Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Protocol for CRISPR/Cas9-based knock-in using the VIKING method

Shigeo S. Sugano (shigeohu@gmail.com)
R-GIRO, Ritsumeikan University

Shun Sawatsubashi (shun-sawa2@umin.ac.jp)
Department of Molecular Endocrinology, Tokushima University

Yudai Joko
Department of Molecular Endocrinology, Tokushima University

Seiji Fukumoto
Department of Molecular Endocrinology, Tokushima University

Toshio Matsumoto
Department of Molecular Endocrinology, Tokushima University

Method Article

Keywords: CRISPR, gene targeting, knock-in, knock-out, VIKING, cell culture

Posted Date: January 24th, 2019

DOI: https://doi.org/10.1038/protex.2019.010

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Abstract

CRISPR/Cas9-based genome editing has dramatically accelerated genome engineering. We recently developed the versatile non-homologous end joining-based knock-in module for genome editing \(VIKING) method1. In this method, cells are simultaneously transfected with three vectors \(donor vector, donor cleavage vector, and target cleavage vector). The VIKING method uses the “VKG1 sequence” which avoids the need for customization of the donor vector. This method has the following three advantages: 1. The donor and donor cleavage vectors do not need to be modified if they contain the VKG1 sequence. Various vectors \(such as pUC19, pcDNA3, pENTR/D-TOPO) contain this sequence. By contrast, other techniques involve the design and construction of new donor vectors, which is complicated. 2. We optimized vector transfection for non-homologous end joining repair-dependent knock-in. Other technologies are hampered by random integration. 3. DNA sequences longer than 10 kbp can be inserted using the VIKING method. Therefore, a longer sequence can be inserted using this method than using a viral vector. Here, we describe the knock-in/knock-out and knock-in procedures using the VIKING method.

Reagents

Cell culture reagents Dulbecco's modified Eagle's medium \(043-30085; Wako, Osaka, Japan) Fetal Bovine Serum, Irish Origin \(S1780-500; Biowest, Nuaille, France) Penicillin-Streptomycin-Amphotericin B Suspension \(×100) \(Antibiotic-Antimycotic Solution) \(161-23181; Wako) Phosphate-buffered saline. \(164-25511; Wako, Osaka, Japan) Trypsin \(201-16945; Wako, Osaka, Japan) **Transfection** Opti-MEM™ I Reduced Serum Medium, no phenol red \(11058-021; Life Technologies, California, USA) TurboFect transfection reagent \(R0531; Thermo Fisher Scientific, Waltham, MA) Puromycin dihydrochloride \(P8833; Sigma-Aldrich Japan, Tokyo, Japan) Blasticidin S, Hydrochloride \(KK-400; Kaken Pharmaceutical, Tokyo, Japan) **Plasmids** Target genome cleavage vector \(we use pX330*; https://www.addgene.org/42230/) \(Fig. 1a blue) AAVS1 locus-specific cleaving vector \(see Remark 1) \(Fig. 2 blue) Donor vector* \(pVKG1-PURO, Puromycin resistance, or pVKG1-BSD, Blasticidin resistance, which are distributed by Addgene \(108670 and 108680, respectively)) \(Fig. 1a magenta) FLAG-tagged VDR variant \(Gln259 > Pro) expression vector \(pcDNA3- FLAGhVDRQ259P****) Donor cleavage vector***** \(VKG1-gRNA-pX330, which is distributed by Addgene \(108671)) \(Fig. 1a green) *A human codon-optimized SpCas9 and chimeric guide RNA expression plasmid. ****This pCDNA3 backbone contains a VKG1 sequence for cleavage by Cas9-gRNA. *****The donor cleavage and donor vectors are called the VIKING module and do not need to be modified according to the target sequence. **DNA extraction** Dulbecco’s phosphate-buffered saline 50 mM NaOH 1 M Tris HCl, pH 8.0 **Genotyping** GoTaq Polymerase \(M3001; Promega, Wisconsin, USA) Primers \(change according to the target locus for knock-in) VKG 1_4561 F, 5'-GCCTATGGAAAAACGCCAGC-3' VKG 1_250 R, 5'-TTCCTGTCTAGCGGTACGCG-3' VDR genome_del 3 F, 5'-GGTGGGCCTCATGTCTTCTG-3' VDR genome_del 1 R, 5'-CCTTCATCATGCCGATGTCC-3' pEN_ObLi_40_For, 5'-TACCGCCTTTGAGTGAGCTG-3' pEN_ObLi_190_Rev, 5'-AAACCTGTCGTGCCAGCTGC-3' AAVS _ F5, 5'-GGAAATGGGGGTGTGTCACC-3' AAVS_R6, 5'-CCCTACCCCCCTTACCTCTC-3'

Equipment

Cell culture incubator \($5\%\ CO_2$/37°C) BSL-2 biosafety cabinet 1.5, 15, and 50 mL tubes Cell culture plasticware \(e.g., dishes, plates, and pipettes) Electroporator \(CUY21 EDITII; Bex, Tokyo, Japan) Cloning ring \(AS ONE, Osaka, Japan) Agarose gel electrophoresis equipment PCR thermal cycler

Procedure

Protocol I: Procedure for knock-in/knock-out in cultured cells using the VIKING method **Day 1: Cell culture** • Incubate HaCaT or human embryonic kidney 293F \(HEK293F) cells in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1 U/mL penicillin-streptomycin \(D10 medium). \(see Remark 2). **Day 2: Transfection \(Fig. 1a)** - Electroporation method \(HaCaT cells) • Suspend cells in 400 µL Opti-MEM. • Transfect cells with the VIKING module and the target genome cleavage vector using a total of 15 µg DNA \(molar ratio of donor cleavage vector \(VKG1-gRNA-pX330):donor vector \(pVKG1-PURO):target genome cleavage vector \(pX330) = 1:1:17) using an electroporator. • Transfer cells to 100 mm dishes and culture in D10 medium lacking antibiotics for 24 h. - Lipofection method \(HEK293F cells). • Suspend cells in 400 µL Opti-MEM. • Transfect cells with the VIKING module and the target genome cleavage vector using a total of 15 µg DNA \(molar ratio of donor cleavage vector \(VKG1-gRNA-pX330):donor vector \(pVKG1-PURO):target genome cleavage vector \(pX330) = 1:1:17) using TurboFect transfection reagent. • Transfer cells to 100 mm dishes and culture in D10 medium lacking antibiotics for 24 h. \(see Remark 3). **Day 4: Selection** • Refresh D10 medium. • Add puromycin \(0.3 µg/mL) to the D10 medium and culture cells for 1–2 weeks. **Day 14: Clone picking \(Fig. 1b)** • Wash cells with phosphate-buffered saline. • Place the cloning ring on a single colony. Pick at least 20 colonies. Limiting dilution is also possible� • Add 20 µL trypsin to each ring. • Incubate cells at 37ºC for 2 min \(HEK293F cells) or 10 min \(HaCaT cells). • Transfer cells to a 24-well or 48-well plate. • Incubate cells at 37ºC for several days. • Transfer cells to a 12-well plate and incubate. • Isolate a portion of the cells for DNA extraction. **Day 16: DNA extraction** • Add 180 µL of 50 mM NaOH and transfer to a 1.5 µL tube. • Incubate at 95ºC for 30 min. • Add 20 µL of 1 M Tris HCl, pH 8.0. • Centrifuge at 12,000 rpm for 10 min. • Transfer the supernatant to a new tube. **Day 17: Genotyping \(confirmation of knock-in by PCR) \(Fig. 1c)** Knock-in and random insertion are confirmed by PCR using the extracted DNA as a template \(see Remark 4 and 5). There is no need for a special polymerase. GoTaq polymerase is used in this protocol. - Design primers to amplify the VKG1 sequence. Example: Primer 1 \(VKG 1_4561 F), 5'-GCCTATGGAAAAACGCCAGC-3'. Primer 2 \(VKG 1_250 R), 5'-TTCCTGTCTAGCGGTACGCG-3'. - Design primers that specifically bind to the target genome sequence. Example: Primer 3 \(VDR genome_del 3 F), 5'-GGTGGGCCTCATGTCTTCTG-3'. Primer 4 \(VDR genome_del 1 R), 5'-CCTTCATCATGCCGATGTCC-3'. **Genotyping by PCR** • PCR sample 2× GoTaq GreenMaster Mix, 12.5 µL 10 µM Primer A, 2.5 µL 10 µM Primer B, 2.5 µL Nuclease-free water, 6.5 µL Genomic DNA, 1 µL Total, 25 µL • Thermocycle 95ºC for 2 min. 30 cycles of 95°C for 30 sec, 55°C for 1 min, and 72°C for 2 min. 72ºC for 4 min. 12ºC hold. Step 1. Confirm random insertion. PCR is performed using Primer 1 and Primer 2 for Amplicon1. Random insertion is confirmed by amplification of Amplicon1. Step 2. Confirm knock-in at the target locus. -

Forward direction PCR is performed using Primer 1 and Primer 3 for Amplicon2, or Primer 2 and Primer 4 for Amplicon3. Knock-in in the forward direction is confirmed by amplification of Amplicon2 and Amplicon3. - Reverse direction PCR is performed using Primer 2 and Primer 3 for Amplicon4, or Primer 1 and Primer 4 for Amplicon5. Knock-in in the reverse direction is confirmed by amplification of Amplicon4 and Amplicon5. **Protocol II: Procedure for knock-in in cultured cells using the VIKING method \(Fig. 2)** **Day 1–16** Same as Protocol I. **Day 17: Genotyping** - Design primers to amplify the VKG1 sequence. Same as Protocol I. - Design primers to amplify the VKG1 sequence. Example: Primer 1 \(pEN_ObLi_40_For), 5'-TACCGCCTTTGAGTGAGCTG-3'. Primer 2 \(pEN_ObLi_190_Rev), 5'-AAACCTGTCGTGCCAGCTGC-3'. - Design primers that specifically bind to the target genome sequence. Example: Primer 3 \(AAVS_F5), 5'-GGAAATGGGGGTGTGTCACC-3'. Primer 4 \(AAVS_R6), 5'-CCCTACCCCCCTTACCTCTC-3'. **Genotyping by PCR** Same as Protocol I. **Remarks** 1. This vector is selected according to the target locus for knock-in. 2. In addition to HaCaT and HEK293F cells, the VIKING method has been validated in the C4-2 \(human prostate cancer), UMR-106 \(rat osteogenic), and MC3T3-E1 \(mouse preosteoblastic) cell lines. 3. These two transfection methods are examples and should be changed depending on cell lines to attain a high transfection efficiency. 4. The backbone of the donor vector is integrated into the genome in principle. 5. Tandem insertions of the donor vectors sometimes occur.

Anticipated Results

Establishment of a deleterious variant of VDR-expressing cell line as an example was demonstrated by using this protocol \(Fig. 3). VDR knock-out cells were produced using knock-in-mediated gene disruption. Subsequently, VDR variant-expressing cell lines were established by following knock-in of VDR point mutant \(Q259P) vector into a safe harbor site of these VDR-knock-out HaCaT cells \(Fig. 3a). Immunoblot results were shown the VDR deficiency in VDR knock-out cells and the expression of VDR variant in the knock-in cells \(Fig. 3c).

Figures

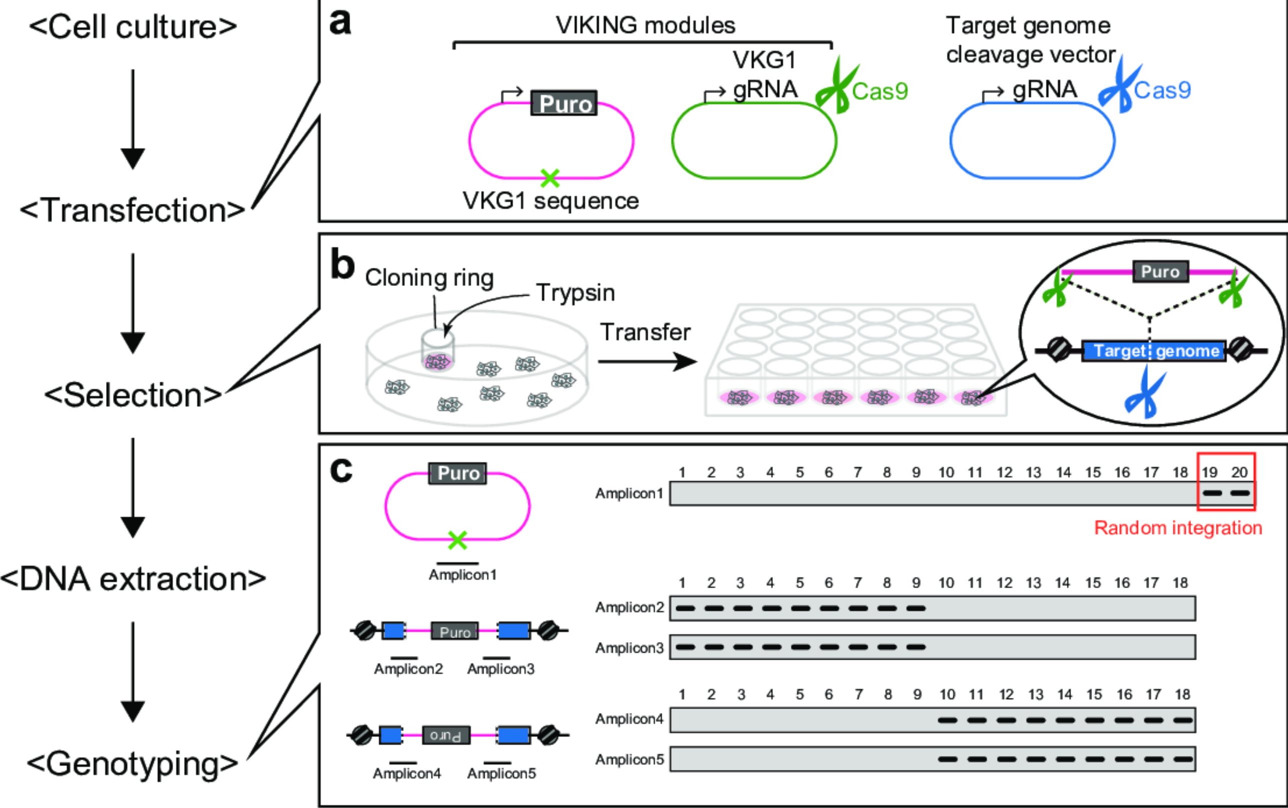

Figure 1

Workflow of VIKING method. Schematic image of electrophoresis shows typical results of genotyping (Protocol I). <img src="http://www.nature.com/protocolexchange/system/uploads/7477/original/Figure_1.pdf?1548150775/for/the/thumbnail/image" >

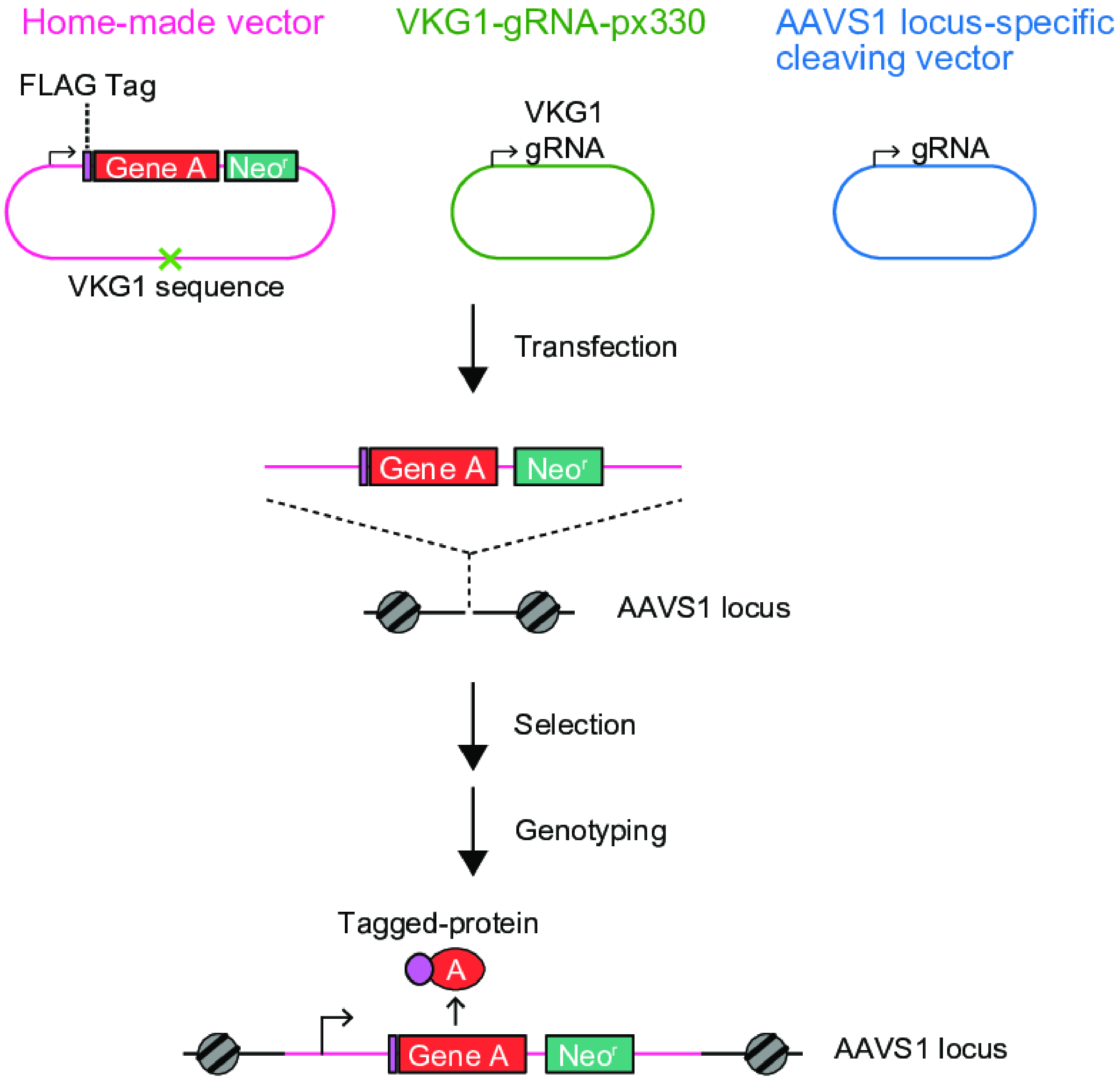

Figure 2

Schematic diagram of protocol for knock-in in cultured cells using the VIKING method. (Protocol II) <img src="http://www.nature.com/protocolexchange/system/uploads/7479/original/Figure_2.pdf?1548150841/for/the/thumbnail/image" >

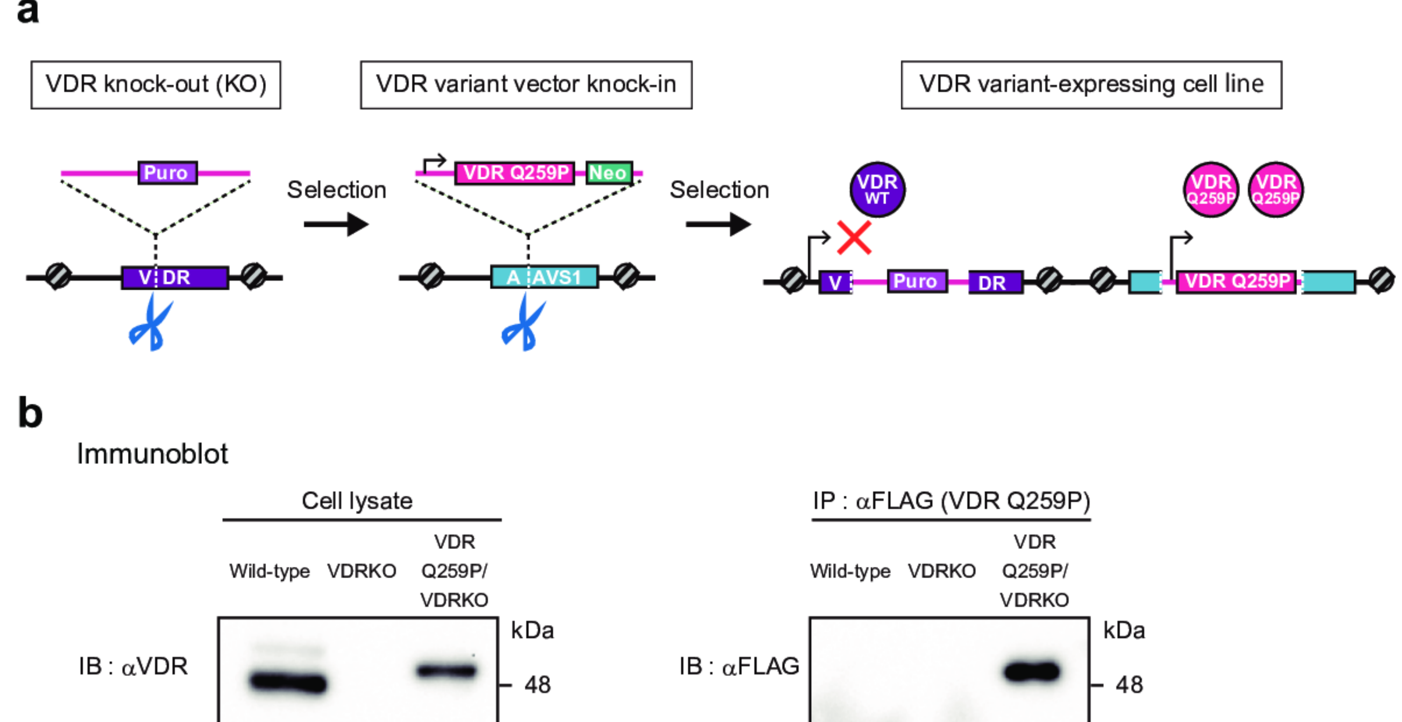

Figure 3

Knock-in of a VDR variant (Q259P) in VDR-knock-out HaCaT cells using the VIKING method. <img src="http://www.nature.com/protocolexchange/system/uploads/7481/original/Figure_3_SS.pdf?1548150885/for/the/thumbnail/image" >