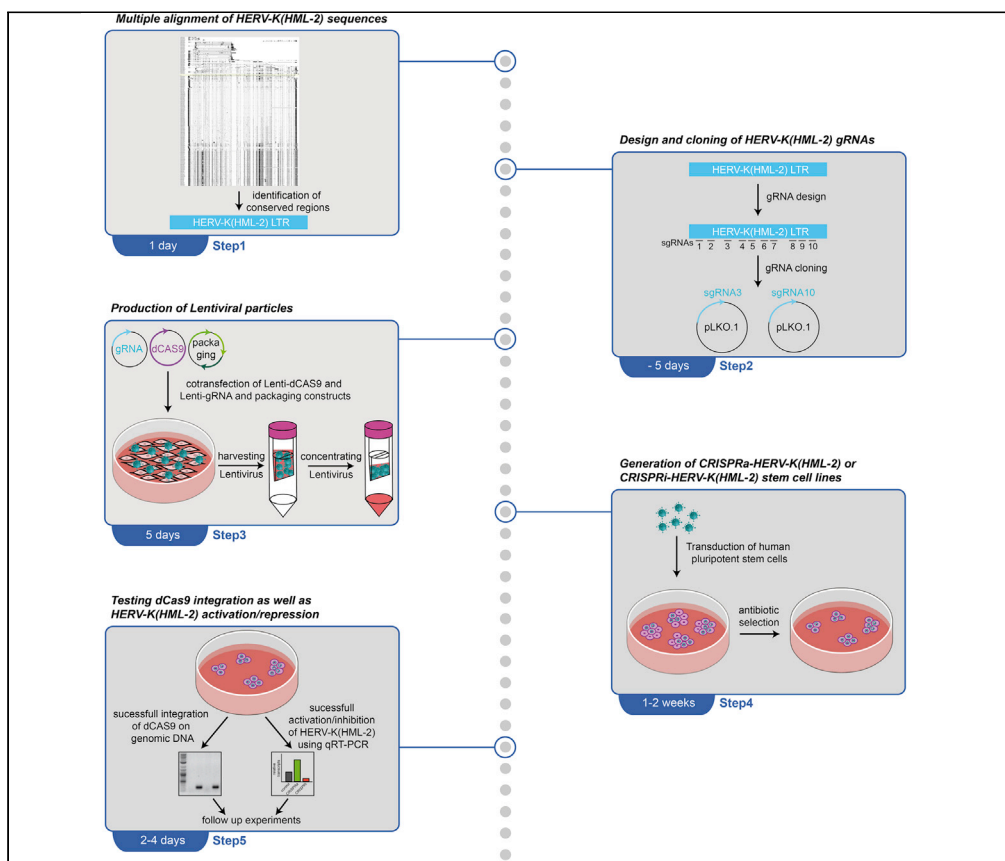


## Protocol

# A protocol for CRISPR-mediated activation and repression of human endogenous retroviruses in human pluripotent stem cells



Human endogenous retroviruses (HERVs) comprise many regulatory elements and can regulate host gene activity at different expression levels via multiple mechanisms. Here, we introduce a step-by-step protocol to activate or repress transcription of HERV-K(HML-2) elements using the CRISPRa and CRISPRi technologies in human embryonic stem cells. This protocol can help deciphering the functional role of HERV-K(HML-2) elements in critical biological processes. The protocol may easily be adapted to other cell lines and HERV groups with relatively low sequence heterogeneity.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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### Highlights

Detailed protocol to manipulate HERV-K(HML-2) expression in human stem cells

The Protocol uses CRISPRa and CRISPRi to modulate transcription of HERV-K(HML-2)

Describes design of HERV-K(HML-2)-targeting gRNAs till validation of transcript levels

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## Protocol

## A protocol for CRISPR-mediated activation and repression of human endogenous retroviruses in human pluripotent stem cells

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<https://doi.org/10.1016/j.xpro.2022.101281>

## SUMMARY

Human endogenous retroviruses (HERVs) comprise many regulatory elements and can regulate host gene activity at different expression levels via multiple mechanisms. Here, we introduce a step-by-step protocol to activate or repress transcription of HERV-K(HML-2) elements using the CRISPRa and CRISPRi technologies in human embryonic stem cells. This protocol can help deciphering the functional role of HERV-K(HML-2) elements in critical biological processes. The protocol may easily be adapted to other cell lines and HERV groups with relatively low sequence heterogeneity.

For complete details on the use and execution of this protocol, please refer to Padmanabhan Nair et al. (2021).

## BEFORE YOU BEGIN

## Protocol overview

In this protocol, we attempt to provide a detailed description of the CRISPR activation and inhibition system to manipulate expression of human endogenous retroviruses (HERVs) in human pluripotent stem cells (hPSCs). Here, we have used HERV-K(HML-2) as an example for activation as well as repression, which is described in detail in Nair V.P et al. (Padmanabhan Nair et al., 2021). Two single guide RNAs (gRNA) were used in combination to specifically target the HERV-K(HML-2) group for CRISPR activation (CRISPRa) or CRISPR inhibition (CRISPRi). Each experiment was complemented with a control gRNA. The protocol describes the specific steps necessary for designing specific gRNAs against HERV-K(HML-2), gRNA cloning and subsequent generation of a CRISPRa/CRISPRi HERV-K(HML-2) targeting human embryonic stem cell line H9 (WA09) using lentiviral transduction. This protocol can be easily adapted for use in other cell lines and modulation of other HERV groups. We have used this protocol to target various HERV groups, for example, HERV-W, HERV-H or HERV-K(HML-2), in various cell lines including HEK293T, HeLa, HT1080, HNSC.100, SH-SY5Y and human induced pluripotent stem cells (iPSCs). Although this protocol is highly adaptable, alterations may significantly change the parameters of the experimental set up and time required for an experiment. Hence, proper planning of the experiment should be done in advance to ensure feasibility. To ensure biologically meaningful and reproducible results, care must be taken throughout the experiments regarding consistent amounts of reagents and cell numbers used, and to avoid any sort of bacterial, fungal or mycoplasma contamination. Clean and consistent tissue culture conditions, reagents and high-quality culture dishes are highly recommended.

△ **CRITICAL:** Work with lentiviruses may have to be performed in a BSL-2 certified laboratory with class A2 biosafety cabinets.



### Preparation of primers, gRNAs and plasmids

⌚ Timing: ~2 days

1. Order qPCR primers and gRNA oligos listed in the [key resources table](#) and dissolve them in the desired amount of nuclease-free water to produce a final concentration of 100  $\mu$ M.
2. Prepare high-quality preparations of the following lentiviral plasmids, psPAX2 (Addgene #12260); pMD2.G (Addgene #12259); pHAGE EF1 $\alpha$  dCas9-KRAB (Addgene #50919); pHAGE EF1 $\alpha$  dCas9-VP64 (Addgene #50918); pLKO.1-puro U6 sgRNA BfuAI stuffer (Addgene #50920); pLKO.1-puro U6 sgRNA CAG (Addgene #50927), to improve transfection efficiency and obtain high lentiviral titers.

⚠ **CRITICAL:** For the preparation of lentiviral transfer as well as packaging plasmids, use the *E. coli* strains Stb13 or DH5 $\alpha$  as these strains reduce the frequency of homologous recombination.

### Preparation of LB agar plates

⌚ Timing: ~4 h

3. Prepare 8-10 plates of LB Agar with Ampicillin resistance.
  - a. Take 500 mL of autoclaved LB Agar.
  - b. Thaw the agar in a microwave oven until the agar turns liquid.
  - c. Allow the agar medium to cool down to 45°C and add 100 mg/mL of Ampicillin antibiotic into the melted agar medium.
  - d. Mix and pour the agar medium into 10 cm bacterial plates and allow it to cool and solidify under the bacterial culture hood.

**Note:** You may keep the lid half open to speed up solidification and minimize condensation.

### Preparation of complete essential 8 flex media

⌚ Timing: ~2 h

4. Thaw the frozen Essential 8 Flex Supplement at 20°C–25°C (takes about one hour). Transfer the entire content of the Essential 8 Flex Supplement to the Essential 8 Flex Basal Medium. Swirl the bottle to mix solutions thoroughly. Complete Essential 8 Flex Medium can be stored at 2°C–8°C for up to three weeks.

⚠ **CRITICAL:** Do not warm the Essential 8 flex medium at 37°C and do not thaw the frozen Essential 8 Media supplement at 37°C.

### Modification of lentiviral transfer vector

⌚ Timing: ~2 days

5. Select the desired lentiviral vector, which is to be used for the cloning of the sgRNAs. We used the lentiviral vector pLKO.1 puro U6 sgRNA BfuA1 stuffer (kindly provided by Rene Maehr and Scott Wolfe, (Kearns et al., 2014), Addgene Plasmid #50920). Besides the puromycin selection cassette, we also used blasticidin. Therefore we replaced the puromycin selection cassette with a blasticidin antibiotic resistance cassette to ensure proper selection of the two guide RNAs following standard cloning techniques.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Cas9 8G4 1:100	Monoclonal Antibody Core Facility Helmholtz Zentrum Munich	Cas9 8G4
Alexa Fluor goat anti rat 568 1:500	Thermo Fisher Scientific	A11077
<b>Bacterial and virus strains</b>		
<i>E. coli</i> strain Stb13	Thermo Fisher Scientific	C737303
Subcloning efficiency DH5 alpha	Thermo Fisher Scientific	18265017
<b>Chemicals, peptides and recombinant proteins</b>		
BfuA1	New England BioLabs	R0701S
Tris-EDTA	Sigma-Aldrich, Darmstadt, Germany	93283
T4 DNA ligase	New England BioLabs	EL0011
Ampicillin	Sigma-Aldrich, Darmstadt, Germany	A0166
Puromycin	Sigma-Aldrich, Darmstadt, Germany	P8833
Blasticidin S Hydrochloride	Santa Cruz	sc-204655A
S.O.C. media	Sigma-Aldrich, Darmstadt, Germany	S1797
Agarose	Sigma-Aldrich, Darmstadt, Germany	A4718
LB Media	Carl Roth, Karlsruhe, Germany	X968.1
LB Media with agar	Sigma-Aldrich, Darmstadt, Germany	L3272
Bovine Serum Albumin	Sigma-Aldrich, Darmstadt, Germany	A4503
Recombinant Vitronectin (r-VTN)	Thermo Fisher Scientific	A14700
Polybrene	Sigma-Aldrich, Darmstadt, Germany	H9268
Trizol	Thermo Fisher Scientific	15596026
Essential 8 Flex Medium Kit	Thermo Fisher Scientific	A2858501
DMEM	Thermo Fisher Scientific	21063-029
Fetal Bovine Serum	Thermo Fisher Scientific	10500-064
Sodium Pyruvate	Thermo Fisher Scientific	11360070
Penicillin/Streptomycin (100x)	Thermo Fisher Scientific	15140-122
0.05% Trypsin-EDTA	Thermo Fisher Scientific	25300-054
Xtreme Gene DNA transfection Reagent	Sigma-Aldrich, Darmstadt, Germany	6366244001
Opti-MEM	Thermo Fisher Scientific	31985062
<b>Critical commercial assays</b>		
Nucleospin Plasmid DNA Isolation Kit	MACHEREY-NAGEL, Dueren, Germany	740588.50
RevertAid First Strand cDNA Synthesis Kit	Thermo Fisher Scientific	K1621
Promega GoTaq PCR	Promega, Madison, USA	M300
Expand High Fidelity PCR system	Sigma-Aldrich, Darmstadt, Germany	11732641001
NucleoSpin Gel and PCR purification kit	MACHEREY-NAGEL, Dueren, Germany	740609.50
QIA-amp DNA mini-Genomic DNA isolation Kit	QIAGEN, Hilden, Germany	51306
DNase I, RNase-free	Thermo Fisher Scientific	EN0521
<b>Oligonucleotides</b>		
HERV-K HML-2 gRNA3 U: AAATGGATTAAGGGCGGTGC	(Padmanabhan Nair et al., 2021)	N/A
HERV-K HML-2 gRNA3 L: GCACCGCCCTTAATCCATTTC	(Padmanabhan Nair et al., 2021)	N/A
HERV-K HML-2 gRNA10 U: ATCCTCCATATGCTGAACGC	(Padmanabhan Nair et al., 2021)	N/A
HERV-K HML-2 gRNA10 L: GCGTTCAGCATATGGAGGATC	(Padmanabhan Nair et al., 2021)	N/A
Primer HERV-K(HML-2) Forward: GGCCATCAGAGTCTAAACCACG	(Padmanabhan Nair et al., 2021)	N/A
Primer HERV-K(HML-2) Reverse: CTGACTTTCTGGGGGTGCCCG	(Padmanabhan Nair et al., 2021)	N/A
Primer dCas9 Forward: TCGGATCTGCTACCTGCAGGAGATCTTTAG	(Padmanabhan Nair et al., 2021)	N/A

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Primer dCas9 Reverse: CAGCCTTATCAGTACTGTCTACCAGCTTCT	(Padmanabhan Nair et al., 2021)	N/A
Primer RNA Polymerase II Forward: GCACCCAGTCCAATGACAT	(Padmanabhan Nair et al., 2021)	N/A
Primer RNA Polymerase II Reverse: GTGCGGCTGCTTCAATAA	(Padmanabhan Nair et al., 2021)	N/A
pLKO.1-puro U6 sgRNA sequencing Primer: CGTGACGTAGAAAGTAATAATTC	This paper	N/A
<b>Recombinant DNA</b>		
pLKO.1-puro U6 sgRNA BfuAI stuffer	Addgene (a gift from Rene Maehr & Scot Wolfe)	Plasmid #50920
pLKO.1-puro U6 sgRNA CAG	Addgene (a gift from Rene Maehr & Scot Wolfe)	Plasmid #50927
pHAGE EF1 $\alpha$ dCas9- VP64	Addgene (a gift from Rene Maehr & Scot Wolfe)	Plasmid #50918
pHAGE EF1 $\alpha$ dCas9- KRAB	Addgene (a gift from Rene Maehr & Scot Wolfe)	Plasmid #50919
pMD2.G	Addgene (a gift from Didier Trono)	Plasmid #12259
pSPAX2	Addgene (a gift from Didier Trono)	Plasmid #12260
pLKO U6 HERV-K G3	This paper	N/A
pLKO U6 HERV-K G10	This paper	N/A
<b>Software and algorithms</b>		
Optimized CRISPR design tool	Zhang Lab, MIT	<a href="https://zlab.bio/guide-design-resources">https://zlab.bio/guide-design-resources</a>
CRISPR-ERA	Lei Stanley Qi Lab, Xiaowo Wang Lab, Stanford	<a href="http://crispr-era.stanford.edu">http://crispr-era.stanford.edu</a>
Graph Pad Prism	GraphPad	<a href="https://www.graphpad.com/scientific-software/prism/">https://www.graphpad.com/scientific-software/prism/</a>
SnapGene	Insightful Science	<a href="https://www.snapgene.com">https://www.snapgene.com</a>
LC480 SW 1.5.1	Roche	<a href="https://lifescience.roche.com/en_de/products/lightcycler14301-480-software-version-15.html">https://lifescience.roche.com/en_de/products/lightcycler14301-480-software-version-15.html</a>
Adobe Illustrator	Adobe Inc.	<a href="https://www.adobe.com/products/illustrator.html">https://www.adobe.com/products/illustrator.html</a>

**MATERIALS AND EQUIPMENT**

- Preparation of Oligo Annealing Buffer: Required for annealing the respective gRNA oligos before cloning; 1 M Tris-HCl, pH 8.0; 5 M NaCl; 500 mM EDTA, pH 8.0.
- Prepare EDTA solution for splitting hPSCs. In a 500 mL filter bottle, add approx. 450 mL 1 × PBS, 500  $\mu$ L 0.5 M EDTA solution and 0.9g NaCl. Filter the EDTA solution into the bottom receiver bottle add 1 × PBS to reach a final volume of 500 mL.

**Oligo Annealing Buffer**

Reagent	Final concentration	Amount
Tris-HCL pH 8.0	10 mM	1 mL
NaCL	50 mM	1 mL
EDTA pH 8.0	1 mM	0.2 mL
<b>Total</b>	n/a	100 mL

Can be stored for several months at 20°C–25°C.

**EDTA solution for splitting human stem cells**

Reagent	Final concentration	Amount
EDTA	0.5 mM	500 $\mu$ L

(Continued on next page)

### Continued

Reagent	Final concentration	Amount
NaCL	n/a	0.9 g
PBS	n/a	450 mL
<b>Total</b>	<b>n/a</b>	<b>500 mL</b>

Filter the EDTA solution before use. EDTA solution can be stored at 20°C–25°C for several months. <https://www.stembook.org/node/748>.

## STEP-BY-STEP METHOD DETAILS

### Designing guide RNAs

⌚ Timing: ~1 day

To modulate expression of HERV-K(HML-2) elements, guideRNA (gRNA) sequences need to be designed targeting, ideally, as many 5'LTRs of the HERV-K(HML-2) group as possible. Various sequence databases may be considered as sources for HERV-K(HML-2) sequences of interest, for instance, Dfam (Storer et al., 2021) and UCSC Table Browser (Karolchik et al., 2004) to retrieve sequences and subsequently generate multiple alignments that aid in gRNA design.

1. Compile HERV-K(HML-2) sequences of interest from a suited database.
  - a. Dfam provides group-specific consensus sequences and pre-generated multiple sequence alignments available for download.
  - b. As for UCSC Table Browser,
    - i. Select a recent human reference genome assembly (e.g., hg19 or hg38).
    - ii. Select group:Repeats; track:Repeatmasker; table:rmsk.
    - iii. Define the region of interest as "genome".
    - iv. Create a filter depending on your HERV sequences of interest. For instance, the HERV-K(HML-2) group comprises different LTR subgroups, specifically LTR5, LTR5A, LTR5B, and LTR5\_Hs. Thus identify respective LTR elements by applying those designations to "repName does match" each.
    - v. Optionally, identify elements on plus and minus strands separately by applying "strand does match + or -".
    - vi. Retrieve data as output format:sequence (or "all fields from selected table" in case elements compiled shall be further inspected before actual sequence retrieval).
    - vii. Retrieve data by selecting "get output".
    - viii. Designations of sequences retrieved in fasta format may be further modified.
2. Multiply align sequences compiled from UCSC Table Browser employing a suited algorithm, for instance, MAFFT (Katoh and Standley, 2013) or MUSCLE (Edgar, 2004). Potentially optimize the resulting multiple alignment manually using a suited software.
3. Use the consensus sequence generated from the multiple alignment (or as provided by Dfam) for gRNA design. Software such as CRISPR-ERA (<http://crispr-era.stanford.edu/>) may be employed for gRNA design.

**Note:** The Cas9 protein used in this study recognizes NGG as the PAM site.

4. Potentially further assess (dis)similarities of gRNA sequences with target sequences in order to identify best suited gRNAs.
5. Select at least two gRNAs suggested by the software, with an emphasis on least off-target effects and maximum efficiency.

**Note:** We usually choose 2–3 different gRNA sets and test their efficiency in a HEK293T-dCAS9-VP64 and HEK293T-dCAS9-KRAB cell line before moving into human stem cells.

HEK293T-dCAS9-VP64 as well as HEK293T-dCAS9-KRAB can be generated in the same way as explained in this protocol.

△ **CRITICAL:** gRNA design is crucial and care should be taken in order to limit off-target effects. We employed two gRNAs targeting HERV-K(HML-2) LTRs to ensure proper activation or repression, as described in Kearns et al. (Kearns et al., 2014).

### Cloning of guide RNAs (gRNAs)

⌚ **Timing:** ~3–4 days

Designed gRNAs are cloned into lentiviral transfer vector pLKO.1-puro U6 sgRNA BfuAI stuffer (Addgene #50920) to ultimately generate a hPSC line stably expressing dCAS9 and the respective gRNA pair.

#### 6. Digestion of lentiviral plasmid vector.

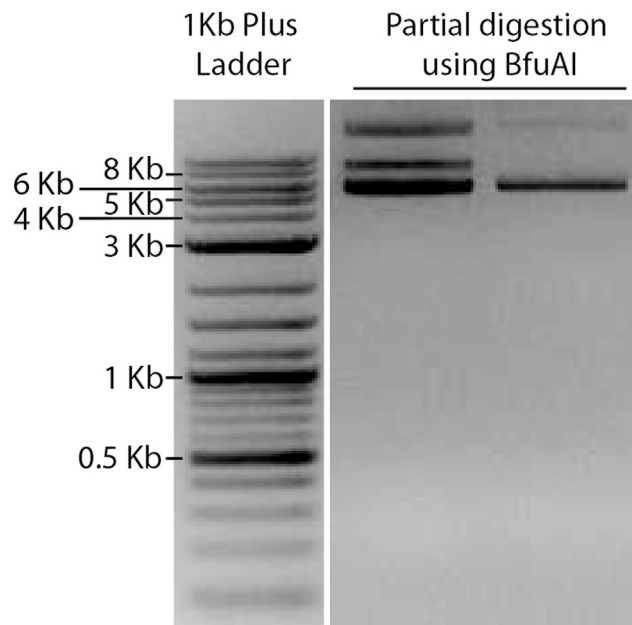
- a. Assemble the restriction digestion reaction set up on ice as shown in Table 1. Digest 1 µg of lentiviral vector (pLKO.1-puro U6 sgRNA BfuAI stuffer) with 1.5 units of *BfuA1* enzyme in a 50 µL reaction. Pipette and combine all the reagents into a 1.5 mL microcentrifuge tube, mix well by tapping the tube or by gently pipetting up and down and spin them down to collect the entire reaction mixture at the bottom of the tube. Incubate the reaction at 50°C for 1.5–2 h in a benchtop heating block or a water bath. Terminate the reaction by heat inactivation at 65°C for 20 min.
- b. Double-check proper digestion by electrophoresis of 3 µL of the digestion in a 0.8% [w/v] agarose gel.
- c. For preparing the agarose gel for purification of the remaining plasmid vector, mix 0.8 grams of electrophoresis grade agarose and 100 mL of 1 × TAE buffer and boil in a microwave until the agarose dissolves completely. Cool the agarose solution to approx. 45°C. Add Ethidium Bromide and pour the gel in a clean standard gel casting tray with a comb with a sufficiently large well volume. Allow the gel to solidify.

**Note:** Extreme care should be taken while working with Ethidium Bromide by complying with all the safety requirements.

- d. Load the digested vector sample along with a tracking dye (e.g., Bromophenol Blue) and a suited DNA size standard.
- e. Use undigested vector as a control for proper and complete digestion.
- f. Perform electrophoresis in 1 × TAE buffer at approx. 100–120 V. Ensure adequate separation of restriction fragments by electrophoresis for 20–30 min. The restriction enzyme-treated plasmid should linearise and produce a single DNA fragment. Visualize DNA fragments on a standard UV table.
- g. Once complete digestion of the vector is confirmed, purify the remaining digested vector using a PCR purification kit, following the manufacturer's instructions <https://www.mn-net.com/media/pdf/02/1a/74/Instruction-NucleoSpin-Gel-and-PCR-Clean-up.pdf>.

**Table 1. Restriction digestion**

Reagent	Amount
Vector	1 µg
10× NEBuffer 3.1	3 µL
<i>BfuA1</i>	1.5 µL
Water	Add to reach 50 µL final



**Figure 1. Cloning of guide RNAs (gRNAs)**

Agarose Gel electrophoresis showing partial digestion of plasmid pLKO.1-puro U6 sgRNA BfuAI stuffer using *BfuAI* enzyme.

- i. We usually use Macherey Nagel NucleoBond PCR purification kit. Elute the digested plasmid DNA in 20  $\mu$ L of nuclease-free water.
- ii. You may also load the entire reaction product onto an agarose gel and, after electrophoresis, extract the digested vector DNA from the agarose gel. Excise the digested product using a clean razor blade by placing the gel on a UV transilluminator and transfer the gel slices into a clean 1.5 mL microcentrifuge tube. Purify DNA from extracted gel pieces using a suited Gel extraction Kit, following the manufacturer's instructions.

**Note:** As for gel extraction of restriction fragments, remove as much excess gel as possible. Protect eyes and skin from UV light while handling the agarose gel on the UV transilluminator.

**Note:** The agarose gel should be exposed to UV for as short as possible to minimize UV induced DNA damage. Nucleic acid stains other than ethidium bromide, not requiring illumination by UV, may be used instead.

7. After the purification step, measure the concentration of the digested plasmid vector DNA using a Nanodrop.
8. Load and electrophorese 100 ng of the purified vector sample in a 0.8% agarose gel to control for proper digestion and purification.

**△ CRITICAL:** Digestion of plasmid vector with *BfuA1* is extremely crucial for successful cloning of single guide RNAs into the lentiviral backbone. The vector should be completely digested. After 2 h of digestion, check 3  $\mu$ L of the digested product on an agarose gel along with the undigested vector. If it is not completely linearized (as shown in [Figure 1](#)), prolong the digestion for another hour. Excess amounts of *BfuA1* enzyme might also hamper the digestion efficiency. Always perform the digestion in a total volume of 30–50  $\mu$ L.

9. Annealing of single guide RNA oligonucleotides.



**Table 2. Annealing mixture for gRNA cloning**

Reagent	Final concentration	Amount
HERV-K HML-2 G3 Upper	10 M	1 $\mu$ L
HERV-K HML-2 G3 Lower	10 M	1 $\mu$ L
5 $\times$ annealing buffer		2 $\mu$ L
Water		6 $\mu$ L
<b>Total</b>		<b>10 <math>\mu</math>L</b>

- a. Upper and lower strands of single guide RNA oligonucleotides must be annealed before proceeding for cloning. The annealing reaction set up for the first pair of gRNA (G3) for HERV-K(HML-2) is given in the Table 2. The same is to be done for the second gRNA (G10). Refer to the “before you begin” section for the composition of the annealing buffer.
- b. Add 1  $\mu$ L of the upper strand of the oligonucleotide and 1  $\mu$ L of the lower strand of the oligonucleotide. Incubate the reaction mixture at 95°C for 5 min and then ramp down to 25°C at 5°C/min. For annealing the desired oligos, set up a reaction in a 1.5 mL microcentrifuge tube as shown in Table 3.
- c. The annealed oligos are to be used as the insert for the ligation reaction.

10. Ligation of the gRNAs.

To ligate the annealed gRNA oligos into the pLKO.1-puro U6 sgRNA BfuAI stuffer vector, set up a ligation reaction (Table 3).

- a. Use 50 ng of the digested and purified pLKO.1-puro U6 sgRNA BfuAI stuffer.
- b. As for the insert, pre-dilute the annealed gRNA oligonucleotides 1:10 using nuclease free water, add 4  $\mu$ L of the 1:10 dilution per reaction mix.
- c. Add 1  $\mu$ L of 10 $\times$  NEB ligase buffer and 1  $\mu$ L of T4 DNA ligase.

**Note:** Include a “vector-only” reaction as a control. Add 4  $\mu$ L of nuclease free water instead of the gRNA insert for that control.

11. Transformation of the ligation reaction.

- a. Pre-warm ampicillin-agar plates in a 37°C incubator.
- b. Set a water bath to 42°C.
- c. Thaw a required amount of *E. coli* competent cells, stored at –80°C, on ice. We used *E. coli* strain Stbl3. For each transformation, use 30  $\mu$ L of Stbl3 cells.

**Note:** Competent cells can also be self-made as described in, for example, (Inoue et al., 1990).

- d. Add 5  $\mu$ L of vector-plus ligation reaction to 30  $\mu$ L of Stbl3 competent cells kept on ice. The same for the ligation control reaction.
- e. DO NOT mix with a pipette, but gently tap the tube, then incubate on ice for 30 min.
- f. Subject the reaction to a heat shock in a water bath at 42°C for 45 s.
- g. Snap-cool the reaction for 5 min on ice.

**Table 3. gRNA ligation mixture**

Reagent	Amount
Vector	50 ng
Insert (1:10 dilution of the annealed oligos)	4 $\mu$ L
10 $\times$ NEB Ligase buffer	1 $\mu$ L
T4 DNA ligase	1 $\mu$ L
Water	add to 10 $\mu$ L final
<b>Total</b>	<b>10 <math>\mu</math>L</b>

- h. Add 200  $\mu$ L of prewarmed S.O.C nutrient medium and grow the cells in a 37°C shaking incubator for 45 min.
- i. Plate the transformation mixes (“vector-only” as well as “vector-plus ligation”) onto prewarmed LB Agar plates with the respective antibiotics, here Ampicillin (100  $\mu$ g/mL), using a sterile loop and incubate at 37°C for 16–18 h in a bacterial incubator.
- j. On the next day, check for growth of bacterial colonies on the agar plate.
- k. The vector-only ligation control plate is expected to show no colonies.

**Note:** Some colonies might grow on the “vector-only” ligation control plate due to sporadic religation events.

- l. The vector-plus ligation should show at least 90% more colonies on the plate in comparison with the vector-only control.
- m. Pick at least 5–8 single colonies from the vector-plus ligation plate and inoculate in 5 mL of LB media supplied with 5  $\mu$ L of Ampicillin.
- n. Incubate in a shaker at 37°C shaker for 16–18 h.
- o. On the next day, the culture should appear cloudy.
- p. Spin down the bacterial culture for 5 min at 1,000  $\times$  g at 20°C–25°C.
- q. Discard the supernatant and store the pellet in a -80 deep freezer or proceed to plasmid DNA isolation directly.

**△ CRITICAL:** The transformation may be done according to standard protocols. Choice of suited competent cells for transformation is crucial in this cloning method, especially when working with lentiviral plasmids. Therefore *E. coli* competent cells with low recombination rate should be used.

12. Isolation of Plasmid DNA. Isolation of plasmid DNA may be done according to the manufacturer’s instructions, for instance, Machery Nagel NucleoSpin Plasmid DNA isolation Kit <https://www.mn-net.com/media/pdf/45/51/02/Instruction-NucleoSpin-Plasmid.pdf>.
  - a. Prepare 5–6 bacterial culture tubes with 5 mL of LB medium supplied with 5  $\mu$ L of ampicillin (100 mg/mL) each.
  - b. Pick a single colony from the bacterial transformation agar plate using a pipette tip and transfer into culture medium.
  - c. Incubate the culture at 37°C in a shaker for about 16 h.

**Note:** Care should be taken not to overgrow the culture as it can affect plasmid DNA yield negatively.

- d. On the next day, pellet bacteria at 11,000  $\times$  g for 30 s, and start plasmid DNA isolation.
- e. Discard supernatant.
- f. Add resuspension buffer A1 and vortex.

**Note:** Vortexing the pellet after adding resuspension buffer will increase the DNA yield.

- g. Add buffer A2 and invert tube 6–8 times.
- h. Incubate at 20°C–25°C for 5 min.

**Note:** Do not vortex at this step, as this will release chromosomal DNA into the suspension.

- i. Add pre-cooled buffer A3 and invert tube 6–8 times.
- j. Centrifuge the suspension at 11,000  $\times$  g for 10 min at 4°C.
- k. Load the cleared lysate onto the column.
- l. Centrifuge the column at 11,000  $\times$  g for 1 min.

- m. Wash the column with buffer AW.
- n. Centrifuge the column at  $11,000 \times g$  for 1 min.
- o. Wash the column with buffer A4.
- p. Centrifuge the column at  $11,000 \times g$  for 1 min.
- q. Dry the silica membrane by centrifugation of the column at  $11,000 \times g$  for 2 min.
- r. Elute the plasmid DNA in 30–50  $\mu\text{L}$  of nuclease-free water at  $11,000 \times g$  for 1 min.
- s. Measure the DNA concentration in a Nanodrop.
- t. The successful insertion of the guide RNA sequence into the vector needs to be verified by sequencing plasmid DNA using a U6 promoter primer.

**△ CRITICAL:** Before proceeding to generation of stable cell lines it is important to confirm successful integration of the respective gRNA into the lentiviral transfer vector, here pLKO.1-puro U6 sgRNA BfuAI stuffer vector, by sequencing.

**△ CRITICAL:** We usually employ HEK293T cells to test the efficiency of the cloned gRNAs and define the most efficient gRNA combinations to activate or repress specific HERV groups before further proceeding in the protocol. HEK293T cells can be transfected efficiently with the respective plasmids, instead of lentiviral infections. This usually takes a week. After defining the most efficient gRNA set we proceed to generation of stable stem cell lines.

### Production and concentration of lentiviruses

**⌚ Timing:** ~1 week

Here we describe how lentiviruses carrying gRNAs, dCas9-VP64 or dCas9-KRAB are produced and concentrated. Once the lentiviruses are ready they can be used to transduce human pluripotent stem cells.

13. Cell culture. HEK293T cells are maintained in DMEM, supplemented with 10% [v/v] FBS and 5% [v/v] Penicillin/Streptomycin, in an incubator at  $37^\circ\text{C}$  and 5% [v/v]  $\text{CO}_2$ . Cells should be passaged every 3 days.

**△ CRITICAL:** It is important to use an early (less than 30) passage of HEK293T cells for viral production. Moreover care needs to be taken that HEK293T cells are not to be overgrown at any time.

14. Seeding HEK293T cells for transfection.
  - a. Start with a 80%–90% confluent T75 flask of HEK293T cells. Discard the cell culture medium, wash cells with 10 mL of  $1 \times$  PBS and trypsinize cells using 2 mL of 0.05% Trypsin-EDTA solution and incubate for 3–4 min at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ .
  - b. Resuspend the detached cells in 5 mL of DMEM medium and collect in a 50 mL Falcon.
  - c. Pellet cells at  $1,000 \times g$  for 5 min at  $20^\circ\text{C}$ – $25^\circ\text{C}$ .
  - d. Discard the medium and resuspend cells gently but thoroughly in fresh 5 mL of DMEM medium.
  - e. Take 10  $\mu\text{L}$  of the cell suspension and count cells in a hemocytometer or another suited cell counting device.
  - f. Seed  $2 \times 10^6$  cells in 7 mL of fresh cell culture medium in a 10 cm cell culture dish.
15. Plasmid transfection.
  - a. On the next day, HEK293T cells (at approx. 70% confluency) can be transfected with the packaging plasmid, psPAX, pMD2.G along with the transfer plasmids which contain the dCas9 fusion constructs or the respective gRNAs. In this protocol, fusion constructs pHAGE EF1 dCas9-VP64 and pHAGE EF1 dCas9-KRAB are used.

- b. For transfection mixture preparation, pipet 250  $\mu$ L of Opti-MEM in a 1.5 mL microcentrifuge tube supplied with 1.5  $\mu$ g of psPAX, 1  $\mu$ g of pMD2.G and 1  $\mu$ g of the respective transfer vector, mix the Opti-MEM and DNA by vortexing, then add 8  $\mu$ L of Xtreme gene DNA transfection reagent.
- c. Incubate the transfection mixture for 30 min at 20°C–25°C.
- d. In the meantime, replace the medium in cell culture dish with 7 mL of fresh culture medium.
- e. Add the transfection mixture to the medium in a dropwise manner. Swirl the plate to evenly distribute the mixture.
- f. Culture the cells for 72 h in a cell culture incubator at 37°C and 5% CO<sub>2</sub>.

**Note:** Transfection efficiency can be tested by including a transfection of HEK293T cells with a transfer plasmid encoding a fluorescent marker of choice.

16. Collection and concentration of viral particles.
  - a. After 72 h, using a 10 mL syringe, collect the cell culture medium from the 10 cm dish, pass it through a 0.45-micron filter into a fresh 50 mL Falcon.
  - b. Transfer the filtered medium into 100 kDa Amicon filter tubes.
  - c. Centrifuge the Amicon filter tubes at 5,000 g for 20 min at RT.

**Note:** Do not centrifuge Amicon filter tubes at more than 5,000 g to prevent tearing of the Amicon filter membrane.

- d. The medium should be concentrated to approx. 250  $\mu$ L.
- e. Transfer concentrated medium into fresh 1.5 mL microcentrifuge tubes.
- f. Concentrated media containing virus particles can be used immediately or stored at –80°C for up to 3 months.

**Note:** Storage of concentrated virus at –80°C for more than 3 months might reduce viral titers and transduction efficiency.

### Transduction of human pluripotent stem cells

⌚ **Timing:** ~1–2 weeks

For successful establishment of CRISPR activation and CRISPR inhibition method in human pluripotent stem cells (hPSCs) (H9/ WA09) in order to manipulate expression of HERV-K(HML-2) elements, cells need to be transduced with the generated viruses carrying gRNAs as well as dCAS9-VP64 or dCas9-Krab, respectively. A detailed protocol for transducing hPSCs is described below.

17. Cell Culture. Human PSCs (H9/ WA09) are maintained in Essential E8 Flex medium at 37°C and 5% CO<sub>2</sub>. Cells are passaged when one of the following occurs: (1) PSC colonies are becoming too dense or too large; (2) colonies cover more than approx. 85% of the surface area of the culture dish. Passaging is done usually every 3–4 days and replacing fresh media every second day following the manufacturer's instructions [https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Fessential\\_8\\_Flex\\_Medium\\_UG.pdf](https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Fessential_8_Flex_Medium_UG.pdf).
18. Seeding of human pluripotent stem cell.
  - a. Coat a 6-well culture plate with vitronectin at a coating concentration of 0.5  $\mu$ g/cm<sup>2</sup>.
  - b. Thaw vitronectin at 20°C–25°C.

**Note:** You will need approx. 60  $\mu$ L per 6-well plate.

- c. Add 6 mL of sterile 1 × PBS (without Calcium and Magnesium) into a 15-mL conical tube at 20°C–25°C and add 60 μL of vitronectin.
- d. Gently resuspend by pipetting the mix up and down several times.

**Note:** This results in a working concentration of 5 μg/mL.

- e. Add 1 mL of the diluted vitronectin solution to each well of a 6-well plate.
- f. Incubate the coated plate at 20°C–25°C for 1 h. After 45 min, proceed with step g.

**Note:** The coated plate can be used directly or stored at 2°C–8°C, and wrapped in parafilm, for up to one week.

- g. Discard the medium from an approx. 80% confluent culture of hPSCs (H9/WA09) cells in a 10 cm dish.
- h. Wash once with 10 mL 1 × PBS.
- i. Discard 1 × PBS and add 8 mL of EDTA solution. Refer to the “before you begin” section for the recipe of the EDTA solution.
- j. Incubate for exactly 2 min at 37°C and 5% CO<sub>2</sub> in an incubator.
- k. Discard the EDTA solution.
- l. Detach cells with 7 mL of E8 flex medium. Refer to the “before you begin” section for preparation of E8 flex medium.
- m. Carefully resuspend cells 2–3 times.

**Note:** Resuspend with great care as it is important that cells remain in patches to avoid cell death as well as spontaneous differentiation.

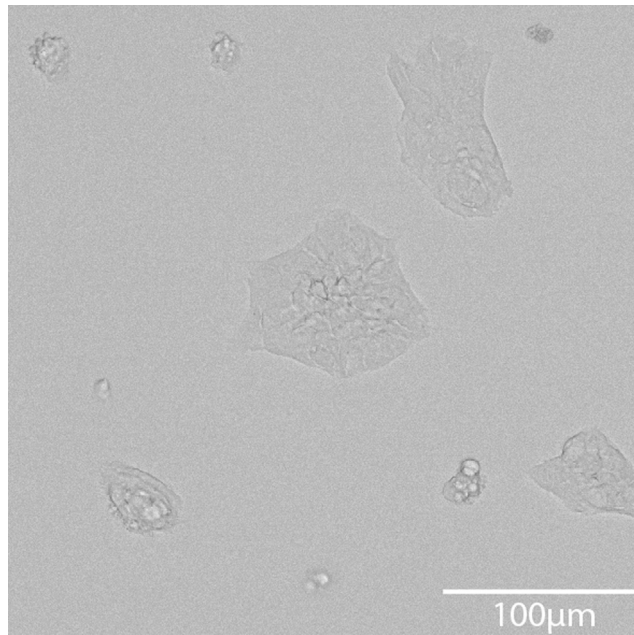
- n. Aspirate and discard the vitronectin solution (step f). It is not necessary to rinse the plate after removal of vitronectin. Cells can be seeded directly onto the vitronectin-coated plate surface.
  - o. Seed 0.5 mL of the cell suspension (H9/WA09) into a vitronectin coated well of the 6-well plate each.
  - p. Add another 1.5 mL E8 flex Medium per well to reach a total volume of 2 mL per well.
19. Transduction of human pluripotent stem cells (H9/WA09).
- a. On the next day, check for the optimal confluency (30%–40%) of the hPSC (H9/WA09) culture (as shown in [Figure 2](#)).
  - b. Discard the medium and replace with 1 mL of fresh E8 Flex medium each.
  - c. Mix 250 μL of the concentrated virus with 8 μg/mL polybrene.

**Note:** The amount of concentrated virus as well as the number of cells seeded before transduction needs to be tested for different cell lines.

- d. Add the virus/polybrene mixture to the cells.
- e. Do not add virus to one of the wells, this will serve as a transduction control.
- f. Culture the cells at 37°C and 5% CO<sub>2</sub> for 24 h.
- g. On the next day, replace medium with 2 mL of fresh E8 flex medium.

**Note:** Cell death will occur due to viral infection.

20. Selection of transduced cells.
- a. Allow the cells to grow for 3–4 days before selection.
  - b. Remove medium and wash the cells twice with 1 × PBS to remove dead cells.
  - c. Detach cells using EDTA and transfer them into a vitronectin-coated 10 cm cell culture dish.



**Figure 2. Transduction of human pluripotent stem cells**  
Bright field picture of hPSCs before transduction. Scale bars, 100  $\mu\text{m}$ .

- d. When cells reach at least 50% confluency subject transduced as well as untransduced control cells to 2  $\mu\text{g}/\text{mL}$  puromycin and 200  $\mu\text{g}/\text{mL}$  blasticidin for 48 h.

**Note:** Suited puromycin as well as blasticidin concentrations vary depending on the cell line used.

**Note:** Transduction efficiency varies, depending on the virus preparation, but usually is within 50%–60% range.

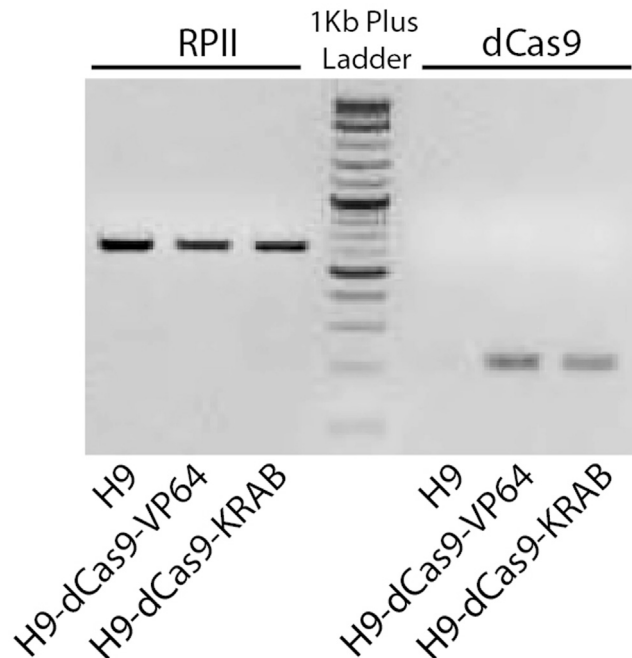
- e. On the next day, wash cells with 1  $\times$  PBS to remove dead cells and replace medium with fresh medium containing antibiotics.
- f. After three days of selection, wash cells and add fresh medium without antibiotics and allow the cells to grow until reaching 80% confluency.
- g. Further expand the cell culture and perform verification of successful integration. Freeze cell lines in liquid nitrogen following standard procedures.

### **Verification of successful dCAS9-VP64 or dCAS9-KRAB integration as well as expression in hPSCs**

⌚ Timing: ~1–2 weeks

After the lentiviral transduction of hPSCs (H9/WA09) with dCas9-VP64 or dCas9-KRAB, cell lines must be verified for successful integration as well as expression of dCas9. In this section we will focus on the isolation of genomic DNA, followed by PCR to verify successful dCas9 integration into genomic DNA (Figure 3), as well as immunofluorescence analysis to detect successful dCas9 expression (Figure 4).

21. Genomic DNA isolation.
  - a. Seed  $4 \times 10^5$  cells of the cell line to be tested into a well of a vitronectin coated 6-well plate.
  - b. On then next day, discard the media.



**Figure 3. dCas9 integration in hES cells**

Genomic DNA was isolated from hPSCs (H9/WA09) transduced with dCas9-VP64 or dCas9-KRAB, and subjected to a PCR experiment. Generated samples were run on a 1.5% agarose gel in TAE buffer at 100 V for 20 min.

- c. Wash the cells with 2 mL of 1× PBS.
- d. Detach the cells from the surface using 1 mL of EDTA at 37°C for 2 min.
- e. Discard the EDTA solution and resuspend the cells in 1 mL of E8 Flex medium.
- f. Collect the cell pellet by centrifugation at 1,000 × g for 5 min.
- g. Process the pellet for genomic DNA isolation using, for instance, QIAamp DNA isolation kit, following manufacturer's instructions <https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/qiaamp-dna-kits/>.
- i. Wash cell pellet with 500 μL of 1× PBS.
- ii. Resuspend the cells in 200 μL lysis buffer (AL buffer).

**Note:** If the lysis buffer develops a precipitate, incubate at 56°C until the precipitate dissolves completely.

- iii. Add 20 μL of Proteinase K.
- iv. Incubate at 56°C for 10 min.
- v. Precipitate the DNA using 200 μL of 100% ethanol and vortex well.
- vi. Add the suspension to the spin column and spin down for 1 min at 10,000 × g to bind the DNA to the silica membrane.
- vii. Discard the flow through and wash the membrane with 600 μL of AW1 buffer.
- viii. Centrifuge for 1 min at 10,000 g.
- ix. Discard the flow through and wash the membrane with 500 μL of AW2 buffer.

**Note:** AW1 and AW2 wash buffers should only be used after adding the indicated amount of ethanol.

- x. Centrifuge for 1 min at 10,000 × g.
- xi. Dry-spin for 2 min.

- xii. Elute the DNA in 30  $\mu\text{L}$  of nuclease-free water.
- xiii. Measure the concentration of isolated genomic DNA in a Nanodrop. The 260/230 ratio should be between 1.8 and 2.0 for a high-quality gDNA isolation.
- xiv. Genomic DNA can be stored at  $-20^{\circ}\text{C}$  for several months.
- xv. Proceed with the PCR.

**Note:** Genomic DNA isolation can be done at  $20^{\circ}\text{C}$ – $25^{\circ}\text{C}$ .

## 22. Polymerase Chain Reaction.

- a. We usually use the Promega GO Taq polymerase Kit.

**Note:** Use only sterile working conditions for PCR. Usage of filter tips and DNase/RNase and pyrogen-free consumables is highly recommended.

- b. Thaw reaction buffer and dNTPs on ice.
- c. Dilute forward and reverse primers to produce a concentration of 10  $\mu\text{M}$ , for instance, from a 100  $\mu\text{M}$  stock.
- d. Dilute the genomic DNA template to 50 ng/ $\mu\text{L}$  from the stock concentration.
- e. Set up a PCR program as given in Table 4.

**Note:** Determine the optimal annealing temperature by calculating the  $T_m$  of the primer pairs used.

- f. Set up the PCR reaction as given in Table 5.
  - i. Use dCas9 primers to amplify the integrated dCas9 in the transduced cell lines. As an internal control for the PCR reaction, a housekeeping gene, for instance, RNA polymerase II was used (Radonić et al., 2004).
  - ii. For preparing the master mix, take fresh DNase/RNase and pyrogen free 1.5 mL micro-centrifuge tubes.
  - iii. Prepare the reaction as per the manufacturer's instructions <https://www.promega.de/products/pcr/taq-polymerase/gotaq-dna-polymerase/?catNum=M3001> - protocols.
  - iv. Run the reaction in a PCR machine programmed as indicated.

**Note:** If the thermal cycler does not have a heating lid, overlay the reaction mixture with 50  $\mu\text{l}$  of mineral oil to avoid evaporation of samples.

- v. Include a reaction with gDNA from an untransduced sample as a negative control for the dCas9 primer pairs. As a positive control, include a reaction with 10 ng of a plasmid DNA which harbors the dCas9 sequence.

**Note:** Always include a negative control reaction to ensure specificity of amplified products. Include an untransduced sample as a negative control as well as a no template water control to determine if there is primer dimer formation or contamination of PCR reagents.

**Table 4. PCR program**

Steps	Temperature	Time	Cycles
Initial Denaturation	$94^{\circ}\text{C}$	2 min	1
Denaturation	$94^{\circ}\text{C}$	30 s	32 cycles
Annealing	$57^{\circ}\text{C}$	45 s	
Extension	$72^{\circ}\text{C}$	30 s	
Final extension	$72^{\circ}\text{C}$	5 min	1
Hold	$4^{\circ}\text{C}$	forever	



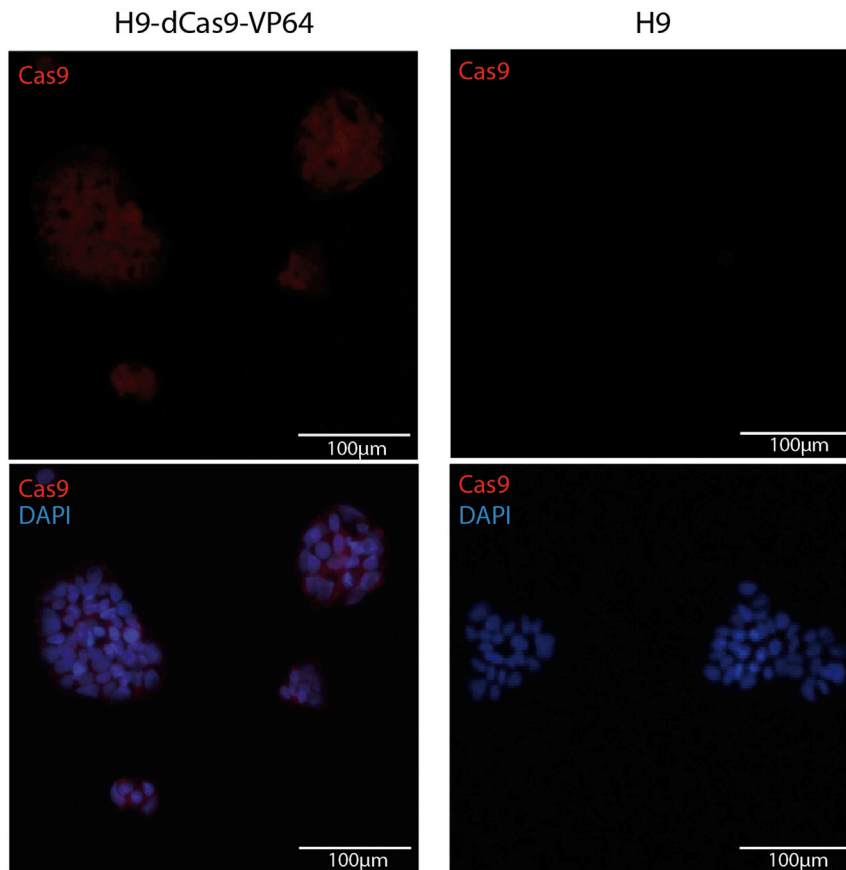
**Table 5. PCR reaction**

Reagent	Amount
gDNA	50 ng
5× Green GoTaq Reaction Buffer	10 μL
10 mM dNTPs	1 μL
10 μM forward primer	1 μL
10 μM reverse primer	1 μL
GoTaq DNA Polymerase	0.25 μL
Nuclease-free Water	to 50 μL
<b>Total</b>	<b>50 μL</b>

- vi. Run the PCR program for 32 cycles and electrophorese reactions in an agarose gel to verify successful dCas9 integration (Figure 3).

**Note:** The PCR reaction can be electrophoresed in a 1.5% agarose gel to detect the dCas9 amplicon. Make sure to include a properly sized DNA standard to detect the size of the amplicon reliably. The dCas9 primers produce a PCR product of approx. 200 bp. The primers targeting RNA polymerase II amplify an approx. 650 bp PCR product (Figure 3).

23. Seeding cells for dCas9 Immunofluorescence.
  - a. Start with a confluent 10 cm dish of H9-dCas9-VP64 or/and H9-dCas9-KRAB cells.
  - b. Discard the growth medium.
  - c. Wash cells with 8 mL of 1× PBS.
  - d. Remove the 1× PBS and detach cells using 6 mL of EDTA.
  - e. Incubate at 37°C for 2 min.
  - f. Remove the EDTA and detach cells from the surface of the 10 cm dish using 8 mL of Essential 8 Flex growth medium.
  - g. Add 2 mL of fresh E8 Flex medium to the wells of a 6-well plate coated with vitronectin.
  - h. Add 0.5 mL of the detached cells per well.
  - i. Allow the cells to grow for 24 h at 37°C and 5% CO<sub>2</sub> in an incubator.
  - j. On the next day, check for cell growth and subject cells to immunofluorescence staining.
24. Immunofluorescence staining.
  - a. Wash cells with 2 mL of 1× PBS.
  - b. Fix cells using 4% paraformaldehyde in PBS (pH 7.4) for 10 min at 20°C–25°C.
  - c. Afterwards the cells will be washed three times in 1× PBS for 5 min.
  - d. For permeabilization samples are incubated for 10 min with PBS containing 0.25% Triton X-100.
  - e. Wash cells in 1× PBS three times for 5 min.
  - f. Incubate cells with 1% BSA in PBST (PBS+ 0.1% Tween20) for 30 min to block unspecific binding.
  - g. Incubate cells with the diluted Cas9 antibody (1:100) in 1% BSA in PBST for 2 h at 20°C–25°C or 16–18 h at 4°C.
  - h. Decant the solution and wash the cells three times in PBS, 5 min each wash.
  - i. Incubate cells with the fluorescently labeled secondary antibody (1:500) in 1% BSA for 1 h at 20°C–25°C in the dark.
  - j. 30 min before incubation end add DAPI (1:10,000) to stain the cell nuclei.
  - k. Decant the secondary antibody solution and wash the cells three times with 1× PBS for 5 min each in the dark.
  - l. Cells are now ready for microscopic inspection (Figure 4).



**Figure 4. Immunostaining of dCas9 in dCas9-VP64 expressing hPSCs**

hPSCs (H9/WA09) expressing dCas9-VP64 were stained for dCas9 expression using immunofluorescence and a Cas9 specific antibody. Scale bars, 100  $\mu$ m.

### Validation of activation or repression of HERV-K(HML-2) elements in hPSCs

⌚ Timing: ~1–2 weeks

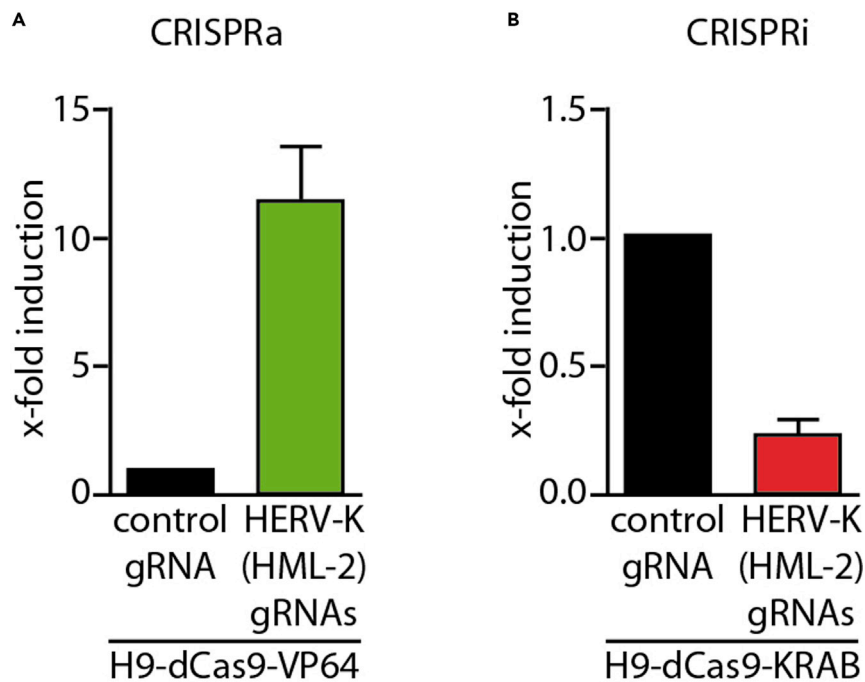
After lentiviral transduction of hPSCs (H9/WA09) with dCas9-VP64 or dCas9-KRAB, as well as the HERV-K(HML-2) gRNAs, cell lines must be verified for successful activation or repression of HERV-K(HML-2). This final section will focus on isolation of RNA, followed by quantitative real-time PCR to verify transcriptional activation/repression of HERV-K(HML-2) (Figure 5).

25. Seeding cells for subsequent RNA isolation.

- a. Start with a confluent 10 cm dish of H9-dCas9-VP64-HERV-K(HML-2) or/and H9-dCas9-KRAB-HERV-K(HML-2) cells.

**Note:** Include H9-dCas9-VP64 or/and H9-dCas9-KRAB cells expressing a control gRNA.

- b. Discard the growth medium.
- c. Wash cells with 8 mL of 1  $\times$  PBS.
- d. Remove the 1  $\times$  PBS and detach cells using 6 mL of EDTA.
- e. Incubate at 37°C for 2 min.
- f. Remove the EDTA and detach cells from the surface of the 10 cm dish using 8 mL of Essential 8 Flex growth medium.



**Figure 5. CRISPR-mediated regulation of HERV-K(HML-2) transcription**

HERV-K(HML-2) transcript levels were quantified by qRT-PCR, targeting a HERV-K(HML-2) *gag* region, in hPSCs (H9/WA09) stably expressing dCas9-VP64 (A) or dCas9-KRAB (B), respectively, together with HERV-K(HML-2) specific gRNAs, or control gRNAs. Results from 3 biological replicates are shown. Data are presented as mean $\pm$ SD.

- g. Add 2 mL of fresh E8 Flex medium to the wells of a 6-well plate coated with vitronectin.
- h. Add 0.5 mL of the detached cells per well.
- i. Allow the cells to grow for 24 h at 37°C and 5% CO<sub>2</sub> in an incubator.
- j. On the next day, check for cell growth and subject cells to total RNA isolation when approx. 80% confluent.

26. RNA isolation.

**Note:** For RNA work, following precautions should be taken. (1) Always work in sterile conditions. Clean the work bench, pipettes and microcentrifuge stand with 80% Ethanol. (2) Always use RNase-free tubes and filter tips for the experiments. Care should be taken to prepare all solutions using DEPC-treated water. (3) Wear gloves while working with RNA. (4) While working with TRIZOL, follow all the precautionary measures and good laboratory practices. Wear proper gloves, lab coats, etc. (5) Always work under a chemical fume hood while working with TRIZOL.

- a. Transfer the 6-well plate to the fume hood.
- b. Discard Media.
- c. Add 1 mL of TRIZOL per well.

**Note:** The solution appears slimy when TRIZOL is added.

- d. Resuspend 4–5 times.

**Note:** The cell suspension should become less slimy.

- e. Add 200  $\mu$ L of chloroform to the mixture.
- f. Vortex vigorously for at least 2 min until the solution becomes bright pink.
- g. Incubate at 20°C–25°C for 2 min.

**Note:** Vortexing after the addition of Chloroform is extremely important.

- h. Centrifuge at 4°C for 20 min at 13,000  $\times$  g.
- i. The solution separates into 3 phases; an aqueous, interphase and organic phase.
- j. Collect the (upper) aqueous phase into a fresh 1.5 mL tube.

**△ CRITICAL:** Make sure the aqueous phase collection is completely free of phenol. We usually collect 300–350  $\mu$ L of the aqueous phase to avoid phenol contamination. Phenol in the aqueous phase can affect the quality of the RNA and inhibit cDNA synthesis.

- k. Add 500  $\mu$ L of ice cold 2-Propanol, vortex briefly, and precipitate the total RNA at –20°C for 1–2 h.

**Note:** Precipitation of RNA can be done at 20°C–25°C for 10 min. However, we have optimized the time and temperature to maximize RNA yield. Incubation at –20°C for 16–18 h for H9/WA09 cells and 1–2 h for other cell lines has been found most efficient.

- l. Centrifuge at 4°C for 45 min at 13,000  $\times$  g.
- m. The RNA forms a white pellet.
- n. Wash the RNA pellet with 1 mL of 75% Ethanol by centrifuging at 4°C for 5 min at 7,500  $\times$  g.
- o. Air-dry the pellet until the ethanol evaporated completely.

**△ CRITICAL:** Dry the pellet until the ethanol completely evaporates. Presence of ethanol can result in low RNA quality and can hinder the efficiency of cDNA synthesis.

- p. Add 30  $\mu$ L of nuclease-free water.
- q. Dissolve the RNA at 55°C for 15 min.
- r. Store the RNA at –80°C.

## 27. cDNA synthesis.

For cDNA from synthesis from isolated RNA, we use the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher).

**Note:** For cDNA synthesis, follow precautions listed for the work with RNA. (1) Always work in sterile conditions. Clean the work bench, pipettes and microcentrifuge stand with 80% Ethanol. (2) Always use RNase/DNase free tubes and filter tips for the experiments. Care should be taken to prepare all solutions in DEPC-treated water.

- a. DNase digestion of residual DNA. For DNase digestion we used the DNase digestion kit (Thermo Fisher).
  - i. Pipette the following components into an Eppendorf tube as shown in [Table 6](#).
  - ii. Incubate the mix at 37°C for 30 min.

**Note:** Make sure you pipette RNA kept on ice.

- iii. Terminate the DNase reaction by adding 1.0  $\mu$ L of EDTA and incubating at 65°C for 10 min.
- iv. DNase-treated RNA can be directly used for cDNA synthesis or can be stored at –80°C until use.

**Table 6. DNase digestion**

Reagent	Amount
RNA	1 $\mu$ L (diluted to 1 $\mu$ g/ $\mu$ L)
10 $\times$ Reaction buffer	1 $\mu$ L
DNase	1 $\mu$ L
Nuclease free water	7 $\mu$ L
Total	10 $\mu$ L

**△ CRITICAL:** Even though the efficient handling of the Phenol-Chloroform method for RNA isolation considerably reduces the risk of gDNA contamination of RNA, a DNase digestion is very important especially when working with high-copy human endogenous retroelements since even trace amounts of gDNA contamination can affect experimental results.

b. Quantitative Real-Time PCR (qRT-PCR).

We use the Roche LC480 II in combination with the Roche LC480 SYBR Green mix. QRT-PCR was performed using HERV-K(HML-2) specific primers, as well as primers targeting mRNA of house-keeping gene RNA Polymerase II (RPII). Primer sequences can be found in the [key resources table](#).

- i. In a 1.5 mL reaction tube prepare, on ice, a PCR mix (Table 7) for each primer pair and sample:

**Note:** Always prepare a PCR Master mix for more than one reaction including a negative control. For a negative control, replace cDNA with PCR-grade water.

- ii. Mix the PCR mix carefully by pipetting up and down.

**Note:** Do not vortex.

- iii. Pipet 9  $\mu$ L of the PCR mix into each well of a qPCR multi-well plate.
- iv. Add 1  $\mu$ L of the respective cDNA template to each well. Include a water negative control.
- v. Seal the multi-well plate with multi-well sealing foil.
- vi. Place the sealed multi-well plate in a centrifuge equipped with a suited rotor.
- vii. Centrifuge at 1,500 g for 2 min at 4°C.
- viii. Load the multi-well plate into the Roche LC480 Instrument.
- ix. Start the PCR program (Table 8).

**△ CRITICAL:** Be sure to adapt the right volume in the PCR protocol of the LC480 machine, if you use a reaction volume different from 20  $\mu$ L.

**△ CRITICAL:** Pipet reaction components on ice, centrifuge at 4°C and transfer the multi-well plate on ice to the LC480 machine to reduce primer dimer formations.

**Table 7. qRT-PCR mix (LC480)**

Reagent	Amount
LightCycler® 480 SYBR Green I Master	5 $\mu$ L
10 $\mu$ M forward primer	0.5 $\mu$ L
10 $\mu$ M reverse primer	0.5 $\mu$ L
Nuclease-free H <sub>2</sub> O	3 $\mu$ L
cDNA	1 $\mu$ L
Total	10 $\mu$ L

**Table 8. qRT-PCR program**

Steps	Temperature	Time	Cycles
Pre-Incubation	95°C	5 min	1
Denaturation	95°C	10 s	45 cycles
Annealing	60°C	10 s	
Extension	72°C	10 s	
Melting Curve	95°C	5 s	1
Melting Curve	65°C	1 min	1
Melting Curve	Increase continuously to 97°C	5 min	measurement fluorescence every 0.11°C
Hold	4°C	forever	

### 28. Relative quantification.

- The relative expression of HERV-K(HML-2) in relation to the reference gene RPII was calculated using the 'delta Cp' method (Livak and Schmittgen, 2001; Pfaffl, 2001).
- The difference in the Cp values were determined as follows:

$$\Delta C_p = C_p_{\text{HERV-K(HML-2)}} - C_{p\text{RPII}}; \text{Fold change} = 2^{-(\Delta C_p)}.$$

- This method gives the relative mRNA transcript levels of HERV-K(HML-2) for a sample normalized to the housekeeping gene RNA polymerase II (See Figure 5 for CRISPRa and CRISPRi).

## EXPECTED OUTCOMES

The protocol is expected to generate a human pluripotent stem cell line stably expressing dCAS9-VP64 or dCas9-KRAB together with gRNAs targeting HERV-K(HML-2) elements, or a control gRNA. HERV-K(HML-2) should be activated or repressed efficiently in these cell lines. The generated hPSC cell lines can be used for further analyses of functional roles of HERVs in biological processes, for example, pluripotency or neuronal development. Moreover, this protocol has already been applied to multiple other cell lines as well as other HERV groups in our lab.

## LIMITATIONS

The current protocol uses two guide RNAs to modulate transcriptional expression of HERV-K(HML-2) in hPSCs. The activation and repression can be even more efficient by using a vector that can hold multiple gRNAs. HERV groups other than HERV-K(HML-2) display greater within-group sequence heterogeneity potentially complicating to some extent gRNA design and placement of gRNAs within a HERV group's LTRs.

## TROUBLESHOOTING

### Problem 1

BfuA1 digestion is incomplete (steps 6–8).

### Potential solution

If the agarose gel shows an incomplete digestion with BfuA1, increase the digestion time by an extra hour. Too little and too much of the enzyme can hamper the digestion efficiency. Use only 1 unit of enzyme per microgram of DNA. Additionally, purify the DNA prior to digestion. Contaminants in the DNA can also reduce digestion efficiency. Digest DNA for 2–3 h at 50°C to ensure complete digestion.

### Problem 2

No colonies on the agar plate after transformation (step 11).

#### Potential solution

If there are no colonies on the Agar plate after transformation, double-check the antibiotic. Make fresh agar plates with fresh stock of antibiotic. Make sure you are using the right antibiotic marker corresponding to the vector. Do not use too little or too high concentration of the antibiotic. Double-check efficiency of competent cells. Use Agilent XL10 Gold in case other competent cells do not produce the desired results. Finally, double-check whether the BfuA1 digestion was complete. Improper digestion of the vector with BfuA1 will hamper the ligation and subsequently the transformation.

#### Problem 3

Too many colonies on the control plate after transformation (step 11).

#### Potential solution

If there are too many colonies on the Agar plate after transformation of the gRNA ligation product, double-check efficiency of competent cells used. Agilent XL10 Gold competent cells can be used instead of Stbl3 competent cells for the sgRNA cloning. Perform a BfuA1 digestion on the ligated sgRNA product prior to transformation to avoid re-ligation.

#### Problem 4

All cells die after antibiotic selection (step 20).

#### Potential solution

Double-check the optimal antibiotics concentration for your cell line used as antibiotics concentrations can vary between cell lines. Perform an antibiotic killing curve ahead of time. Excess cell death can also be caused by too low transduction efficiencies. Several factors can impact viral titers. 1) Transfection efficiency of plasmids is too low due to, for example, poor plasmid quality. If the latter is the case, prepare fresh plasmid DNA. 2) HEK293T cells are not 70% confluent on the day of transfection. If so, let the cells grow until they reach 70% confluency. Besides, adequate antibiotic concentration needs to be established for every cell line in order to avoid too high concentrations. Also, transduced hPSCs should be at least 50% confluent on the day of transduction with lentiviral particles to avoid excess cell death.

#### Problem 5

PCR or qRT-PCR does not work (steps 21–25).

#### Potential solution

Double-check the quality of the genomic DNA. A too high high salt concentration or residual phenol can inhibit the PCR reaction. In case of such contaminations, re-purify samples using Phenol-chloroform-Isoamyl alcohol and increase the amount of washing steps. If gDNA appears degraded, perform all the steps under a clean safety cabinet and using nuclease-free reagents.

#### Problem 6

No activation or repression of the respective HERV group (steps 25 and 26).

#### Potential solution

After transduction of the desired cell lines with the cloned gRNAs, the CRISPR activation and/or CRISPR repression can be checked by a quantitative real time qPCR reaction. If there is no observable activation or repression of the specific HERV group, check for the optimal binding of the sgRNA. Ideally, use several pairs of gRNAs and try different sets/combinations of gRNAs located in different regions of the chosen HERV LTRs.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to, and will be fulfilled by, the lead contact, Michelle Vincendeau, ([michelle.vincendeau@helmholtz-muenchen.de](mailto:michelle.vincendeau@helmholtz-muenchen.de)).

#### Materials availability

This study did not generate new unique reagents or cell lines. All reagents listed here can be found in the main article ([Padmanabhan Nair et al., 2021](#)).

#### Data and code availability

This study did not generate unique databases.

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### AUTHOR CONTRIBUTIONS

M.V. conceived and initiated the project. M.V. and V.P.N. planned the experiments and conducted most experimental work with the support of J.M. V.P.N., J.M., and M.V. drafted and edited the manuscript. M.V. finalized the manuscript and all authors approved the manuscript before submission.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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