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Workflow to Enhance Virus Reconstitution of Human Herpesvirus 6A (HHV-6A)

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We use this protocol and it's working

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Abstract

Human herpesvirus 6A (HHV-6A) is a T-lymphotropic betaherpesvirus that establishes lifelong latency in its host. Virus reactivations have been implicated in several diseases, including multiple sclerosis, encephalitis, myocarditis, and chronic fatigue syndrome, but HHV-6A biology remains poorly understood. Despite the availability of HHV-6A bacterial artificial chromosomes (BACs), it remains challenging to reconstitute the virus, hampering virological studies. Here, we describe an optimized protocol that enhances BAC transfection efficiency and accelerates virus propagation, facilitating virus reconstitution within two to three weeks. We used an HHV-6A GFP reporter virus, but the protocol also worked equally well for other HHV-6A BACs and mutants.

Guidelines

Troubleshooting Guide

	A	B	C	D
		Problem	**Potential Cause**	**Potential Solution**
	1.1	Low cell viability before nucleofection	Too high passage	Use low-passage but well proliferating JJHan cells
	1.2		Too early after thawing	Compare cell growth to Figure 1A before starting
	1.3		Cell density is too low or too high	Maintain a cell density between min. 105/ml and max. 106/ml
	1.4		Too high DMSO concentration	Make sure to use 1% DMSO. Ensure equal distribution of the DMSO using an orbital shaker at low speed (~20 rpm)
	1.5		biological contaminations	test for potential microbial, yeast, or fungal contamination
	2.1	Low cell viability after nucleofection	Low viability before nucleofection	Perform a viability staining before starting nucleofection (<90%)
	2.2		Low BAC DNA quality, contaminated with nicked, damaged BAC DNA or genomic DNA	Ensure high-quality BAC DNA at an optimal concentration of ~1 µg/µl, 260/280 ratio of ~1.8, and 260/230 ratio of 2.0 – 2.2. Avoid vigorous pipetting and use wide bore tips. Perform exonuclease V digestion before nucleofection (see *2. DMSO addition and Exonuclease V treatment*)
	2.3		Contaminating endotoxins	Perform an endotoxin removal step after the exonuclease digestion
	2.4		Cells have been kept too long in nucleofection buffer	Keep the cells for max. 15 min in nucleofection buffer
	2.5		Rigorous pipetting of cells after nucleofection	Very gentle and cautious handling is required; set a timer to ensure slow pipetting.

	A	B	C	D
	2.6		unsuitable medium (low FCS, no glutamine or presence of antibiotics)	Use preconditioned medium with 20 % FCS without antibiotics
	3.1	Low transfection efficiency	Low cell viability	*See No. 1*
	3.2		Low BAC DNA quality, contaminated with nicked, damaged BAC DNA or genomic DNA	*See No. 2.2*
	3.3		Too low or high BAC DNA concentration	The protocol is optimized for 2 µg of HHV-6A BAC DNA
	3.4		Too old nucleofection buffer	Store larger stocks of the homemade nucleofection buffer at -20 °C long term. Thaw them only once and keep at 4°C for max. three months. Let aliquots for nucleofections come to RT before use.
	4.1	No Virus Spread	Low transfection efficiency	*See No. 3*
	4.2		Low cell viability	*See No.2*
	4.3		Missing pp71 expression	Check the DNA quality and/or prepare fresh DNA. Co-transfect 1 µg of pp71 expressing plasmid
	4.4		Non-potent stimuli	Store stocks of stimuli at -20°C. Thaw fresh aliquots no more than three times. Keep thawed aliquots at 4 °C for max. one week.
	4.5		Mutation in the BAC that abrogates virus replication	Repeat the protocol in parallel with the WT HHV-6A BAC as a control.

Materials

DNA Preparation

- Purified high-quality HHV-6A GFP BAC DNA (NucleoBond Xtra BAC Kit, Macherey-Nagel, Cat.: 740436.25) in TE buffer (optimal concentration ~1 µg/µl) stored at 4°C
- Plasmid expressing Human Cytomegalovirus protein pp71
- Exonuclease V (RecBCD, NEB, Cat.: M0345S) digestion to remove contaminating DNA
- DNA LoBind® Tubes (1.5 mL, Cat. 00030108051 Eppendorf) to reduce loss of DNA
- DNA low binding, wide bore tips (ART 200 µl Tip, wide bore, Thermo Scientific, Cat. 2069G) to avoid shearing of fragile BAC DNA

Nucleofection

- Wide bore tips (ART Tips 1000 µl barrier tip, Thermo Scientific, Cat. 2079GPK) to transfer cells from cuvette to culture plate
- Preconditioned medium: 20 % FCS, RPMI without any antibiotics, stable glutamine, 25 mM HEPES, taken from about 24 - 48 h cultured JJHan cells, 0.2 µm filtered
- Electroporator and corresponding materials to perform nucleofection:

	A	B	C	D
	Nucleofector	**Nucleofection Buffer**	**Cuvettes**	**Program**
	Lonza Amaxa 2b	CellLine Nucleofector Kit Amaxa V (Lonza, Cat.: VCA-1003) *alternatively:* self-made buffer Amaxa V (dx.doi.org/10.17504/protocols.io.64mhgu6)	Fisherbrand Electroporation Cuvettes Plus (Fisher Scientific, Cat.: FB102)	**U-014** (T-008)
	Lonza Amaxa 4D X Unit	SF Cell Line 4D-Nucleofector™ X Kit L (Lonza, Cat.: V4XC-2024) *alternatively:* self-made buffer Amaxa V (dx.doi.org/10.17504/protocols.io.64mhgu6)	Included in the kit	**EN-138** (EH-100, EH-109, ER-137, EW-113)

	A	B	C	D
	Cells per nucleofection	**BAC DNA per nucleofection**	**pp71 plasmid DNA per nucleofection**	**Amaxa buffer per nucleofection**
	5×106	2 µg	1 µg	100 µl

Virus Propagation

- Virus culture medium: 5% FCS, RPMI, stable glutamine, 25 mM HEPES
- Hydrocortisone (Sigma, Cat.: H0888), stock concentration 9 mg/ml in 80% EtOH
- IOX2 (Sigma, Cat.: SML0652), stock concentration 10 mM stock in DMSO

Alternatively:
- Ruxolitinib (Invivogen, Cat.: Tlrl-rux), stock concentration 10 mM in DMSO
- PHA-L (Merck, Cat.: 11249738001) stock concentration 3.75 mg/ml in RPMI

RPMI medium

	A	B
	FCS	20%
	HEPES, glutamine without antibiotics	25 mM

NucleoBond Xtra BAC kit for large construct plasmid DNA **Macherey-Nagal Catalog #**740436.25

Exonuclease V (RecBCD) - 1,000 units **New England Biolabs Catalog #**M0345S

DNA LoBind® Tubes **Eppendorf Catalog #**00030108051

ART™ Barrier Specialty Pipette Tips, 200, wide bore **Thermo Fisher Catalog #**2069G

ART™ Barrier Specialty Pipette Tips, 1000, wide bore **Thermo Fisher Catalog #**2079GPK

Cell Line Nucleofector® Kit V **Lonza Catalog #**VCA-1003

Electroporation Cuvettes Plus™, 2mm gap with imbedded aluminum electrodes, Capacity: 400μL **Thermo Fisher Catalog #**FB102

SF Cell Line 4D-Nucleofector® X Kit L **Lonza Catalog #**V4XC-2024

Hydrocortisone **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H0888

IOX2 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**SML0652

Ruxolitinib **InvivoGen Catalog #**tlrl-rux

Phytohemagglutinin-L (PHA-L) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**11249738001

Troubleshooting

Preparation of Cells (15 min)

1

Note

2 days before transfection

Due to the preference of the virus to establish latency, it is essential to utilize cells that support viral replication. CD4+ T cell lines, such as JJHan cells, are known to support HHV-6A replication (1-4). Viable, proliferating cells are a fundamental prerequisite for optimal transfection efficiency. For optimal nucleofection, JJHan cells should be in their exponential growth phase and double within 24 h. They should settle at the bottom of the culture vessel and form small clumps of proliferating cells.

To achieve optimal viability, culture the cells in RPMI with 20% FCS, [M] 25 millimolar (mM) HEPES, without any antibiotics in a 37 °C incubator under a 5% CO_2 atmosphere.

2 Seed about 2 × 10^6 cells/nucleofection in a T75 flask to reach min. 5 ×10^6 cells on the day of transfection. To ensure the cells are in the log growth phase on the day of nucleofection, seed them two days prior at a density of approximately 5×10^4 cells/ml (equals about 40 ml/nucleofection).

3 Seed an additional flask T25 of JJHan cells (5 × 10^4 – 5 × 10^5 cells/ml) in fresh medium (min. 2 ml/nucleofection) that will be used later for preparing preconditioned medium. (see *Reconstitution* section)

DMSO addition and Exonuclease V treatment (2 h 30 min)

1h 30m

4

Note

1 day before transfection

Treatment of the cells with 1% DMSO prior to nucleofection will significantly improve the transfection efficiency of the HHV-6A BAC. Exonuclease V digestion of the BAC improves DNA quality and transfection efficiency by removing damaged BAC DNA and contaminating genomic *E. coli* DNA (5). The combination of these two steps increases transfection efficiency, thereby accelerating virus reconstitution.

Add 1% DMSO to the culture medium of the cells used for transfection (400 µL DMSO for 40 ml total volume per nucleofection) 24:00:00 prior to the nucleofection

and resuspend carefully.

4.1 To avoid DMSO sedimentation, place the cells in the incubator Overnight on an orbital shaker at low speed (20 rpm).

5 Thaw all reagents for the exonuclease V treatment On ice .

6 Set up the following reaction for the exonuclease V treatment based on the manufacturer's protocol, assuming about 20% of contaminating DNA within the HHV-6A BAC preparation:

	A	B	C
		Concentration	**Per Nucleofection**
	BAC DNA	2 µg per reaction	2 µg HHV-6A BAC DNA
	ATP 10 mM	1 mM	1 µl
	NEB Buffer 10x	1 x	1 µl
	dH_2O	Up to total volume	Fill up to 10 µl
	Exonuclease V	10 Units/1 µg target DNA	1 µl
	Total		10 µl

7 Incubate the reaction for 01:00:00 at 37 °C in a thermal cycler, then heat inactivate the enzyme for 00:30:00 at 70 °C . Store the DNA at 4 °C . 1h 30m

Note

Notes: Depending on the BAC DNA concentration, the reaction volume can be adjusted. Beware not to exceed a total volume of 20 µl/nucleofection, as this will later dilute the final concentration of the nucleofection buffer. The exonuclease V treatment can theoretically be performed on any day. If the digestion is performed immediately before nucleofection, allow the mix to cool down to Room temperature before proceeding.

Reconstitution Protocol: Preparation (45 min per nucleofection)

8 Let all reagents, including exonuclease V treated DNA, pp71 plasmid, and nucleofection buffer, reach Room temperature .

9 Meanwhile, prepare preconditioned medium by taking 48-h-old medium from JJHan cells, and filter it using a 0.2 µm filter. Mix the medium in a 1:1 ratio with fresh RPMI medium.

RPMI medium

	A	B
	FCS	20%
	HEPES, glutamine without antibiotics	25 mM

10 Prepare a 12-well (or 6-well) plate with 2 mL of preconditioned medium per nucleofection and prewarm the plate to 37 °C in an incubator.

Note

Notes: The choice of the well size depends on the viability of your cells after nucleofection. Start using a 12-well plate and change to a larger size if needed.

11 Prepare separate tubes with DNA for each reaction using wide bore tips:

	A	B
	exonuclease V treated HHV-6A BAC DNA	2 µg
	pp71 plasmid	1 µg

12 Turn on the nucleofector and select program U-014 (Lonza Amaxa 2/2b) or EN-138 (Lonza Amaxa 4D X Unit).

13 Unpack as many cuvettes as needed and label them appropriately.

Reconstitution Protocol: Nucleofection

10m

14

10m

Note

The key to a successful virus reconstitution is to obtain optimal transfection efficiency while maintaining high cell viability. The use of wide bore tips, as well as gentle handling of the BAC DNA and fragile cells, especially post nucleofection, is crucial to avoid mechanical stress. Avoid bubbles and vigorous pipetting.

Count cells and spin down 5 × 10^6 JJHan cells per nucleofection at 90 x g, Room temperature, 00:10:00 .

Note

Notes: Process each sample separately to ensure cautious and consistent handling.

15 Discard the supernatant and remove as much leftover liquid as possible with a pipette without disturbing the cell pellet.

16 Resuspend the cells gently but thoroughly in 100 µL nucleofection buffer. Use regular tips to allow an even resuspension of the cells.

Note

Notes: Ensure the cells do not sit in pure nucleofection buffer for more than 00:15:00 .

17 Add the cell suspension to the DNA mix in the prepared tube without resuspending.

- Change to wide bore tips and resuspend the cells thoroughly by pipetting up and down 4 times.
- Try to reach a homogenous mixture of cell suspension and DNA, avoid bubbles and shearing by rigorous pipetting up and down.

18 Transfer the suspension to a cuvette, avoid bubbles, and slightly tap the bottom of the cuvette to bring the entire cell/DNA suspension to the bottom of the cuvette.

19 Place the cuvette in the nucleofector and electroporate using the respective program (U-014 or EN-138).

20 Immediately, add 800 µL of prewarmed preconditioned medium very slowly from the prepared plate to the cuvette.

- To do so, turn the cuvette carefully towards a more horizontal position and slowly but steadily fill it with the medium alongside the wall.
- Do not hastily drop the medium onto the cells, and do not pipette up and down to resuspend.
- Turn the cuvette to its upright position and put the cap on.

21 For the recovery of the cells after the electrical pulse, place the cuvette in the incubator at 37 °C for 10 - 15 min before transferring the cells to the plate.

22 Remove the prepared culture plate from the incubator and transfer cells using a 1000 µl wide bore tip into the respective well. To do so, turn the cuvette carefully to a horizontal position.

- Place the tip at the bottleneck of the cuvette and very slowly and gently take up the suspension into the pipette.
- During this process, rotate the cuvette to allow the remaining suspension to gradually flow towards the pipette tip without letting it to drop out of the cuvette.
- When the entire volume is taken into the pipette tip, carefully and slowly transfer the suspension into its respective well.

Note

Notes:

- This step is very critical for the success of the transfection, and the handling will influence the cell viability, and thus, efficiency of the BAC transfection.
- To achieve optimal results, it takes about 00:03:00 to pipette the cells out of the cuvette and into the well.
- Do not rinse the cuvette to avoid transferring cell debris.

23 Incubate the cells at 37 °C and check transfection efficiency (GFP) 1 - 2 days post-transfection (dpt).

Note

Notes: Due to the large size of the HHV-6A BAC and minor inconsistencies in handling, the transfection efficiency can vary between 0.5–30%. This high inherent variability can be observed even when performing the procedure in replicates with cells and BAC DNA from the same origin (Figure 2A). We have found that the self-made nucleofection buffer performs as efficiently as the commercially available buffer (Figure 2B).

Propagation of HHV-6A Virus (2 to 3 weeks)

24

Note

High cell viability and transfection efficiency provide the basis for the stimulation strategy to enhance virus replication. The timing of stimulation primarily depends on the viability of the transfected cells, as the cells are in a fragile state following nucleofection, and stimulation can induce cell death. We have tested many stimulation protocols and identified the ones that work best for the HHV-6A reconstitution. Among those are the combinations of hydrocortisone + PHA, hydrocortisone + ruxolitinib, and hydrocortisone + IOX2. Specifically, the use of hydrocortisone + IOX2 showed the highest efficiency, resulting in full recovery within two weeks post-nucleofection (5).

Depending on the success of the nucleofection, initiate the following stimulation protocol at 2 dpt:

	A	B	C
	Transfection Efficiency	**Viability**	**Stimulation**
	high (>10 %) to medium (<10%)	high (cells are round, in good shape, dense, some clumps)	add 9 µg/ml hydrocortisone + 10 µM IOX2
	medium (<10%)	medium (some debris, but most cells are loosely attached to the bottom and some clump)	add 9 µg/ml hydrocortisone, check after three days: if cell viability is good, then add 10 µM IOX2

	A	B	C
	Low (only a few single cells are transfected)	Low to high	not suitable for efficient virus reconstitution, discard
	Low to high	Low (a lot of debris, membrane integrity changes, clumps due to cell death)	not suitable for efficient virus reconstitution, discard

25 Check the cells regularly and monitor the viral spread closely.

Note

Notes: Consider that the HHV-6A replication cycle takes about two days before the infection spreads to the neighboring cells. Avoid disturbing the cells while they are in a fragile state. If they maintain good viability, resuspending the cells occasionally can enhance virus spread.

26 Based on the cell density, add 2 mL fresh medium and adjust the respective stimulant accordingly about every 2 - 3 days. Reduced FCS (5%) ensures that the uninfected cells do not overgrow the infected cells.

Note

Notes: In the beginning, propagate the transfected cells without removing the old medium, as the supernatant may contain cell-free virus. After one week of stimulation, the whole medium can be replaced with fresh medium and fresh stimulants.

Fresh medium

	A	B
	RPMI	5%
	HEPES	25 mM

27 When the cell density drastically increases (to about 3×10^6 cells /ml), transfer the cells to a larger well or split them into two wells.

Note

Notes: A close cell-to-cell contact is recommended to enhance virus spread. This can also be accomplished by angling the plate or flask at a slight angle (e.g. onto another plate). The minimum cell density of the culture should be 5 x 10^5 cells/ml.

28 If the cells are highly infected (>80% GFP+), add fresh, uninfected JJHan cells to grow large virus cultures. Start with a 1 : 1 ratio (infected: uninfected) twice per week.
- If the virus replicates faster, increase the ratio to 1 : 3 or up to 5. Track the virus spread and adjust the stimulation to the volume.

29 If the culture is highly infected, hydrocortisone addition alone is sufficient to allow further replication. When virus reactivation is needed, stimulate again with hydrocortisone + IOX2.

30 A minimum of 10 mL with highly infected cells is recommended before transferring the virus culture into a cell culture flask.

31 Freeze virus stocks with a medium to high infection level (2–10 × 10^6 cells/vial, ~50–80% infected).

Protocol references

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