Applications of CRISPR technology in Epstein-Barr virus research and therapy

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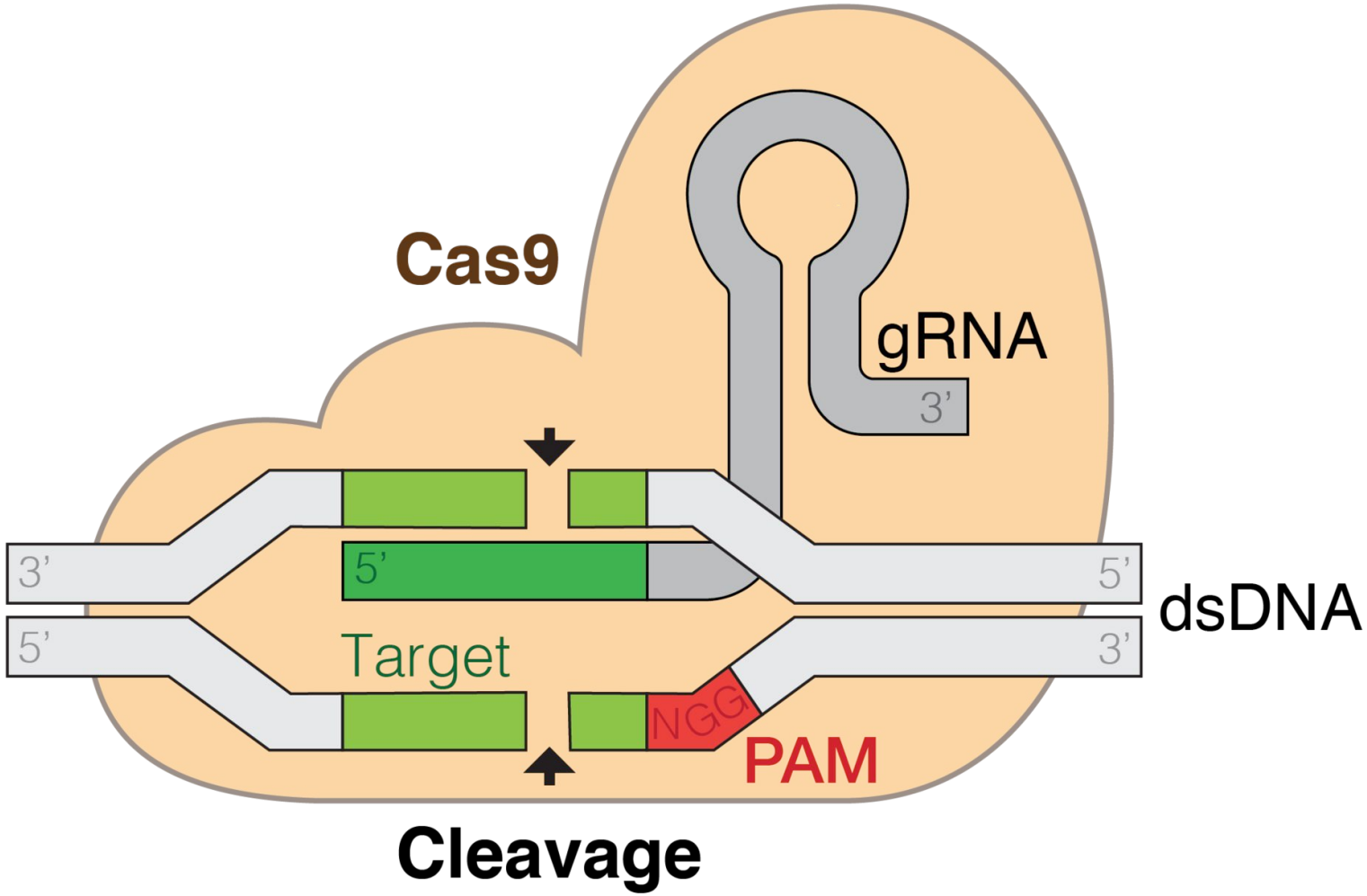
CRISPR-Cas9 is a game-changing technology in biology and medicine



CRISPR applications in EBV research and therapy

1. Mutant construction for genetic study and vaccine development

2. Marker insertion and rapid cloning of EBV

3. Eradication of EBV from infected cells

4. CRISPR screening for host restriction/dependency factors

5. CRISPRa activation of EBV gene expression

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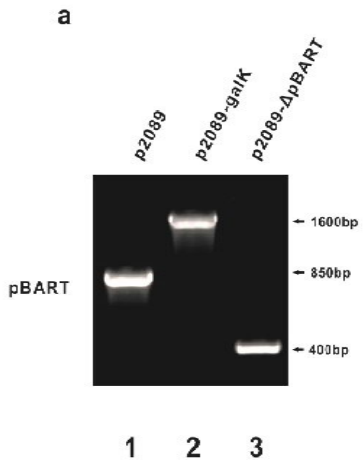
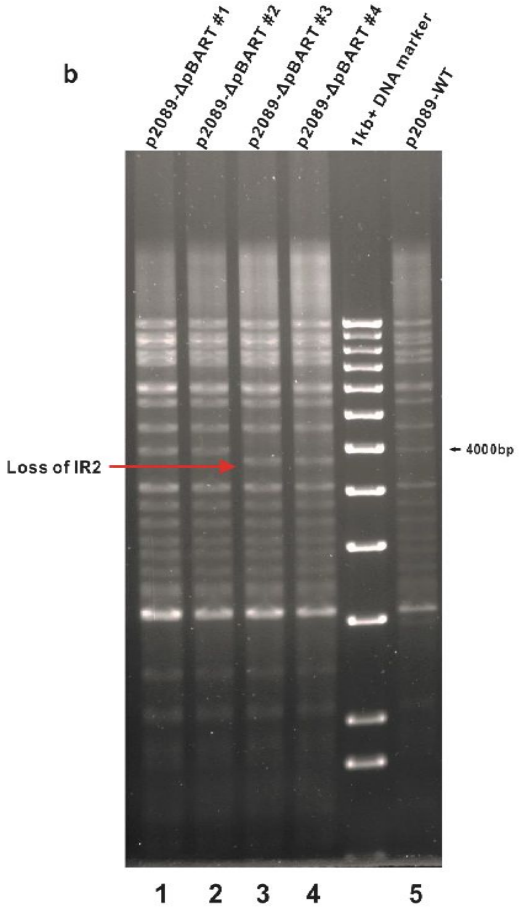
Conventional method for EBV mutant construction in BAC

1. galK-mediated recombination



Deletion of target region

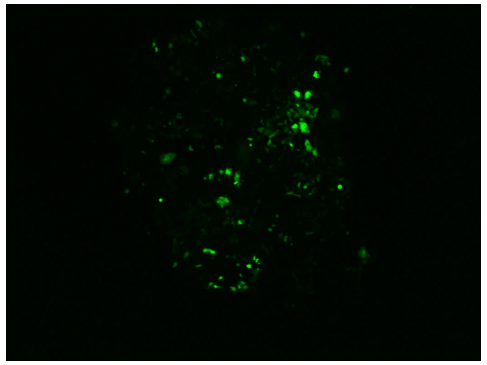
2. BamHI Restriction Patterning



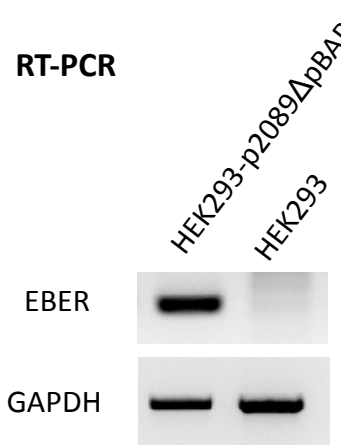
EBV genome integrity

3. Stable cell establishment

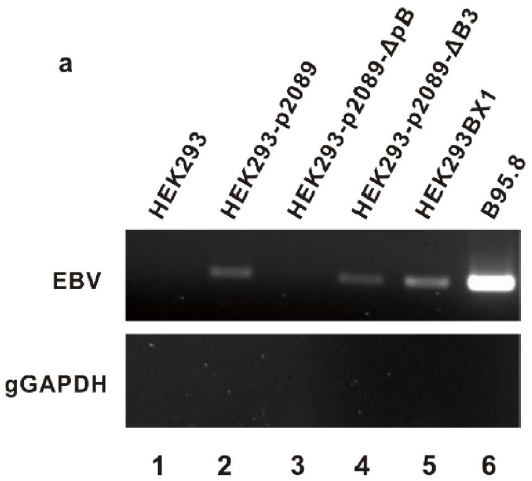
GFP+



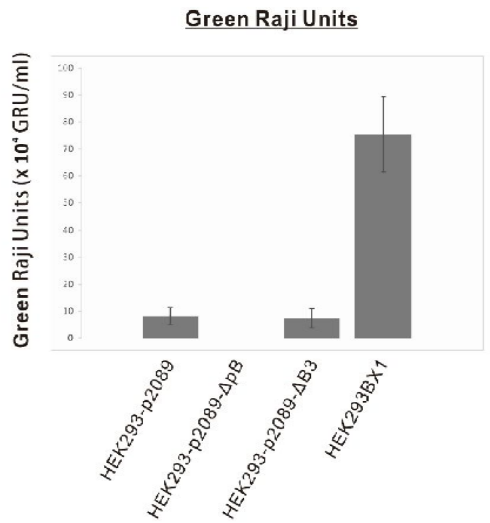
RT-PCR



4. PCR detection for cell-free virions from culture medium



5. EBV titer measurement



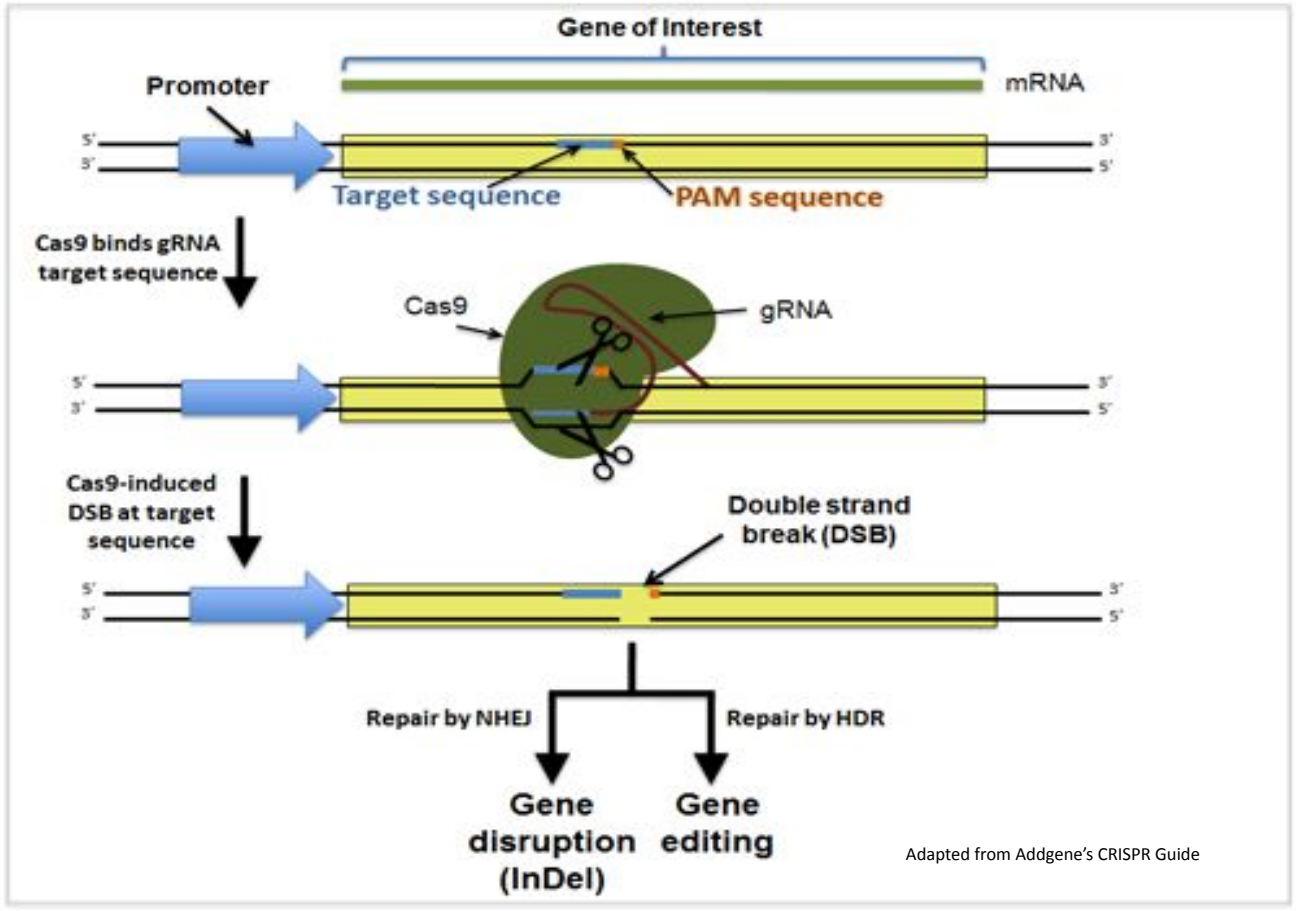
Bottleneck for EBV BAC technology

When virus producer cell lines are generated, viral titers are usually very low and some of these cells even lose their ability to support virus lytic replication. The low viral yield constitutes a barrier to its wider use.

∴ EBV BAC technology is not commonly used in NPC study.

CRISPR/Cas9-mediated genomic engineering

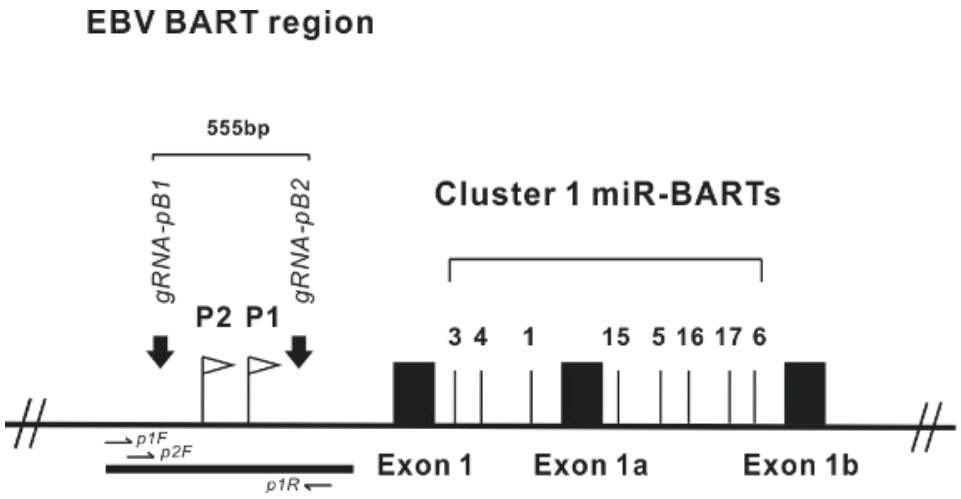
RNA-guided genomic editing



Double Strand Breaks Repair Pathways (NHEJ / HDR)
** deletions/ insertion**

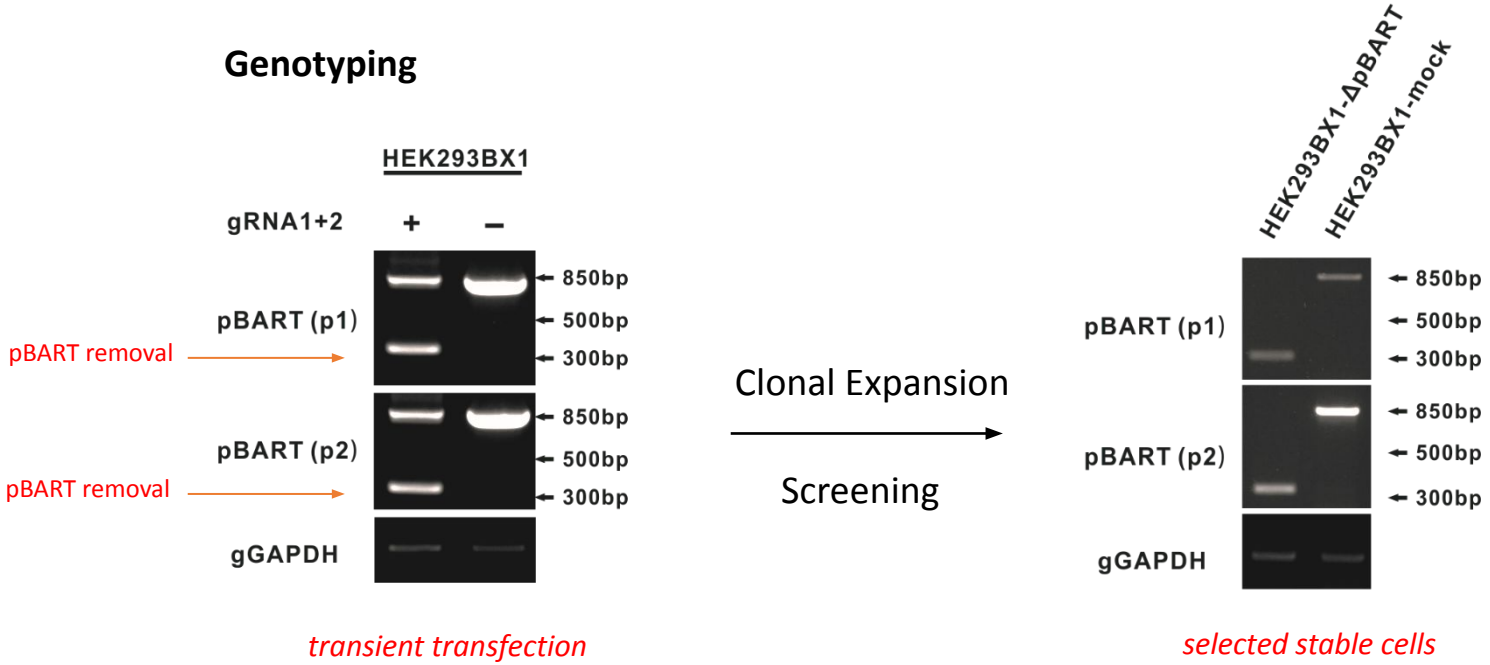
CRISPR/Cas9-mediated EBV editing?

CRISPR/Cas9-mediated EBV genome editing

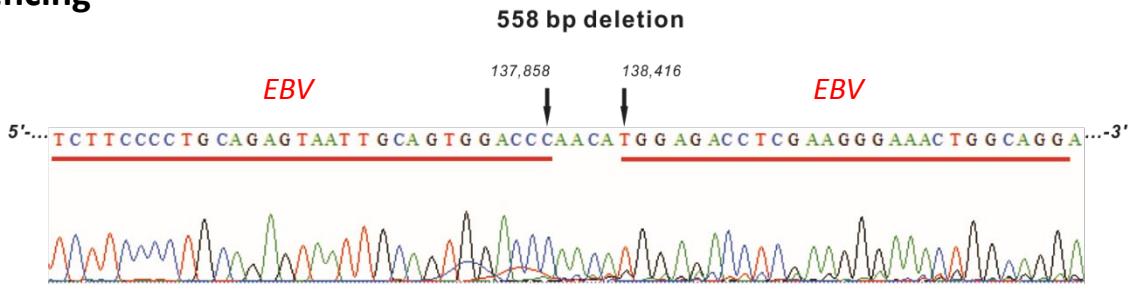


CRISPR/Cas9-mediated BART promoter removal

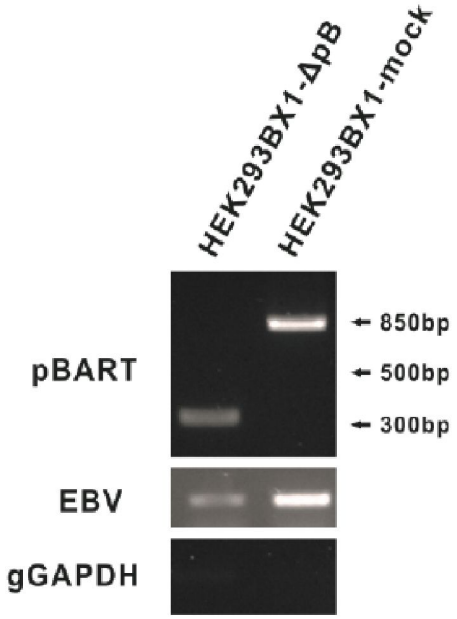
CRISPR/Cas9-mediated EBV editing



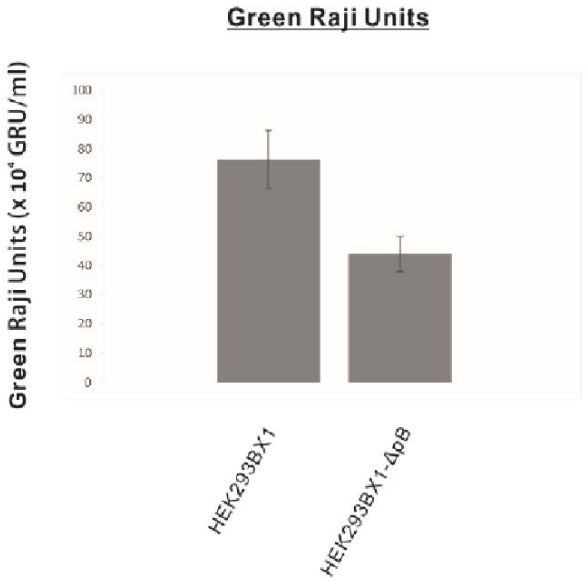
Sequencing



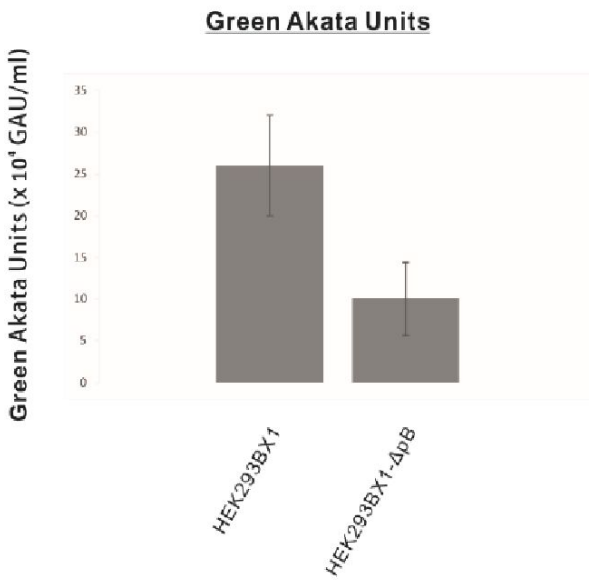
Characterization of miR-BART-deficient EBV



PCR detection for cell-free virions from culture medium



EBV titer measurement



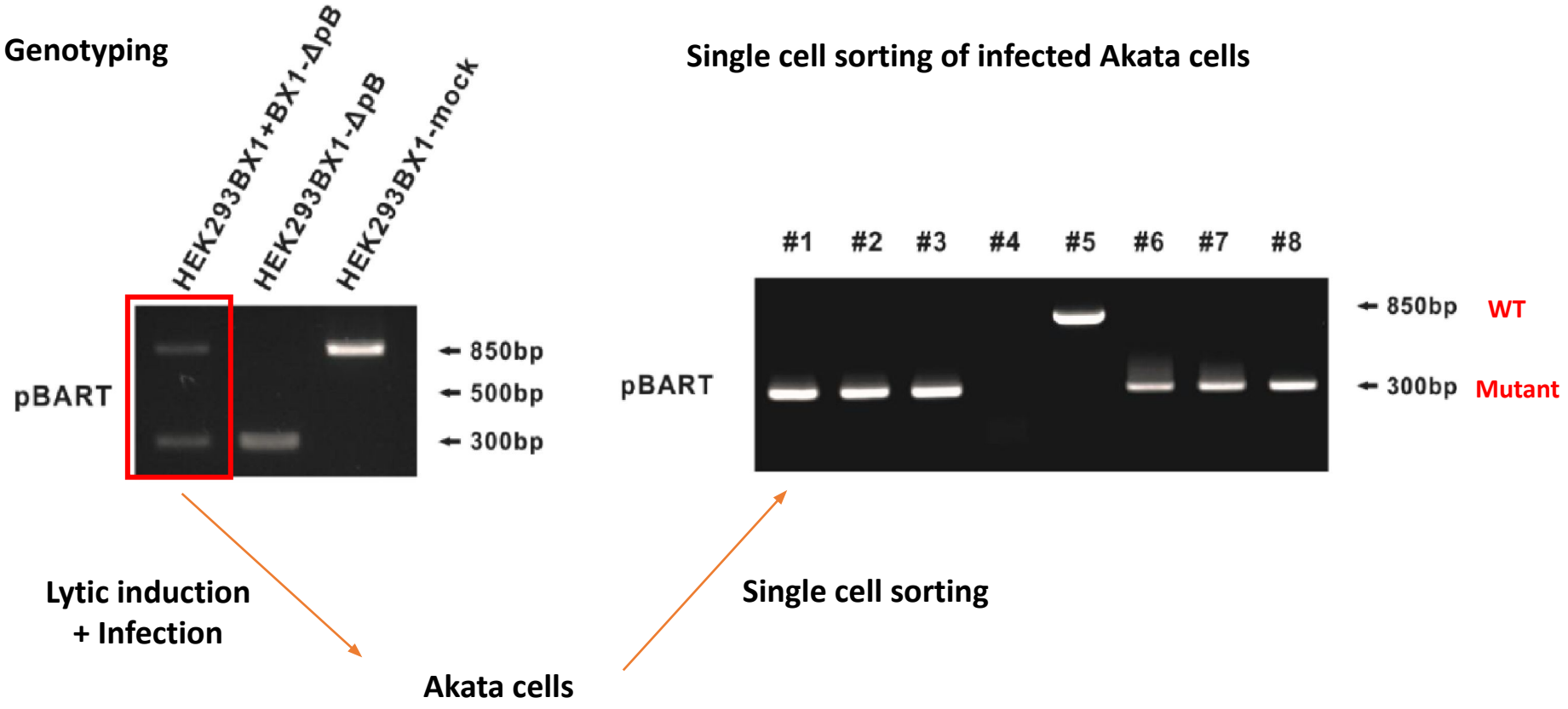
infection of Akata B cells

CRISPR/Cas9-mediated editing successfully generated the mutant virus for Akata cell infection.

Technical challenges for CRISPR-mediated EBV genome editing

- It is hard to separate edited from unedited virus.
- EBV is a multicopy episome which usually maintains 5-100 copies of cccDNA in latently infected cells (Adams and Lindahl 1975).
- It is difficult to completely edit all EBV episomes in one single infected cell by CRISPR.
- Editing was complete in only 1 out of 30 clones.

Selection of EBV mutant by single cell sorting of Akata cells



Summary 1

A novel CRISPR-mediated EBV genome editing method was established for recombinant EBV generation. This novel method provides an alternative way for recombinant EBV construction and has advantages over the conventional EBV BAC technology.

Shall an EBV vaccine be made?

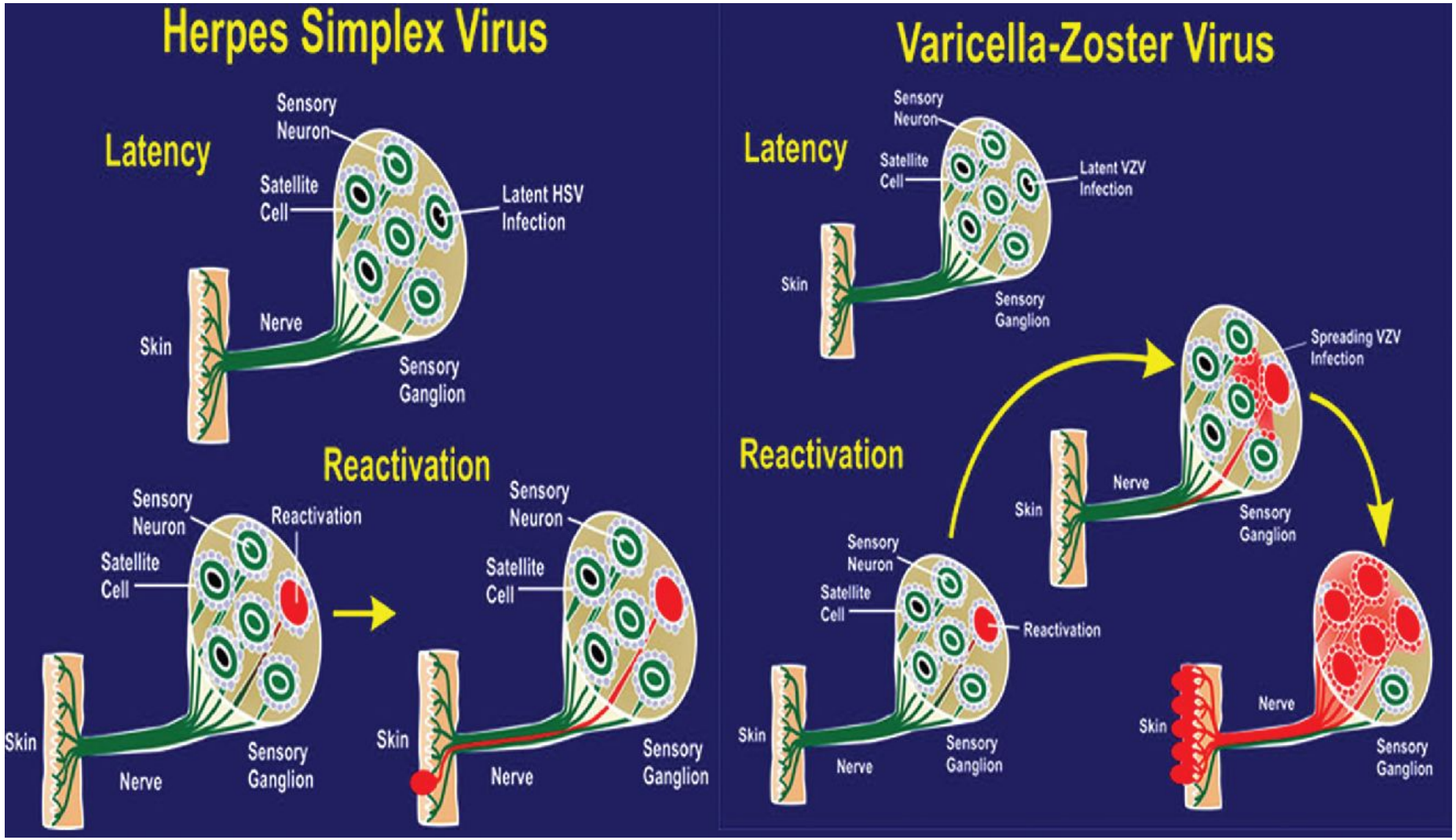
1. Shall an EBV vaccine be made to protect against NPC, GC, IM and other diseases?

2. Is live attenuated EBV vaccine a viable option?

3. How does a VZV vaccine work?

4. What lessons can we learn from the successful VZV vaccine?

HSV, VZV and EBV: How similar and different are they?



(CID 51:197-213, 2010)

VZV vaccine: The history

History	The Oka strain of VZV was isolated from a healthy Japanese child with varicella and attenuated by serial passage at 34°C in human and guinea pig cells
Properties	Mixture of genotypically distinct VZV strains
	Forty-two SNPs distinguish the Oka vaccine from the wild-type Oka parent strain of VZV
	Twenty of the 42 SNPs specify amino acid changes
	Although each is genotypically unique, all strains of VZV in the Oka vaccine share a subset of the 42 SNPs
	Three SNPs (at positions 106262, 107252 and 108111 in open reading frame 62, which encodes a transactivator of viral genes required for VZV replication) distinguish the Oka vaccine from all wild-type strains of VZV
	Differences in strain content are observed among Oka vaccines produced by different manufacturers and even between different batches from the same manufacturer
Varicella vaccine	Clinical studies in Japan demonstrated the safety, immunogenicity and clinical efficacy of Oka vaccine, which protected susceptible immunocompetent and immunocompromised children against varicella, even when administered shortly after exposure
	Vaccine virus establishes latency and reactivates to cause HZ, but at a lower frequency than wild-type VZV
	Oka vaccine also boosted VZV-specific cell-mediated immunity in immunocompetent and immunocompromised adults

VZV vaccine: The history

	In the United States, the safety and efficacy of Oka vaccine was documented in healthy children and adults and in several groups of immunocompromised children and adolescents
	Varicella vaccine (Varivax; Merck) was licensed by the FDA in 1995
	Routine childhood vaccination has markedly reduced the incidence of varicella in the United States
Zoster vaccine	Same preparation of live, attenuated Oka/Merck VZV as used in varicella vaccine
	Minimum potency at least 14 times greater than that of varicella vaccine (higher potency is necessary to induce a significant increase in VZV-specific cell-mediated immunity in older adults, who are already immune to varicella)
Zoster vaccine safety and efficacy	The Shingles Prevention Study demonstrated the safety and efficacy of zoster vaccine in immunocompetent adults ≥60 years of age: reduced the burden of illness caused by HZ by 61% (Table 3); reduced the incidence of HZ by 51% (Table 3); reduced the incidence of clinically significant postherpetic neuralgia by 67% (Table 4); and neither caused nor induced HZ
	Zoster vaccine (Zostavax; Merck) was licensed by the FDA in 2005
	Recommended for routine use in immunocompetent adults aged ≥60 years
	As of March 2010, ~6 million doses have been distributed in the United States, sufficient to immunize ~12% of the population of ≥60 year-old persons for whom it is recommended

Zoster vaccine: non-sterile but effective

- It is a live attenuated vaccine.
- The vaccine does not provide sterile immunity.
- The vaccine is safe and highly effective in preventing herpes zoster.
- VZV-CMI, but not VZV antibodies, is critical in limiting reactivation and replication of latent VZV.

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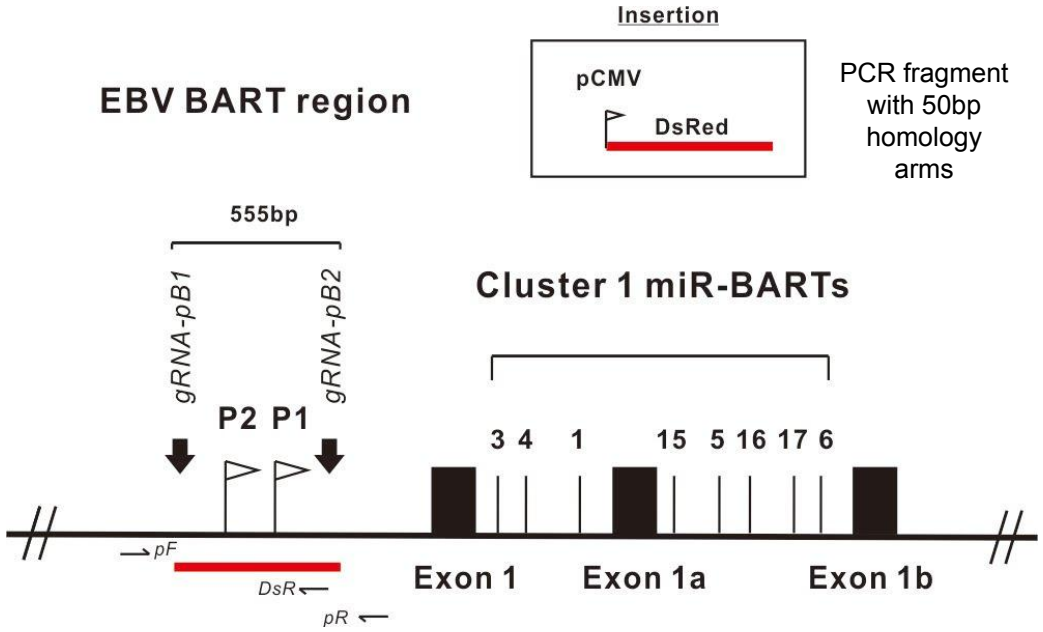
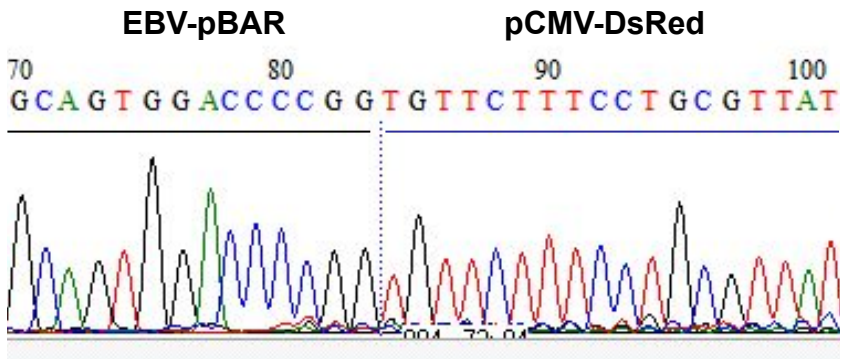
CRISPR insertion of a DsRed marker into EBV genome

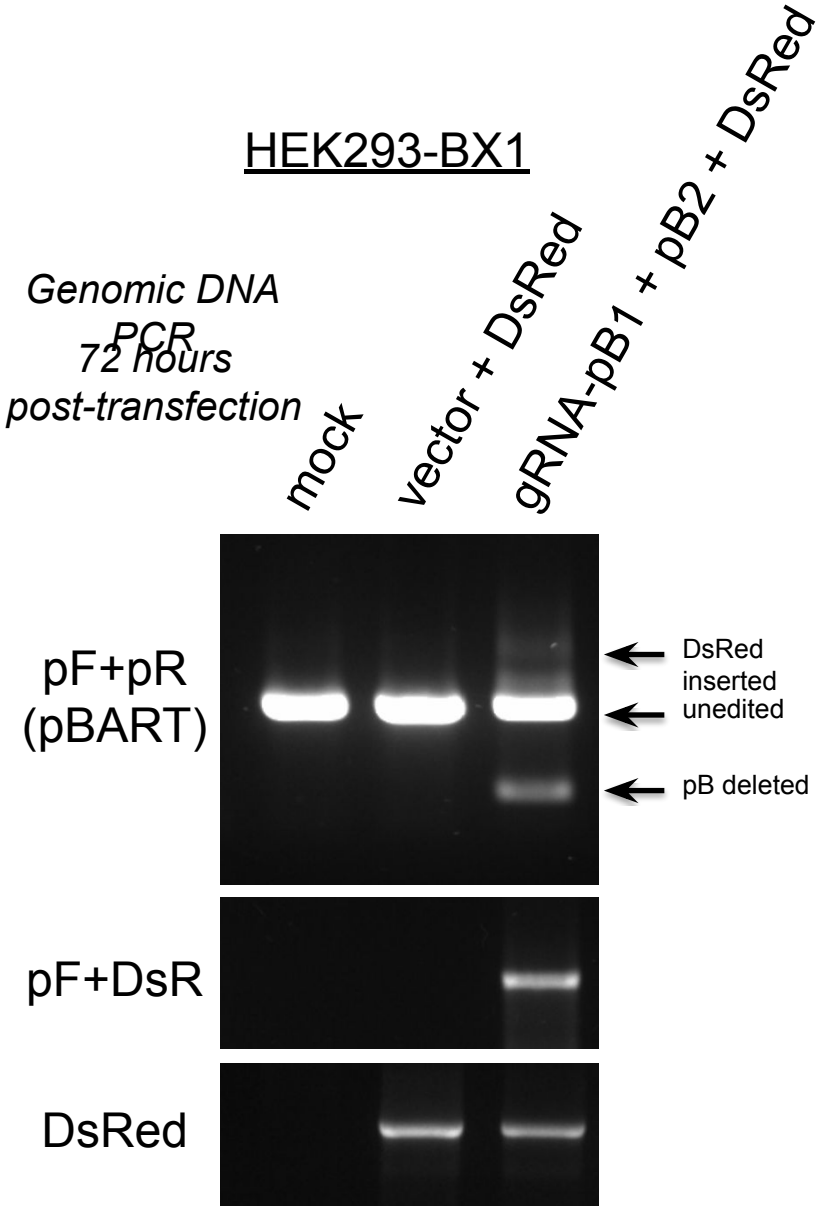




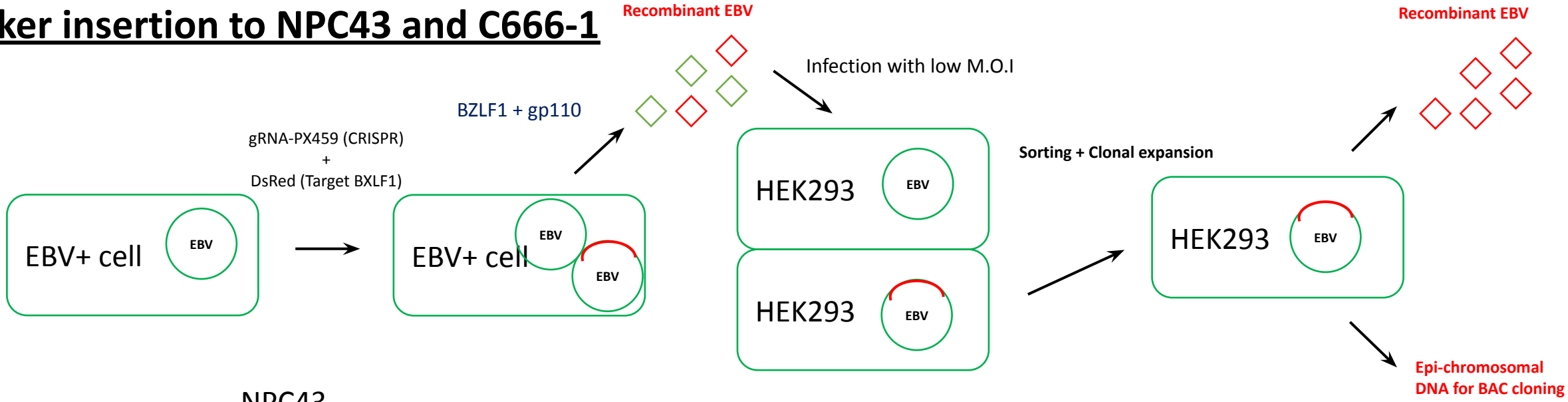
Sequencing

HEK293-BX1

Genomic DNA PCR
72 hours post-transfection

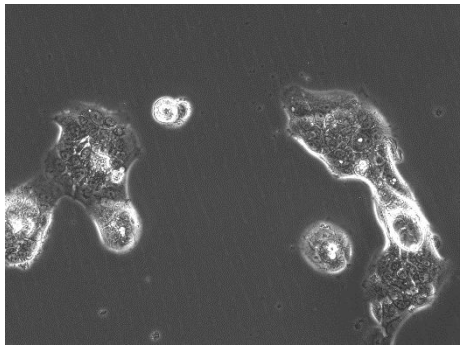


Marker insertion to NPC43 and C666-1

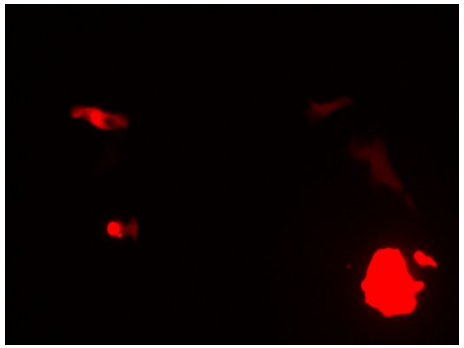


NPC43

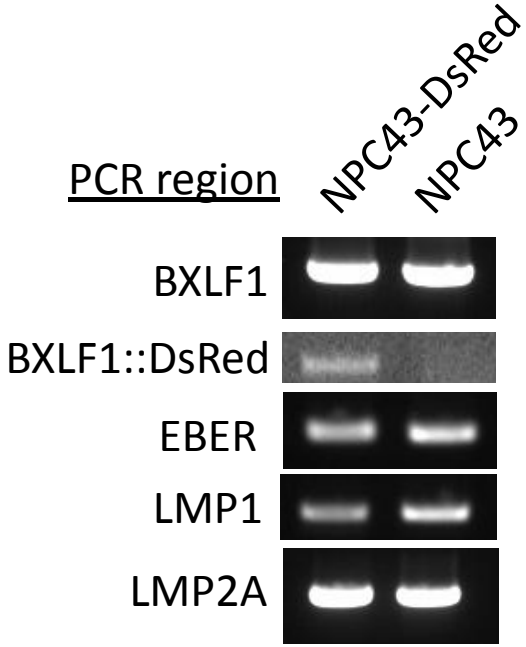
Bright field



DsRed

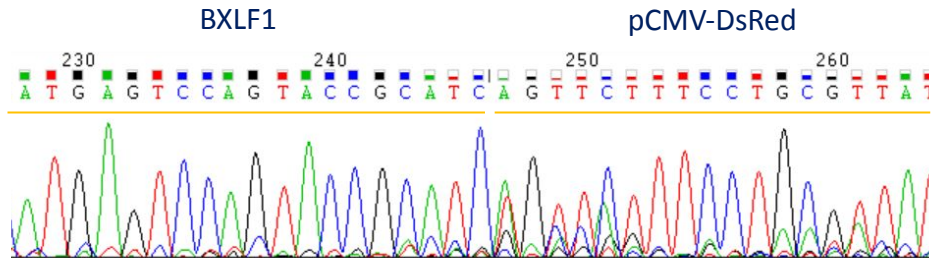


PCR region

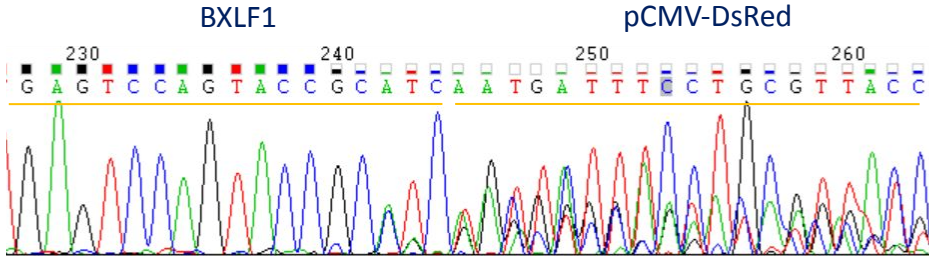


Supernatant Virion

NPC43-DsRed



C666-1-DsRed

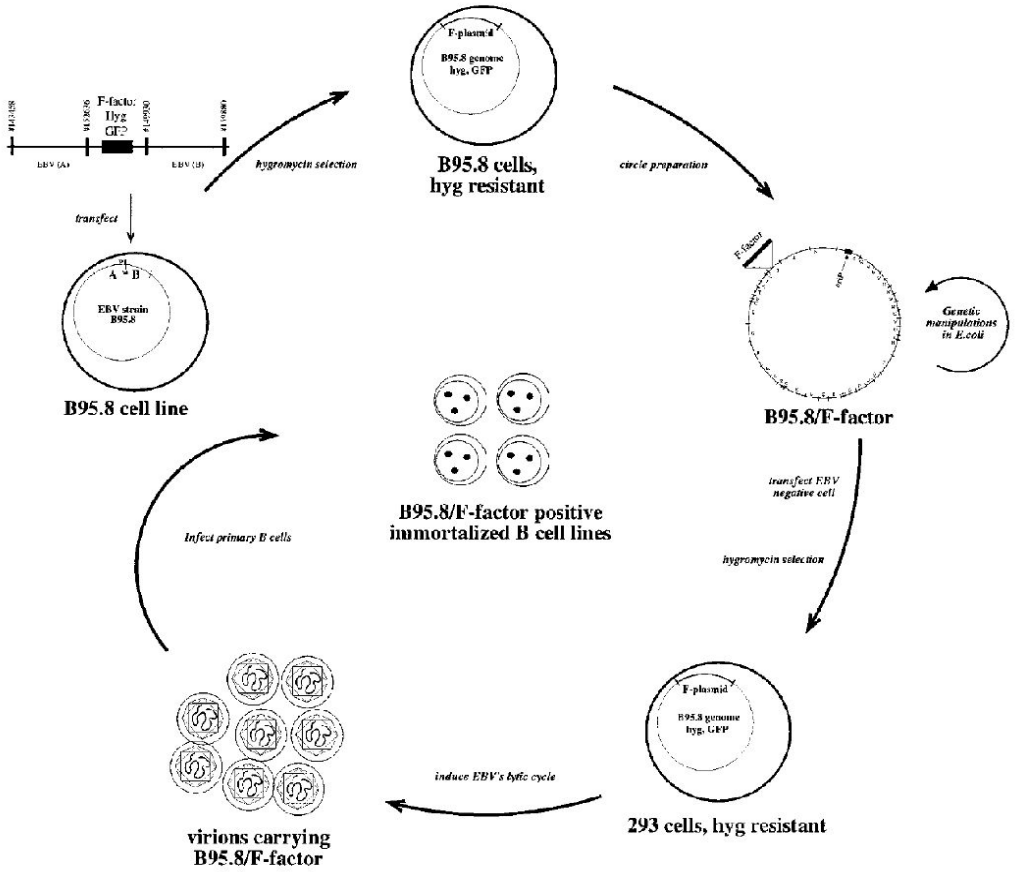


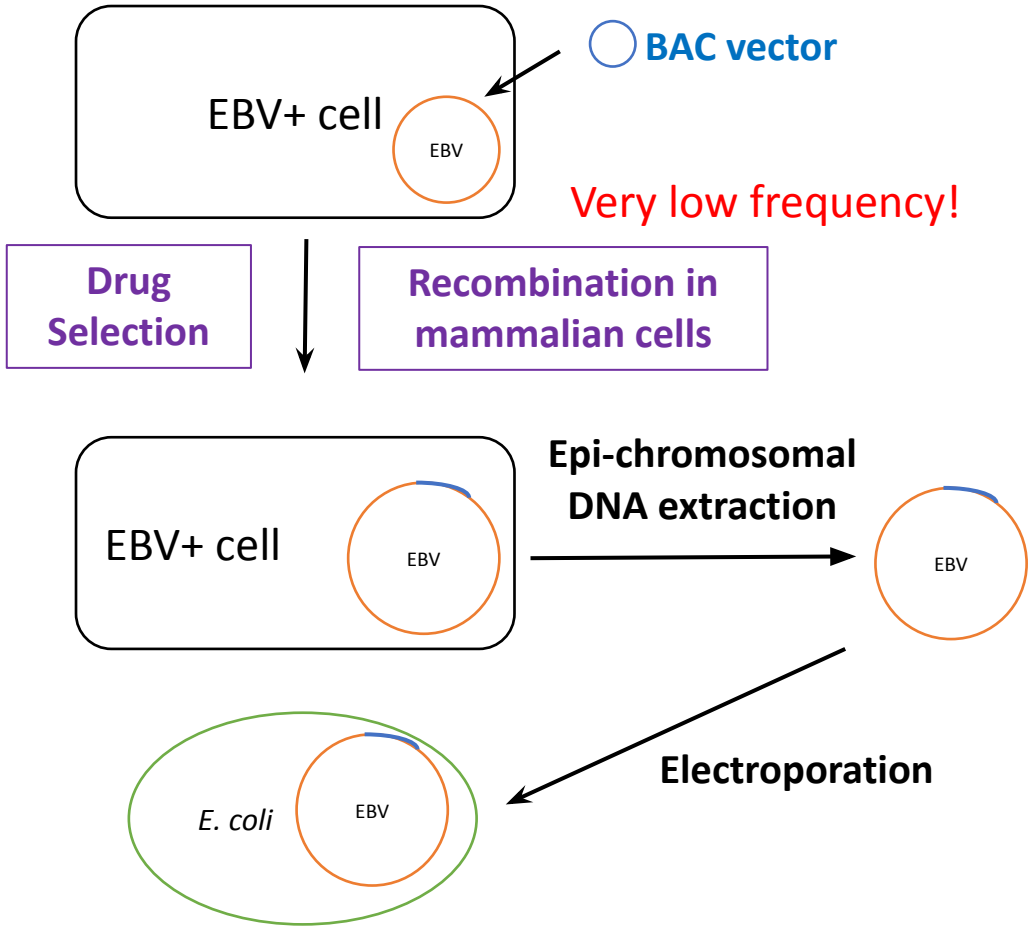
Conventional method for EBV BAC cloning

Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 8245–8250, July 1998
Medical Sciences

Propagation and recovery of intact, infectious Epstein–Barr virus from prokaryotic to human cells

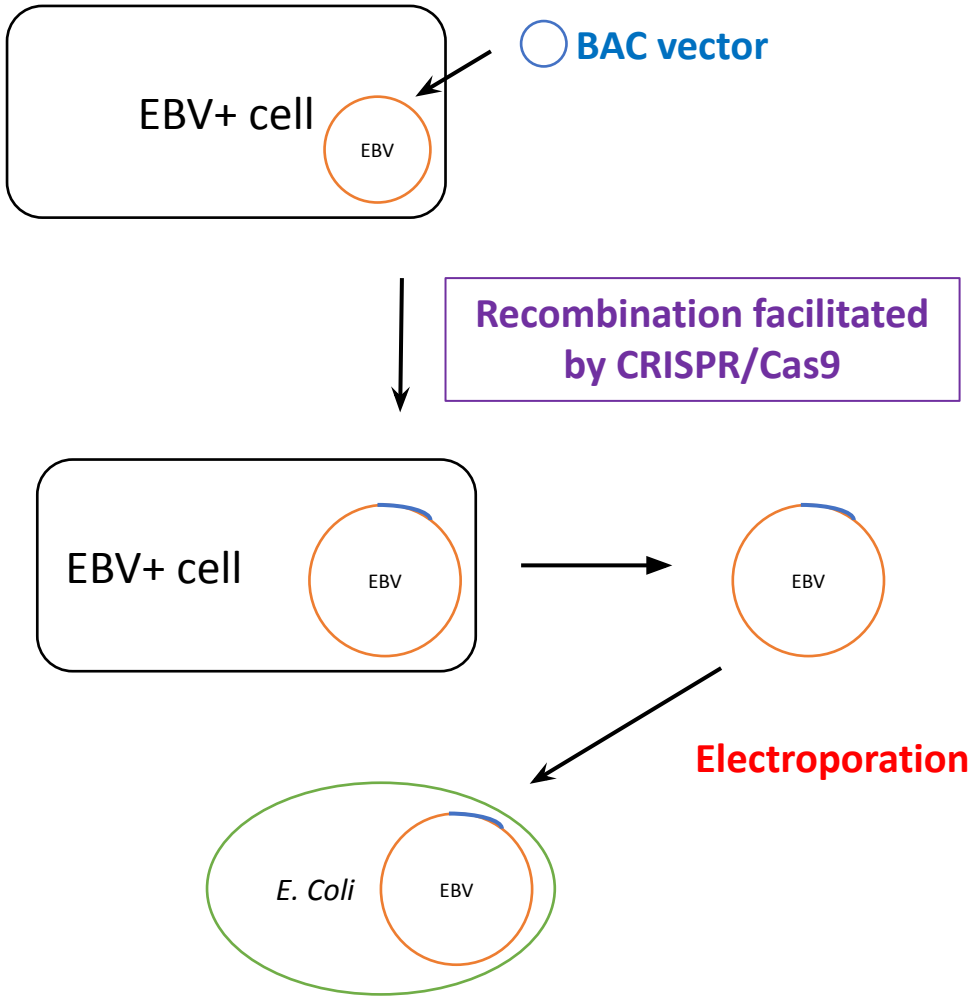
HENRI-JACQUES DELECLUSE*, TANJA HILSENDEGEN*, DAGMAR PICH*, REINHARD ZEIDLER†, AND WOLFGANG HAMMERSCHMIDT*‡

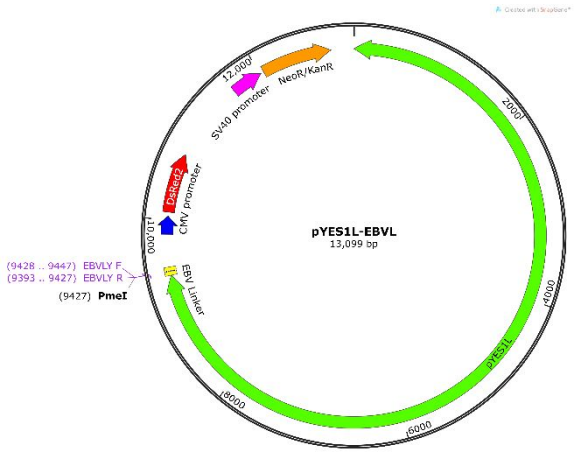




CRISPR-facilitated EBV BAC cloning

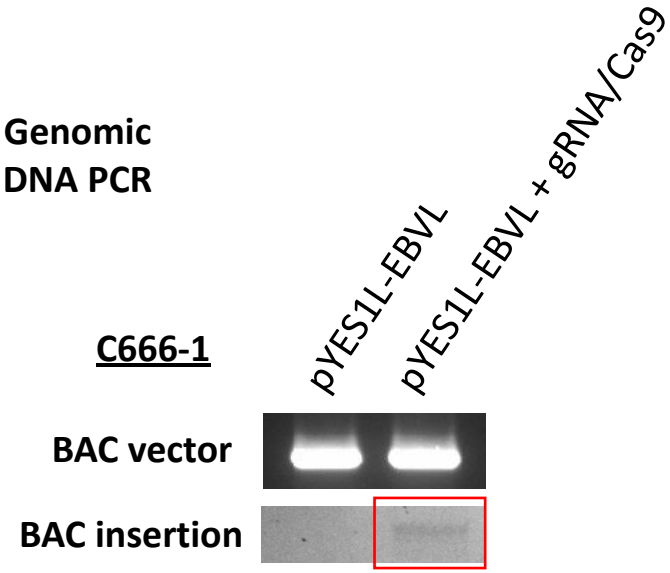
Cell line



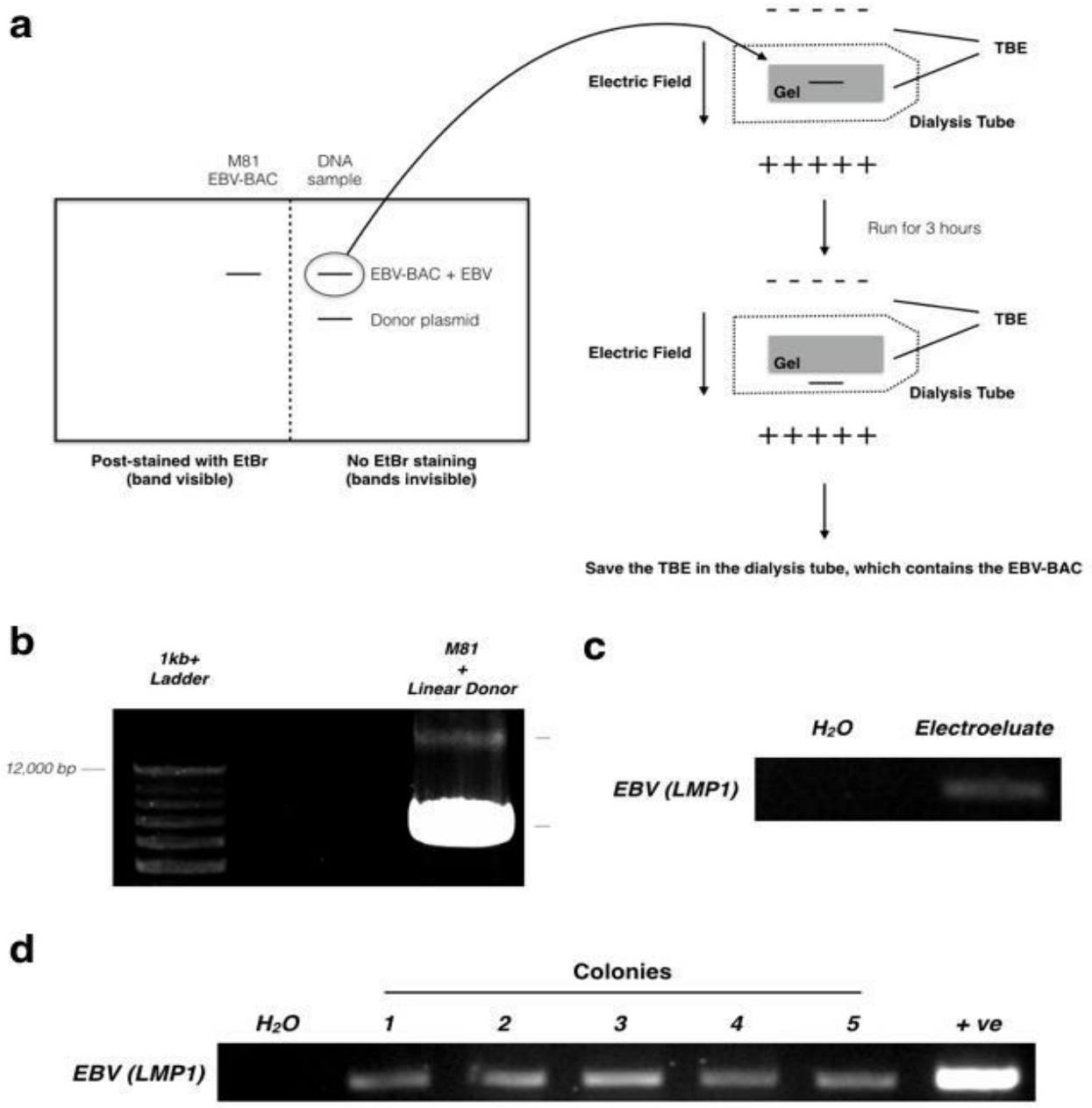


EBV linker-BAC vector

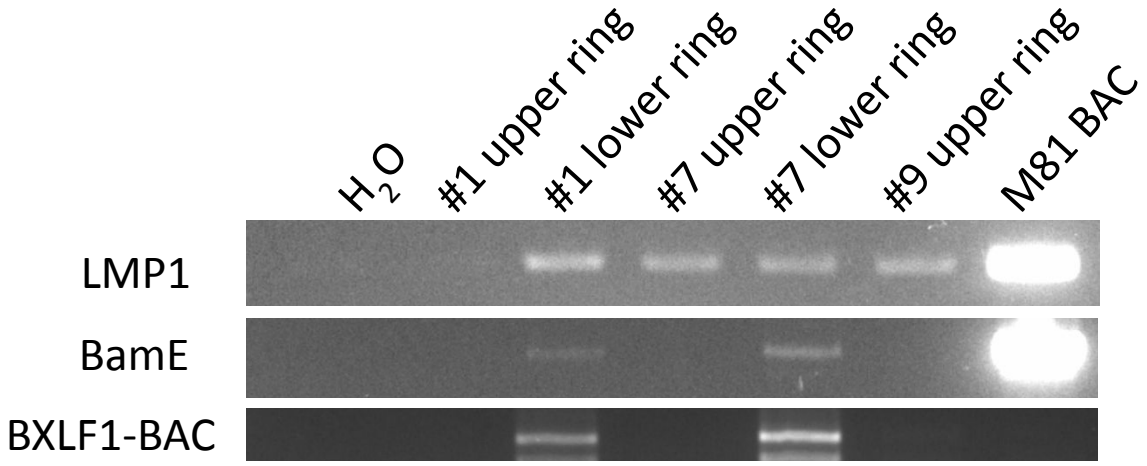
Genomic DNA PCR

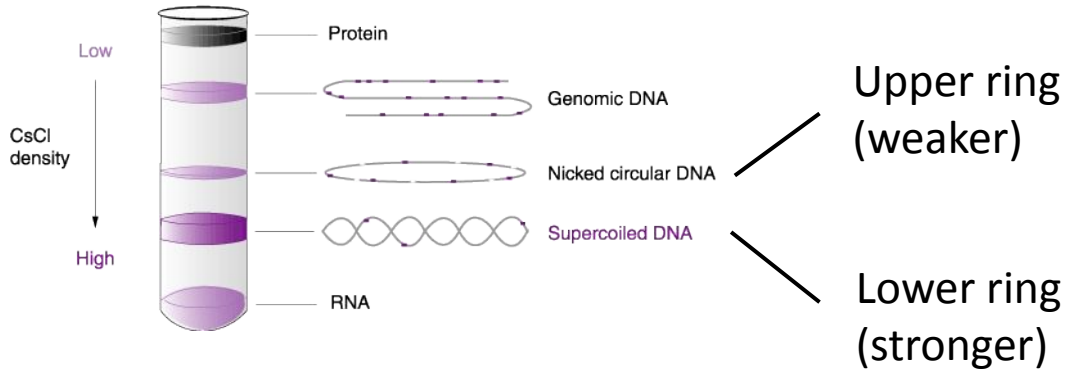


Gel purification of EBV-BAC



Cloning of NPC43 virus





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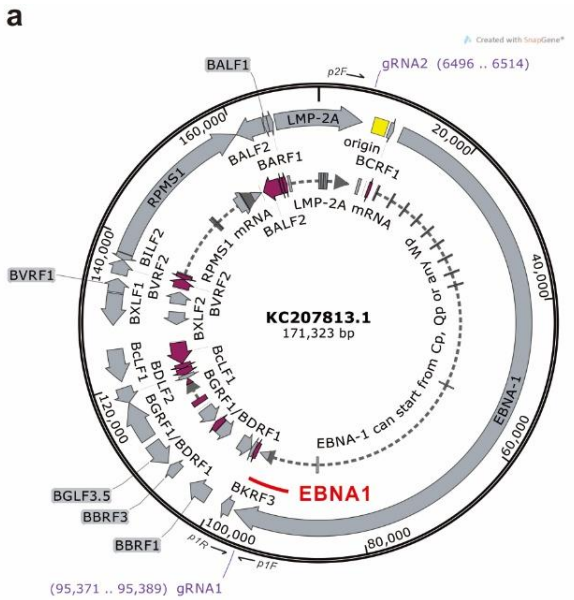
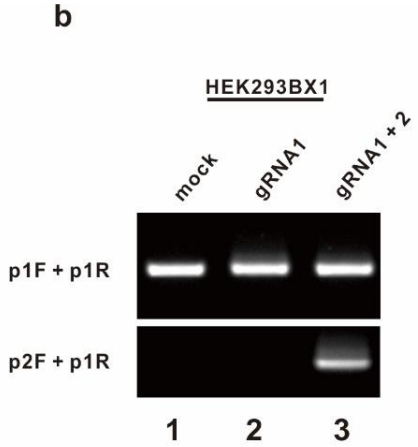
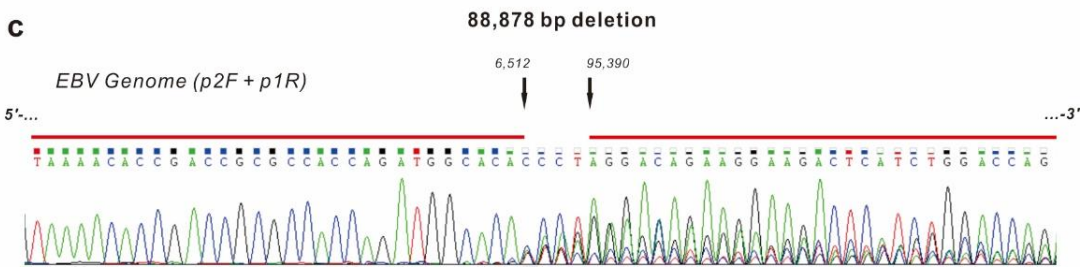
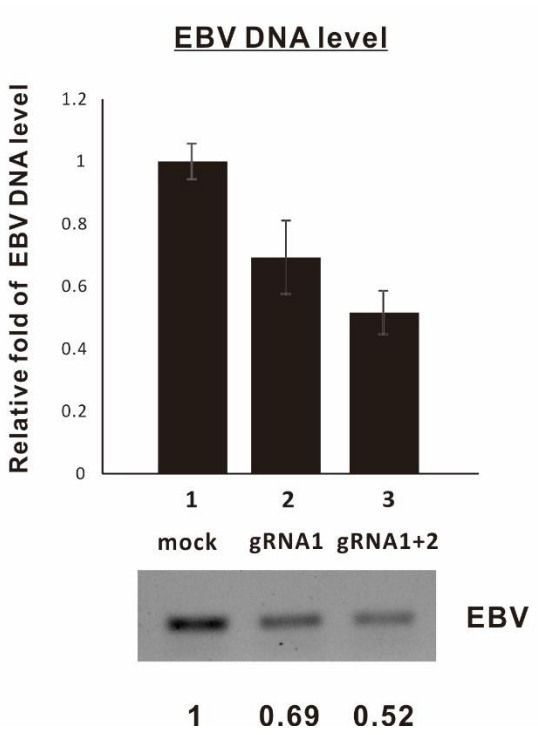
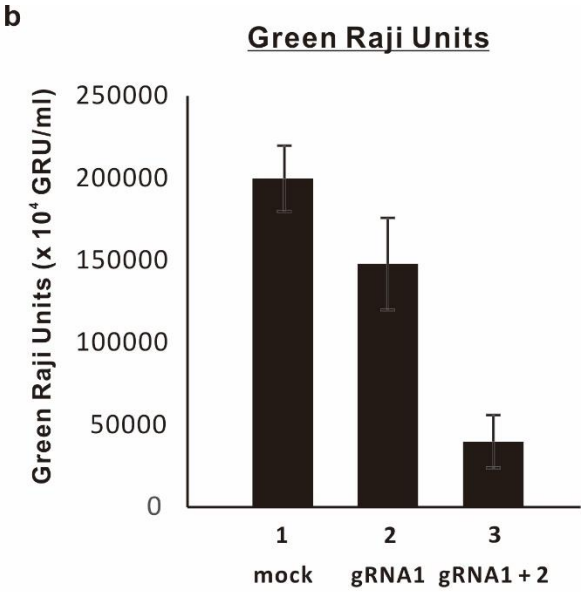
Suppression of EBV through CRISPR/Cas9 targeting of EBNA1



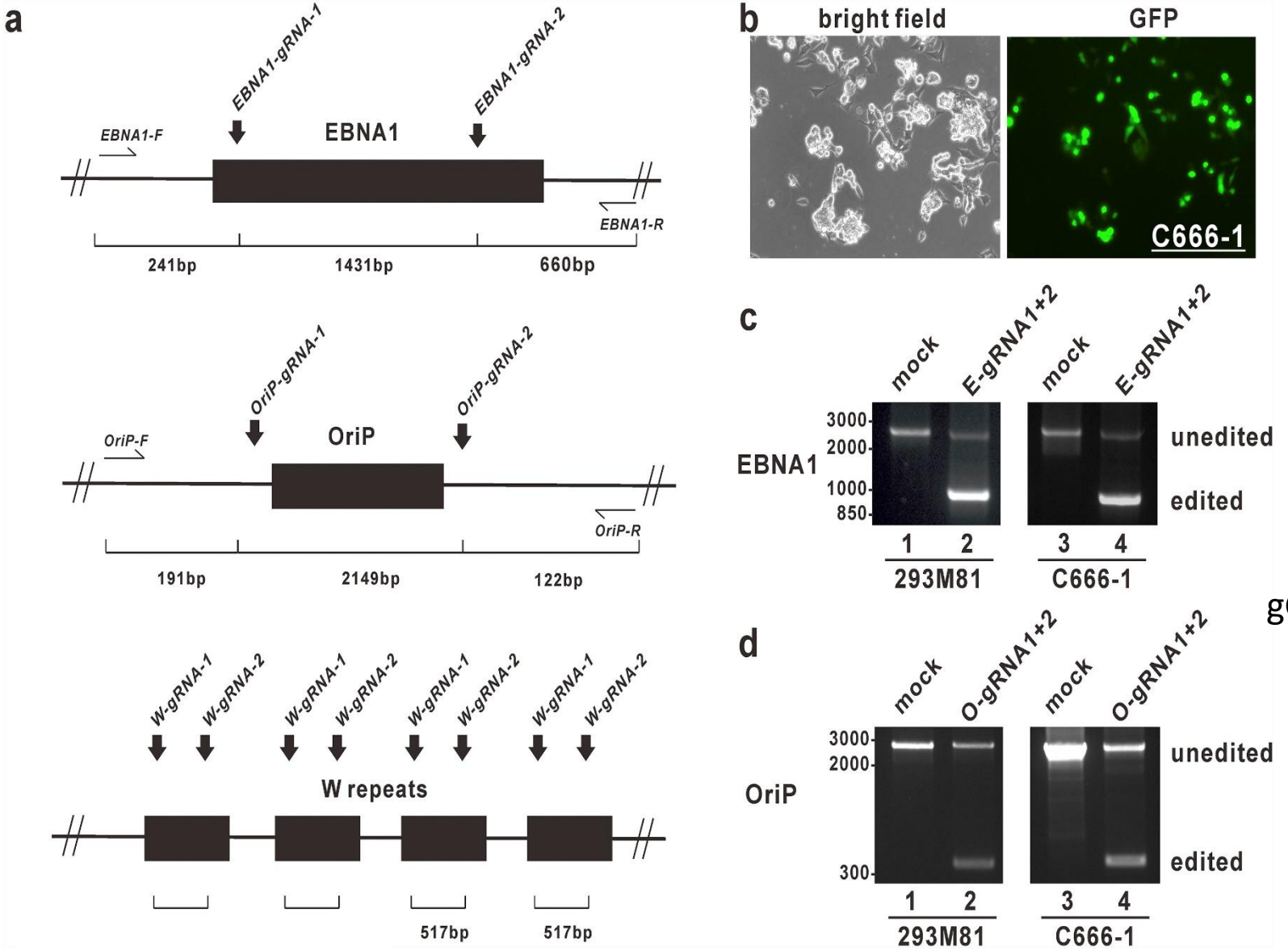




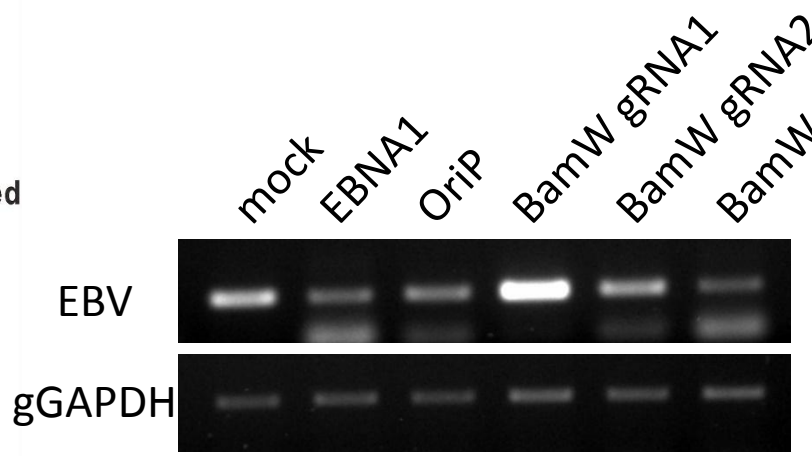




Targeting of EBNA1, oriP, W repeat region reduced EBV productio

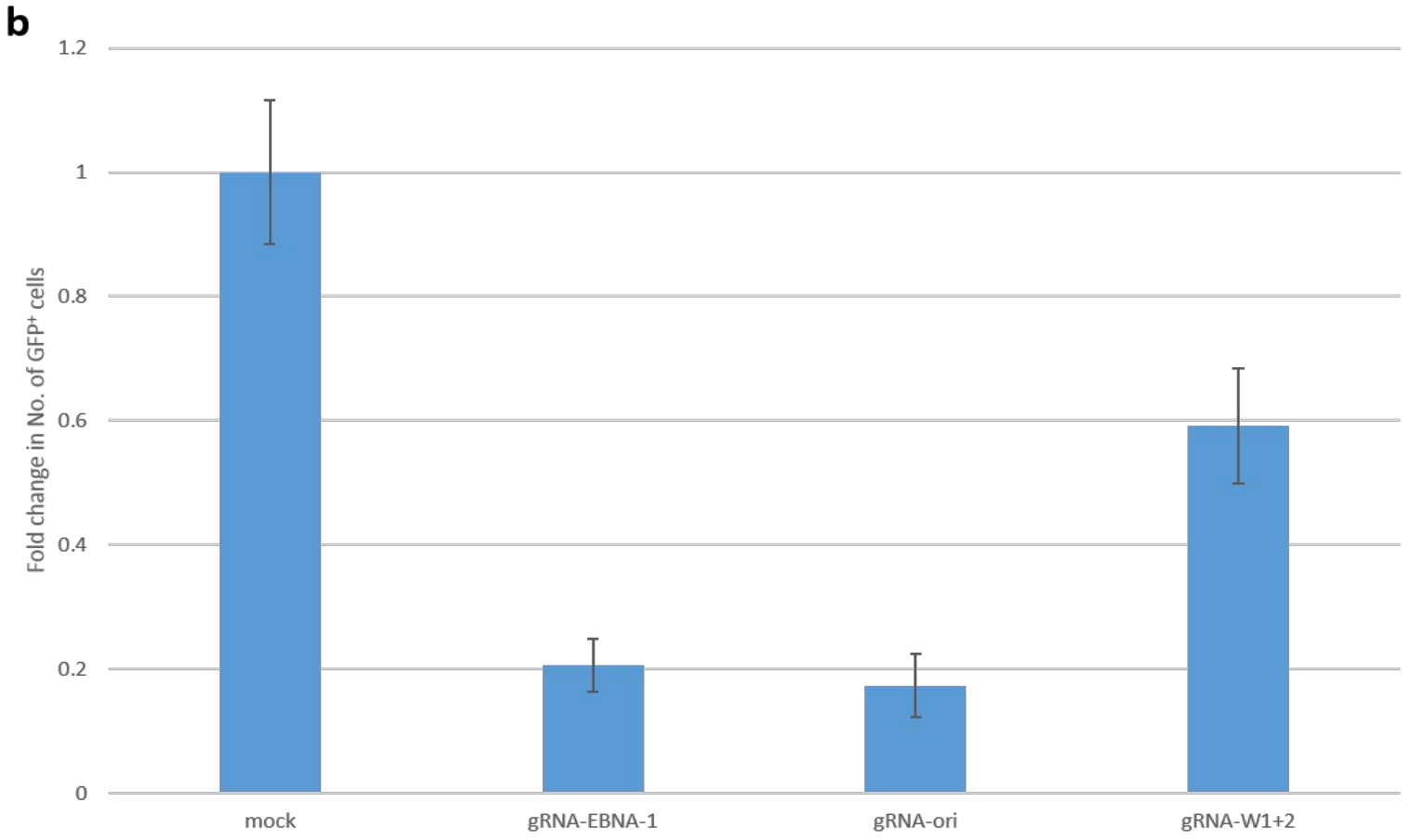


HEK293M81



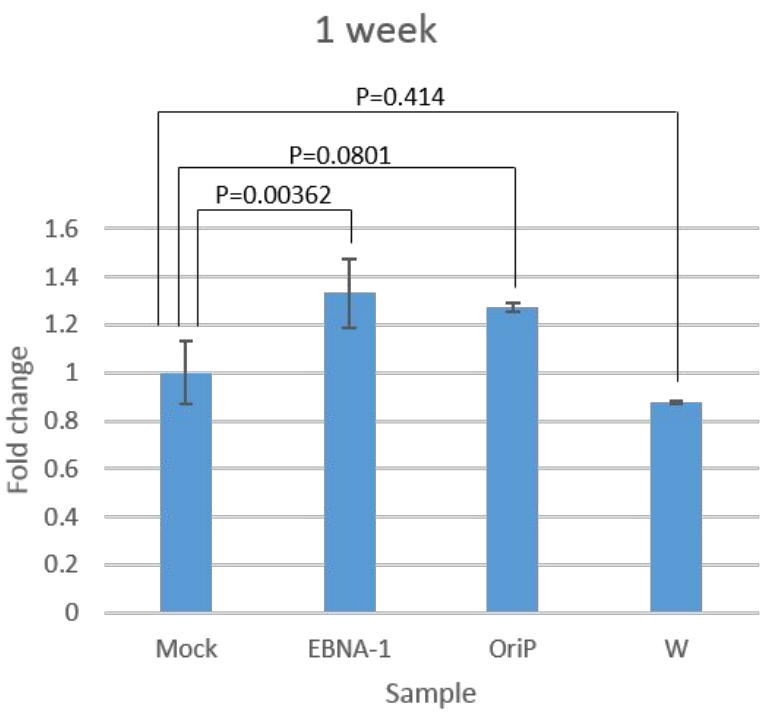
M81 EBV DNA in supernatant

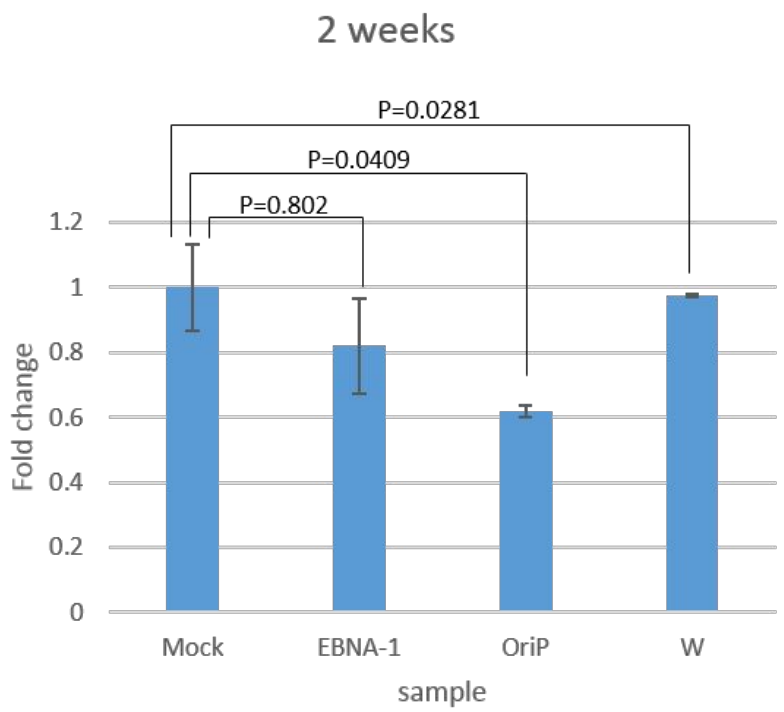
Targeting of EBNA1, oriP, W repeat region inhibited EBV infection in HEK293 cells

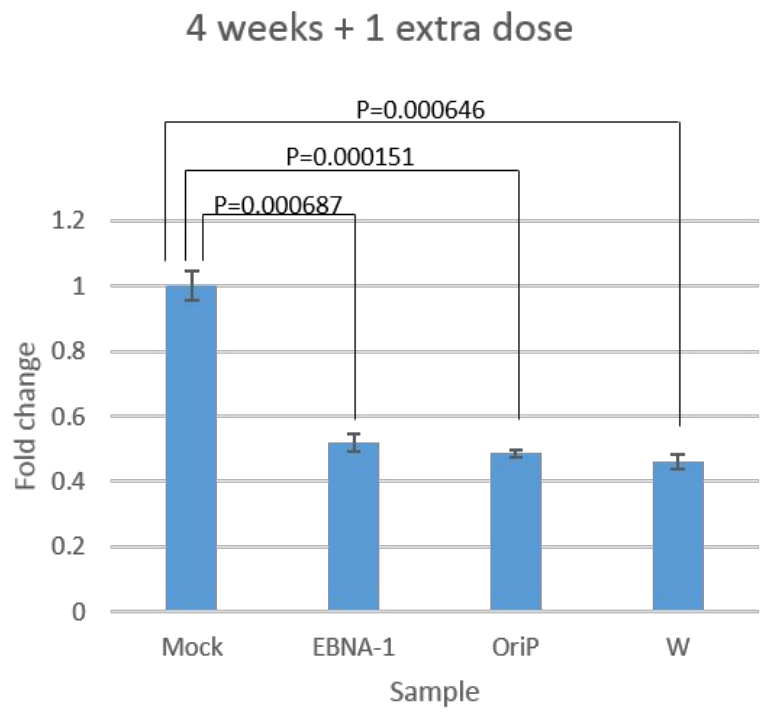


Infectivity assay measured by flow cytometry

Targeting of EBNA1, oriP, W repeat region inhibited EBV infection in HEK293 cells

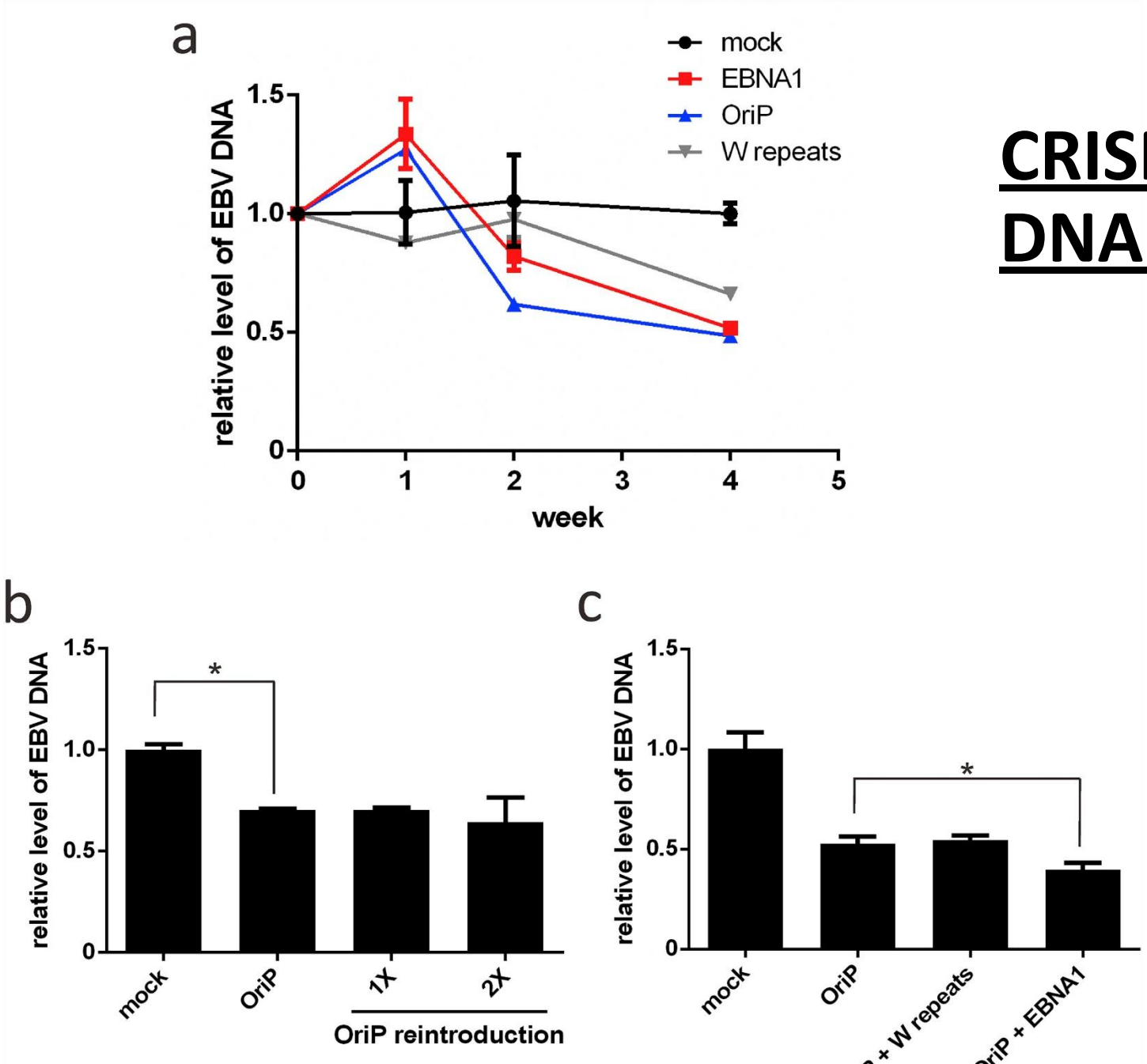




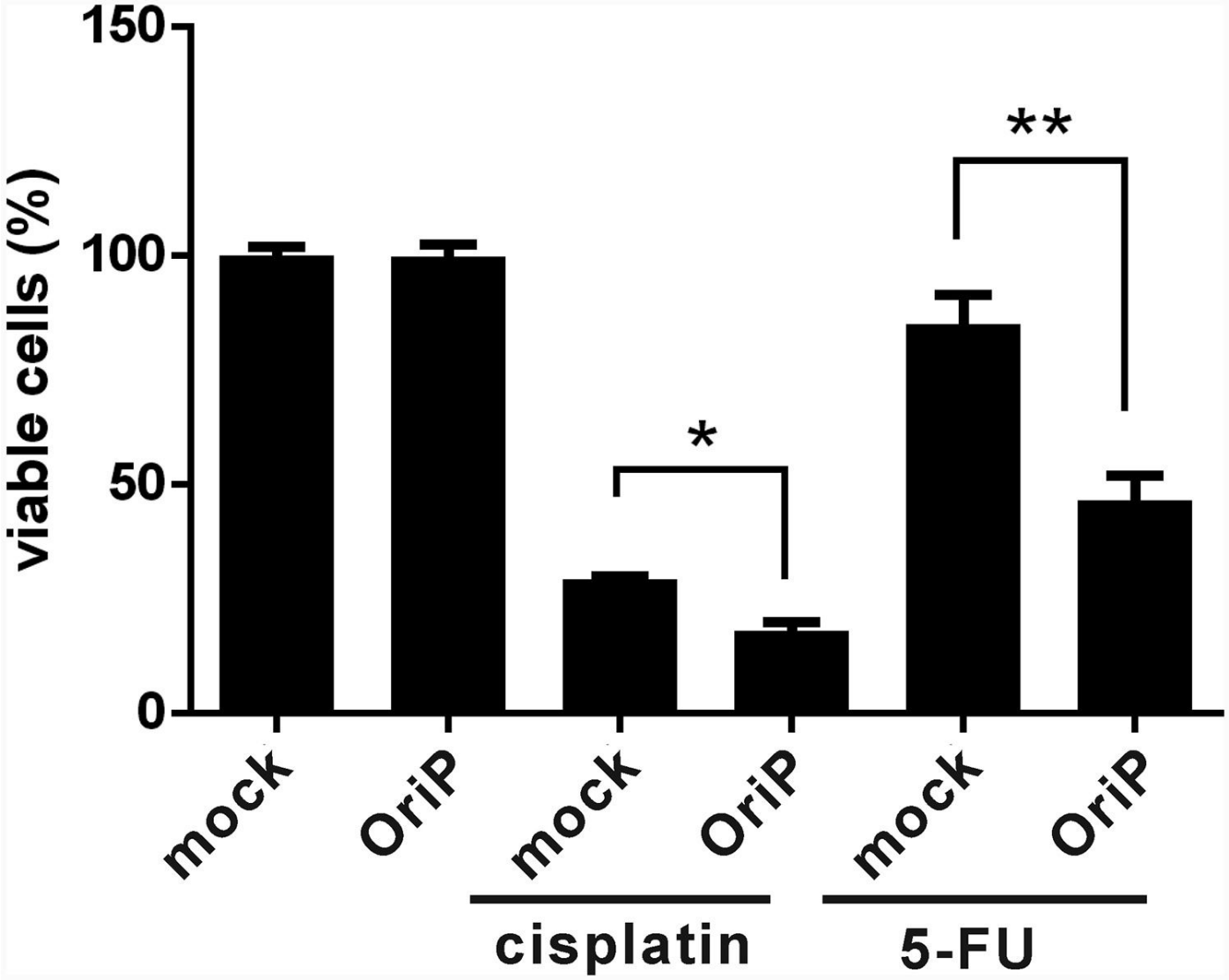


EBV genome copy numbers were measured by Taqman-qPCR

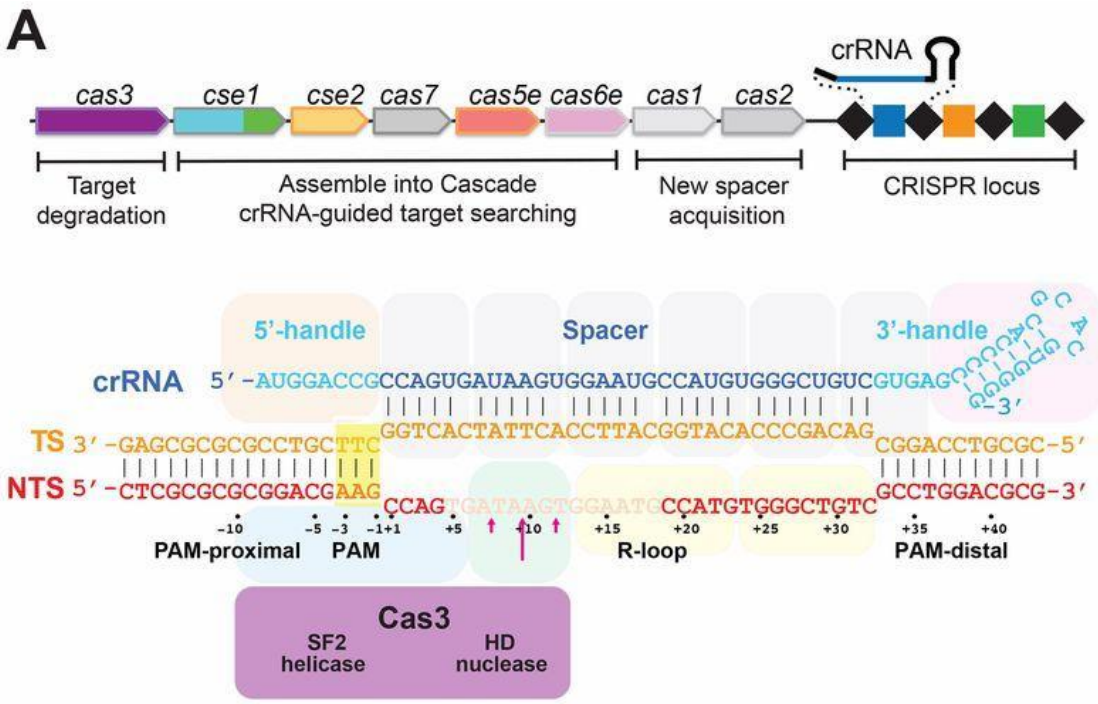
CRISPR suppression of EBV DNA load in C666-1 cells

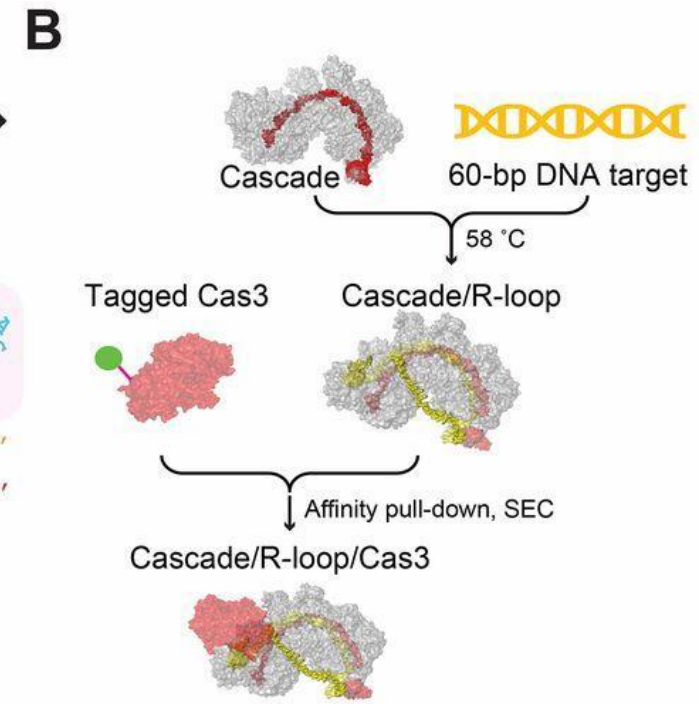


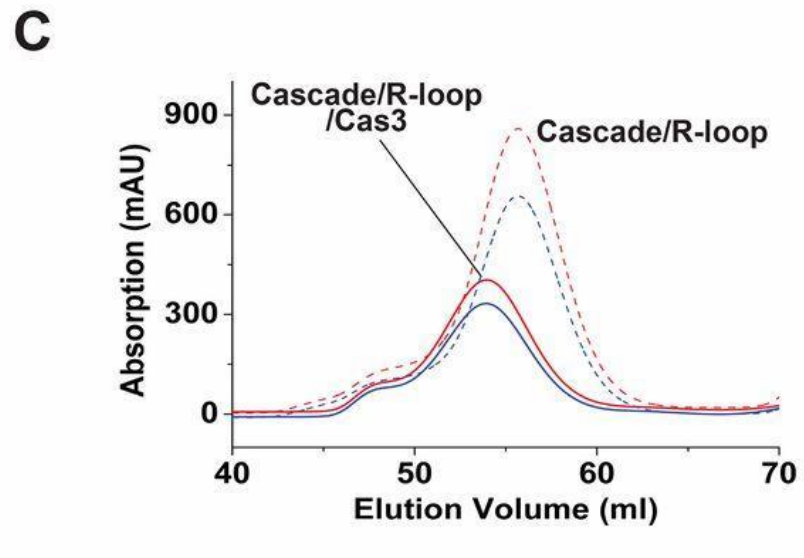
EBV targeting sensitizes C666-1 cells to killing by cisplatin and 5-FU

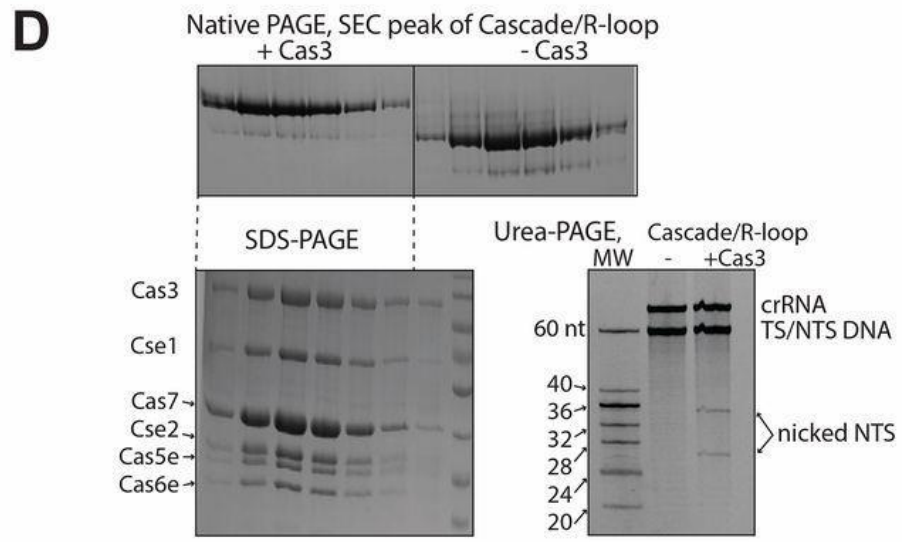


Cas3: RNA-guided DNA degradation









CRISPR applications in EBV research and therapy

1. Mutant construction for genetic study and vaccine development

2. Marker insertion and rapid cloning of EBV

3. Eradication of EBV from infected cells

4. CRISPR screening for host restriction/dependency factors

5. CRISPRa activation of EBV gene expression

CRISPR Libraries

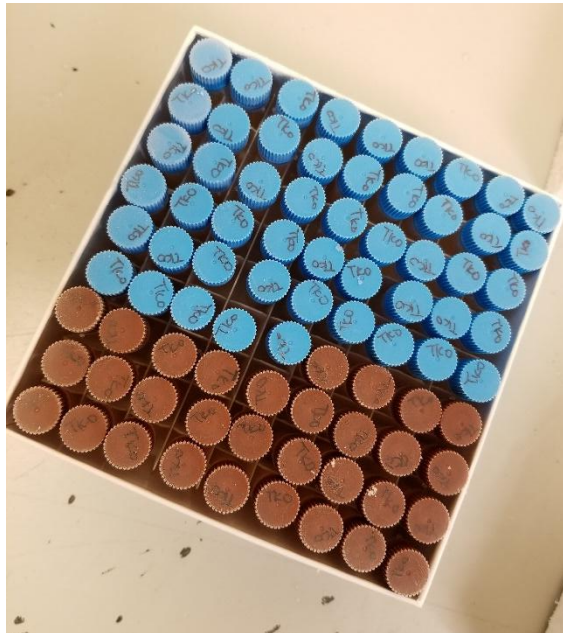
Toronto KnockOut (TKO) CRISPR Library - version 3

- 70,948 guides (4 guides/gene) targeting 18,053 protein-coding genes
- Lentiviral library (Puromycin resistance)

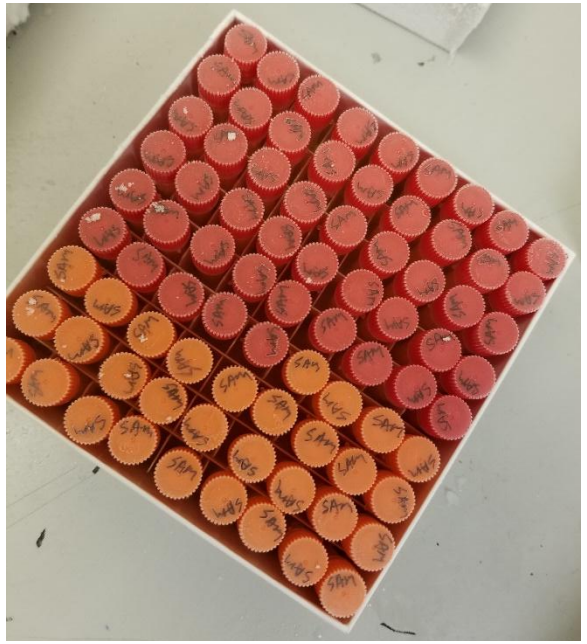
Human CRISPR Activation Library (SAM - 2 plasmid system)

- 70,290 guides targeting 23,430 protein-coding genes
- lenti-SAMv2 (contains dCas9-VP64 and sgRNA, Blasticidin resistance)
- lenti-MPHv2 (contains MS2-P65-HSF1 activator helper, Hygromycin resistance)

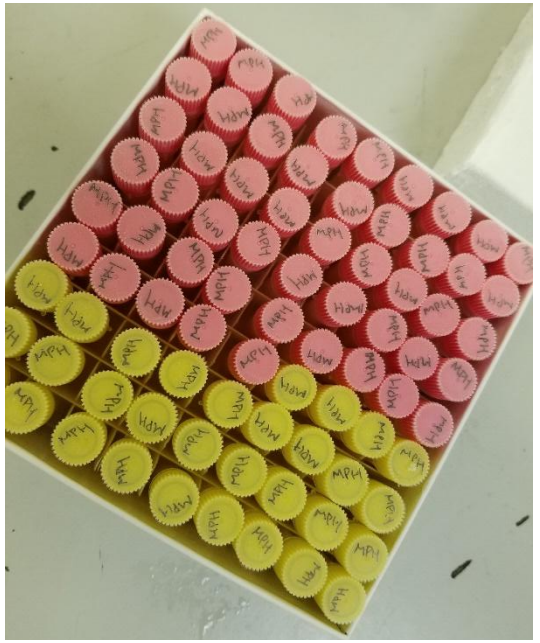
Our CRISPR library stocks



Lenti-TKO
Puromycin resistance
Titer: ~10^5 per ml
Stocks: 1ml per stock



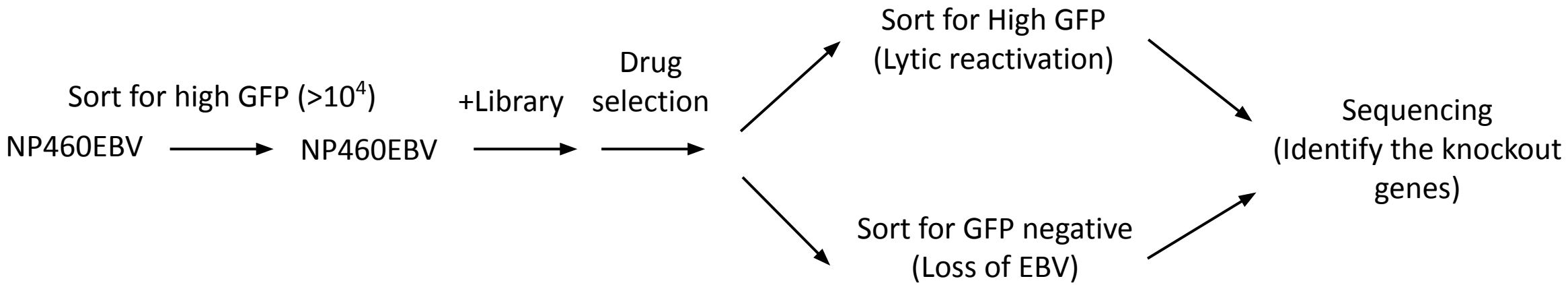
Lenti-SAMv2
Blasticidin resistance
Titer: ~10^5 per ml
Stocks: 1ml per stock



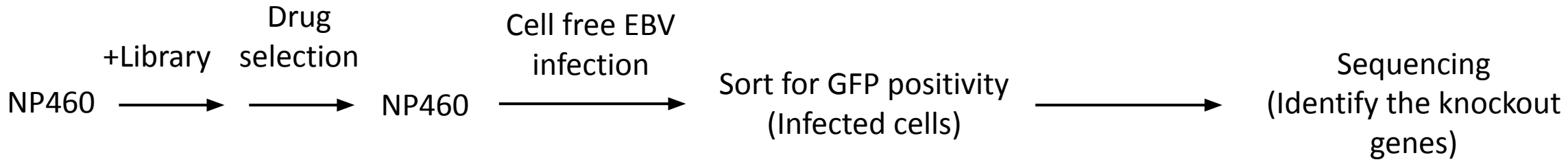
Lenti-MPHv2
Hygromycin resistance
Titer: ~10^5 - 10^6 per ml
Stocks: 1ml per stock

Rationale of experiments

Screening factors for EBV maintenance:



Screening factors for EBV entry:



TKO library screening for maintenance factors

Sort for high GFP (>10^4)

NP460EBV → NP460EBV → +Library → Drug selection

2 days 7 days

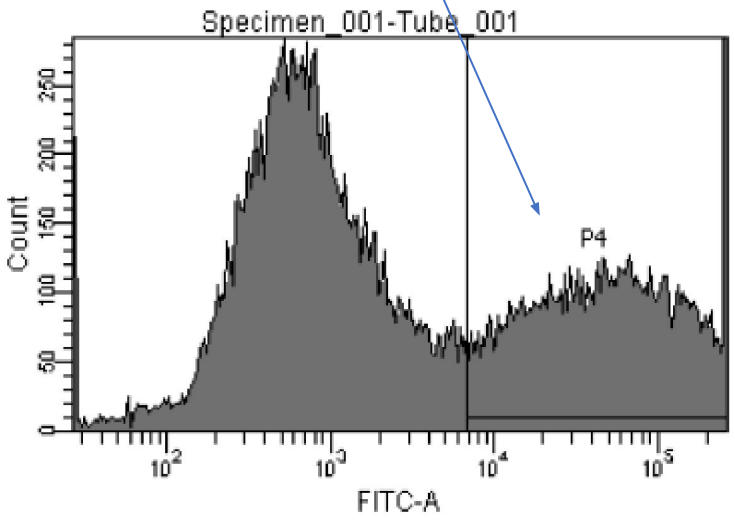
Sort for High GFP

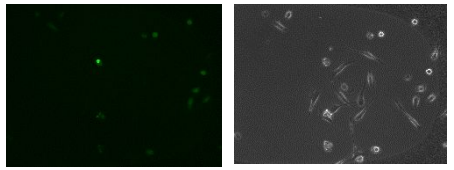
3 days

Sequencing (Identify the knockout genes)

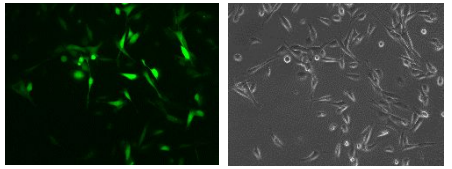
Sort for GFP negative

3 days

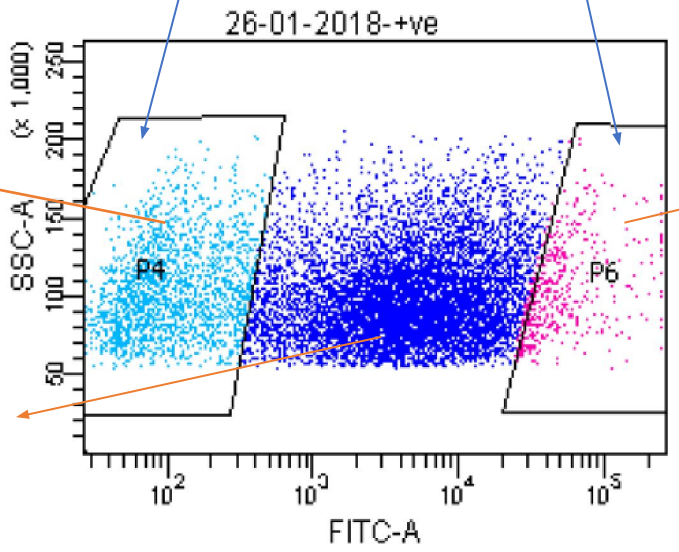


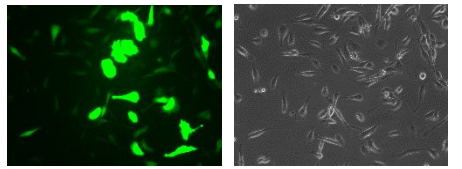


Low GFP

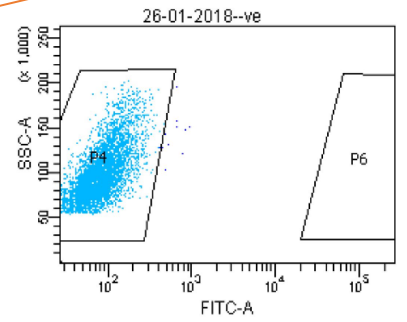


Normal GFP





High GFP



EBV –ve NP460

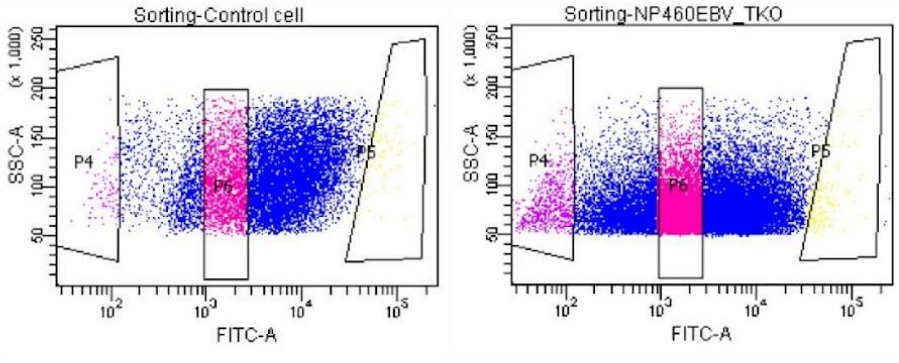
TKO library screening results for NP460EBV

Low GFP	Candidate genes	Reads (out of 45)
IL37	interleukin 37	5
CXorf22	Cilia And Flagella Associated Protein 47	4
C1orf101	Catsper Channel Auxiliary Subunit Epsilon	3
TOPBP1	DNA Topoisomerase II Binding Protein 1	2
SEL1L	SEL1L ERAD E3 Ligase Adaptor Subunit	2
SGCG	Sarcoglycan Gamma	1
FZD2	Frizzled Class Receptor 2	1
TMEM238	Transmembrane Protein 238	1

High GFP	Candidate genes	Reads (out of 45)
PHACTR3	Phosphatase And Actin Regulator 3	3
EN2	Engrailed Homeobox 2	3
SLC25A46	Solute Carrier Family 25 Member 46	3
GALT	galactose-1-phosphate uridylyltransferase	2
ANTXR2	Anthrax Toxin Receptor 2	2
SERPINA1	Serpin Peptidase Inhibitor, Clade A	1
NCBP2L	Nuclear Cap Binding Protein Subunit 2 Like	1
PDE6D	Phosphodiesterase 6D	1
CPE	Carboxypeptidase E	1
CXCR2	C-X-C motif chemokine receptor 2	1
EPB42	Erythrocyte Membrane Protein Band 4.2	1
FXYD5	FXYD Domain Containing Ion Transport Regulator 5	1
EEF1A2	Eukaryotic Translation Elongation Factor 1 Alpha 2	1
DEPDC4	DEP Domain Containing 4	1
INTS7	Integrator Complex Subunit 7	1
EMC7	ER Membrane Protein Complex Subunit 7	1
CAGE1	Cancer Antigen 1	1

TOPBP1 is an EBV dependency factor identified by CRISPR screening

a



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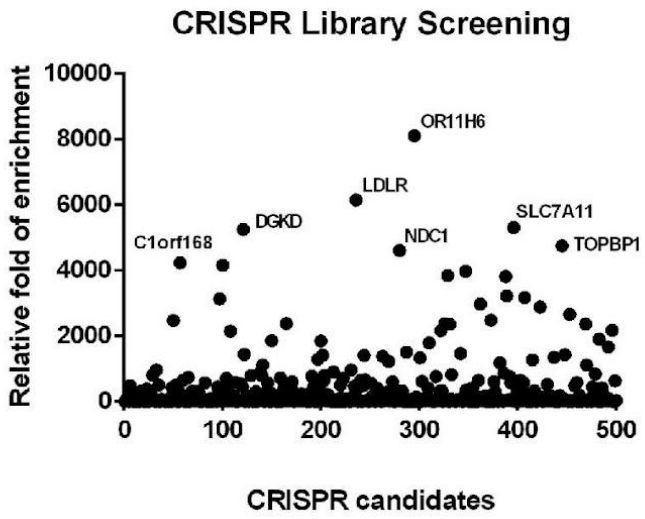
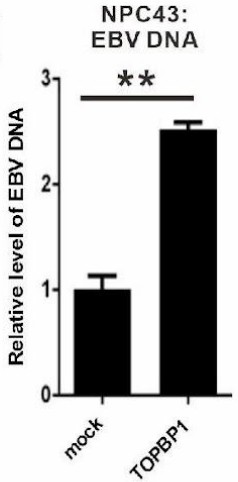


c

Genes	Relative enrichment
OR11H6	8115
LDLR	6152
SLC7A11	5312
DGKD	5251
TOPBP1	4755
NDC1	4611
C1orf168	4234
CPN2	4163
PYCRL	3982
POU4F2	3848
SLAIN1	3822
SLC18B1	3228
SPC24	3181
COMMD5	3138
RIOK3	2978
TBK1	2889
TSKU	2661
SAG	2485
C14orf132	2476
FOCAD	2387

d



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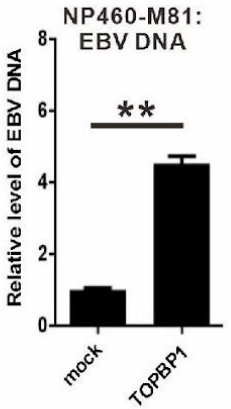

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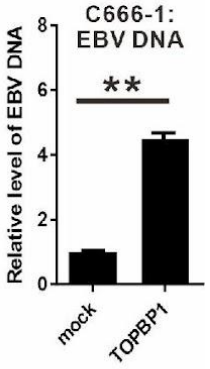
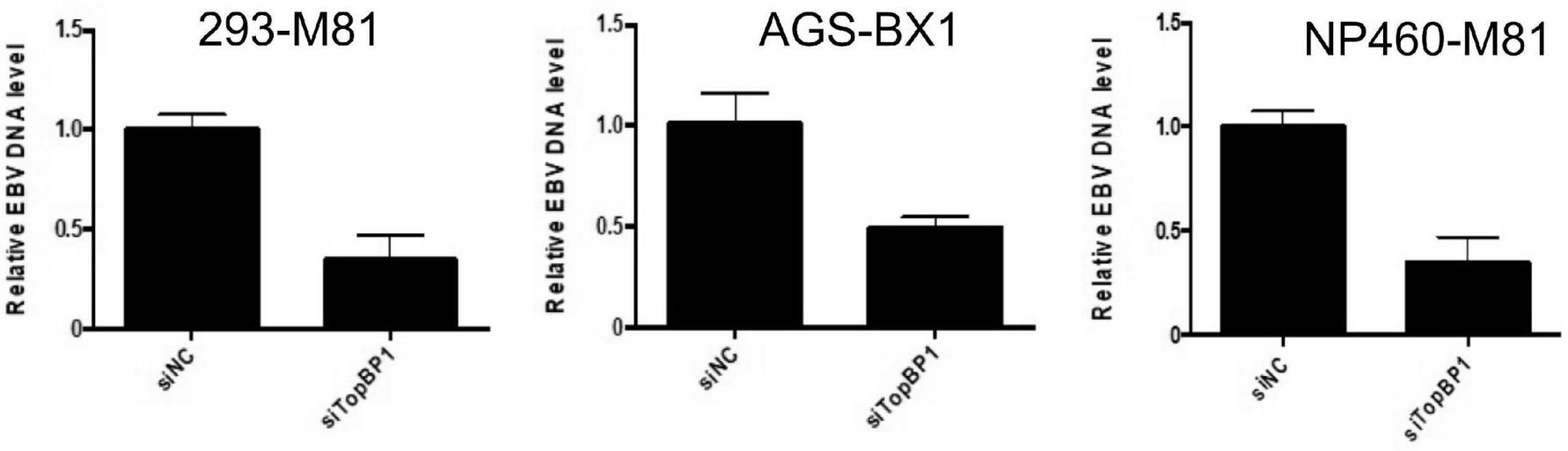
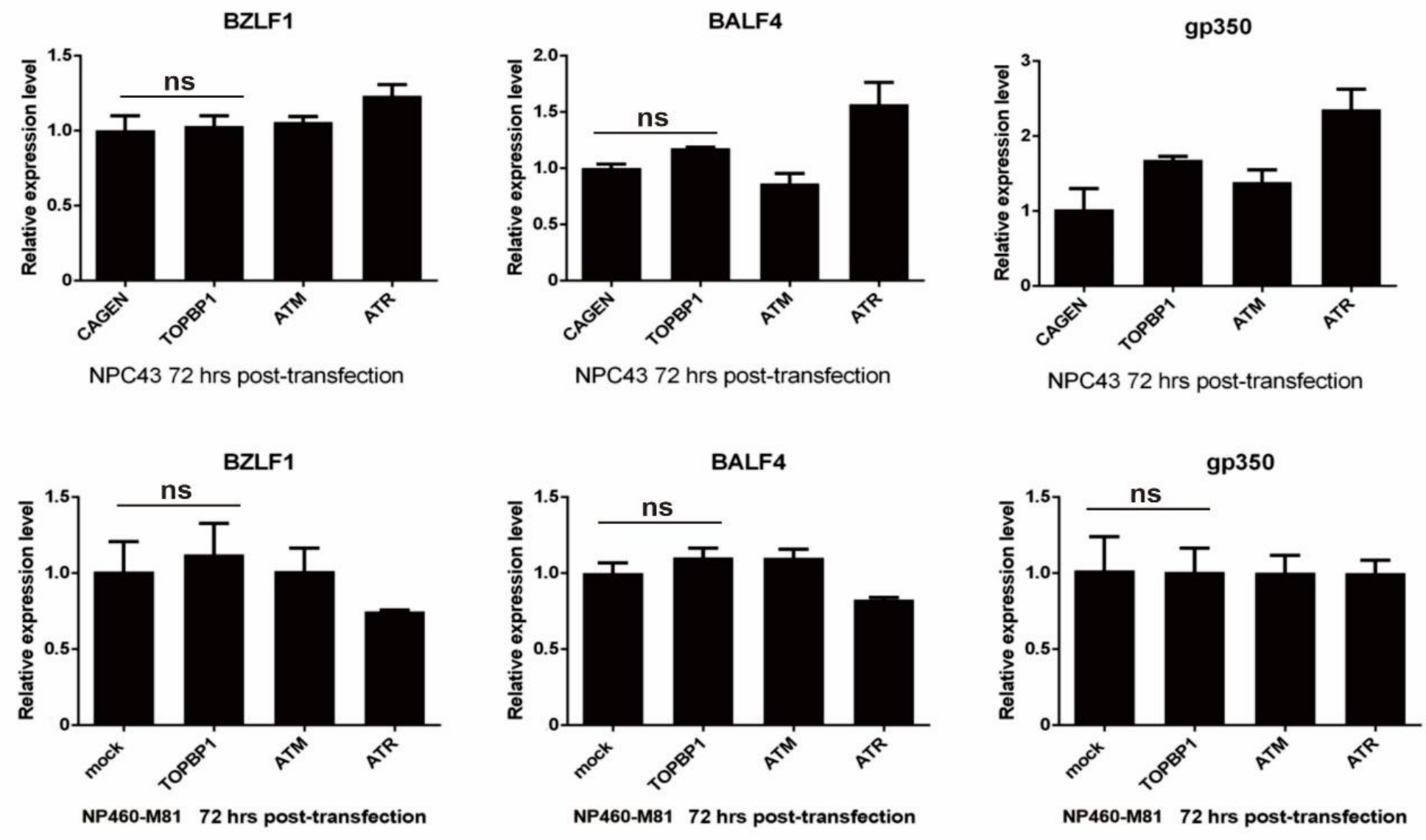


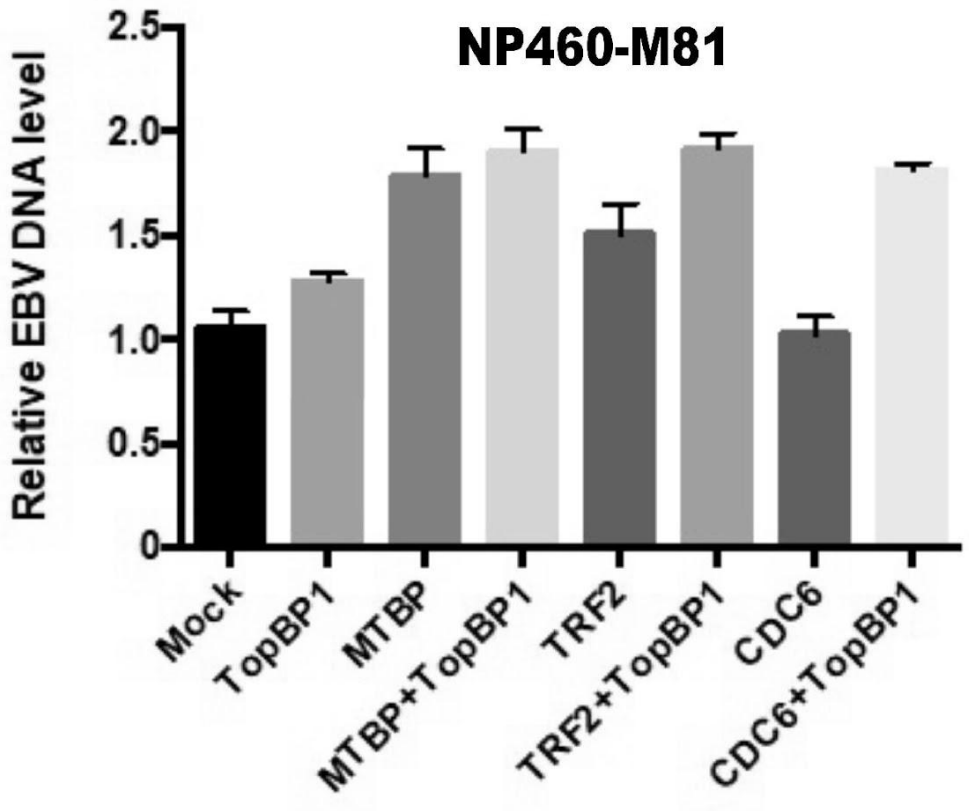
Knockdown of TOPBP1 decreases EBV DNA level

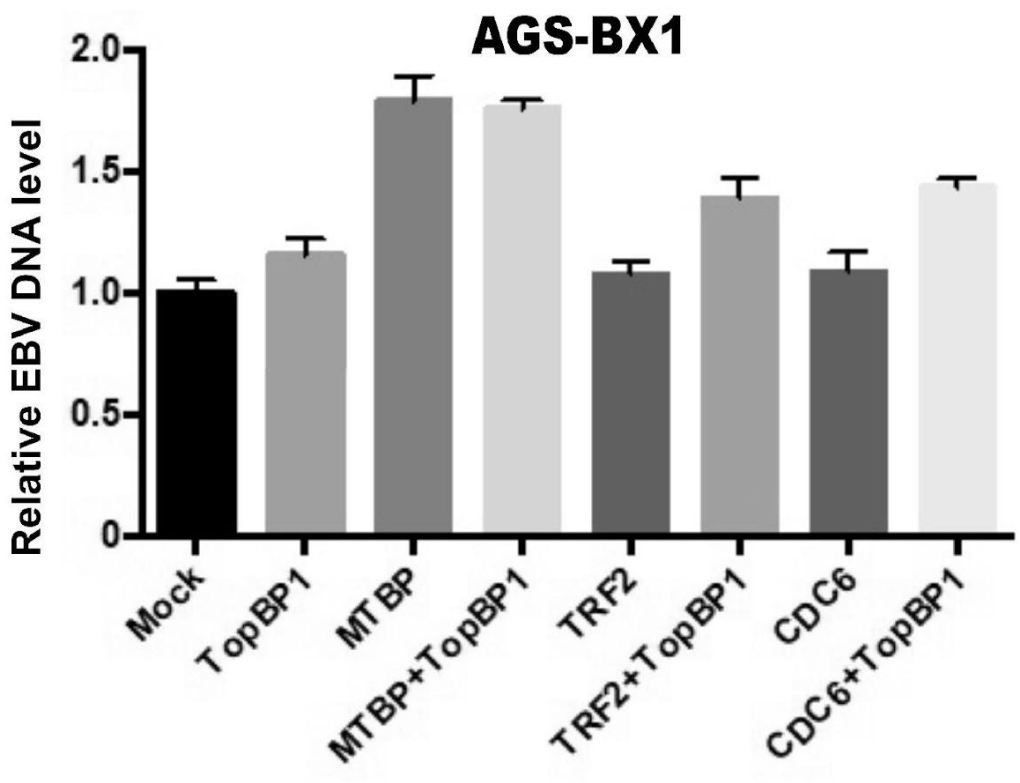


TOPBP1 does not affect lytic transcript expression

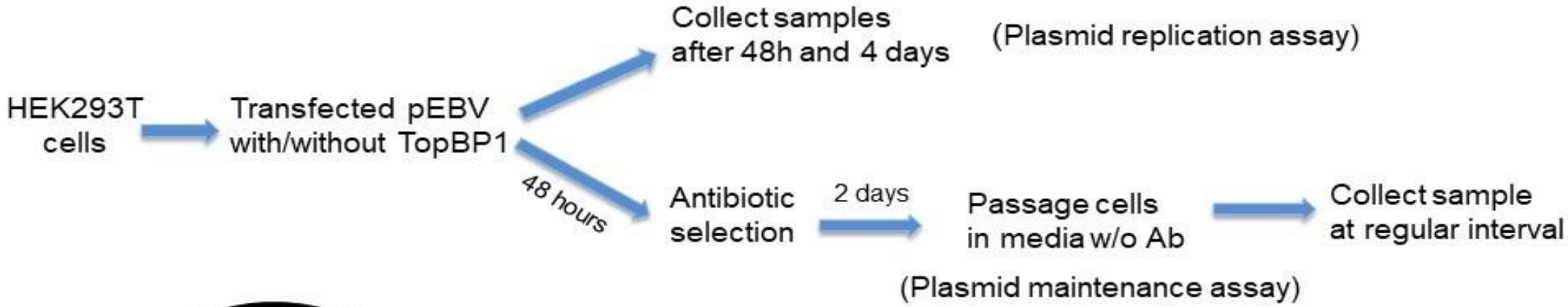


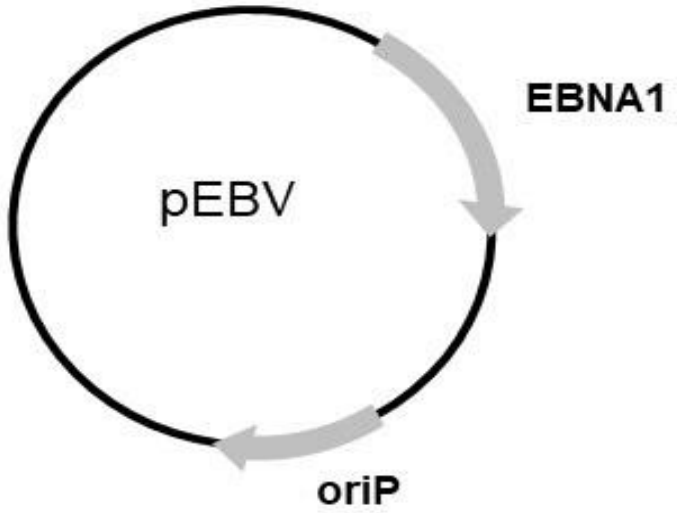
Impact of TOPBP1 and other replication proteins on EBV DNA level



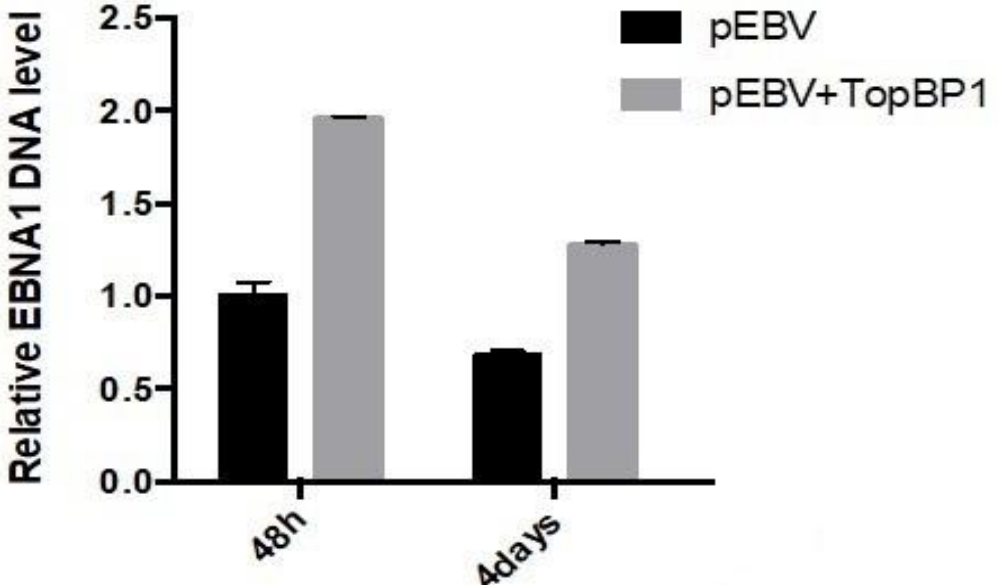


TOPBP1 facilitates oriP replication





Plasmid replication assay



Summary 4: TOPBP1 and EBV infection

- TOPBP1 is an EBV dependency factor that boosts viral DNA content.
- TOPBP1 does not affect lytic induction.
- TOPBP1 and other replication proteins boost EBV DNA level in latently infected cells.
- TOPBP1 facilitates EBV oriP replication.

CRISPR applications in EBV research and therapy

1. Mutant construction for genetic study and vaccine development

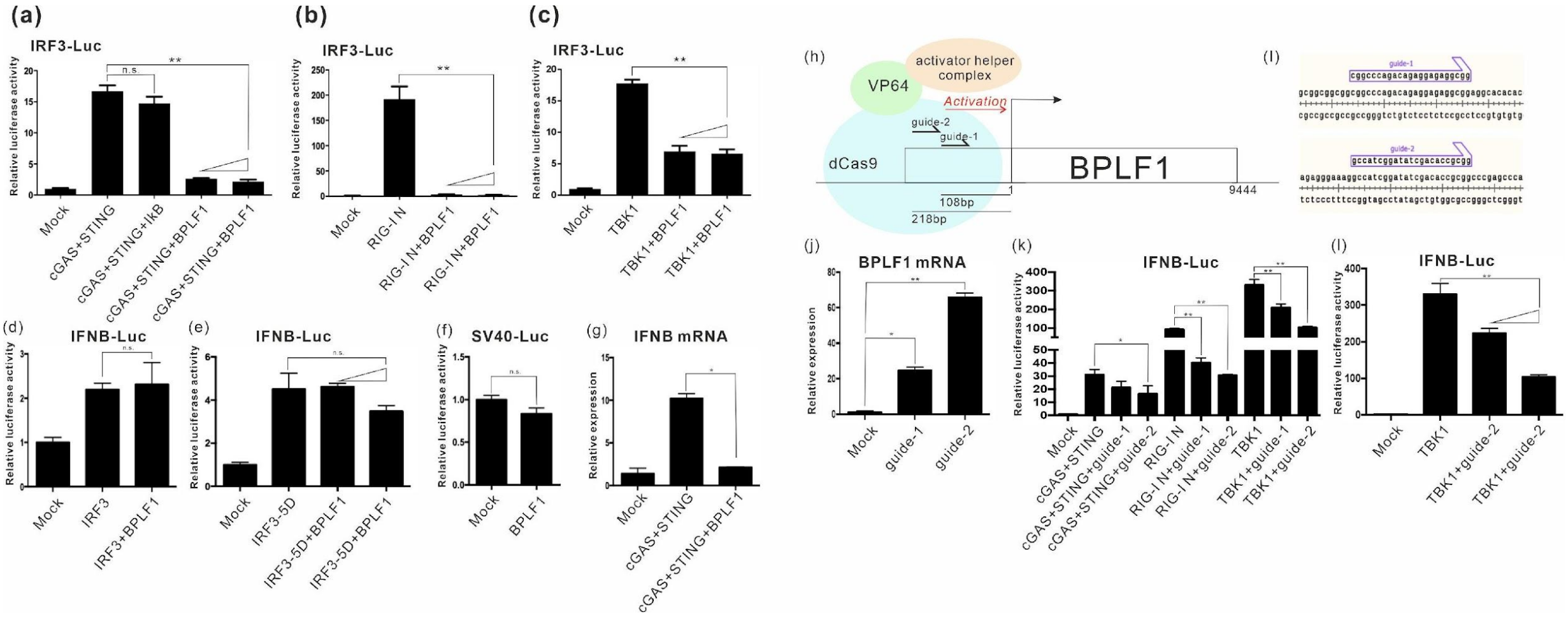
2. Marker insertion and rapid cloning of EBV

3. Eradication of EBV from infected cells

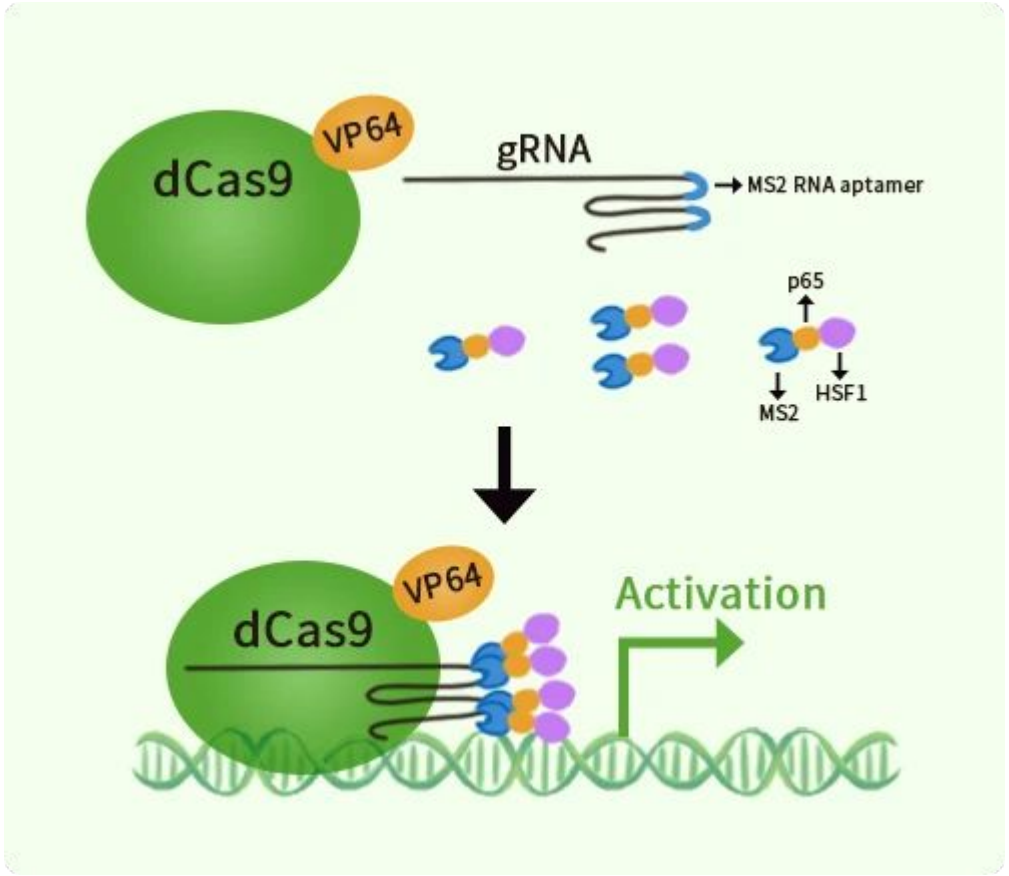
4. CRISPR screening for host restriction/dependency factors

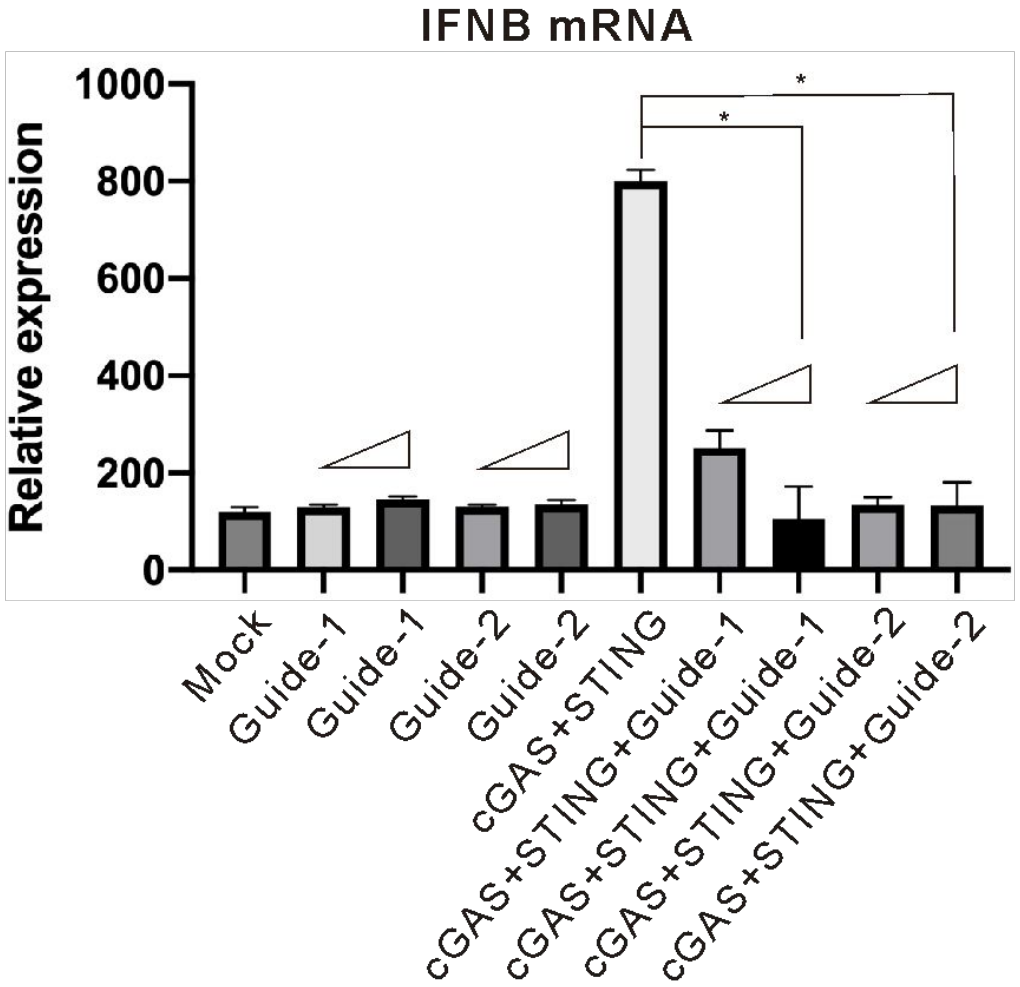
5. CRISPRa activation of EBV gene expression

Inhibition of cGAS-STING-, RIG-I- and TBK1-induced IFN-β-promoter activation by BPLF1



CRISPRa induction of BPLF1 expression in infected cells





BPLF1 is the largest EBV protein (3149 aa).

Summary

- CRISPR is a powerful tool for construction of mutant EBV.
- CRISPR can facilitate rapid cloning of EBV strains.
- CRISPR has the potential to eradicate EBV infection.
- CRISPR screening is a powerful tool for identification of EBV maintenance and restriction factors.
- CRISPRa can be used to activate EBV gene activation.

Future perspectives

- Can we make candidate vaccine strains?
- Can we identify key cellular factor for EBV infection?
- Can we engineer EBV producer cells to achieve high yields?
- Can we define the role of innate immunity in EBV infection and persistence?
- Can we rationally design and develop new anti-EBV agents?

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