Current Progress in Xenotransplantation

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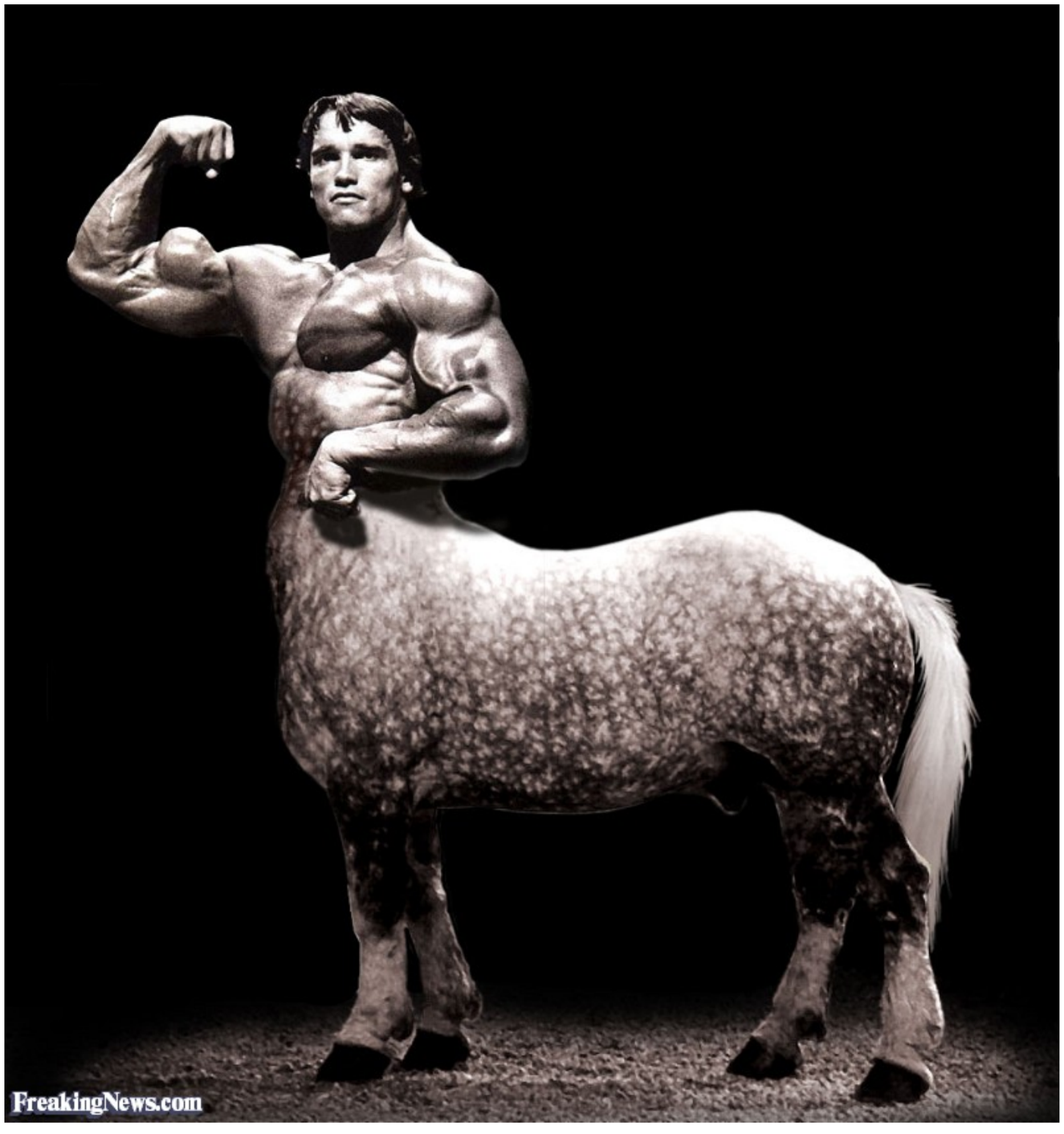
Special Series on Laboratory Animal Science

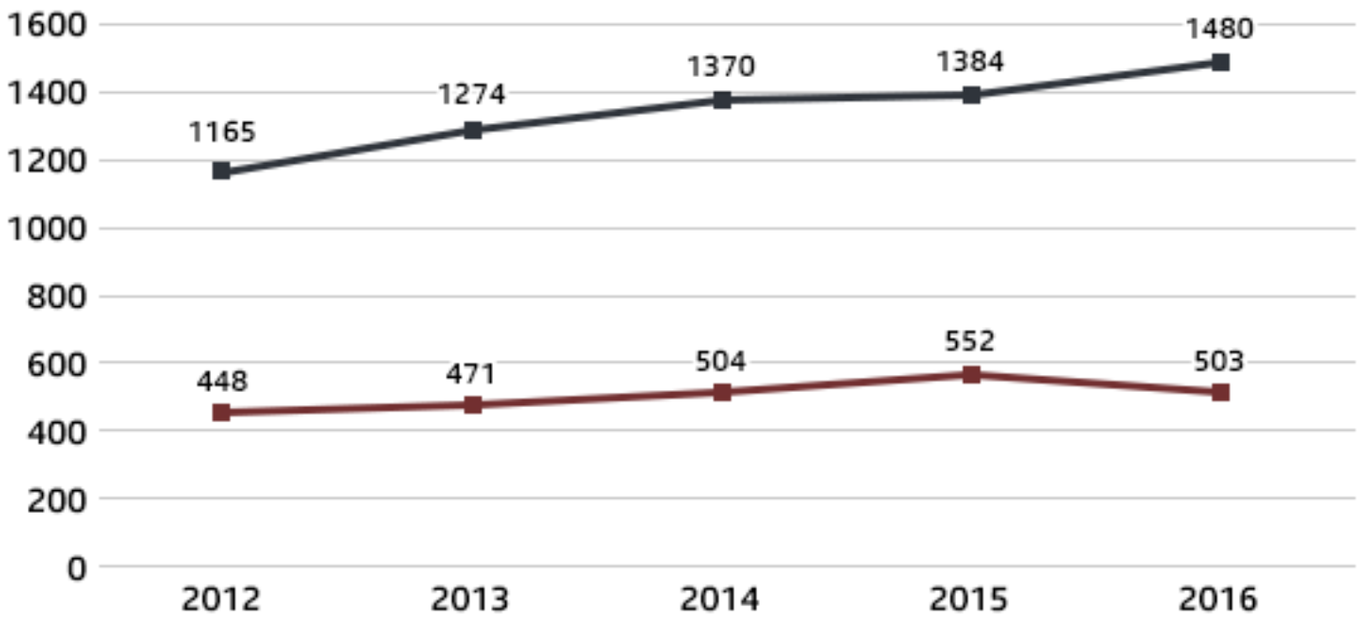
What is xenotransplantation?

- the process of grafting or transplanting organs or tissues between members of different species
  - whole, solid organs, e.g. kidney, liver, heart …
  - tissues, e.g. skin
  - specialised cells, e.g. pancreatic islet cells

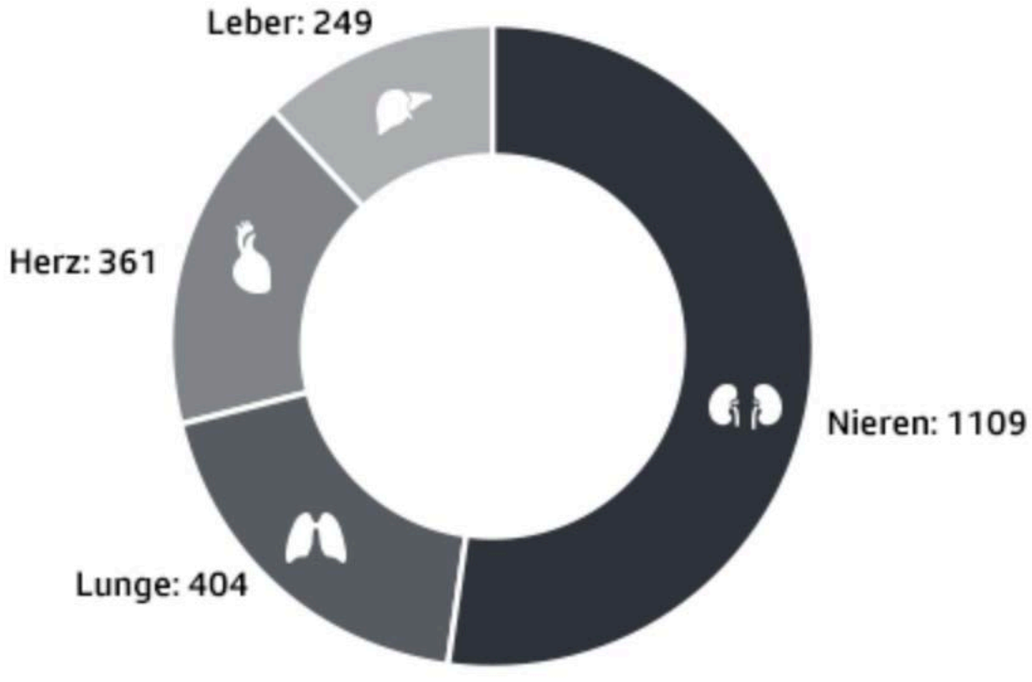


http://www.freakingnews.com/Celebrities-Horses-Pictures--639.asp

Why xenotransplantation?



mean waiting time per organ [days]



www.swisstransplant.org

Pig organs for xenotransplantion

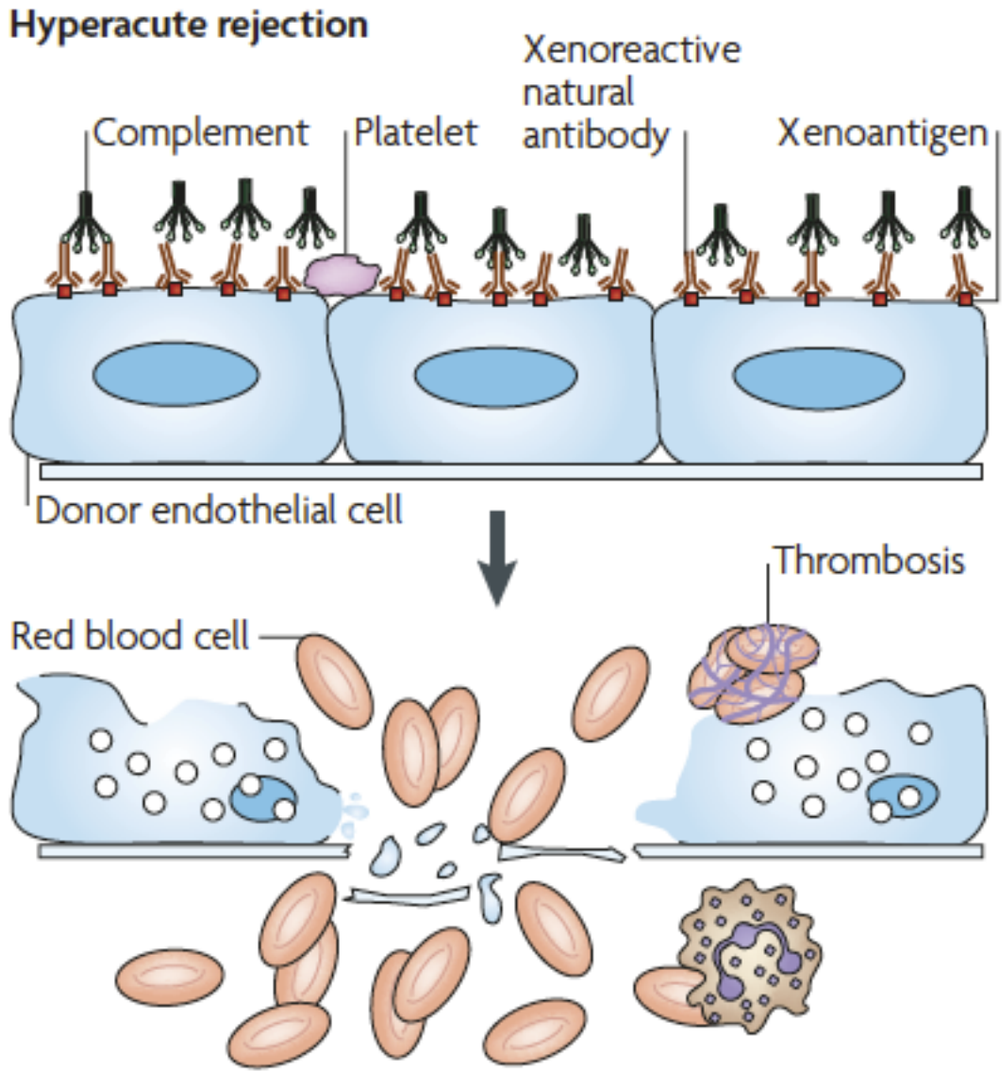
- The pig is considered to date the most appropriate candidate species for humans as recipients due to anatomical similarity, physiological compatibility, breeding characteristics and for ethical reasons
- Hence, ongoing pre-clinical research is concentrating on pigs as donors and nonhuman primates as recipient species

http://todaysimpact.org/scientists-make-part-human-part-pig

Challenges in Xenotransplantation

Xenograft rejection: Hyperacute rejection (HAR)

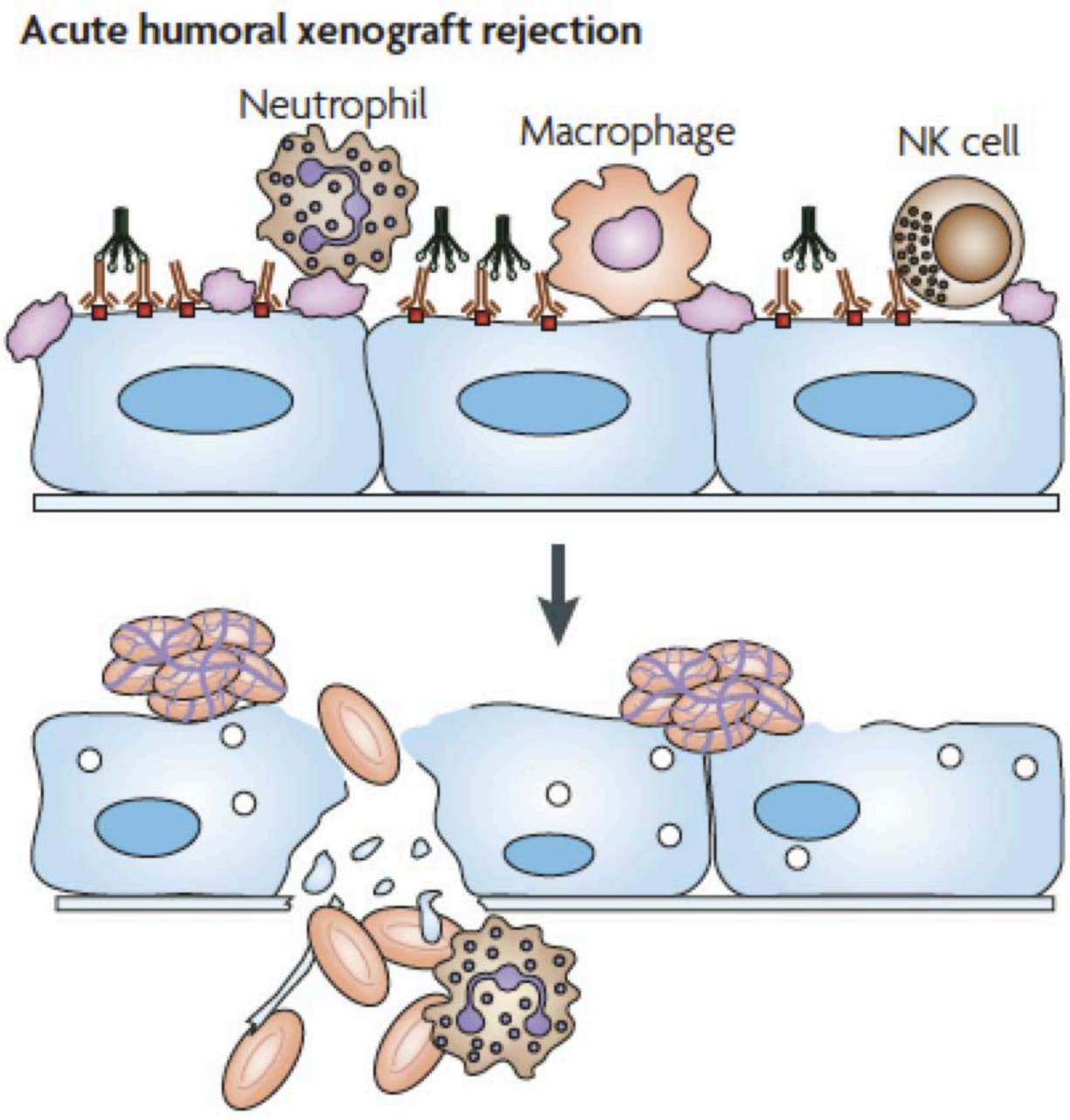
- pre-existing antibodies binding to xenograft antigens prompt complement activation, graft endothelial cell activation and destruction and graft rejection within minutes to hours
- the antigen is believed to be the terminal α3-galactose of the N-acetyllactosamine in glycoprotein and glycolipid carbohydrate chains („αGal epitope“)
- the enzyme responsible for the production of the αGal epitope, α1-3 galactosyltransferase (αGalT), is not active in humans
- allegedly, very high prevalence of αGal autoantibodies in humans (1-8% of total IgM, 1-2.4% of total IgG)



Yang & Sykes, Nat. Rev. Immunol. 2007

Xenograft rejection: acute humoral xenograft rejection (AHXR)

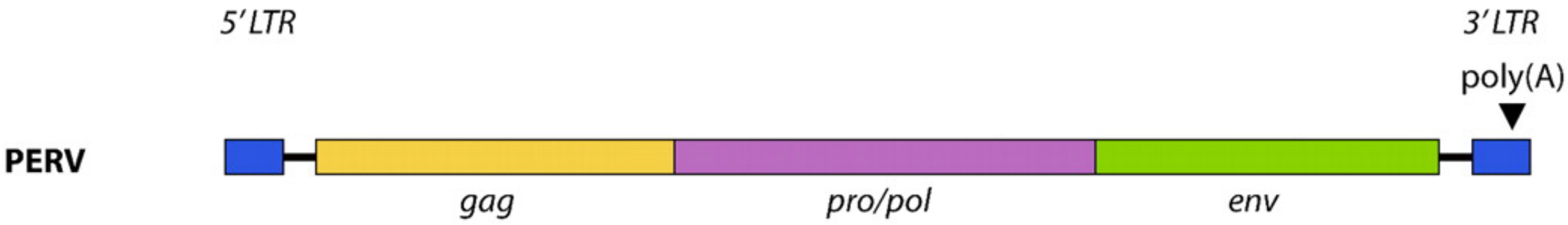
- if HAR can be prevented, AHXR can occur days to weeks after xenotransplantation
- AHXR also occurs in xenotransplants from αGalT-deficient pigs or in the presence of low αGal-antibody levels



Yang & Sykes, Nat. Rev. Immunol. 2007

The role of PERVs in xenotransplantation

- Porcine endogenous retroviruses (PERVs)



modified from: Denner & Tönnjes, Clin. Microb. Rev.

- PERV-A and PERV-B can infect human cells, while PERV-C is restricted to pig cells (recombinated PERV-C can, however, infect human cells)
- PERV mRNAs are expressed in all swine tissues of all swine breeds tested to date
- PERVs infect some nonhuman primate cells, but do not replicate in them (as opposed to human cells), making nonhuman primates less informative regarding PERV infections
- PERVs integrate into the hosts genome using its integrase which can be passed on to the host’s descendants -> genome editing is the only way to inactive PERVs

Clinical consequences of PERV infection in humans

- To date, no direct link between PERV infection and disease was found, clinical trials, however, have to address the potential risks of retroviral infection in the light of other zoonotic retroviruses such as
  - SIV/HIV and the AIDS pandemic
  - Human T-cell leukemia virus (HTLV) 1+2 and leukemia, immunodeficiencies and neurodegeneration (e.g. HAM/TSP)
  - Koala retrovirus (KoRV, closely related to PERV) and lymphomas and immunodeficiencies

Assessing cross-species transmission of PERV *in vivo*

THE LANCET • Vol 352 • August 29, 1998

No evidence of infection with porcine endogenous retrovirus in recipients of porcine islet-cell xenografts

Walid Heneine, Annika Tibell, William M Switzer, Paul Sandstrom, Guillermo Vazquez Rosales, Aprille Mathews, Olle Korsgren, Louisa E Chapman, Thomas M Folks, Carl G Groth

- study of 10 diabetic patients receiving porcine fetal islets between 1990-1993

Study design

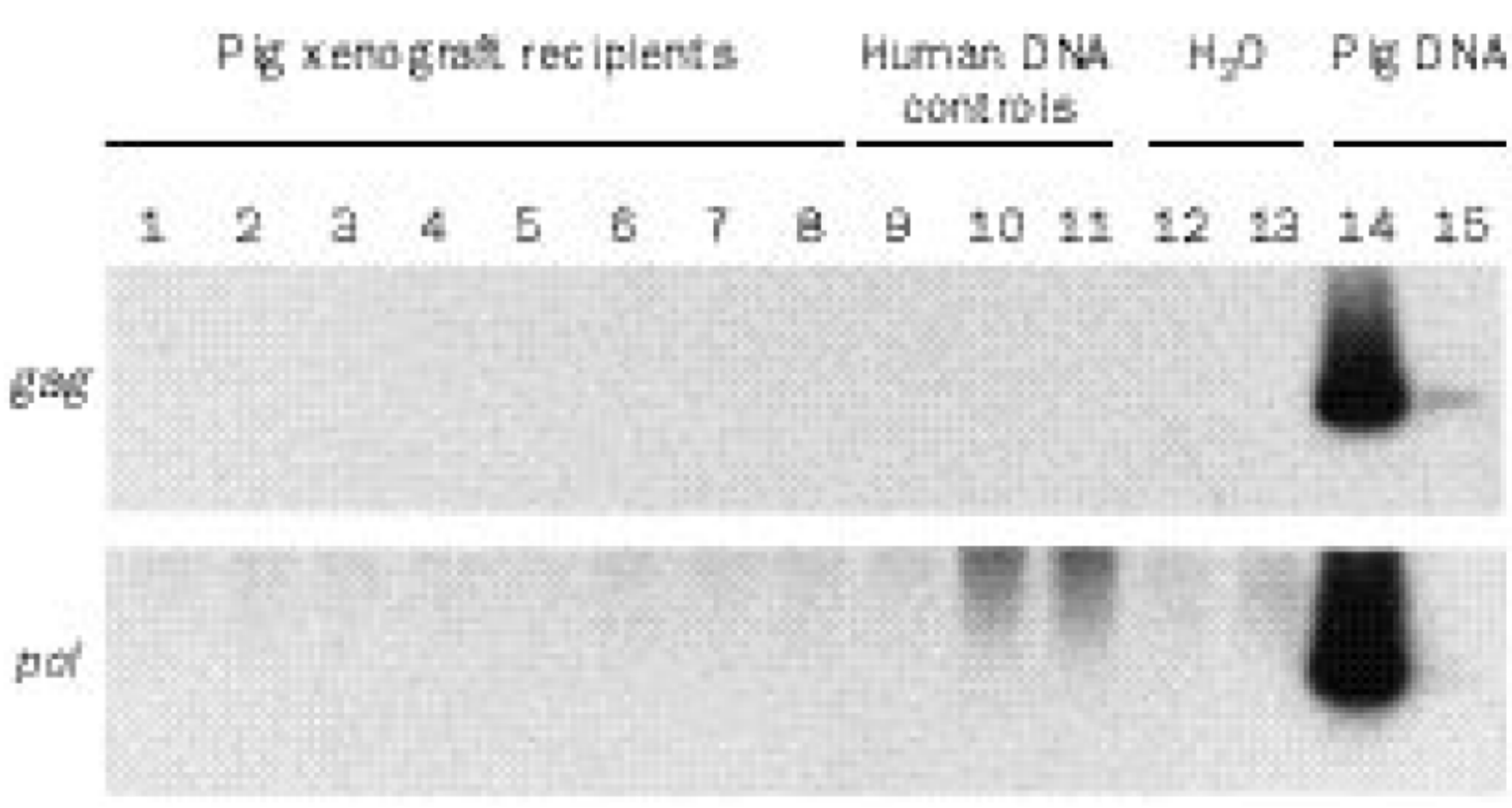
- 10 patients (mean age 40 years) with insulin-dependent diabetes of mean duration = 30 years and end-stage diabetic nephropathy
- patients underwent transplantation with fetal porcine pancreatic isletlike cell clusters (ICC, $4x10^8$ to $2x10^{12}$ cells per patient)
- immunosuppresion with cyclosporin, prednisolone and azathioprine (only one patient received no cyclosporin)
- detection of PERVs by genomic PCR from PBMCs, RT-PCR for PERV mRNA in serum, porcine mtDNA Southern Blot, cell-based antibody screening for porcine antibodies

Clinical results

- Follow-up of patients for 4.5-7.5 years
- n=1 patient with recurrent pneumonia (suffered from asthma before), n=6 patients were treated for chronic diabetic ulcers
- 2 patients died of myocardial infarction 2.5 and 5 years after xenotransplantation
- no signs of lymphoproliferative or neurological disease of the kind associated with type 3 retroviruses

No signs of PERV integration on PCR of PBMCs

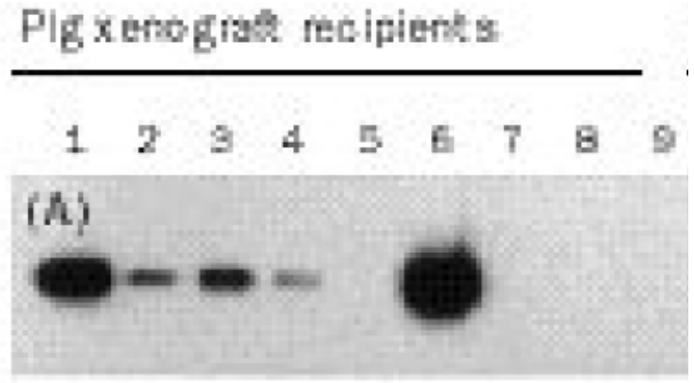
Post-transplantation	Time of sampling	PERV sequences*	
		gag	*pol*
32–60 mo	April, 1995	0/9	0/9
32–60 mo	April, 1997	0/8	0/8
32–60 mo	April, 1997	0/8	0/8



lane 14, 15: serially diluted Pig DNA in human DNA

Persistence of pig mtDNA in xenotransplant recipients for up to 1 year

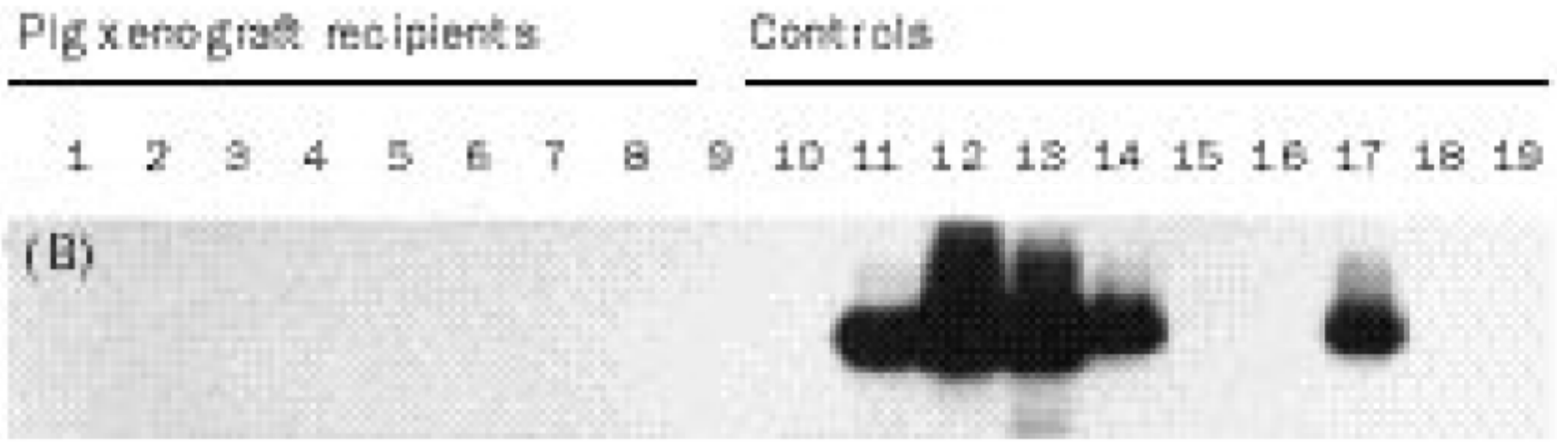
Patient	Pig mtDNA*						Transplant characteristics†		
	2–3 days	2 wk	3 wk	6 mo	1 yr	4–7 yr‡	Evidence of xenoislet survival	Site	ICCs (1000s)
XIT1	+	+	+	+	−	−	C-peptide+	IP	390
XIT2	+	−	−	−	NA	NA		IP	520
XIT3	−	NA	−	−	NA	−		IP	460
XIT4	+	−	−	−	NA	−		IP	410
XIT5	+	−	−	−	NA	−		IP	330
XIT6	−	−	−	−	NA	−	C-peptide+	IP	520
XIT7	+	−	−	+	+	−	C-peptide+	IP	800
XIT8	+	+	−	+	−	−	C-peptide+	IP	1020
XIT9	−	−	−	−	NA	−		RC	200
XIT10	−	+	−	+	NA	NA	Biopsy+	RC	410



#1-5 XIT1, first 5 timepoints

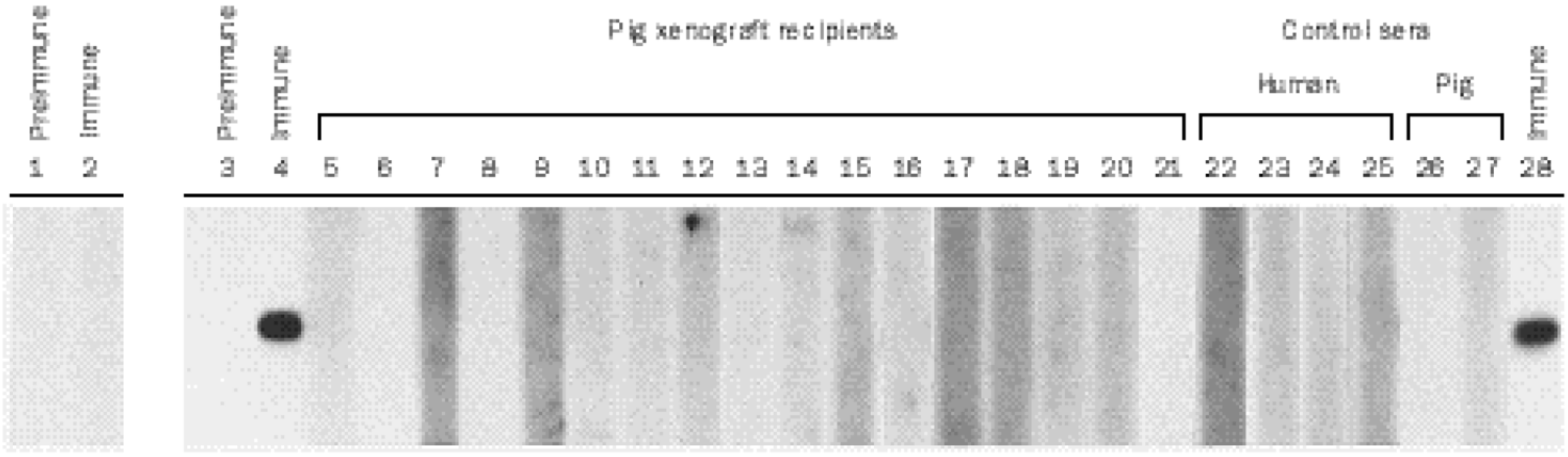
#6-9 XIT2, first 4 timepoints

No evidence of PERV mRNA in blood of ICC recipients



Lanes 1–5=patient XIT1 at days 3, 14, 26, 194, 478 post-xenograft
lanes 6–9=patient XIT2 at days 3, 17, 24, and 178 post-xenograft
lane 10=human control serum
lane 11=pig serum
lanes 12–14=PERV RNA from PK15 tissue culture supernatant diluted 10, 100, and 1000 fold medium
lane 15=uninfected culture medium control
lanes 16, 17=DNase treatment control (16 DNAse +, 17 DNAse -)
lanes 18, 19=water as negative control

No antibodies against p30 PERV protein in ICC recipients



1 uninfected HEK cells

2 HEK cell lysate incubated with antiserum (goat)

3 control serum

4 antiserum

Conclusions of Heneine et al. study

- No evidence of cross-species PERV transmission in 10 ICC transplant recipients up to 7 years post-transplantation
- Establishing a minimum standard for patient screening
- These findings were confirmed in a later study with more patients (n=160) and broader indications for porcine cell exposure: ICC and skin transplantation, whole livers and spleens for extracorporal blood perfusion (Paradis et al., Science 2009)

Inactivation of PERVs by Genome Editing

Using Zinc Finger Nucleases (ZFN) to knock-out PERVs

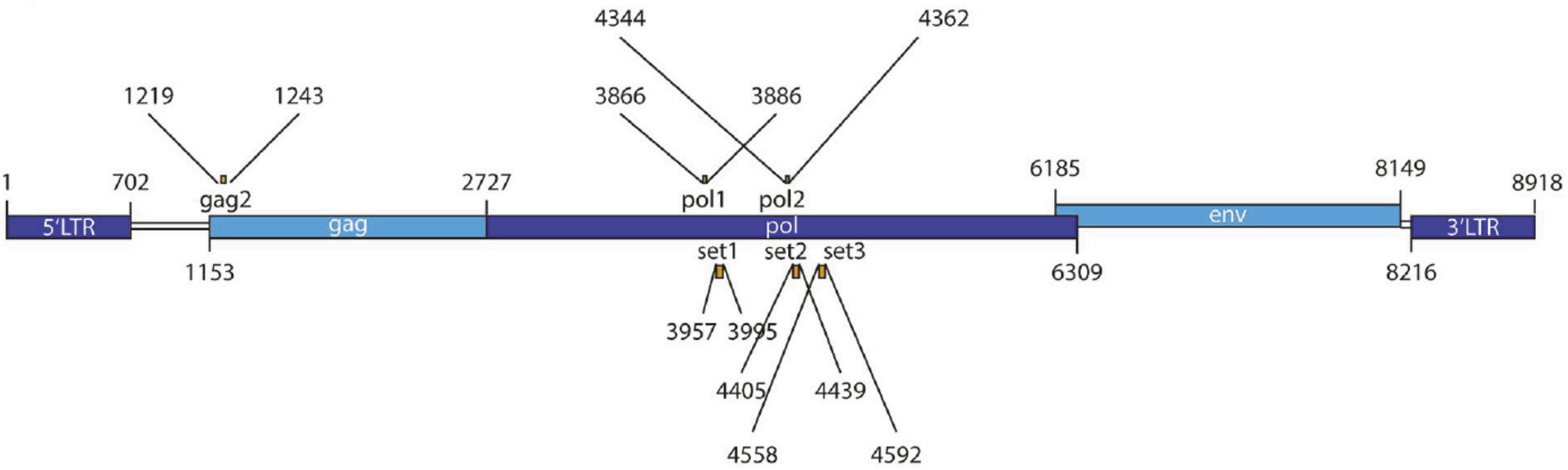
- From 67 ZFN candidates targeting PERV-pol, 10 were found to target highly conserved regions; finally, 3 sets of ZFN were used for the experiment

RESEARCH ARTICLE

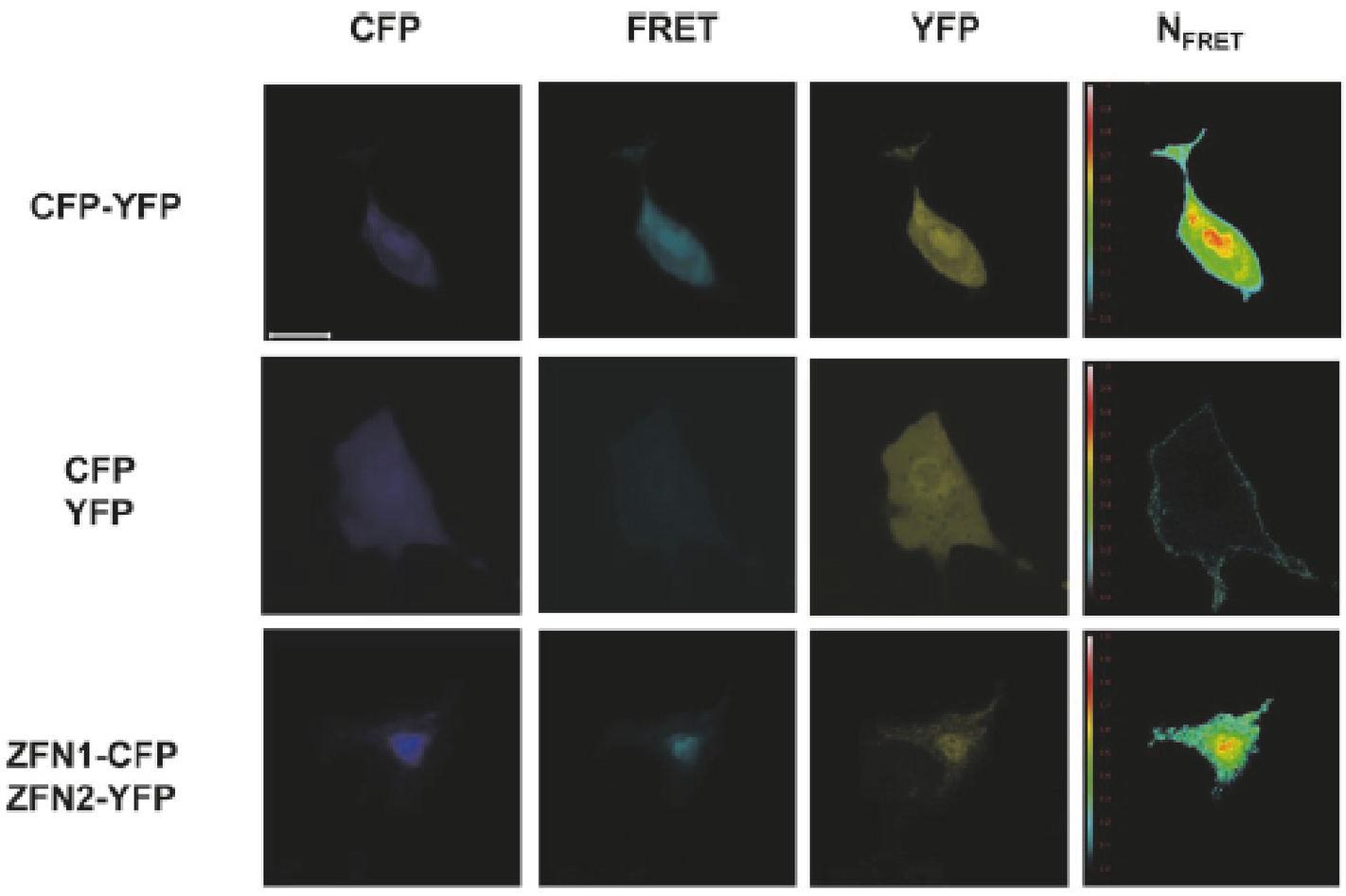
Cytotoxic Effects during Knock Out of Multiple Porcine Endogenous Retrovirus (PERV) Sequences in the Pig Genome by Zinc Finger Nucleases (ZFN)

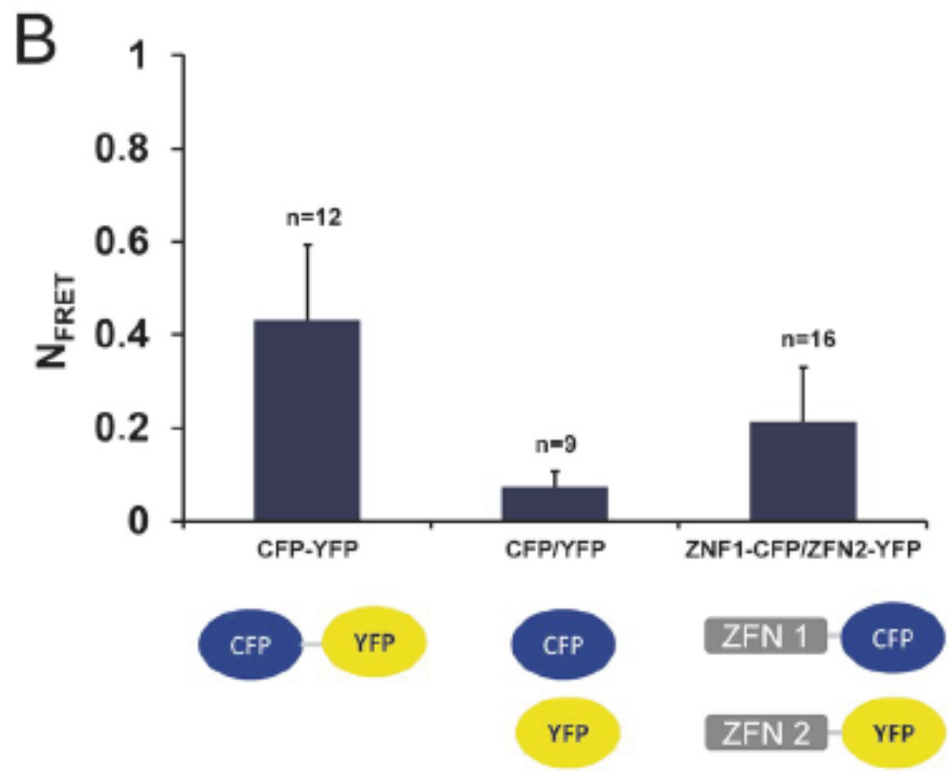
Marwan Semaan[1], Daniel Ivanusic[1,2], Joachim Denner[1]*

1 Robert Koch Institute, Nordufer 20, Berlin, Germany, **2** Freie Universität Berlin, Kaiserswerther Str. 16–18, Berlin, Germany



PERV-ZFN shuttle correctly to the nucleus

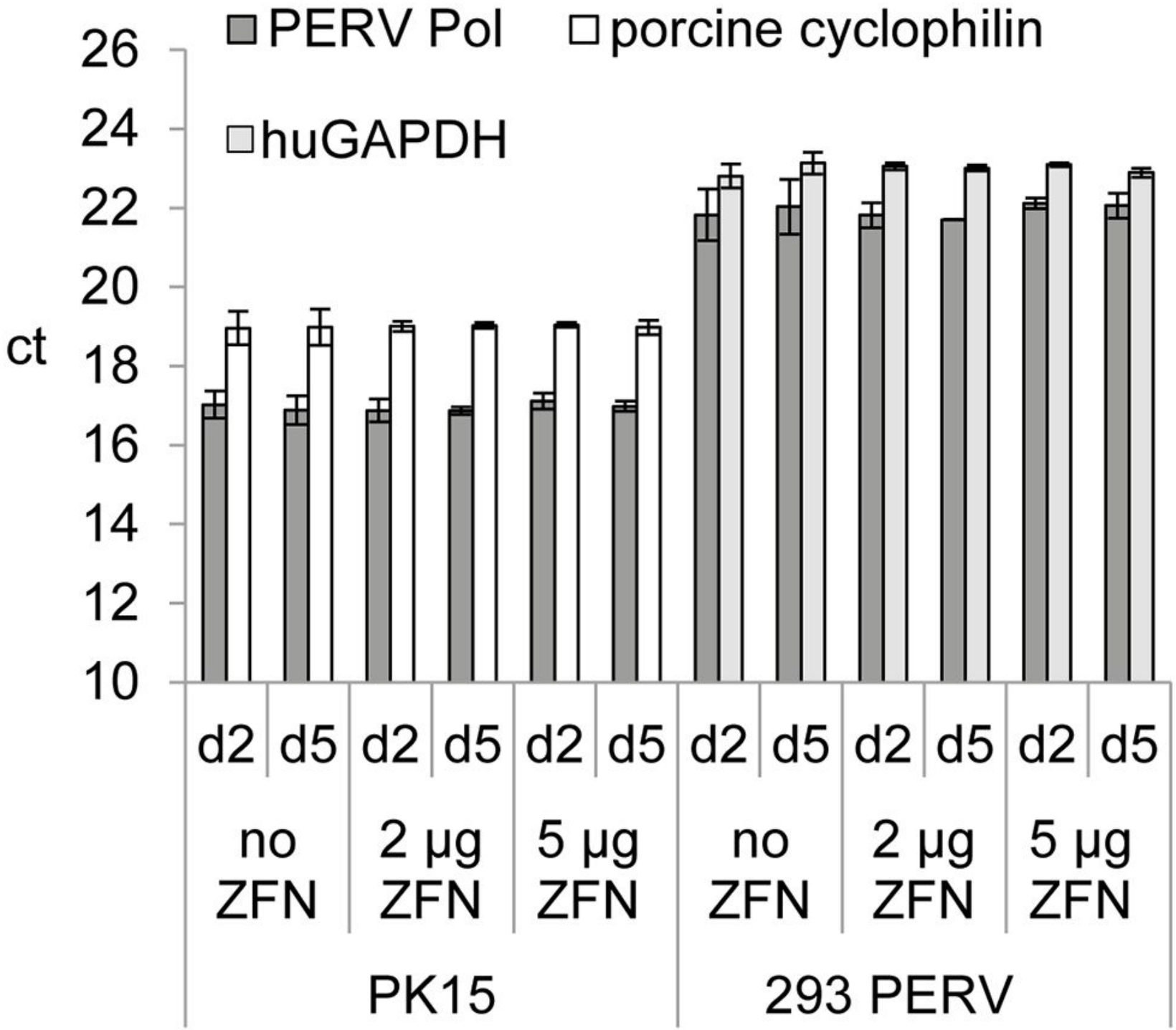




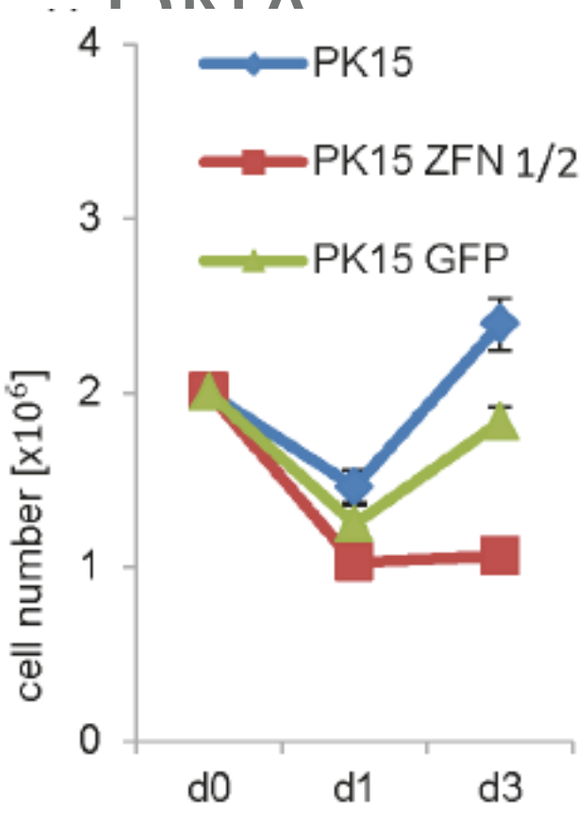
„CFP-YFP“: bicistronic CFP/YFP vector

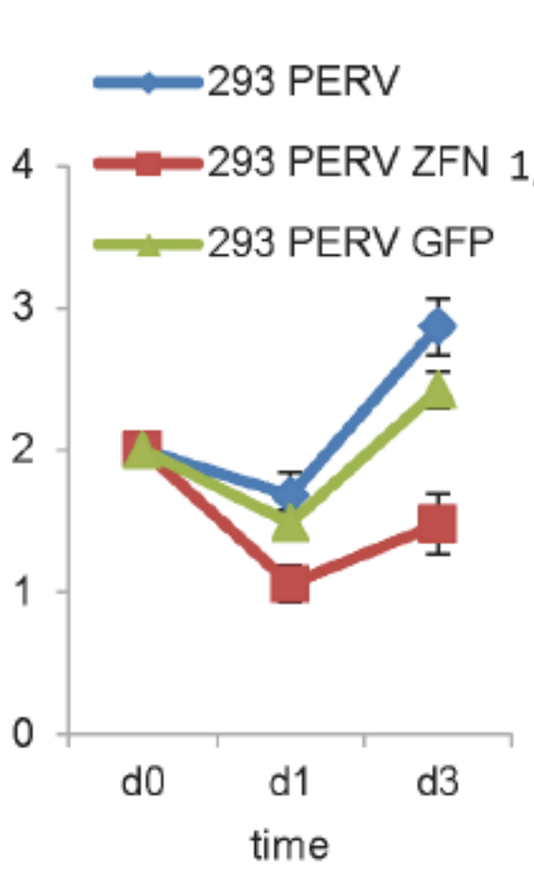
„CFP/YFP“: separately co-transfected CFP/YFP vectors

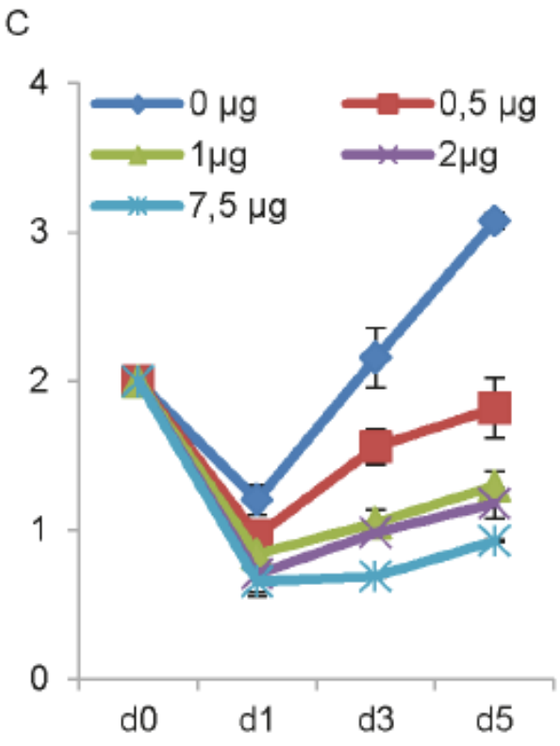
Impact of PERV-ZFN on PERV mRNA levels

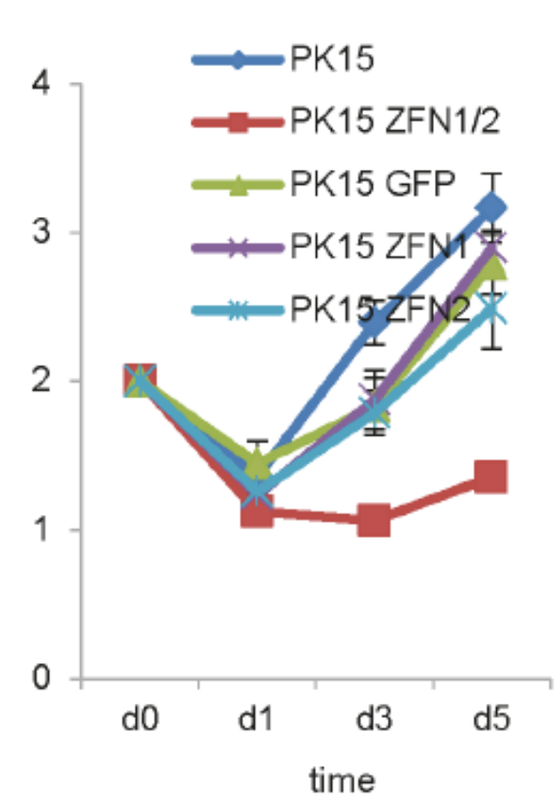


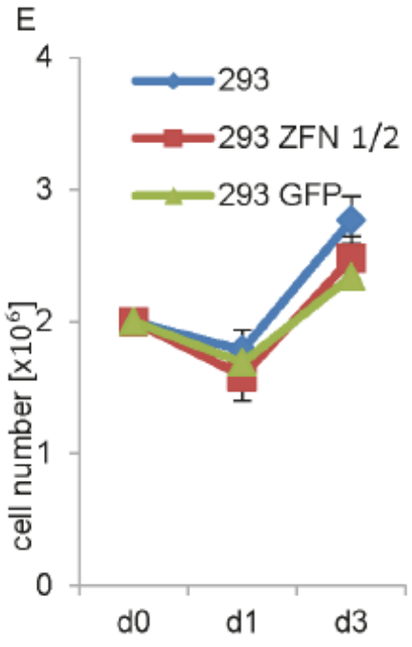
Toxicity of PERV-ZFN is dependent on PERV DNA



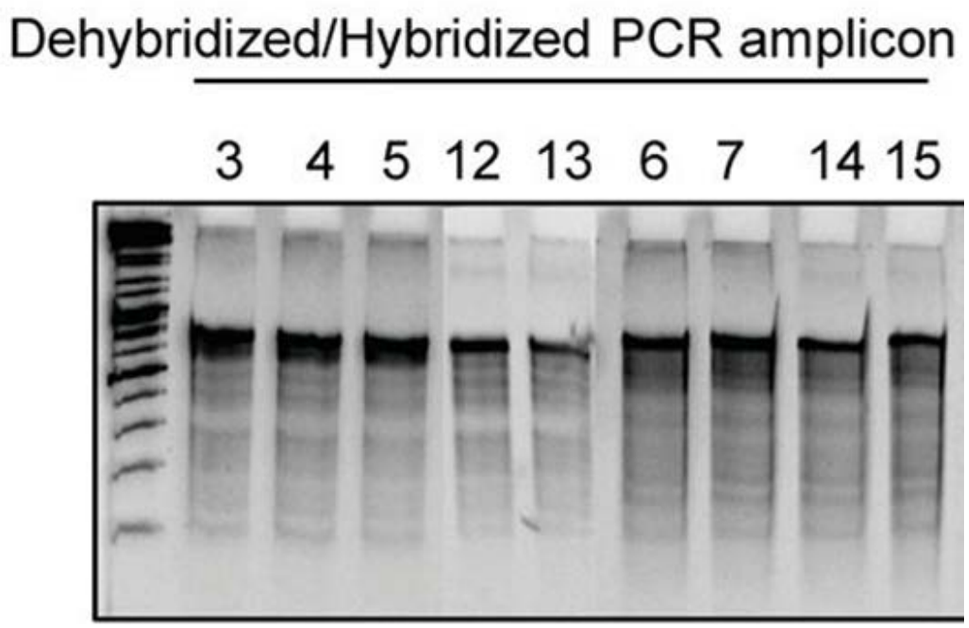








Assessing the functionality of PERV-ZFNs



3 PK15 + 1 µg ZFN1+2
4 PK15 + 2 µg ZFN1+2
5 PK15 + 4 µg ctrl plasmid
12/13 repetition of 4, 5
6 PERV-infected HEK293 + 2 µg ZFN1+2
7 PERV-infected HEK293 + 4 µg ctrl plasmid
14/15 repetition of 6,7

- TOPO cloning showed high grade of polymorphism in integrated PERV DNA -> surveyor nuclease assay not suited for this analysis

Conclusions of Semaan et al.

- Cytotoxicity (growth arrest) of porcine, PERV-bearing PC-15 and PERV-infected HEK293, but not infected HEK293 after transfection with ZFN1+2
- Maybe high number of proviral inserts led to chromosomal destabilisation after ZFN treatment
- The authors suggest replacement of CMV promoter in ZFN plasmid with less active promoter -> lower PERV-ZFN activity could provide more time for cell to repair double-strand breaks

- Semann et al. might have checked the amount of PERV integration or titrate PERV inserts to test their hypothesis

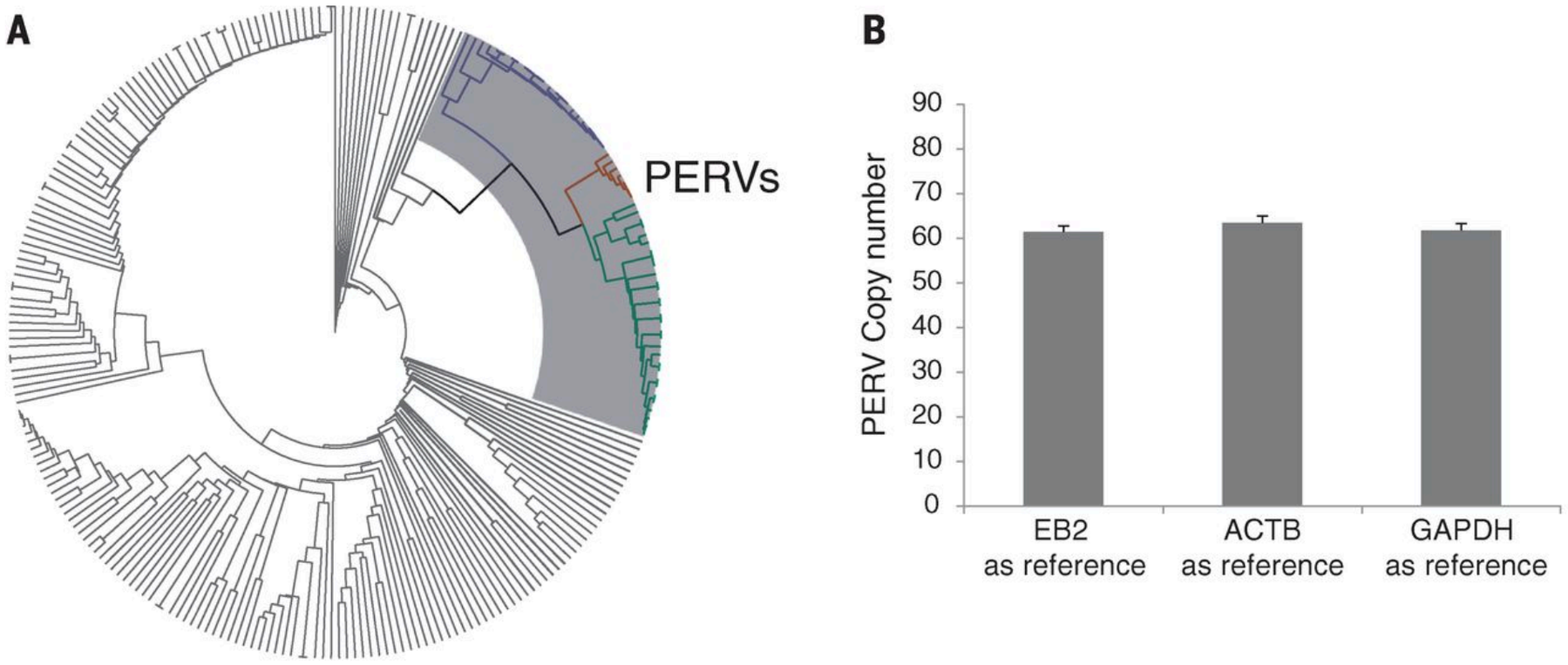
Inactivation of PERVs using CRISPR/Cas9

GENOME EDITING

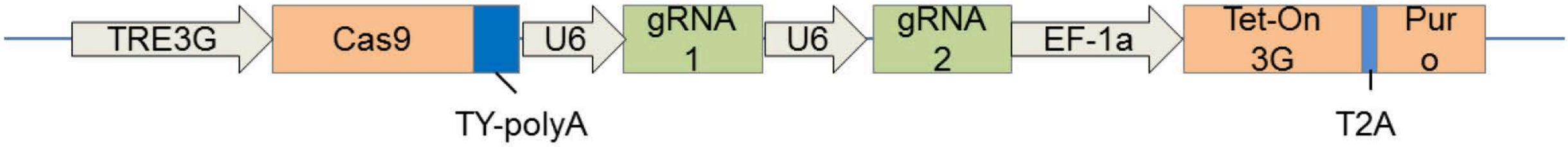
Genome-wide inactivation of porcine endogenous retroviruses (PERVs)

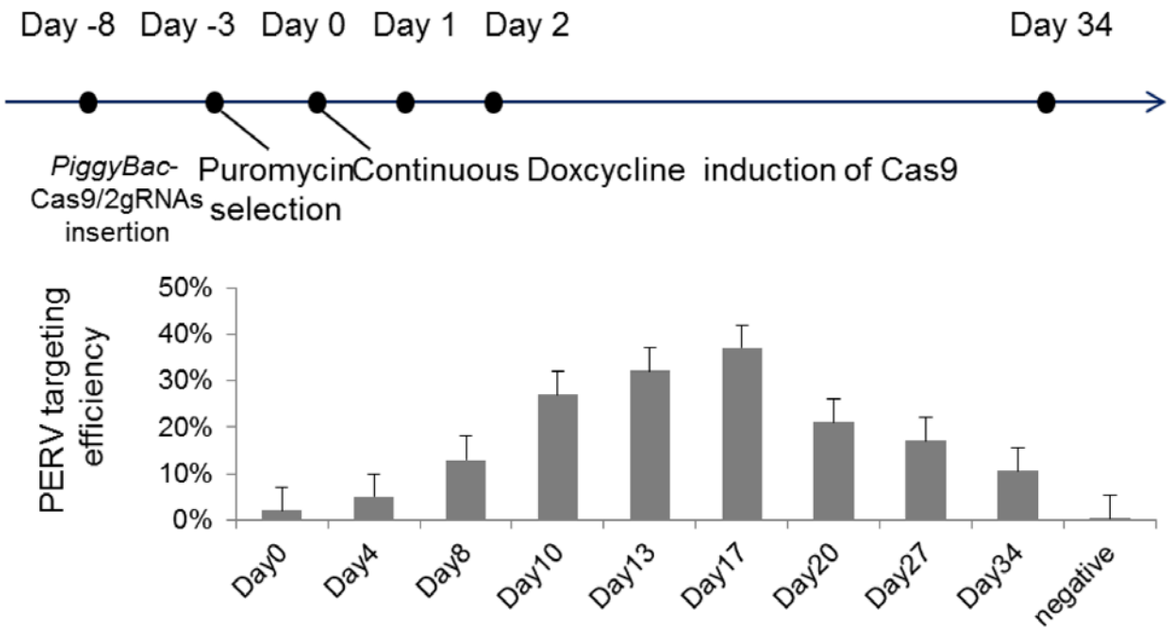
Luhan Yang,[1,2,3]*† Marc Güell,[1,2,3]† Dong Niu,[1,4]† Haydy George,[1]† Emal Lesha,[1] Dennis Grishin,[1] John Aach,[1] Ellen Shrock,[1] Weihong Xu,[6] Jürgen Poci,[1] Rebeca Cortazio,[1] Robert A. Wilkinson,[5] Jay A. Fishman,[5] George Church[1,2,3]*

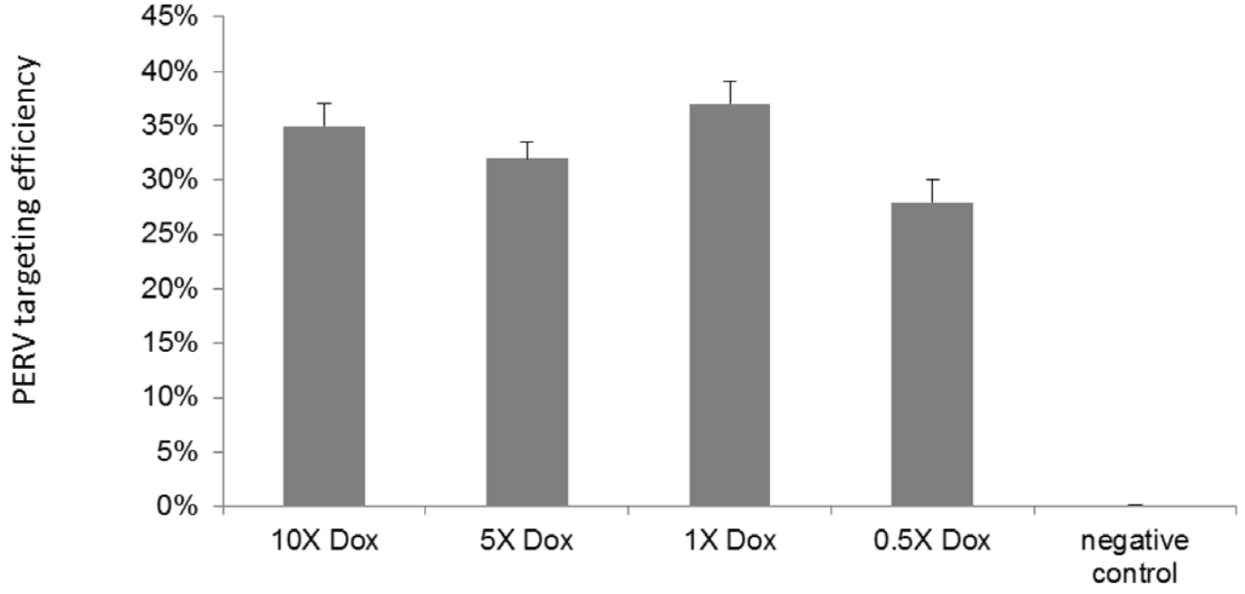
Science 201



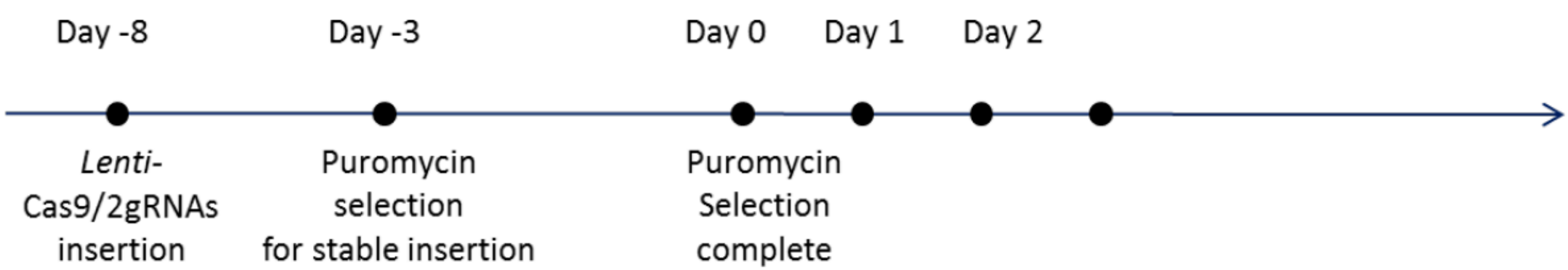
PiggyBac-mediated genomic integration for high editing efficacy

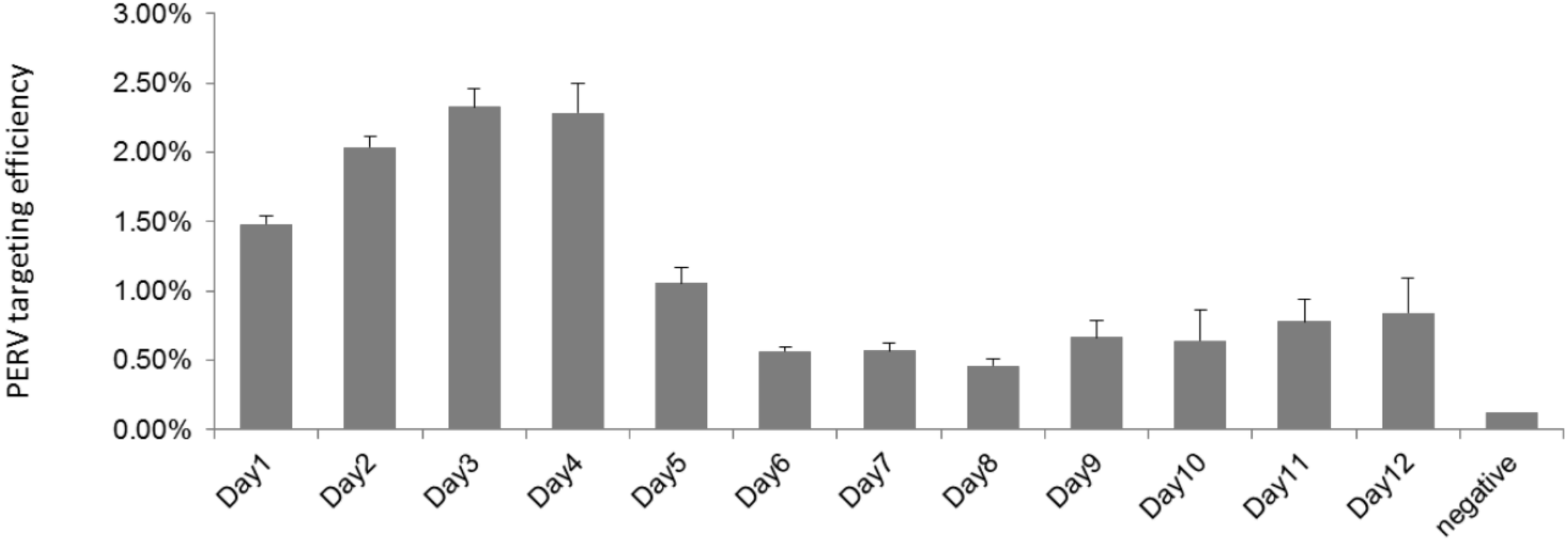




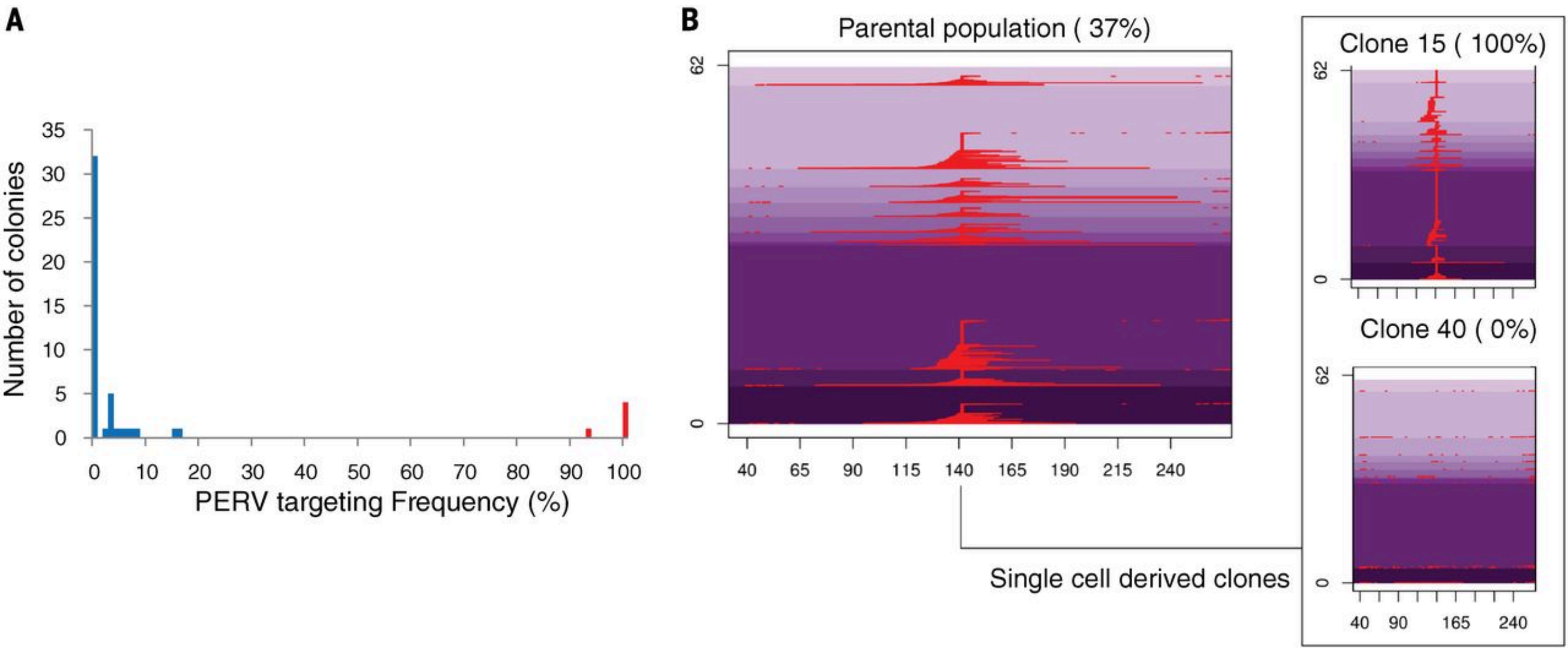


Low editing efficacy when using Lentiviruses



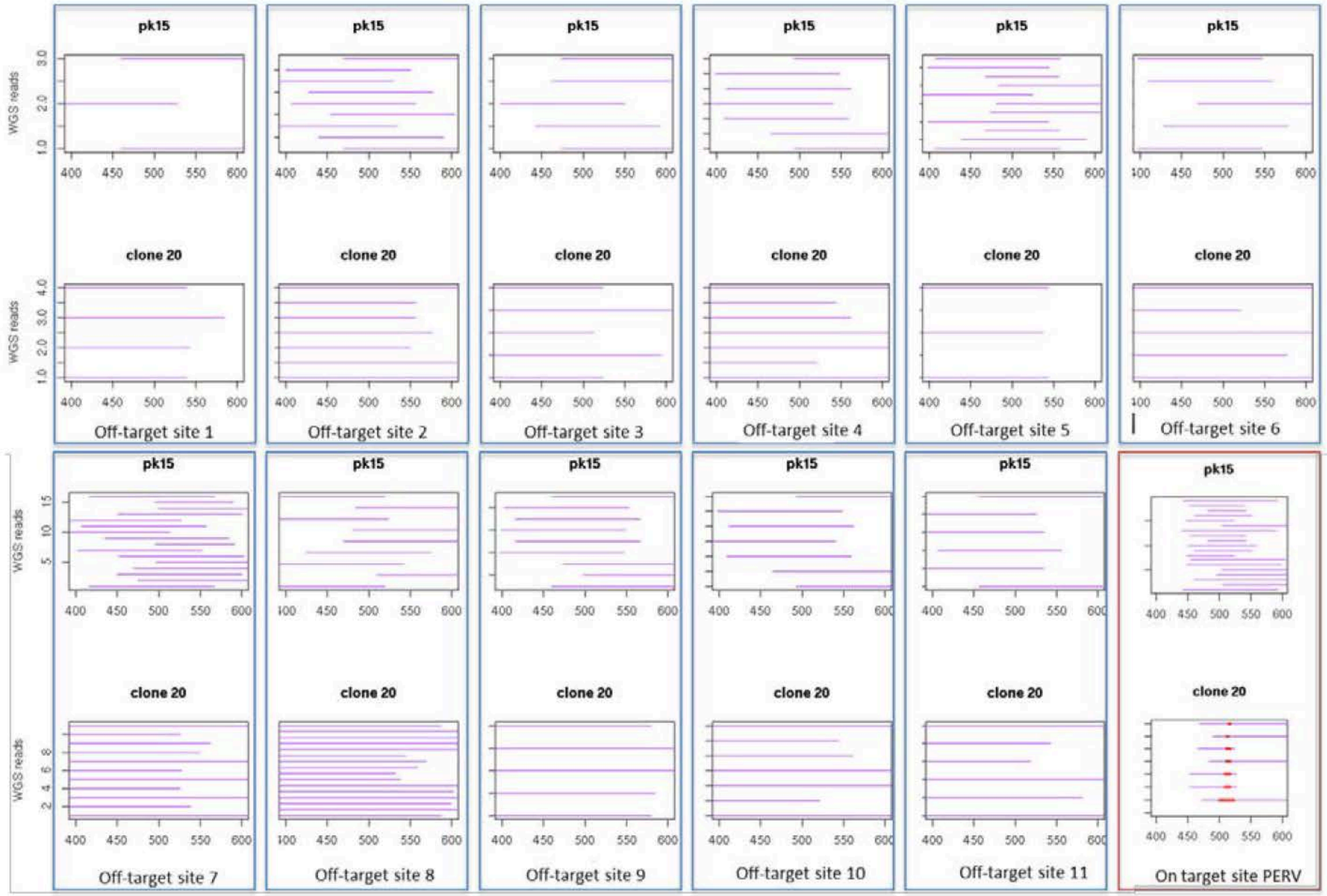


A bimodal distribution of PERV targeting efficacy

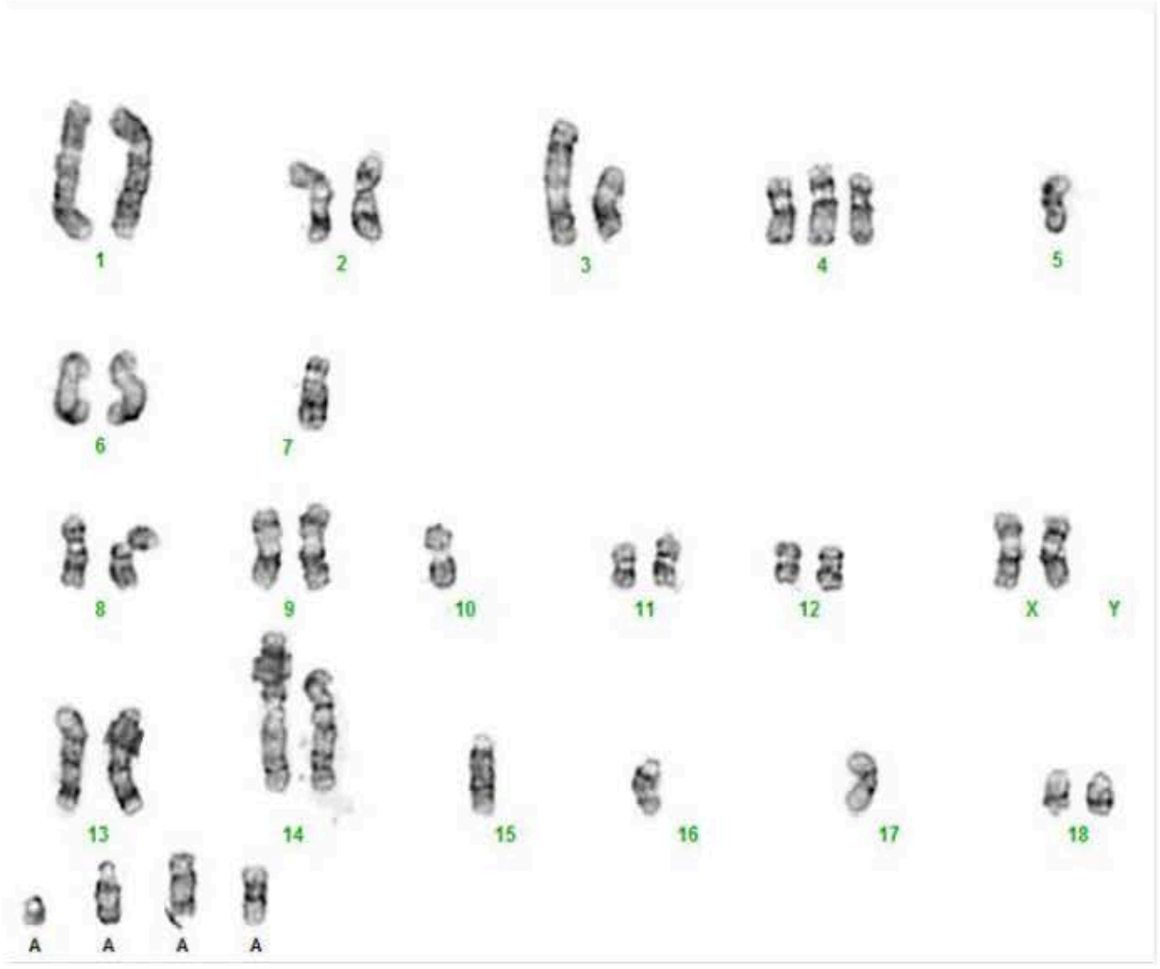


- high degree of repetition in indels from same clone, but not across clones, suggesting a mechanism of gene conversion in which previously mutated PERV sequences were used as templates to repair wild-type PERV sequences

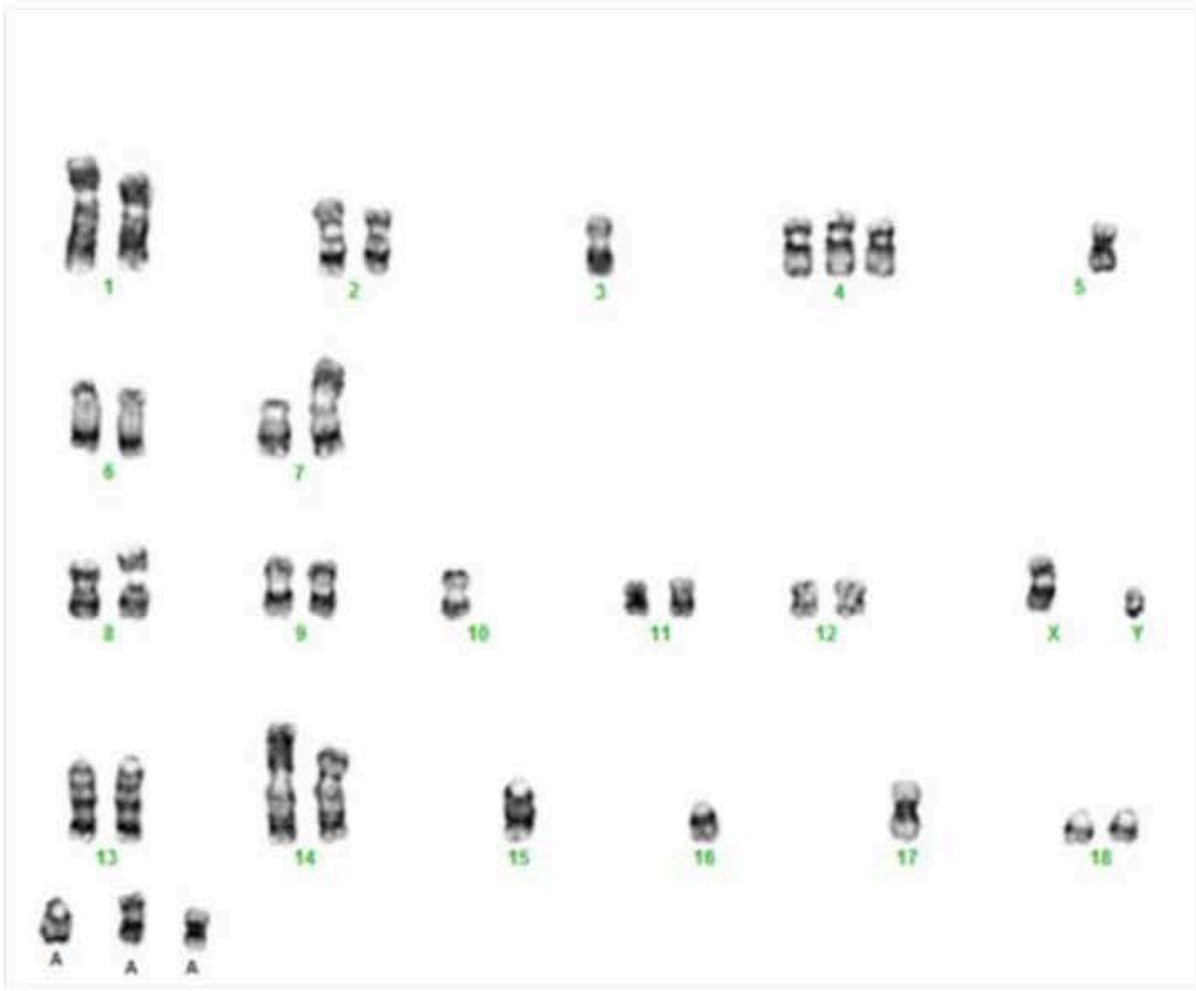
Off-target analysis using whole genome sequencing



No observable genomic rearrangements

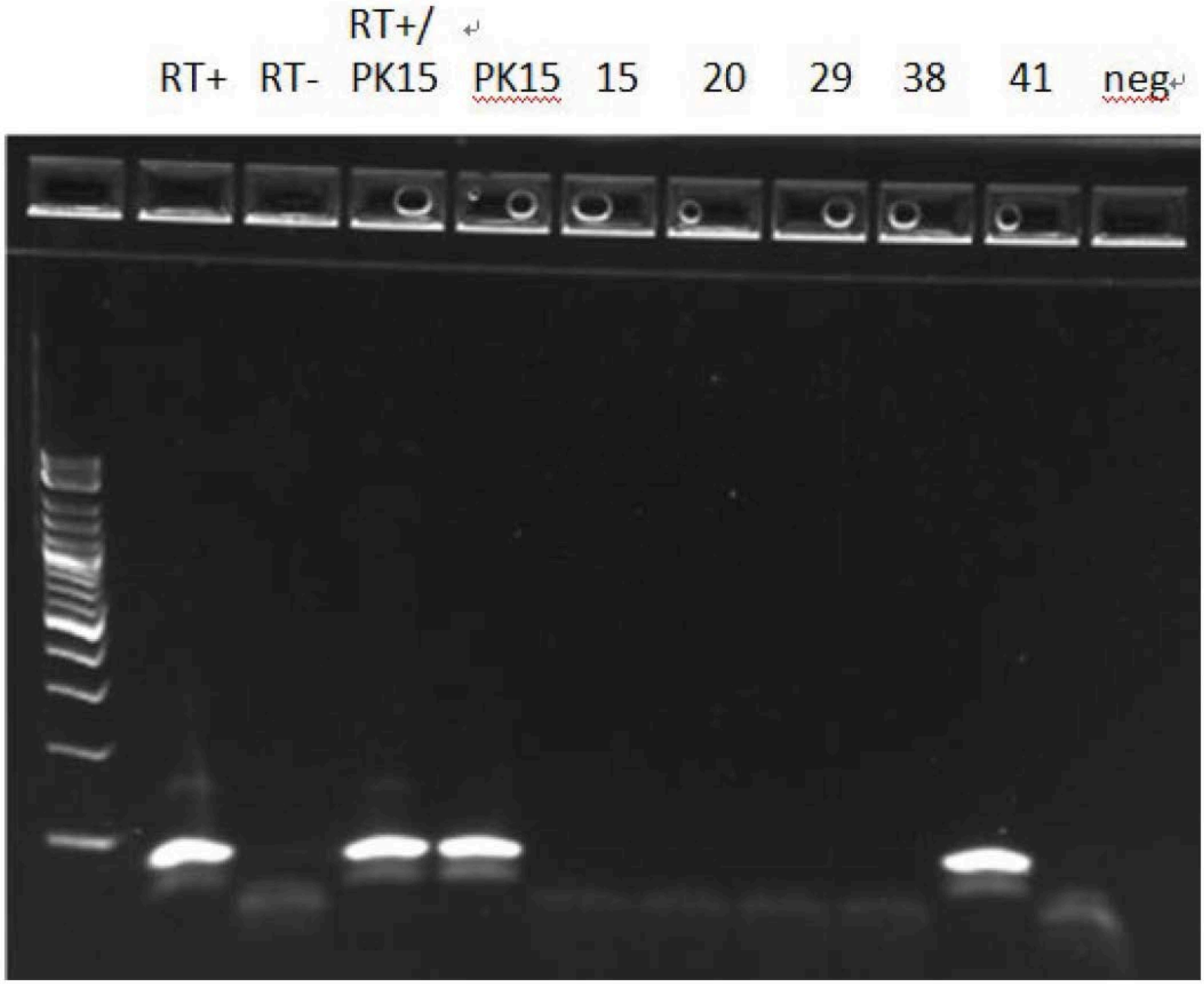


highly modified clone

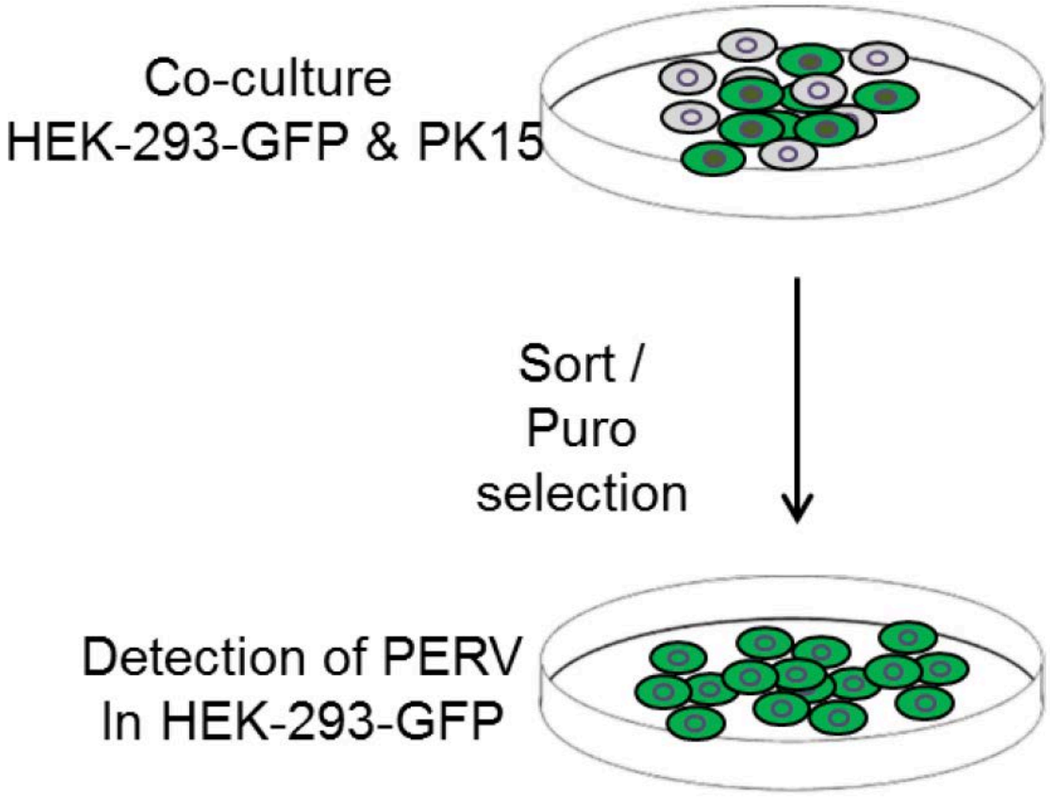


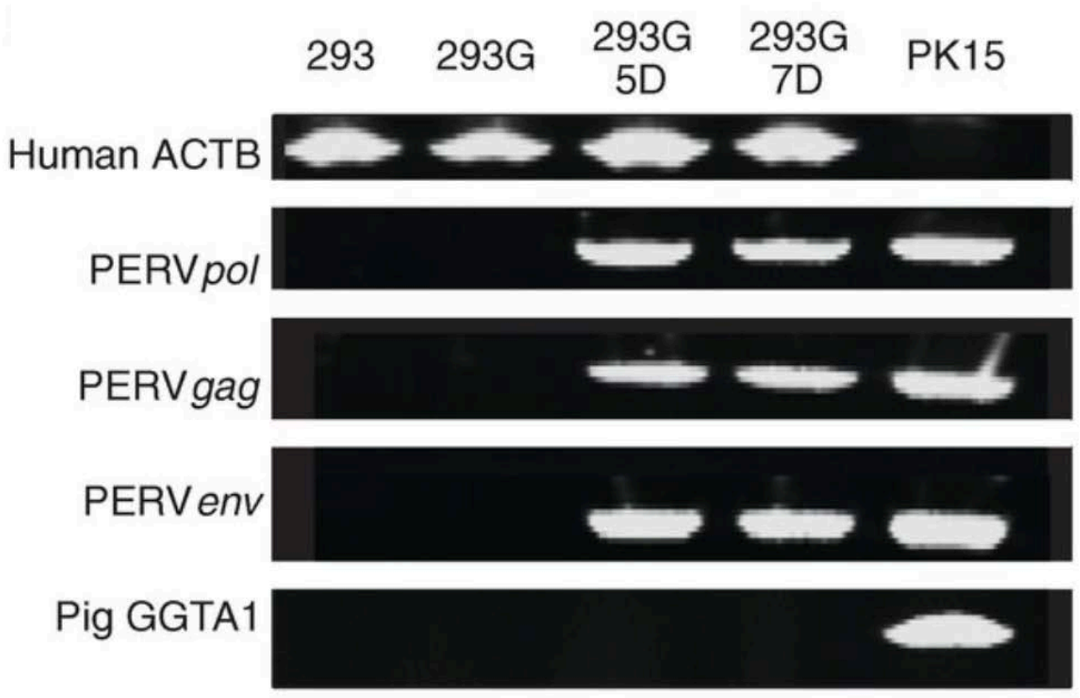
weakly modified clone

Undetectable PERV RT activity in highly edited clones

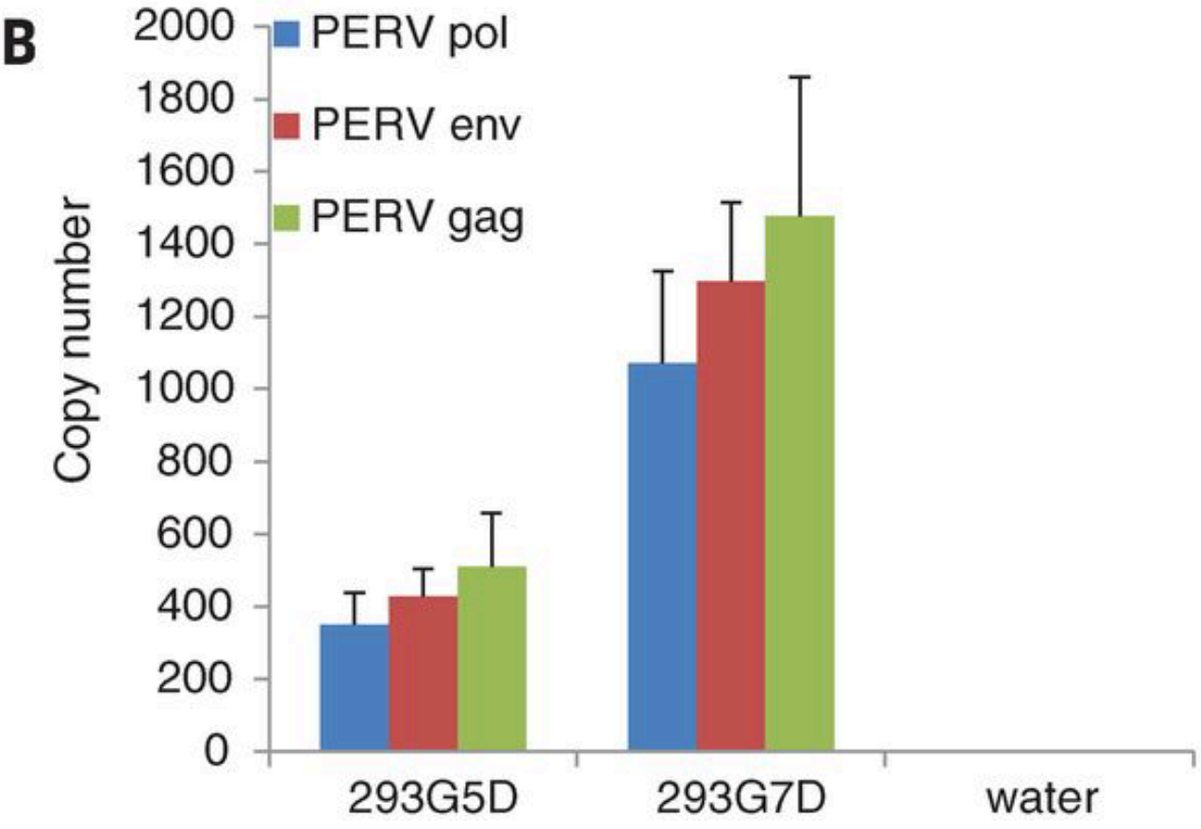


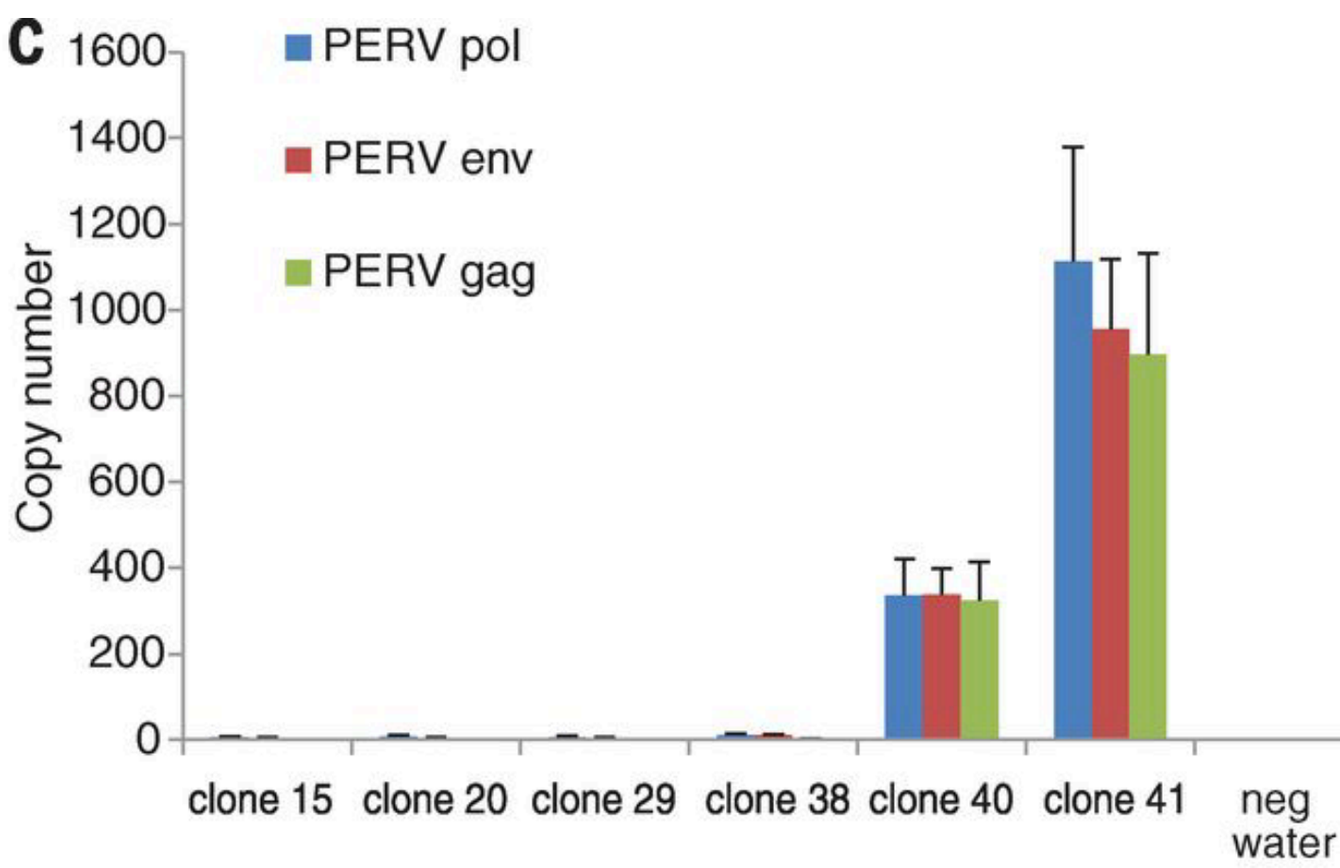
Establishment of a co-culture system to study PERV transmission





Successful ablation of PERV transmission





Conclusions Yang et al.

- Ablation of integrated PERV DNA resulted in efficient (~ 3 logs) reduction of infectivity in PC15 cells
- at that time, maximal number of genes that were edited simultaneously was 6 (here: 62)
- findings need to be repeated *in vivo*

Science

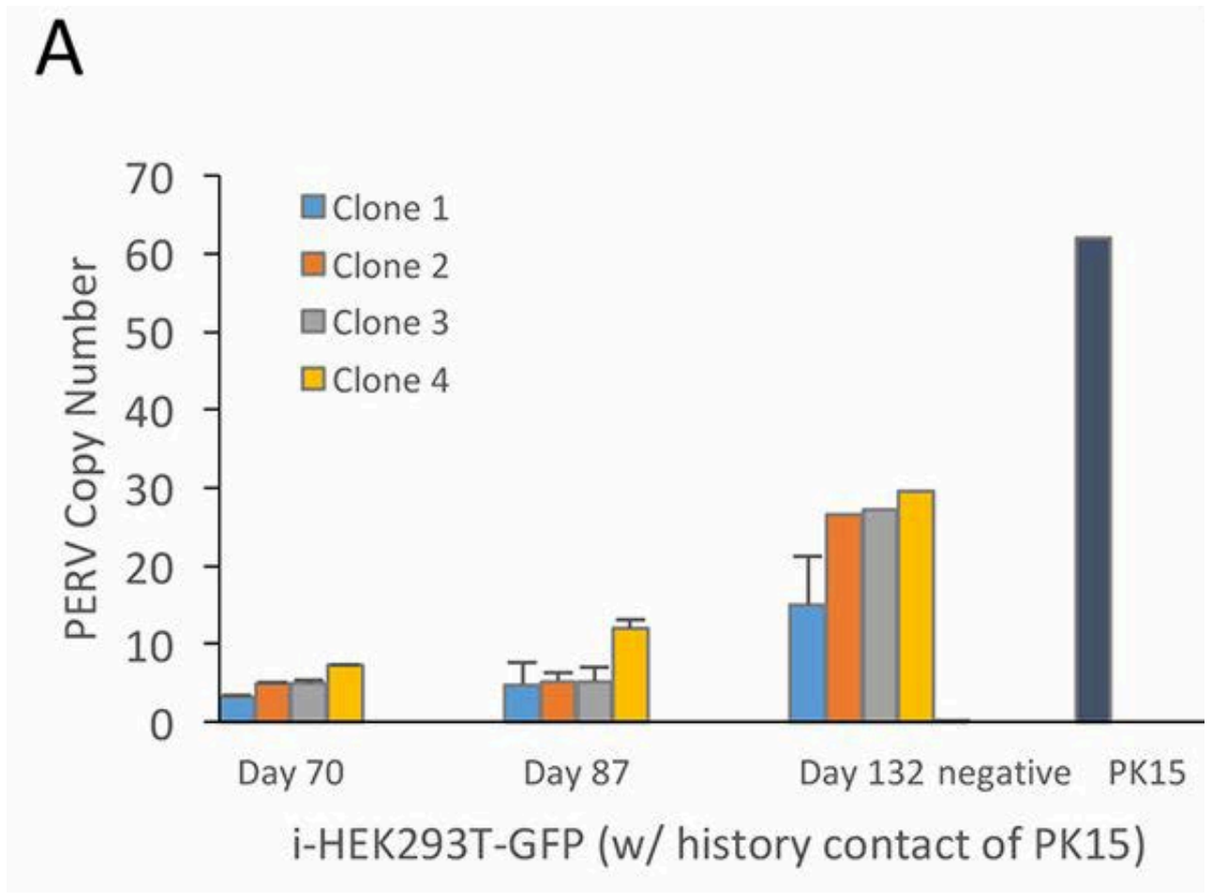
REPORTS

Cite as: D. Niu *et al.*, *Science* 10.1126/science.aan4187 (2017).

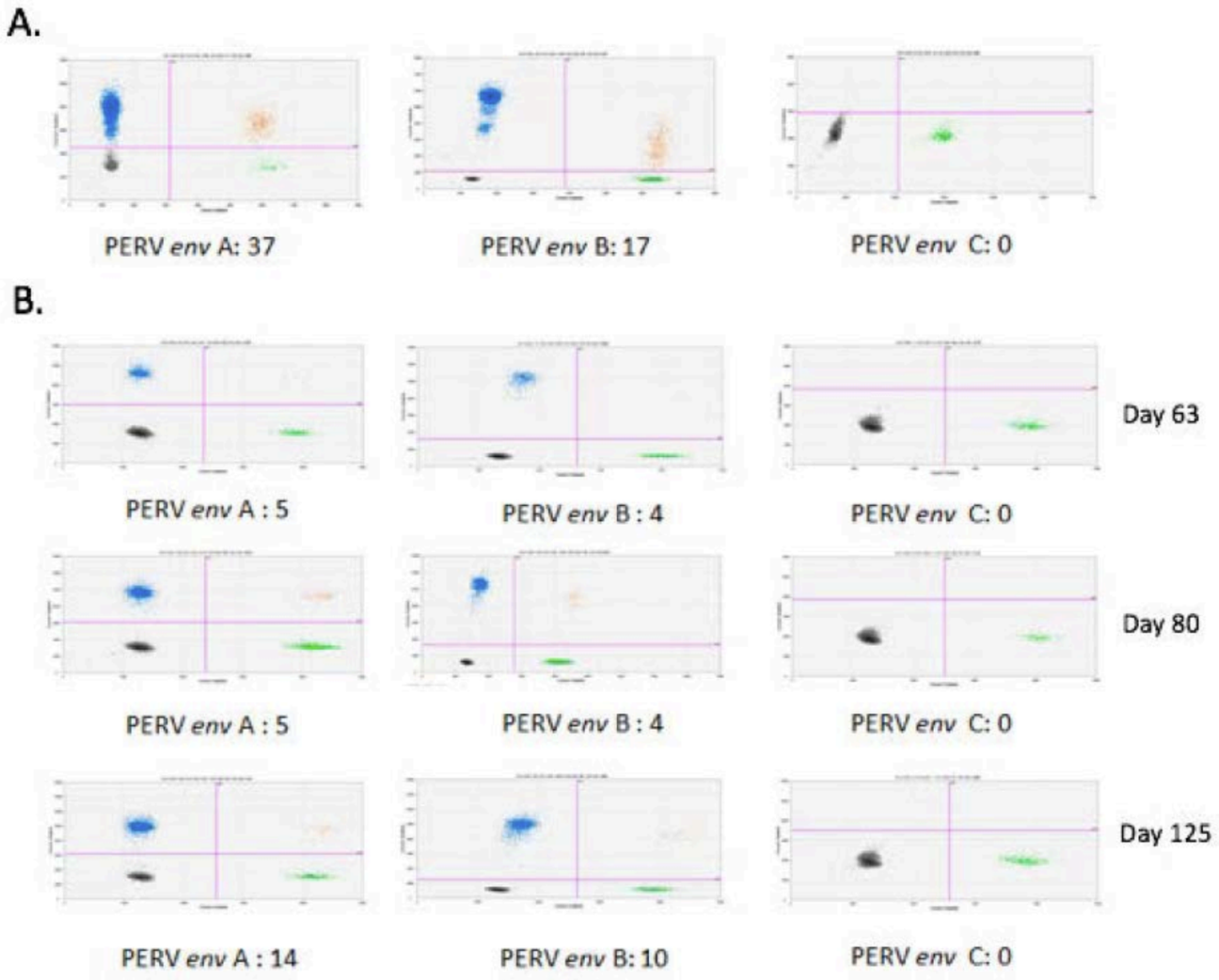
Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9

Dong Niu,[1,2*] Hong-Jiang Wei,[3,4*] Lin Lin,[5*] Haydy George,[1*] Tao Wang,[1*] I-Hsiu Lee,[1*] Hong-Ye Zhao,[3] Yong Wang,[6] Yinan Kan,[1] Ellen Shrock,[7] Emal Lesha,[1] Gang Wang,[1] Yonglun Luo,[5] Yubo Qing,[3,4] Deling Jiao,[3,4] Heng Zhao,[3,4] Xiaoyang Zhou,[6] Shouqi Wang,[8] Hong Wei,[6] Marc Güell,[1†] George M. Church,[1,7,9†] Luhan Yang[1†‡]

- Are PERVs able to replicate in human cells?



PERV-A and PERV-B are the isoforms found in i-HEK293, and are integrated into the genome



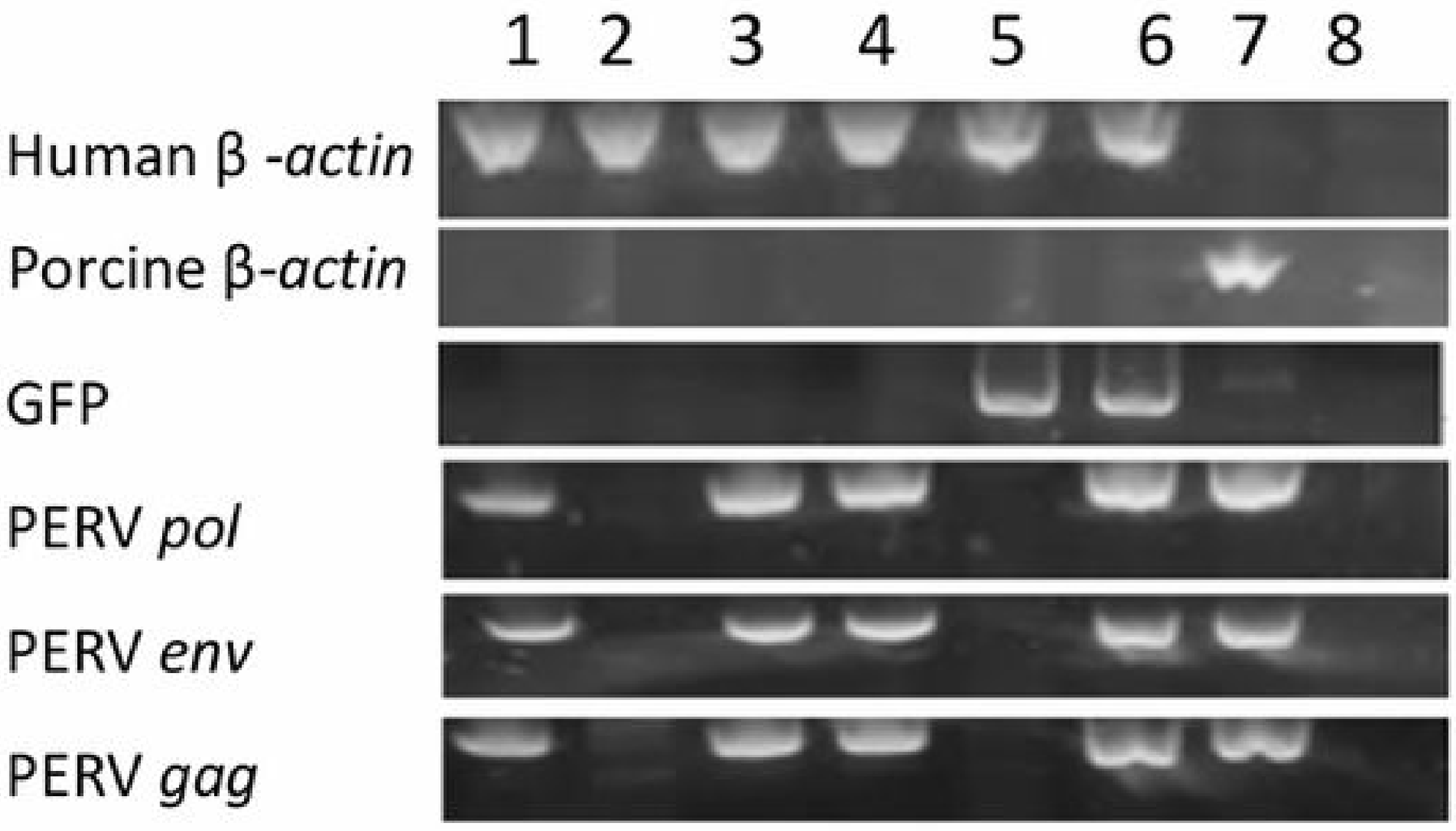
Validated Human PERV junction sequences	Position (GRCh38.p7)	Gene
GACCATTACCAAGCAGGGTCTGACAGGTACTGAAAGGATGAA	chr1:32818962-32818933	S100PBP
ATTAAACAACTCAGCCGGGTGCGGTGCCTCTGAAAGGATGAA	chr6:43630446-43630475	MAD2L1BP
AACTGTATAGTCTTGGATAAACAGTTCAGGTGAAAGGATGAA	chr6:143062056-143062027	AIG1
CGTGGGAAACAGGGGCTCCGCGACTGCGCTTGAAAGGATGAA	chr4:1683715-1683744	FAM53A
GCAAATGTCTTTATTGTTTGTTGCTGGTTTTGAAAGGATGAA	chr14:61764943-61764972	SNAPC1
GGAGTGCAGTGATCTGATCTCCGCTCATGGTGAAAGGATGAA	chr18:58882490-58882519	ZNF532
CCGTTCCTCCCAAGGCGGGCCTTGAAAGGATGAAAGGATGAA	chr5:172984005-172983976	ATP6V0E1

targeted PERV sequenci

A: PK-15

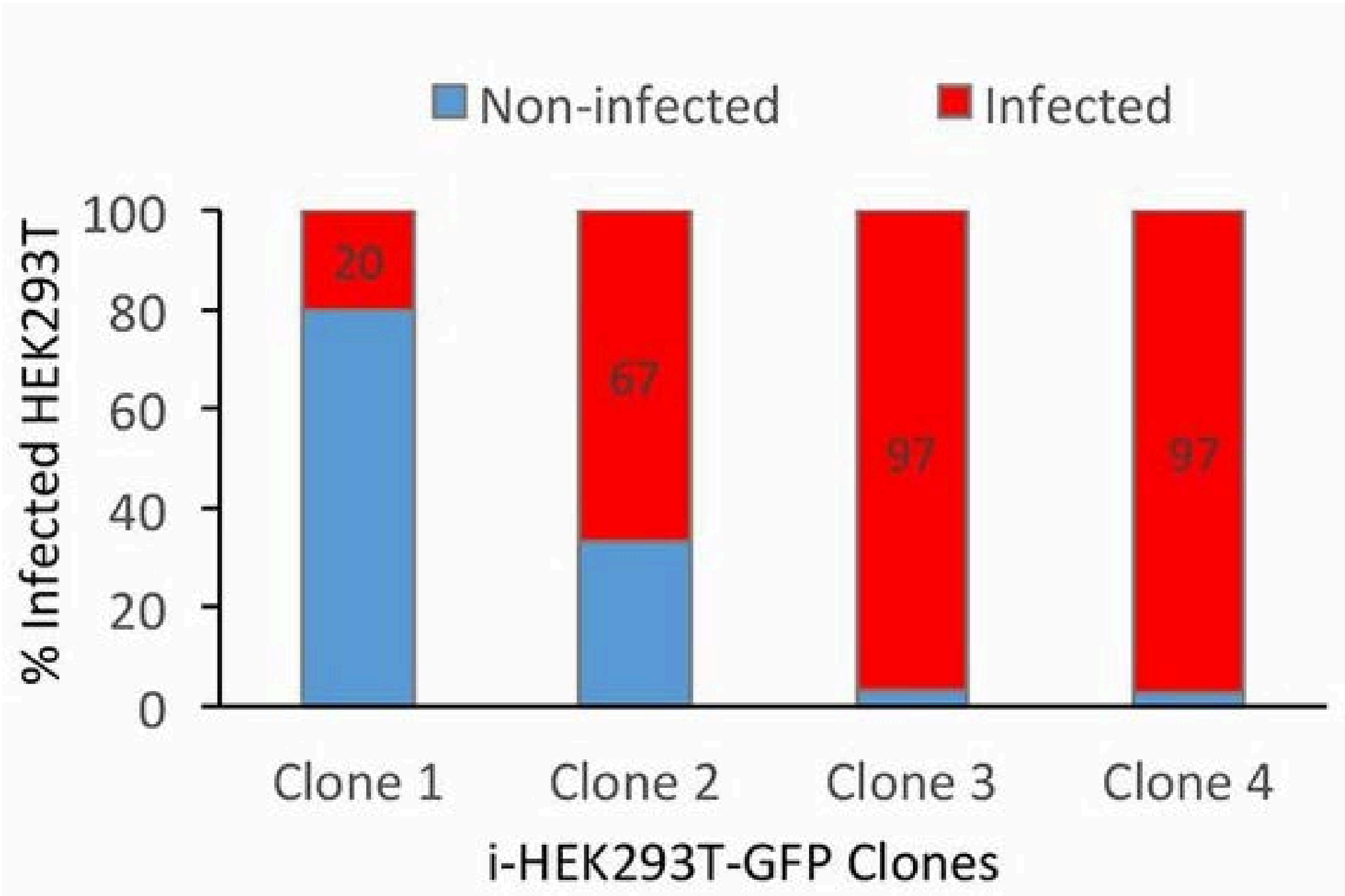
B: i-HEK293

PERV-infected HEK293 propagate infectivity to PERV-naïve HEK293

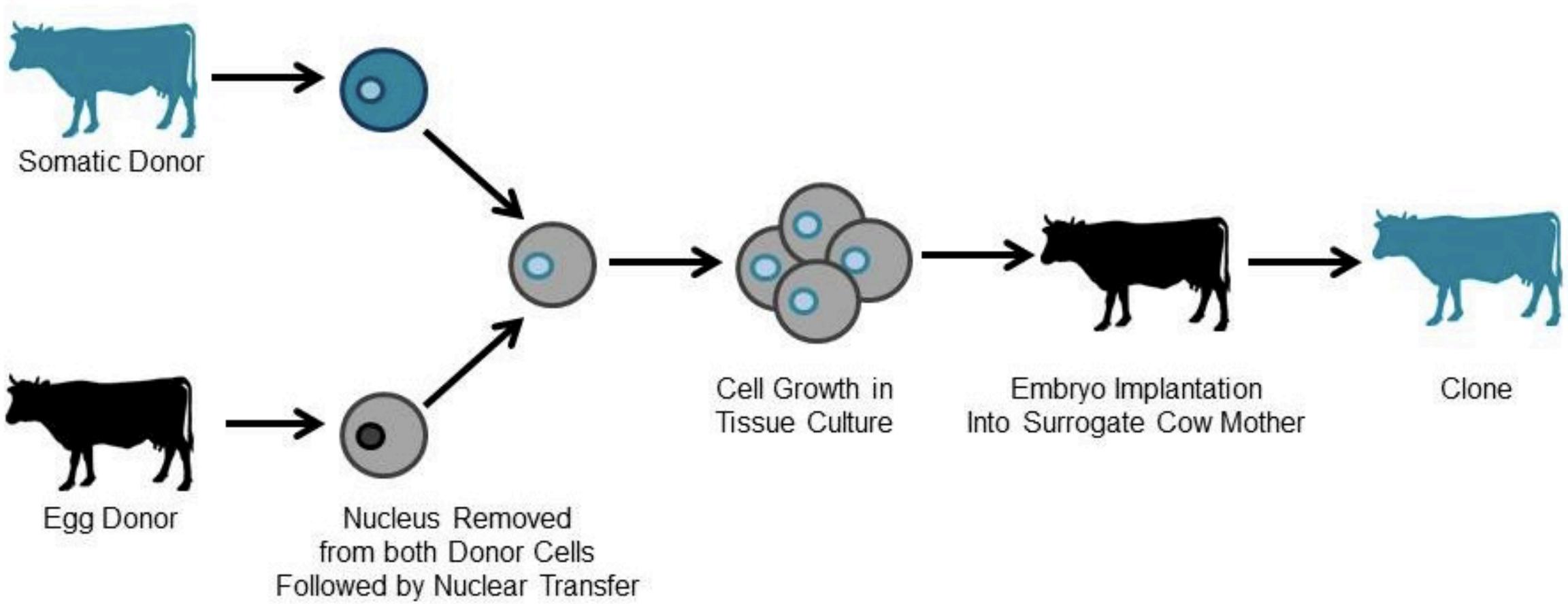


1) HEK293T clone 1
2) HEK293T clone 2
3) HEK293T clone 3
4) HEK293T clone 4
5) HEK293T-GFP control
6) i-HEK293T-GFP
7) PK15 WT
8) negative

Different infectivity potential of i-HEK293

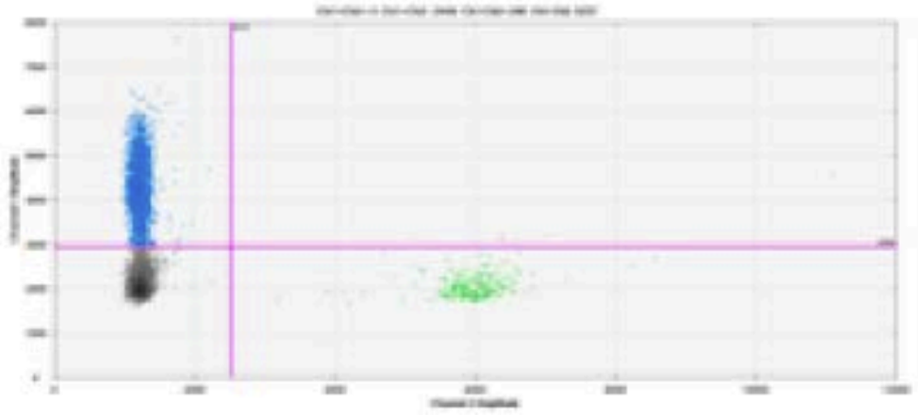


Generation of PERV-deficient primary porcine cells for somatic cell nuclear transfer (SCNT)

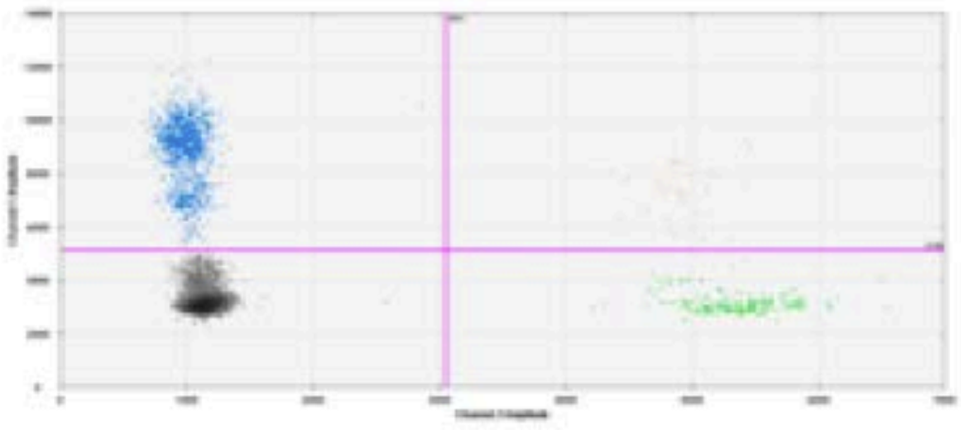


https://wildlifesnpits.wordpress.com/2016/01/05/somatic-cell-nuclear-transfer-the-conservation-genomic-solution-you-havent-heard-of/

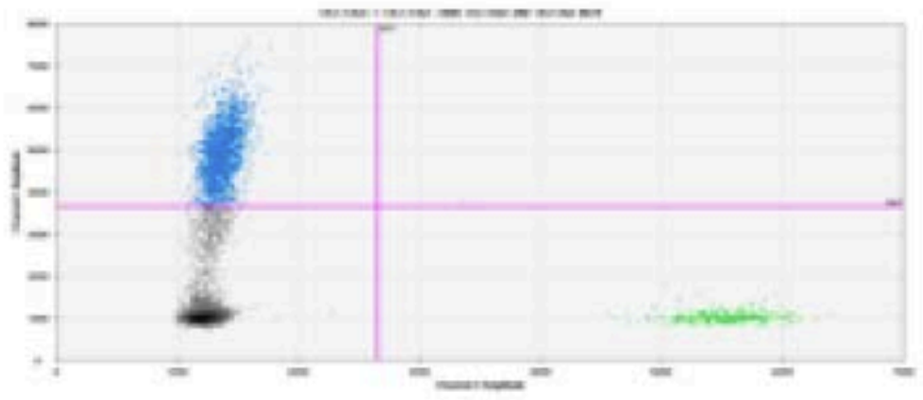
PERV repertoire in the porcine fetal fibroblast cell line FFF3



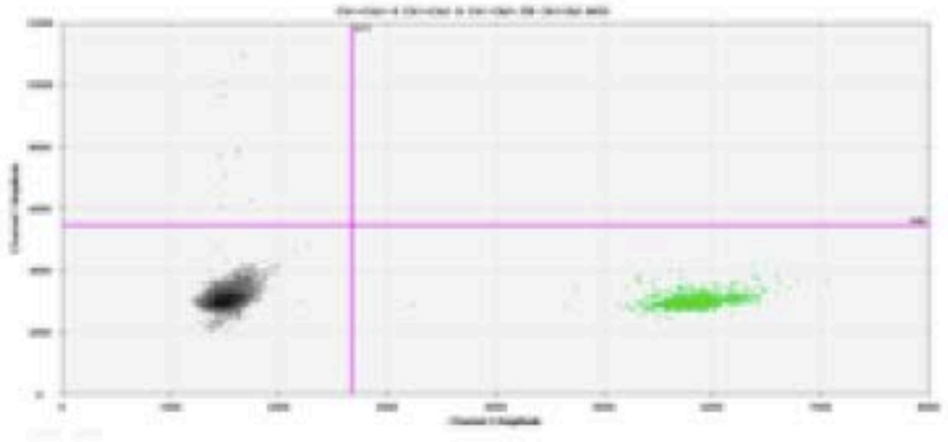
PERV *pol:* 25 copies



PERV *env* A: 10 copies



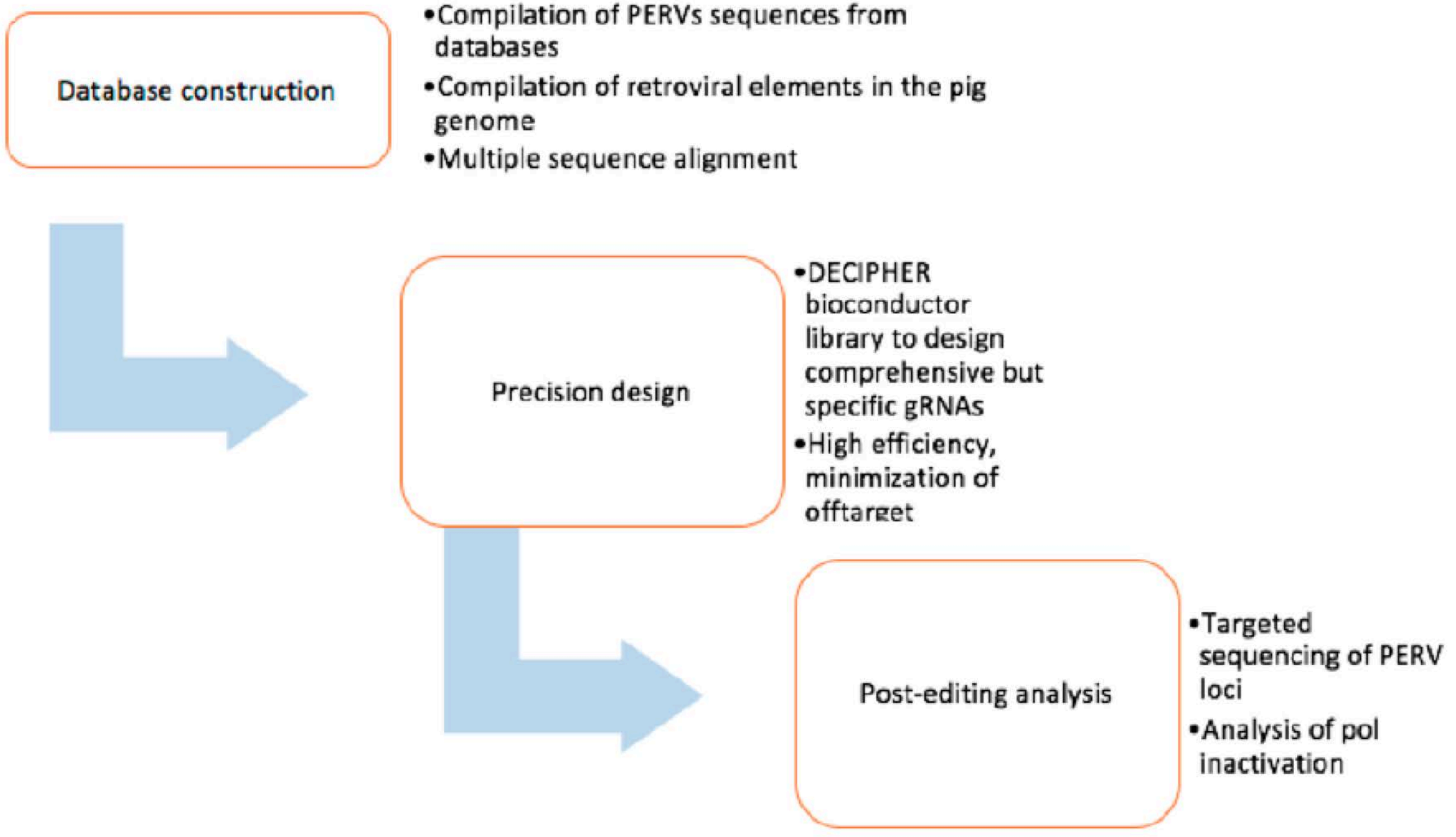
PERV *env* B: 14 copies



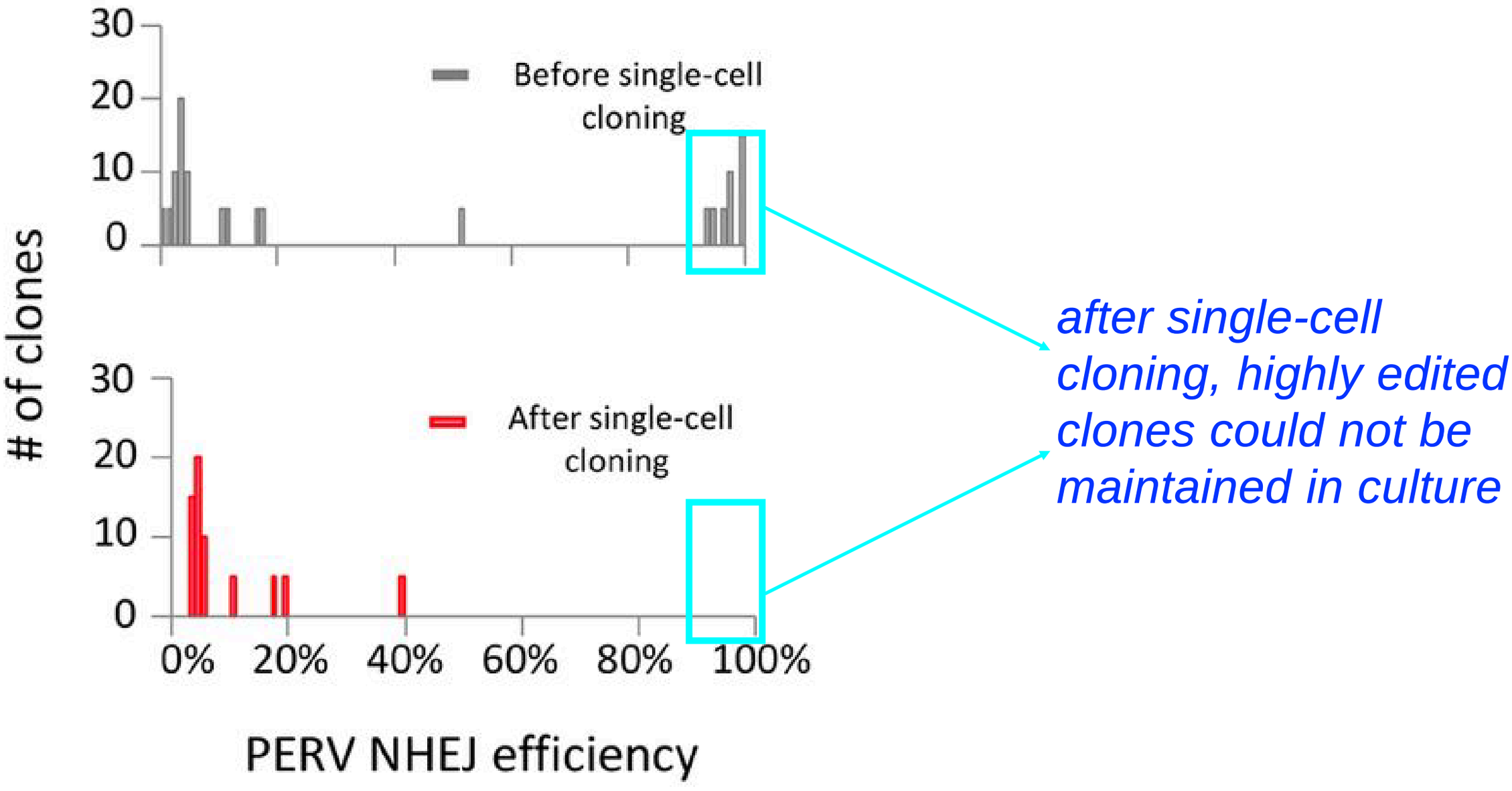
PERV *env* C: 0 copy

one truncated copy of PERV, that was undetectable in ddPCR was picked up during whole genome sequencing

Scheme describing the design process of *pol* targeting gRNAs

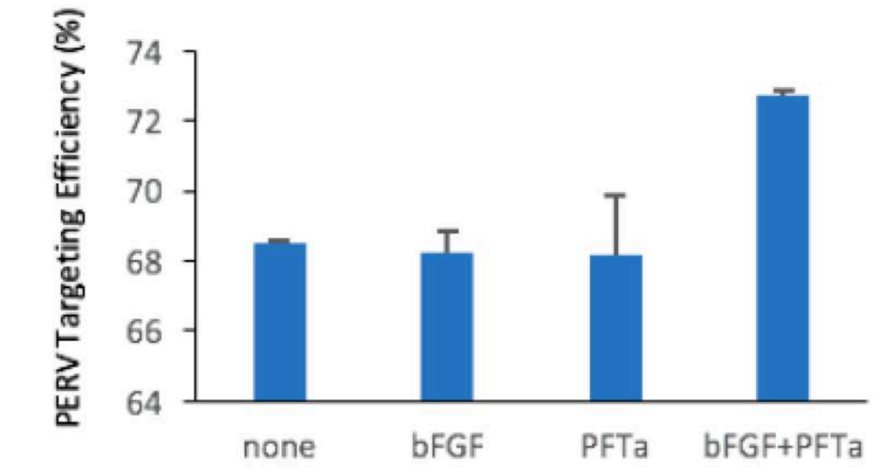


Bimodal distribution of editing efficacies (again)

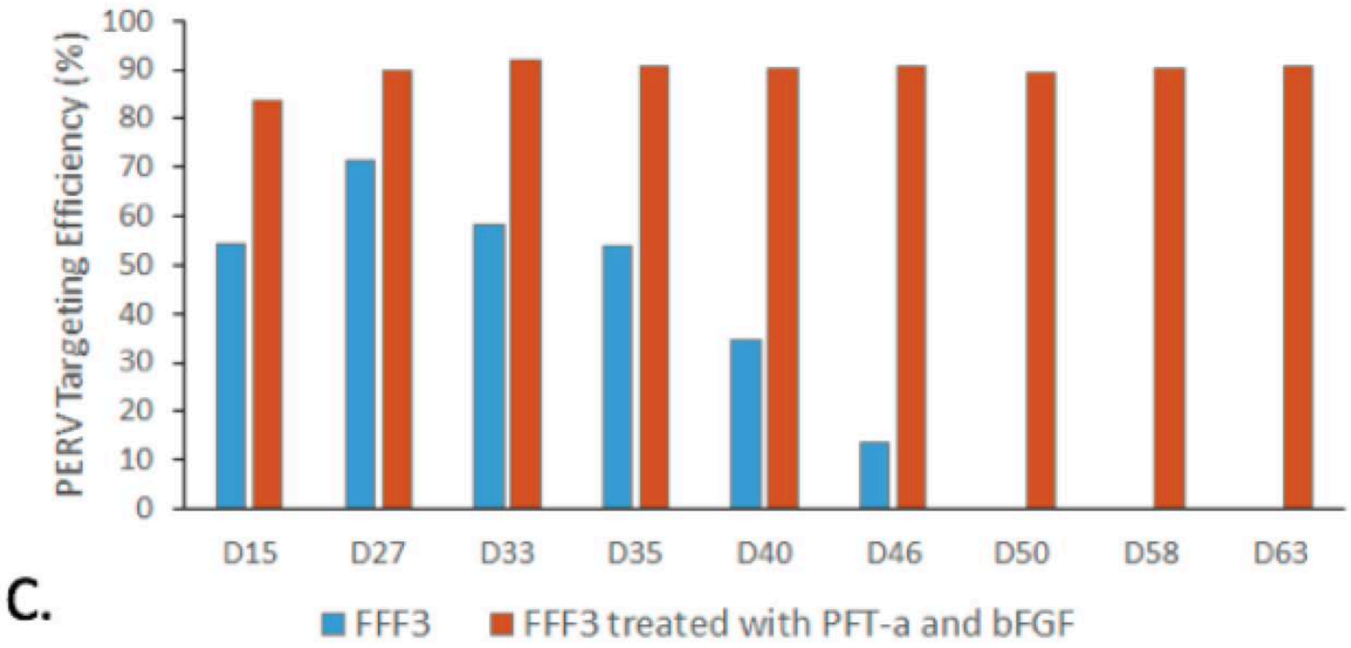


Does simultaneous DNA cleavage at several PERV sites trigger DNA damage-induced senescence or apoptosis?

- Addition of the p53 inhibitor pifithrin alpha (PFTα) and basic fibroblast growth factor (bFGF) significantly increased the targeting efficacy of the resulting FFF3 populations

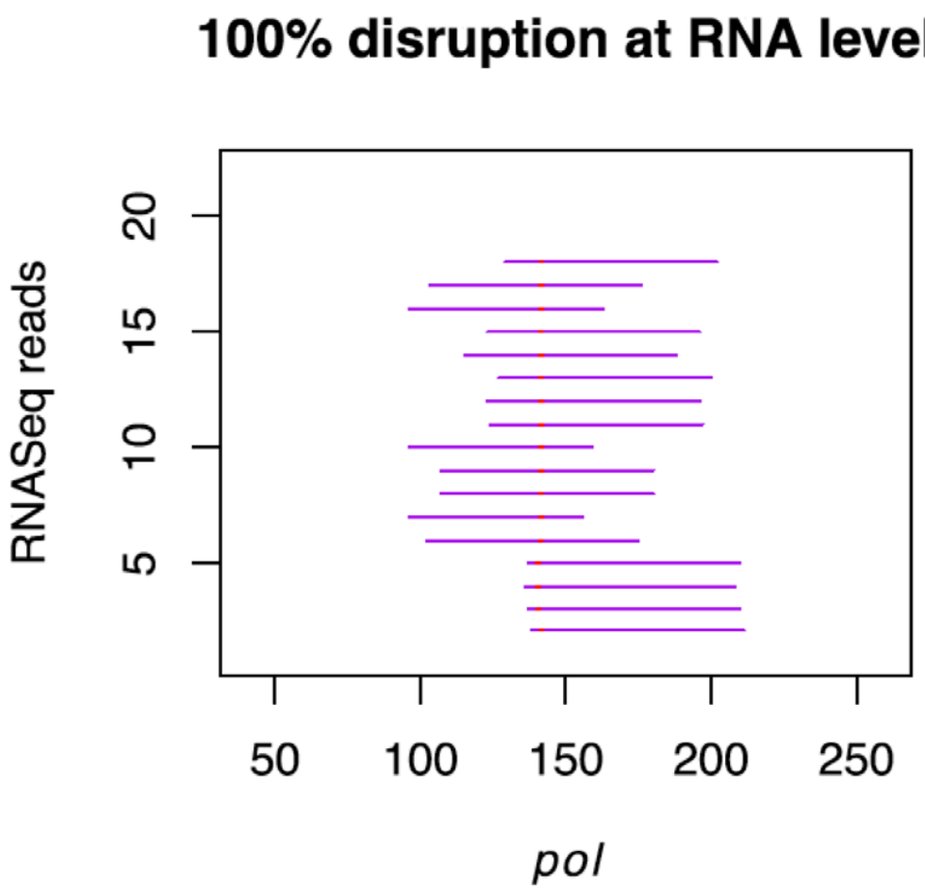


B.

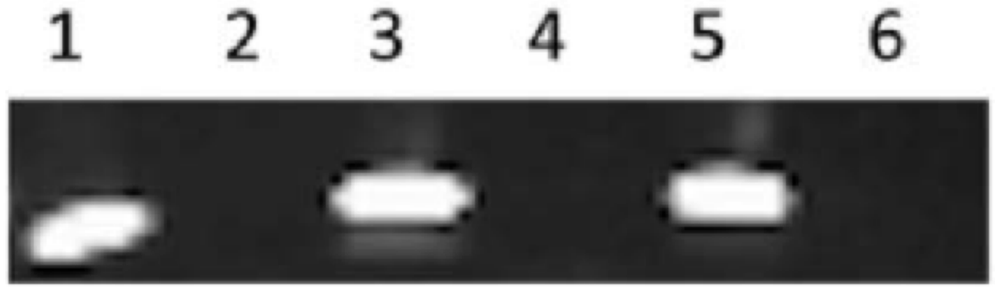


C.

Targeting FFF3 cells with CRISPR/Cas9 is highly efficient and leads to the elimination of PERV production

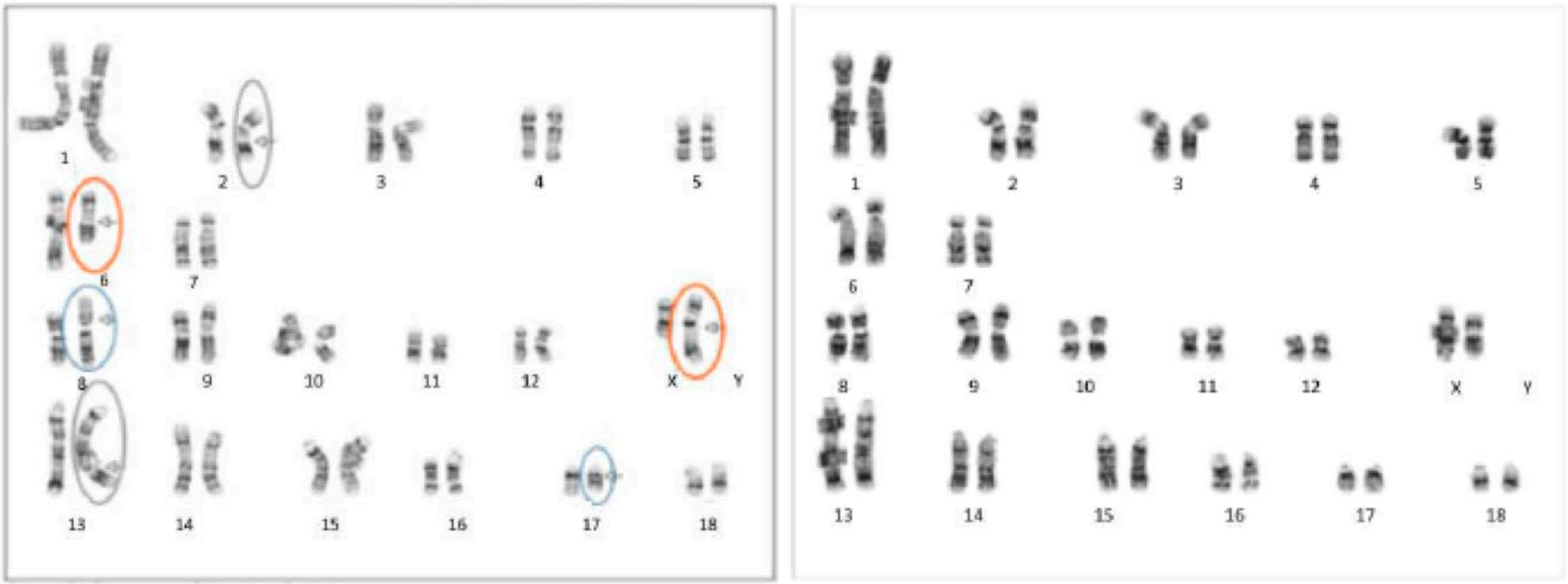


RT assay



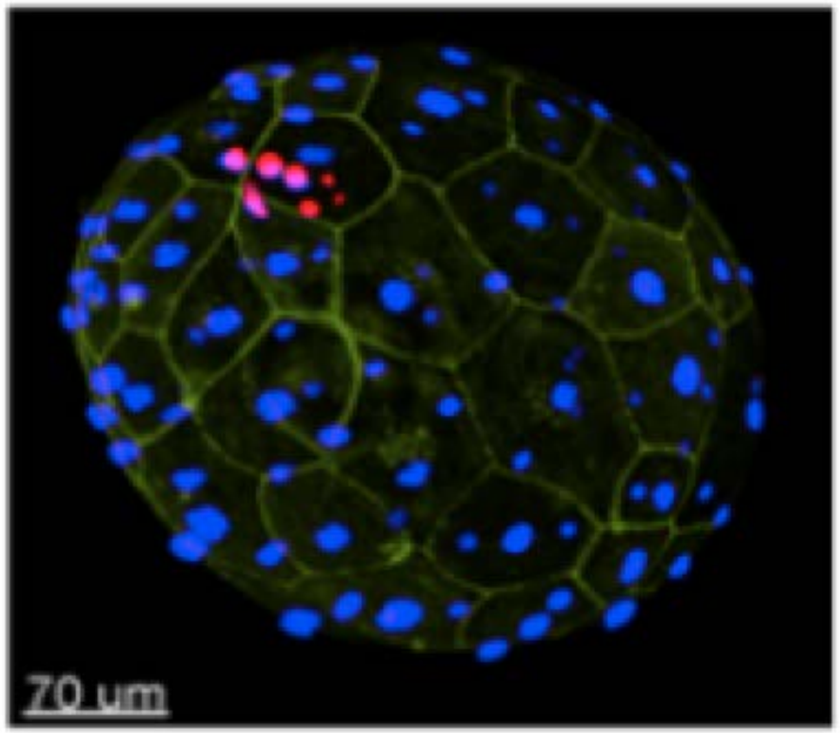
1. RT+ (using commercial reverse transcriptase (RT))
2. RT- (no RT enzyme)
3. RT+/FF WT (commercial RT enzyme plus WT FFF3 lysis of virus pellet from FFF3 culture media)
4. 100% PERV-ko FFF3 (100% PERV-inactivated FFF3 clone lysis)
5. WT FFF3 (WT FFF3 lysis)
6. neg (no lysis or RT enzyme, no RNA template)

Karyotype analysis shows chromosomal aberrations on chromosomes bearing PERVs (n=5) with n=3 100% PERV-free FFF3 showing normal karyotypes

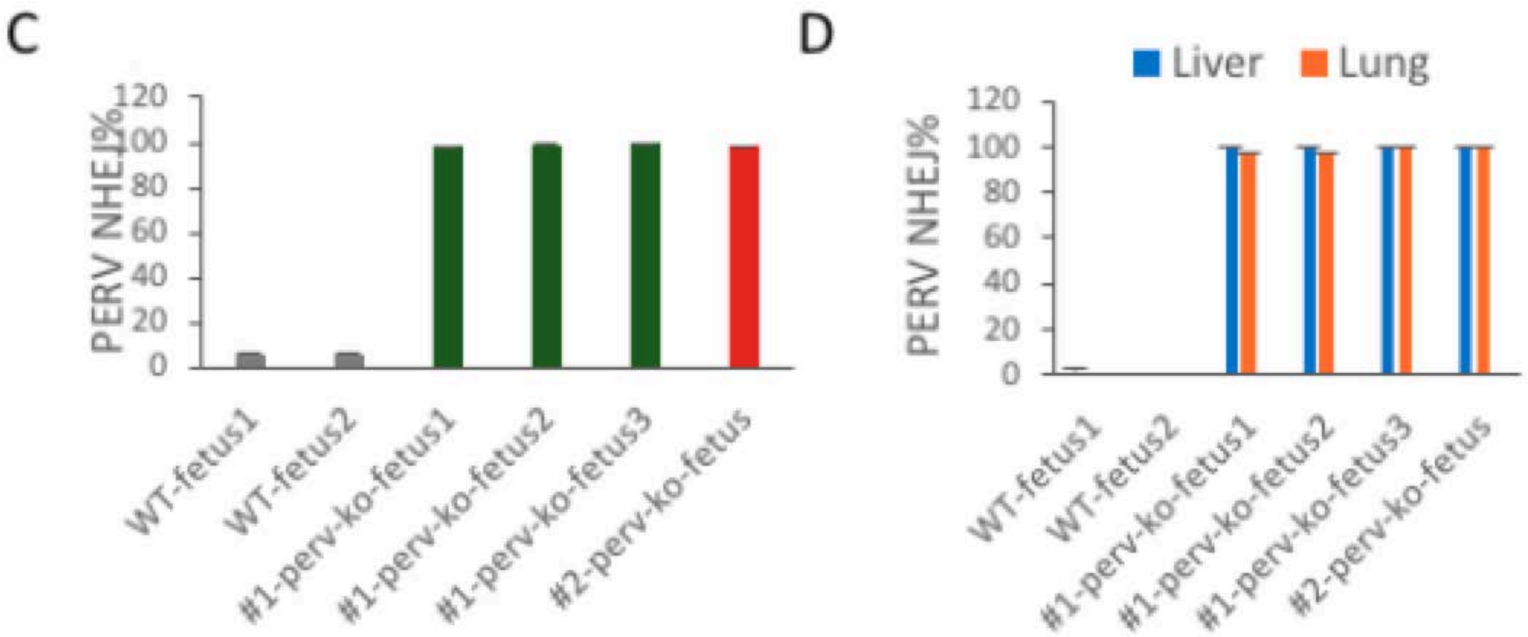


By sequencing of PERV junctions, no macrodeletions, as induced by CRISPR/Cas9, could be found

Production of PERV-inactivated embryos by SCNT

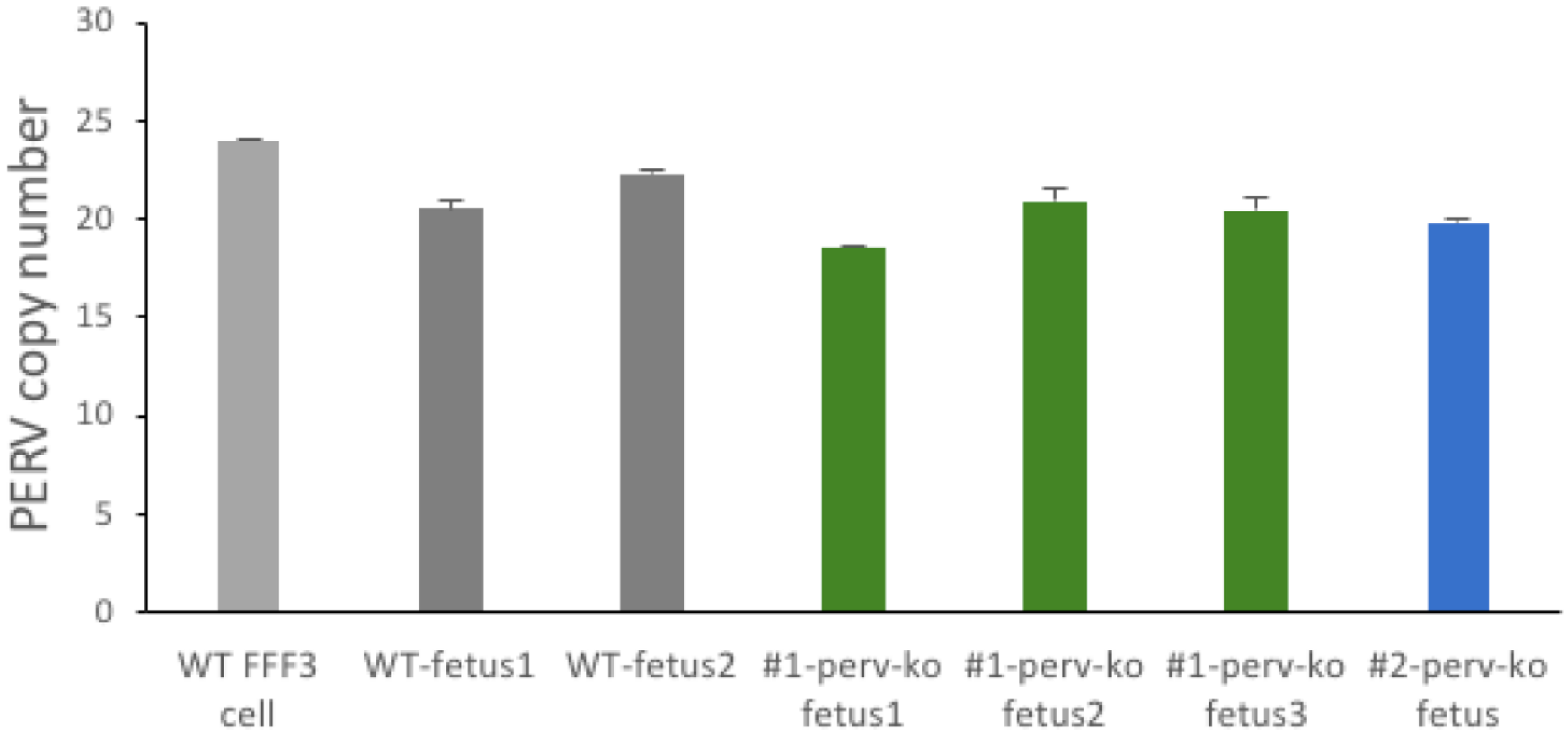


- *20-40% of the constructed, PERV-inactivated embryos reached blastocyst stage after 6 days of culture (normal porcine efficacy)*
- *Sox2 staining demonstrates pluripotency of inner cell mass*



Confirmation of ~100% PERV eradication on DNA (left) and mRNA (right) level

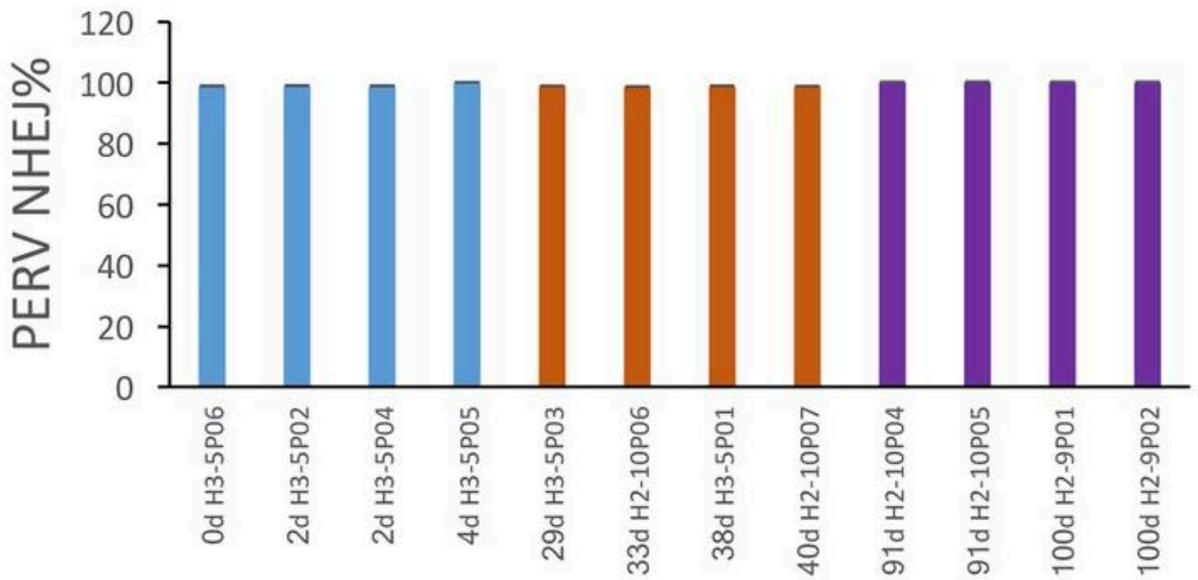
PERV copy numbers remain constant between PERV-inactivated fetuses and WT FFF3



Photoshooting day

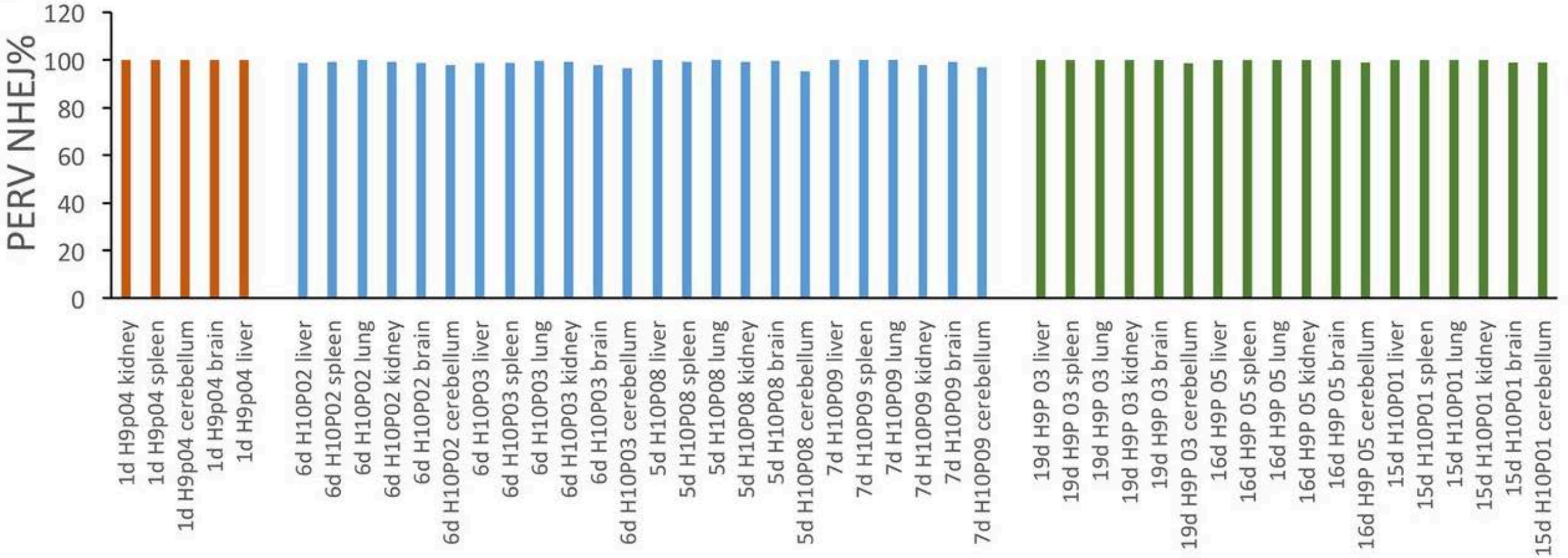
Laika

Newborn pigs upheld PERV inactivation



left: DNA

below: mRNA



Synopsis Niu et al.

- PERVs can replicate in human cells and amongst human cells *in vitro*
- *treatment with p53 inhibitor can mitigate the stress from multiplex DNA damage during multiplexible genome engineering and support clonal expansion of 100% PERV-inactivated cells*
- *successful generation of PERV-inactivated pigs from primary porcine cell lines through SCNT, those pigs could provide safe resources for xenotransplantation*
- *physiological functions of PERVs remain largely unknown*
- *effects of PERVs on human disease remain unknown*

THANK YOU FOR YOUR ATTENTION