4°C.
46. Collect supernatant and incubate with the equilibrated FLAG-agarose beads on a rotating wheel or on a rolling bar shaker for 3 h at 4°C.
47. Centrifuge beads at 2,054×*g* for 5 min at 4°C. Remove supernatant and wash beads 3 times with 10 bed volumes of wash buffer.
48. Equilibrate beads with elution buffer (see materials and equipment table). Wash beads 1 time with 10 bed volumes of elution buffer without 3×-FLAG peptide (e.g., for 1 mL of beads volume use 10 mL of elution buffer).
49. Bead-bound vRNAP is eluted under native conditions with 3×-FLAG peptide. Dilute 3×-FLAG peptide with elution buffer to 200 μg/mL and add 1 bed volume to the beads. Incubate sample 12–18 h at 4°C on a stirring wheel.
50. Collect eluate the next day.
51. Concentrate eluate to 100–200 μL by using a Vivaspin 500 concentrator (Sartorius; 10,000 MWCO PES) at 10,000×*g* at 4°C.
52. Load sample onto a 10%–30% sucrose density gradient. Centrifuge at 165,000×*g* for 16 h at 4°C (use Beckman Coulter; Optima L-80 XP Ultracentrifuge; rotor SW60 Ti or equivalent).
53. Manually collect 200 μL fractions and analyze the protein composition of each fraction by separating the sample on a 12% Bis-Tris denaturing polyacrylamide gel (Figure 8). Usually, the core vRNAP sediments in the middle part of the sucrose gradient (Figure 8, fractions 11–12) and the complete vRNAP sediments in sucrose fractions 15–17 (Figure 8, fractions 15–17). The fractions can be stored at −80°C for a couple of months.

Depending on the sample, its integrity may also be analyzed by other means, for example native (blue native) gels.

⚠ CRITICAL: After cell lysis, the vRNAP complexes isolation has to be carried out on ice or at 4°C.

EXPECTED OUTCOMES

C-terminally HA/FLAG tagged Rpo132 allows purification of early vRNAP complexes. As shown in Figure 8, this affinity isolation making use of the FLAG tag yields to two major vRNAP complexes.

A lighter complex containing all the subunits of the catalytically active vRNAP core enzyme (Figure 8, lanes 11 and 12) and a second heavier complex containing vRNAP core subunits and additional RNA processing and viral transcription factors (Figure 8, lanes 15–17) (Grimm et al., 2019). The final yield of purified core and complete vRNAP complexes is approx. 1–2 mg of protein for 50 confluent dishes of HeLa S3 cells. The isolated complete vRNAP complexes are native and enzymatically active capable of early promoter-dependent transcription of VACV genes (Grimm et al., 2019; Hillen et al., 2019). The particles are well suitable for cryo-EM based structural investigations and allowed the characterization of vRNAP complexes in different phases of transcription including initiation, elongation and co-transcriptional capping (Grimm et al., 2019, 2021; Hillen et al., 2019). This generic procedure can be used as guide for the isolation of other macromolecular complexes of interest.

LIMITATIONS

The generation of new VACV strains requires approximately a month and purification protocols from VACV infected cells must be adjusted to the respective complexes to be isolated. Thus, the effort is rather high and pays off only for proteins and/or macromolecular complexes that cannot conveniently be obtained from prokaryotic expression systems, such as *E. coli*.

Compared to bacterial expression systems, the typical yield of a purification from VACV-infected cells is lower. Liquid cultures are recommended for larger purifications, and this requires special non-standard equipment that may be difficult to establish in regular labs.

The generation of new recombinant VACV strains, with the gene of interest being under control of the VACV endogenous promoter, is not feasible in all cases. Introduction of a tag in a particular position in the VACV genome may result in the generation of a lethal mutation.

TROUBLESHOOTING

Problem 1

After the second homologous recombination, wildtype virus but not the FLAG/HA tagged recombinant virus is repeatedly obtained (step 5, m).

Potential solution

In general, it cannot be ruled out that the tag insertion interferes with the expression and function of the tagged gene and/or the neighboring gene and the recombinant virus is not viable. If possible, a different insertion site should be selected (e.g., N-terminal over C-terminal). Should this not be possible, the target gene could be inserted in a different locus of the VACV genome, for example by selecting recombinant viruses on the basis of plaque formation (Blasco and Moss, 1995).

Problem 2

Difficulties to detect plaques (see step 28, "purification of VACV" section).

Potential solution

Insertion of fluorescent proteins in VACV genome, could be used as tool for detection of plaques, e.g., GFP-tagging of the protein of interest and anti-GFP isolation.

Problem 3

Virus loss during propagation. To get high yields of purified virus, cells should be harvested once the cytopathic effect occurs. Due to their specific morphology, infected HeLa S3 cells do not develop clear cytopathic effects and could detach from the culture plate before the harvest of the cell monolayer (step 21).

Potential solution

To minimize virus loss, collect cell supernatant together with the cells and centrifuge sample for 10 min at 1,800×*g* at 4°C.

Problem 4

Low yield of purified tagged proteins from infected cells (steps 38 and 42).

Potential solution

Low yield of purified complexes could result from poor infection-efficiency. Therefore, confirm the virus titer by plaque assay. Alternatively, infect HeLa S3 cells and incubate them at 32°C instead of at 37°C and prolong the time of infection from 24 h to 40 h. As some proteins may be present only in viral factories, extraction conditions may be adjusted to solubilize expressed proteins.

Problem 5

The expressed protein precipitates after lysis (step 45).

Potential solution

It is possible that the expressed protein becomes insoluble after lysis. In these cases, it is recommended to test different salt and detergent concentrations of the lysis buffer and check the supernatant and pellet fractions after lysis for the presence of the protein of interest.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to, and will be fulfilled by, the lead contact, Utz Fischer (utz.fischer@biozentrum.uni-wuerzburg.de).

Materials availability

This study did not generate new unique reagents. All unique/stable reagents generated in this study are available from the lead contact with a complete Materials Transfer Agreement.

Data code and availability

No new datasets or code were generated in the preparation of this manuscript.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.B., I.L., S.B., C.G., and U.F.; Investigation and validation, J.B.; Writing - Original Draft, J.B. and I.L.; Writing - Reviewing, I.L., S.B., and U.F.; Figures, I.L. and S.B.; Editing, U.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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