Generation of cell lines for the exon-specific isoform expression reporter system (EXSISERS)

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Method Article

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Abstract

Exon-specific isoform expression from alternatively spliced mRNA is a fundamental mechanism that substantially enriches a cell's proteome. However, conventional methods to assess alternative splicing are either consumptive and work-intensive or do not quantify isoform expression over multiple time points and at the protein level. We thus developed an exon-specific isoform expression reporter system (EXSISERS), which non-invasively reports the translation of exon-containing isoforms by scarlessly excising reporter proteins from the nascent polypeptide chain via highly efficient, intein-mediated protein splicing. EXSISERS enables non-invasive monitoring of exon-specific isoform expression with high sensitivity and cellular resolution and enables high-throughput screening of exon-specific therapeutic interventions.

This protocol on how to generate EXSISERS reporter cell lines is related to the publication 'Non-invasive and high-throughput interrogation of exon-specific isoform expression' in Nature Cell Biology.

Introduction

Alternative splicing (AS) occurs in >90% of genes, and disruption of this sensitive regulatory system is associated with diseases such as spinal muscular atrophy and Parkinson's disease[1–3] (**Fig. 1a,c**). Established methods for analyzing splicing isoforms either detect mRNA by endpoint-labeling (RT-qPCR, (sm)FISH[4], RNA-sequencing[5]), protein by single-timepoint immunochemistry (immunoblot analysis, immunofluorescence staining), or minigene analysis [6–8].

Current protein-level methods for measuring isoform-specific expression are limited by available exon-specific antibodies. Analyses at the mRNA level can be misleading since post-transcriptional and co-translational regulation does not necessarily change the mRNA levels, *e.g.,* in cases of translation-arrested[9], ribosomal-frameshift-regulated[10], or locally translated mRNA[11,12]. Furthermore, RT-qPCR and RNA-FISH are inherently consumptive, preventing longitudinal analyses of the sample.

For minigenes, fragments of the genomic sequence, in particular exon-intron fragments, are copied into a plasmid driven by a constitutive promoter and expressed in a cell line of interest. The splicing behavior of the minigene is then read out by RT-qPCR or an embedded reporter gene. However, this method may not reflect the physiological splicing behavior because partial intron/exon motifs may be overexpressed at unphysiological levels, while essential regulatory sequences are truncated.

Consequently, it is nearly impossible to faithfully recapitulate the regulatory machinery outside the precise three-dimensional genomic architecture at the endogenous sites.

We, therefore, developed EXSISERS, which noninvasively quantifies endogenous exon usage at the protein level via scarless posttranslational excision of an exon-resident effector domain. In the case of the inclusion of the exon of interest (EOI), a reporter or effector of choice is co-translated and rapidly

released by an efficient split-intein13 protein splicing event resulting in an unmodified protein-isoform and thereby preserving the original isoform ratios (**Fig. 1b**).

In this protocol, we describe how the genetic constructs for a given EOI (EXSISERS and CRISPR/Cas9 plasmids) are generated in under a week, and how the respective EXSISERS reporter cell line can be conveniently generated within 6 weeks via double selection using puromycin and ganciclovir.

Reagents

Q5® Hot Start High-Fidelity 2X Master Mix (M0494S, New England Biolabs)

BbsI-HF® (R3539S, New England Biolabs)

CutSmart® Buffer (usually provided together with BbsI-HF®, but can be ordered separately, B7204S, New England Biolabs)

Quick Ligation™ Kit (M2200S, New England Biolabs)

NEBuilder® HiFi DNA Assembly Master Mix (E2621S, New England Biolabs)

NEB® Stable Competent *E. coli* (High Efficiency) (C3040H, New England Biolabs)

NEB® 10-beta/Stable Outgrowth Medium (B9035S, New England Biolabs)

Carbenicillin, Ready-Made Solution, 100 mg/mL in ethanol/water, 0.2 µm filtered (C1613-1ML, Sigma-Aldrich)

LongAmp Hot Start Taq 2X Master Mix (M0533S, New England Biolabs)

ROTIPHORESE® 50x TAE Buffer (CL86.1, Carl Roth)

SYBR™ Safe DNA Gel Stain (S33102, Thermo Fisher Scientific)

Quick-Load® 1 kb Plus DNA Ladder (N0469S, New England Biolabs)

Gel Loading Dye, Purple (6X) (B7024S, New England Biolabs)

Nuclease-free water (T143.1, Carl Roth)

Buffer EB (19086, QIAGEN)

LB-broth (10855001, Thermo Fisher Scientific)

DPBS, no calcium, no magnesium (14190144, Thermo Fisher Scientific)

Advanced DMEM (12491015, Thermo Fisher Scientific)

GlutaMAX™ Supplement (35050061, Thermo Fisher Scientific)

Penicillin-Streptomycin (5,000 U/mL) (15070063, Thermo Fisher Scientific)

Fetal Bovine Serum, certified, United States (16000044, Thermo Fisher Scientific)

Accutase® solution sterile-filtered (A6964-100ML, Sigma-Aldrich)

X-tremeGENE™ HP DNA transfection reagent or X-tremeGENE™ 360 DNA transfection reagent (both can be used equivalently) (6366244001 or 8724105001, Sigma-Aldrich)

Opti-MEM™ I Reduced Serum Medium (31985062, Thermo Fisher Scientific)

Ganciclovir (G2536, Sigma-Aldrich)

Puromycin Dihydrochloride (A1113803, Thermo Fisher Scientific)

Plasmids

pMasterBlaster_Cas9:P2A:i53

pEXSISERSgp41-1-BSD-PuroR-HSV-Tk, pEXSISERSgp41-1-NLuc-PuroR-HSV-Tk, or pEXSISERSNrdJ-1:FLuc-PuroR-HSV-Tk

Primer sequences:

Forward and reverse primers containing spacer sequence (normally 24-25 bp including overhang for ligation) (from Integrated DNA Technologies, Inc). Use CRISPOR14 (http://crispor.tefor.net) to search for suitable spacers.

Example: To insert EXSISERSgp41-1-BSD-PuroR-HSV-Tk into *FOXP1* exon 18b following deoxynucleotides were used: CACCGAGGATGAGTTTGGGTCCTTT and AAACAAAGGACCCAAACTCATCCTC. After ligation of the heterodimerized deoxynucleotides into the BbsI digested pMasterBlaster_Cas9:P2A:i53 backbone, the spacer will be G + AGGATGAGTTTGGGTCCTTT followed by the *S. pyogenes* sgRNA scaffold. You can replace the first nucleotide of a 20 bp spacer with a

G or added an additional G in front of the 20 bp spacer if the first natural nucleotide of the chosen 20 bp spacer does not start with a G. Both strategies are equivalently suitable for standard *S. pyogenes* Cas9.

Troubleshooting:

Many so-called “high fidelity” Cas9 variants or other Cas9 orthologues do not tolerate a PAM-distal mismatch by the introduction of an additional G or replacing a non-G nucleotide with a G.

In this case, the introduction of 5’ hammerhead ribozyme or a tRNA sequence (preferred due to easier design) between the starting G and the spacer/sgRNA scaffold will remove this extra G. Optimally, after EXSISERS’ insertion, the sgRNA spacer sequence does not match the target sequence anymore, which avoids repetitive cutting by Cas9 leading to unintended InDels by NHEJ. In contrast to many other protocols, we do not recommend introducing synonymous PAM-blocking or other mutations to prevent Cas9 recutting since uncharacterized splice enhancer/suppressor motifs might be altered, or cryptic splice sites may be unintentionally introduced, as seen in this study (doi:10.1186/s13024-018-0280-6). If an adequate design is not possible, we suggest using Cas9 RNPs to deliver both, Cas9 and sgRNAs, directly. This delivery method has a very short time of action and thereby prevents the long-term expression of Cas9 and, thus, recutting the already integrated donor. If a NGG PAM cannot be found, one can try to use Cas9 orthologues/variants with more relaxed PAM requirements, *e.g.*, Sc++ Cas9 (NNG), SpG (NGN), or SpRY (NRN>NYN) (doi:10.1038/s41587-020-0517-0, doi:10.1126/science.aba8853).

Two primer pairs are required to amplify 5’- and 3’-homology arms (5’- and 3’-HAs) for Gibson isothermal DNA assembly. We suggest homology arms of at least 800 bp for plasmid DNA-based homologous recombination.

Example: Following primers were used to amplify a 0.8 kbp 5’-HA and a 1.6 kbp 3’-HA (underlined: 5’-overlap for DNA assembly, **bold**: primer binding site):

5’-HA *FOXP1* exon 18b: GGCGAATTGGAGCTGAAGAC**TCTGCTGGTCAGTTATTACCAGAGAC** & GCACCTGGGTCTTAAGGTCCAGGC**ACCCAAACTCATCCTCTACTCTGATAAAG**

3’-HA *FOXP1* exon 18b:

CGCCAACGATATCCTGACCCACAAC**TCCTTTTGGACAGTTGATGATGAAGAG** & GAACAAAAGCTGGGTACGAAGACGA**GCCACAATATTCAAAATGCTAAGCATC**

Alternative: Shorter homology arms can be used (300 bp or longer per homology arm) for PCR-based dsDNA donor generation from a plasmid DNA donor (not used in this manuscript),

Genotyping primer for LongAmp Hot Start Taq 2X Master Mix: preferentially, at least one primer should bind outside of the homology arm to excluded false-positive PCR results by randomly integrated plasmids.

Example: For EXSISERS*FOXP1*:18bBSD, the following primers were used (both primers bind to regions outside of the homology arms): GAACCTATTTTGGGCTTGTATGC and AGAGGATGGAAATTATGATACTGCTG.

Troubleshooting: If genotyping fails due to extraordinary high GC-content, we suggest adding up to 10% DMSO or 2 M betaine during PCR. Changing the polymerase/PCR master mix to other polymerases or polymerase master mixes that can handle long amplicons may also solve the problem, *e.g.* Platinum SuperFi II PCR Master Mix (12368010, Thermo Fisher Scientific) or KOD Xtreme™ Hot Start DNA Polymerase (71975, Sigma-Aldrich). Furthermore, decreasing the elongation temperature from 72 °C to 65 °C and increasing the elongation time per kilobases from 30 s per 1 kbp to 1 min per 1 kbp may further increase the success rate.

Sanger sequencing primers:

seq-U6: GAGGGCCTATTTCCCATGATTC

seq-5-HA-fw: GTTTTCCCAGTCACGAC

seq-5-HA-rv-gp41-1 (for gp41-1 intein system): GCCGGTGTTGCTCAGCAC

seq-5-HA-rv-NrdJ-1 (for NrdJ-1 intein system): CACCTGGATGTTCTTGTCGTTG

seq-3-HA-fw-gp41-1 (for gp41-1 intein system): GAAGATCCTGAAGATCGAAGAACTG

seq-3-HA-fw-NrdJ-1 (for NrdJ-1 intein system):

CATCGGCAAGCTGAAGTCC

seq-3-HA-rv: CGGATAACAATTTCACACAG

Genotyping/Sanger sequencing primers:

geno_HsFOXP1cd18b_1_fw: GAACCTATTTTGGGCTTGTATGC

geno_HsMmFOXP1cd18b_1_rv: AGAGGATGGAAATTATGATACTGCTG

Equipment

2.0 ml Eppendorf Safe-Lock Tubes (0030120094, Eppendorf)

1.5 ml Eppendorf Safe-Lock Tubes (0030120086, Eppendorf)

0.2 ml Eppendorf PCR Tubes (0030124332, Eppendorf)

Falcon™ 15 mL Conical Centrifuge Tubes (10263041, Fisher Scientific)

Falcon™ 50 mL Conical Centrifuge Tubes (352070, Fisher Scientific)

14 ml Greiner Bio-One™ Culture Tubes with Two-Position Vent Stoppers (10574991, Thermo Fisher Scientific)

Thermal cycler (Eppendorf)

Centrifuge for Falcons (Eppendorf)

Centrifuge for Eppendorf reaction tubes (Andreas Hettich)

LB-Agar-Ampicillin-100 Platten (l5667, Sigma-Aldrich)

Bacterial cell spreaders L-shaped (PC58.1, Carl Roth)

37 °C incubator for bacteria growth

Shaking incubator with thermoregulator for bacteria culture

Toothpicks, 80 mm (EC48.1, Carl Roth)

Water bath

Erlenmeyer flask

Electrophoresis chamber

FastGene Agarose Gel Band Cutter (FG-830, NIPPON Genetics Europe)

Blue light illuminator

NanoDrop™ One/OneC spectrophotometer

Monarch® DNA Gel Extraction Kit (T1020S, New England Biolabs)

Monarch® Plasmid Miniprep Kit (T1010S, New England Biolabs)

QIAGEN Plasmid Maxi Kit (25) (12163, QIAGEN)

Vortexer

Cell culture hood for aseptic handling of mammalian cells

37 °C incubator with H2O-saturated and 5% CO2 atmosphere for mammalian cell cull culture

6-well plate Costar® Clear TC-treated (3516, Corning)

96-well Flat Clear Bottom Black Polystyrene TC-treated Microplates(3603, Corning)

48-well plate Costar® Clear TC-treated Multiple Well Plates, Individually Wrapped, Sterile (3548, Corning)

T75 EasYFlask, TC Surface, Filter Cap (156499, Thermo Fisher Scientific)

Brightfield microscope

Cell counter

Wizard® SV Genomic DNA Purification System (A2360, Promega)

Procedure

I. Cloning of a spacer sequence into pMasterBlaster_Cas9:P2A:i53 (Fig. 2b)

A) Digestion of pMasterBlaster_Cas9:P2A:i53 with BbsI:

Prepare the following reaction mix in a 1.5 ml Eppendorf reaction tube:

1 µg of pMasterBlaster_Cas9:P2A:i53

5 µl CutSmart

1 µl BbsI-HF

fill up to 50 µl

Mix well by pipetting up and down and incubate at 37 °C for 2 hours and mix every 30 min to ensure full digestion.

Pour a 1% agarose gel with a suitable comb in 1x TAE buffer by cooking, *e.g.*, 0.8 g agarose with 80 ml 1x TAE buffer in a suitable boil-proof Erlenmeyer flask until all agarose is fully dissolved (no agarose clots should be visible).

Let it cool to ~50 °C.

Under a safety hood, put 1:10,000 of SYBR Safe DNA gel stain stock solution into the liquid 1% agarose TAE buffer mixture.

Under a safety hood, mix gently.

Under a safety hood, pour the hot solution into a suitable gel electrophoresis vessel and let it solidify at room temperature for 30 min.

After two hours of incubation of **step I.A)2.**, add 10 µl of DNA Gel Loading Dye (6x) to the mixture and mix well by pipetting up and down.

Load this mixture in the respective well, including a reference well with 10 µl of the DNA ladder and run at 90 V for 30 min (exact voltage and running time depends on the gel size and the chambers used; please see manufacturer's instructions for more details.

Use a suitable blue light illuminator to visualize DNA gel stains to check the gel. A single band of about 8927 bp is expected; if more than one band is visible, the digestion was not fully successful (probably supercoiled and relaxed plasmid isoforms), and the procedure should be repeated from **step I.A)1.**

Cut out the band with the gel cutter and use the DNA Gel Extraction Kit to extract the DNA after the manufacturer's protocol using a final elution volume of 10 µl.

Measure the DNA concentration on a suitable DNA quantification machine, *e.g.*, NanoDrop™ One/OneC spectrophotometer.

Store at -20 °C or continue with the protocol.

Troubleshooting: If the DNA and the DNA ladder are not visible after running the gel, you might have forgotten the DNA gel stain. Please go back to **step I.A)1.** Check the connection if the plus and minus pole is connected correctly to the correct power supply outputs.

Potential danger: During the cooking procedure, always keep the vessel opening in the direction opposite to you to protect yourself and your colleagues. Handle SYBR Safe gel stain always with care under a safety hood, even though it is classified as biosafe, and always wear protective gloves. In the case a UV illuminator is used instead of a blue light illuminator, wear protective eyewear that blocks UV light. Exposure of the DNA gel to UV should be minimized to a few seconds to check the correct size of the digested plasmid since UV light is known to rapidly damage DNA.

B) Heterodimerization of the deoxynucleotides:

Resuspend the two deoxynucleotides for the spacer (5'-CACCGN19-3' and 5'-AAACn19-3', or alternatively 5'-CACCGN20-3' and 5'-AAACn20-3') to a final concentration of 100 µM in a suitable liquid, *e.g.*, in nuclease-free water or buffer EB.

Example: Centrifuge the lyophilized deoxynucleotide briefly for 10 s to sediment potential lyophilisate on the tube lid. Resuspend a tube with exemplary 230 nmol deoxynucleotides with 230 µl of nuclease-free water or buffer EB using a pipette.

Incubate for 10 min at room temperature or 37 °C to fully solubilize the deoxynucleotides. Vortex gently after incubation.

Prepare a 1.5 ml Eppendorf PCR tube with 80 µl buffer EB.

Pipette 10 µl from both solubilized deoxynucleotides from **step I.B)2.** into the tube with 80 µl buffer EB from **step I.B)3.**

Program a suitable PCR cycler with the following conditions

STEP TEMPERATURE RAMP RATE TIME

Initial Denaturation 95 °C 1 minute

Annealing 95–85 °C -2 °C/s

85–25 °C -0.1 °C/s

Hold 4 °C Hold

Place the tube in the cycler and start the program.

Remove the tube from the cycler and store at -20 °C or continue with the protocol.

C) Ligation of the heterodimerized deoxynucleotides with the BbsI-digested pMasterBlaster_Cas9:P2A:i53:

Thaw all components besides the Quick Ligase to room temperature and pipette following reaction in a 1.5 ml Eppendorf reaction tube:

x µl (50 ng) BbsI-digested pMasterBlaster_Cas9:P2A:i53 (product of **step I.A)**)

0.5 µl Heterodimerized product of the spacer (product of **step I.B)**)

10 µl Quick Ligase Reaction Buffer (2X)

fill up to 20 µl with nuclease-free water

add 1 µl of Quick Ligase in mix gently by pipetting up and down

Incubate ligation reaction mixture for 5-10 min at room temperature.

Store at -20 °C or continue with the protocol.

D) Transformation of chemically competent *E.coli* with ligation product from step I.C):

Take one vial of chemical competent NEB stable *E. coli* cells from -80 °C and place it on a sufficient amount of ice to thaw it gently.

Add 2 µL of the ligation mixture from **step I.C)** into the vial with 50 µL of ice-cold competent cells and mix very gently by tapping it a few times with the fingers (Do not vortex).

Incubate on ice for 30 minutes.

Switch on the water bath to set the temperature to 42 °C.

Heat-shock the cells for 30 seconds at 42°C in the pre-warmed water bath.

After heat-shock, immediately place the vial on ice for 5 minutes.

Add 950 µL of NEB 10-beta/Stable Outgrowth Medium, pre-warmed to 37 °C, to the cells without mixing it by pipetting.

Shake at 37°C for 1 hour at 200–300 rpm in an appropriately heated table shaker.

Pre-warm an LB agar plate with ampicillin for plating later.

Spread 50 µL transformed cell mixture onto the pre-warmed LB-agar plate with ampicillin with an L-shaped cell spreader or sterile glass beads.

Place the plate overnight at 37 °C.

E) Picking colonies for plasmid miniprep

In the morning, prepare 4x 14 ml culture tubes with 2-position vent stoppers with each tube containing 2 ml LB-broth with carbenicillin at a final concentration of 100 µg/ml (2 µl of the stock solution per 2 ml LB-broth).

Pick one single colony for each 14 ml vessel using a sterile toothpick and place the toothpick with the colony on the tip into the vessel.

Incubate for 5–6 hours at 37 °C in a shaking incubator (~200–300 rpm)

After this time, the medium should be cloudy, indicating the growth of the picked *E. coli* colonies. Transfer the 2 ml culture to a 2 ml Eppendorf tube and store the near-empty culture tube with the residual bacterial culture in a 4 °C fridge.

Continue with the 2 ml culture in the 2 ml Eppendorf tube. Use the Monarch Plasmid Miniprep Kit to isolate the plasmid DNA for each clone following the instructions of the manufacturer and elute the

plasmid DNA in the final step of the protocol in 30 µl 65 °C hot water (room temperature results in lower yields of DNA).

Measure the plasmid DNA concentration using a spectrophotometer, *e.g.*, NanoDrop™ One/OneC spectrophotometer.

F) DNA Sanger sequencing of pMasterBlaster_Cas9:P2A:i53 with cloned spacer

The plasmid DNA obtained from the miniprep step should have a concentration ranging from 20–100 ng/µl, which is suitable for most Sanger sequencing services. Please ask the respective companies for detailed instructions.

Use the seq-U6 sequencing primer (GAGGGCCTATTTCCCATGATTC) to verify if the 20 bp is inserted as intended.

Download the Sanger sequencing results as *.ab1 files and map them to the designed plasmid reference file, using appropriate software, *e.g.*, Geneious, Benchling, or SnapGene.

Identify correct clones.

G) Inoculation of a Sanger sequencing verified clone into a culture for maxiprep

Fill in an appropriate Erlenmeyer flask with air ventilation 100 ml room temperature LB-broth containing a final concentration of 100 µg/ml carbenicillin (100 µl of the 100 mg/ml stock solution)

Take one drop of the residual liquid in the 14 ml culture tube of a correct clone identified by Sanger sequencing (**step I.E)4.**) and inoculate the 100 ml culture.

Place the vessel overnight at 37 °C in a shaking incubator with 150–200 rpm.

H) Plasmid isolation of the overnight 100 ml culture

Use QIAGEN Plasmid Maxi Kit to isolate the plasmid DNA from the 100 ml overnight culture according to the manufacturer's instructions.

In the final step of the manufacturer's protocol, resuspend the air-dried plasmid DNA pellet in 50–100 µl buffer EB and incubate for 1 h at 37 °C in a shaking incubator to solubilize the plasmid DNA.

Measure the concentration of the plasmid DNA on a spectrophotometer, *e.g.*, NanoDrop™ One/OneC spectrophotometer. The concentration of the plasmid DNA should usually be greater than 200 ng/µl.

II. Cloning of the CRISPR/Cas9 donor plasmid for homologous recombination (Fig. 2a)

A) Digestion of pEXSISERSgp41-1-BSD-PuroR-HSV-Tk plasmids with BbsI:

Prepare the following reaction mix in a 1.5 ml Eppendorf reaction tube:

2 µg of pEXSISERS plasmid

5 µl CutSmart

1 µl BbsI-HF

fill up to 50 µl

Mix well by pipetting up and down, and incubate at 37 °C for 2 hours and mix every 30 min to ensure full digestion.

Pour a 1% agarose gel with a suitable comb in 1x TAE buffer by cooking, *e.g.*, 0.8 g agarose with 80 ml 1x TAE buffer in a suitable boil-proof Erlenmeyer flask until all agarose is fully dissolved (no agarose clots should be visible).

Let it cool to ~50 °C.

Under a safety hood, put 1:10,000 of SYBR Safe DNA gel stain stock solution into the still liquid 1% agarose TAE buffer mixture.

Under a safety hood, mix gently.

Under a safety hood, pour the hot solution into a suitable gel electrophoresis vessel and let it solidify at room temperature for 30 min.

After two hours of incubation of **step II.A)2.**, add 10 µl of DNA Gel Loading Dye (6x) to the mixture and mix well by pipetting up and down.

Load this mixture in the respective well including a reference well with 10 µl of the DNA ladder and run at 90 V for 30 min (exact voltage and running time depends on the gel size and the chambers used; please see manufacturer's instructions for more details.

Use a suitable blue light illuminator to visualize DNA gel stains to check the gel. Two bands of about 2883 (EXSISERS plasmid backbone) and 3438 bp (EXSISERSgp41-1-BSD-PuroR-HSV-Tk insert) are expected; if more than two bands are visible, then digestion was not fully successful (probably supercoiled and relaxed plasmid isoforms) and should be repeated from **step II.A)1.**

Cut out both bands with the gel cutter and use the DNA Gel Extraction Kit to extract the DNA after the manufacturer's protocol using a final elution volume of 10 µl.

Measure the DNA concentration on a suitable DNA quantification machine, *e.g.*, NanoDrop™ One/OneC spectrophotometer.

Store at -20 °C or continue with the protocol.

B) isolation of genomic DNA from mammalian cells

This protocol step is written for the example of *FOXP1* exon 18b.

Aspirate medium from the HEK293T cells growing in T75 flasks with a minimal 30–40% confluency.

Briefly rinse with PBS to remove residual medium containing FBS and aspirate again.

Add 3 ml Accutase detachment solution onto the cells and incubate for 10 min while gently swirling it every 2 minutes.

Transfer 2.5 ml of the Accutase-cell-solution in a sterile 15 ml falcon tube and centrifuge for 5 min at 500 rcf.

During centrifugation, again add 10 ml DMEM advanced supplemented with GlutaMax, 10% FBS, and Penicillin/Streptomycin (see HEK293T maintenance) onto the T75 flask containing the residual 0.5 ml Accutase-cell-suspension and place it back to the 37 °C incubator with 5% CO2 atmosphere and H2O saturated atmosphere.

After centrifugation, remove the Accutase supernatant.

Proceed with the genomic DNA extraction kit Wizard® SV Genomic DNA Purification System according to the manufacturer's instructions and elute DNA from the spin column with 100 µl 65 °C hot nuclease-free water.

Measure the concentration of the plasmid DNA on a spectrophotometer, *e.g.*, NanoDrop™ One/OneC spectrophotometer.

Store at 4 °C or proceed with protocol.

C) PCR-amplification of homology arms

Resuspend the four deoxynucleotide primers to PCR-amplify the 5'- and 3'-HA in a suitable liquid to a final concentration of 100 µM, *e.g.*, in nuclease-free water or buffer EB.

Example: Centrifuge the lyophilized deoxynucleotide briefly for 10 s to sediment potential lyophilisate on the tube lid. Resuspend a tube with exemplary 230 nmol deoxynucleotides with 230 µl of nuclease-free water or buffer EB using a pipette.

Incubate for 10 min at room temperature or 37 °C to fully solubilize the deoxynucleotides. Vortex gently after incubation. The primers should have a concentration of 100 µM.

Prepare a 10 µM solution of each primer by distributing 9 µl of nuclease-free water into each of four separate 1.5 ml Eppendorf tubes and add 1 µl of the 100 µM primer solution.

Thaw the Q5 Hot Start High-Fidelity 2X Master Mix to room temperature and invert it gently until any remaining white flakes (normally $MgSO_4$) are fully dissolved.

Pipette the following reaction mixtures into a 0.2 ml Eppendorf PCR reaction tube to PCR-amplify the 5'- and 3'-homology arms in separate reactions:

Reaction for 5'-HA:

x µl (50–250 ng) genomic DNA from **step II.B)**

2.5 µl 5'-HA forward primer (10 µM)

2.5 µl 5'-HA reverse primer (10 µM)

25 µl Q5 Hot Start High-Fidelity 2X Master Mix

fill up to 50 µl with nuclease-free water

Reaction for 3'-HA:

x µl (50–250 ng) genomic DNA from **step II.B)**

2.5 µl 3'-HA forward primer (10 µM)

2.5 µl 3'-HA reverse primer (10 µM)

25 µl Q5 Hot Start High-Fidelity 2X Master Mix

fill up to 50 µl with nuclease-free water

Program a PCR cycler with the following parameters:

STEP TEMPERATURE TIME

1. Initial Denaturation 98 °C 1 minute

2. Denaturation 98 °C 10 s

3. Annealing x °C 15 s

4. Elongation 72 °C x min (1 min per kilobase*)

5. go back to step 2 and repeat 34x, then continue with 6.

5. Final Extension 72 °C 2 min

6. Hold 4 °C Hold

* Normally Q5 is able to synthesize 1 kbp in 30 s but some complex genomic DNA might require longer extension time, thus we suggest always 1 min per kilobase for homology arms amplification, especially for long homology arms.

Example: For *FOXP1* exon 18b, a Tm of 68 °C and an elongation time of 1 min was used to PCR-amplify the 0.8 kbp 5'-homology arm, and a Tm of 66 °C and an elongation time of 2 min was used to PCR-amplify the 1.6 kbp 3'-homology arm.

Primers used to PCR-amplify 5'-HA for *FOXP1* exon 18b (underlined: 5'-overlap for DNA assembly, **bold**: primer binding site):

GGCGAATTGGAGCTGAAGAC**TCTGCTGGTCAGTTATTACCAGAGAC** &
GCACCTGGGTCTTAAGGTCCAGGC**ACCCAAACTCATCCTCTACTCTGATAAAG**

Primers used to PCR-amplify 3'-HA for *FOXP1* exon 18b:

CGCCAACGATATCCTGACCCACAAC**TCCTTTTGGACAGTTGATGATGAAGAG** &
GAACAAAAGCTGGGTACGAAGACGA**GCCACAATATTCAAAATGCTAAGCATC**

Place the two PCR reaction tubes for 5'- and 3'-HA into the cycler and start the program.

Pour a 1% agarose gel with a suitable comb in 1x TAE buffer by cooking, *e.g.*, 0.8 g agarose with 80 ml 1x TAE buffer in a suitable boil-proof Erlenmeyer flask until all agarose is fully dissolved (no agarose clots should be visible).

Let it cool to ~50 °C.

Under a safety hood, put 1:10,000 of SYBR Safe DNA gel stain stock solution into the still liquid 1% agarose TAE buffer mixture.

Under a safety hood, mix gently.

Under a safety hood, pour the hot solution into a suitable gel electrophoresis vessel and let it solidify at room temperature for 30 min.

Add 10 µl of DNA Gel Loading Dye (6x) to each of the 50 µl PCR reaction and mix well by pipetting up and down

Load this mixture in the respective well including a reference well with 10 µl of the DNA ladder and run at 90 V for 30 min (exact voltage and running time depends on the gel size and the chambers used; please see manufacturer's instructions for more details.

Use a suitable blue light illuminator to visualize DNA gel stains to check the gel. A band of 0.8 kbp is expected for the *FOXP1* exon 18b 5'-homology arm and a 1.6 kbp for the 3'-homology arm; if more than one band is visible on different molecular weights, then the PCR reaction might have resulted in unspecific products; this is not necessarily a problem, because the brightest band may still the one of the expected size.

Cut out the band with the gel cutter and use the DNA Gel Extraction Kit to extract the DNA after the manufacturer's protocol using a final elution volume of 10 µl.

Measure the DNA concentration on a suitable DNA quantification machine, *e.g.*, NanoDrop™ One/OneC spectrophotometer.

Store at -20 °C or continue with the protocol.

D) Gibson isothermal DNA assembly

Pipette following reaction scheme into a 0.2 ml Eppendorf PCR reaction tube, so that every fragment has a 1:1 stoichiometry (calculate it using a web calculator, such as https://nebiocalculator.neb.com/#!/ligation):

An exemplary calculation to insert homology arms to generate a donor to insert EXSISERSgp41-1-BSD-PuroR-HSV-Tk into *FOXP1* exon 18b:

x µl (50 ng) EXSISERS plasmid backbone from **step II.A)** (2883 bp)

x µl (1:1 molar ratio) EXSISERSgp41-1-BSD-PuroR-HSV-Tk insert from **step II.A)** (3438 bp)

x µl (1:1 molar ratio) 5'-HA for *FOXP1* exon 18b from **step II.C)** (847 bp)

x µl (1:1 molar ratio) 3'-HA for *FOXP1* exon 18b from **step II.C)** (1640 bp)

fill up to 10 µl with nuclease-free water

add further 10 µl of NEBuilder HiFi DNA Assembly Master (2x)

Mix the 20 µl reaction mixture up and down at least 5x.

Place it into a PCR thermocycler and incubate the 20 µl Gibson isothermal DNA assembly reaction mixture for 1 h at 50 °C.

Store at -20 °C or continue with the protocol.

E) Transformation of chemically competent cells with ligation product of step II.D):

Take one vial of chemical competent NEB stable *E. coli* cells from -80 °C and place it onto an appropriate amount of ice to thaw it gently.

Add 2 µL of the assembly mixture from **step II.D)** into the vial with 50 µL of ice-cold competent cells and mix very gently by tapping it a few times with the fingers (DO NOT VORTEX).

Incubate on ice for 30 minutes.

Switch on the water bath to set the temperature to 42 °C.

Heat-shock the cells for 30 seconds at 42°C in the pre-warmed water bath.

After the heat-shock, immediately place the vial on ice for 5 minutes.

Add 950 µL of NEB 10-beta/Stable Outgrowth Medium, pre-warmed to 37 °C, to the cells without mixing it by pipetting.

Shake at 37°C for 1 hour at 200–300 rpm in an appropriately heated table shaker.

Pre-warm an LB agar plate with ampicillin for plating later.

Spread 50 µL transformed cell mixture onto the pre-warmed LB-agar plate with ampicillin with an L-shaped cell spreader or sterile glass beads.

Place the plate overnight at 37 °C.

F) Picking colonies for plasmid miniprep

In the morning, prepare 4x 14 ml culture tubes with 2-position vent stoppers with each tube containing 2 ml LB-broth with carbenicillin at a final concentration of 100 µg/ml (2 µl of the stock solution per 2 ml LB-broth).

Pick one single colony for each 14 ml vessel using a sterile toothpick and place the toothpick with the colony on the tip into the vessel.

Incubate for 5–6 hours at 37 °C in a shaking incubator (~200–300 rpm)

After this time, the medium should be cloudy, indicating the growth of the picked *E. coli* colonies. Transfer the 2 ml culture to a 2 ml Eppendorf tube and store the near-empty culture tube with the residual bacterial culture in a 4 °C fridge.

Continue with the 2 ml culture in the 2 ml Eppendorf tube. Use the Monarch Plasmid Miniprep Kit to isolate the plasmid DNA for each clone following the instructions of the manufacturer and elute the plasmid DNA in the final step of the protocol in 30 µl 65 °C hot water (room temperature water can be also used but expect less DNA).

Measure the plasmid DNA concentration using a spectrophotometer, *e.g.*, NanoDrop™ One/OneC spectrophotometer.

G) DNA Sanger sequencing of EXSISERS donor plasmid with cloned homology arms

The plasmid DNA after miniprep should have a concentration ranging from 20–100 ng/µl suitable for most Sanger sequencing companies. Please ask the respective companies for detailed instructions.

Use the following primers to verify if the homology arms are inserted as planned and do not contain any errors:

for gp41-1 intein systems, use the following primers:

seq-5-HA-fw: GTTTTCCCAGTCACGAC

seq-5-HA-rv-gp41-1: GCCGGTGTTGCTCAGCAC

seq-3-HA-fw-gp41-1: GAAGATCCTGAAGATCGAAGAACTG

seq-3-HA-rv: CGGATAACAATTTCACACAG

for NrdJ-1 intein systems, use following primers:

seq-5-HA-fw: GTTTTCCCAGTCACGAC

seq-5-HA-rv-NrdJ-1: CACCTGGATGTTCTTGTCGTTG

seq-3-HA-fw-NrdJ-1: CATCGGCAAGCTGAAGTCC

seq-3-HA-rv: CGGATAACAATTTCACACAG

Download the Sanger sequencing results as *.ab1 files and map it to the designed plasmid reference file, as it should look at the end using appropriate software, *e.g.*, Geneious, Benchling, or SnapGene.

Identify correct clones.

H) Inoculation of a Sanger sequencing verified clone into a culture for maxiprep

Fill in an appropriate Erlenmeyer flask with air ventilation 100 ml room temperature LB-broth containing a final concentration of 100 µg/ml carbenicillin (100 µl of the 100 mg/ml stock solution)

Take one drop of the residual liquid in the 14 ml culture tube of a correct clone identified by Sanger sequencing (**step II.E)4.**) and inoculate the 100 ml culture.

Place the vessel overnight at 37 °C in a shaking incubator with 150–200 rpm.

I) Plasmid isolation of the overnight 100 ml culture

Use QIAGEN Plasmid Maxi Kit to isolate the plasmid DNA from the 100 ml overnight culture according to the manufacturer's instructions.

In the final step of the manufacturer's protocol, resuspend the air-dried plasmid DNA pellet in 50–100 µl buffer EB and incubate for 1 h at 37 °C in a shaking incubator to solubilize the plasmid DNA.

Measure the concentration of the plasmid DNA on a spectrophotometer, *e.g.*, NanoDrop™ One/OneC spectrophotometer. The concentration of the plasmid DNA should usually be greater than 500 ng/µl.

III. Step-by-step protocol for generating an EXSISERS knock-in cell line (Fig. 3).

The procedure uses a customized donor/targeting plasmid, a CRISPR/Cas9 plasmid, and a Flp/Cre-recombinase expressing plasmid. Both the DNA donor and the CRISPR/Cas9 plasmid are delivered to the cell line of choice via transfection or nucleofection. The following steps refer to the knock-in of EXSISERSgp41-1-BSD-PuroR-HSV-Tk into *FOXP1* exon 18b in HEK293T cells.

A) Maintenance of HEK293T

HEK293T cells (ECACC: 12022001, Sigma-Aldrich) were maintained in H2O saturated atmosphere in Gibco™ Advanced DMEM supplemented with 10% FBS, GlutaMAX™ and penicillin-streptomycin at 100 µg/ml at 37 °C and 5% CO2. Cells were passaged at 90% confluency by removing the medium, washing with DPBS, and separating the cells with 2.5 ml of an Accutase® solution (Gibco™, Thermo Fisher Scientific). Cells were then incubated for 5–10 min at room temperature until a visible detachment of the cells was observed. Accutase™ was subsequently inactivated by adding 7.5 ml pre-warmed DMEM,

including 10% FBS and all supplements. Afterward, cells were transferred into a new flask at an appropriate density or counted and plated in 96-well, 48-well, or 6-well format for plasmid transfection.

B) CRISPR/Cas9-mediated knock-in

For CRISPR/Cas9-mediated knock-in, the customized pMasterBlaster_Cas9:P2A:i53_sgRNA (see section I) and the EXSISERS donor plasmid (see section II) are delivered via transfection.

Aspirate medium from the HEK293T cells growing in T75 flasks with a minimal 70% confluency and a maximal confluency of 90%.

Briefly rinse with PBS to remove residual medium containing FBS and aspirate again.

Add 3 ml Accutase detachment solution onto the cells and incubate for 10 min while gently swirling it every 2 minutes.

Count the cell number per ml and prepare a cell solution in warm medium (room temperature to 37 °C) with 200,000 cells/ml for a total volume of 40 ml in a 50 ml Falcon tube. Accutase should not exceed 10% (<= 4 ml) of the total volume.

If the cell concentration is not high enough, transfer the cell Accutase solution in a 15 ml Falcon tube and centrifuge for 5 min at 500 rcf, room temperature. Aspirate the supernatant, resuspend the cell pellet gently in 3 ml medium, count the cells and go back to **step III.B)4.**

Seed HEK293T cells one day before transfection on 2x 6-well plate (600,000 cells/well in 3 ml). Only one plate is used the next day for transfection.

pMasterBlaster and pEXSISERS are transfected in a 1:1 molar stoichiometry using 1.2 µg of each plasmid DNA:

Prepare the transfection mix in 6x 1.5 ml Eppendorf reaction tube by adding OptiMEM and both plasmid dilutions.

Transfection mixture per tube:

pMasterBlaster_Cas9:P2A:i53_sgRNA 1.2 µg

EXSISERS donor/targeting plasmid 1.2 µg

OptiMEM fill up to 240 µl

Repeat for 6 tubes; mix thoroughly.

X-tremeGENE HP or 360 should be stored at -20 °C; place it at room temperature roughly 10 minutes before usage.

Vortex the glass bottle briefly before usage.

Add 7.2 µl of X-tremeGENE HP or 360 to the transfection mixture per tube and tap it gently but thoroughly with the fingers for 10 seconds while avoiding unnecessary drops on the plastic walls of the Eppendorf reaction tube to ensure maximum transfection efficiency.

Let the transfection mix incubate for 20 minutes at room temperature.

Under the cell culture hood, add each transfection mix in a dropwise manner to one single well of a 6-well plate with HEK293T cells seeded 24 hours before and gently shake the plate back and forth.

Place the cell culture plate back to the 37 °C incubator with a 5% CO_2 atmosphere and H_2O saturated atmosphere.

C) Puromycin selection

Culture cells 3–7 days after transfection to establish a puromycin resistance , then replace the medium in both plates (transfected and untransfected plate) with fresh medium (3 ml per well) with 6 different concentrations of puromycin (0.5, 1, 2, 5, 10, and 50 µg/ml final concentration per well for each plate).

Example: For HEK293T cells in *FOXP1* exon 18b locus, we used 50 µg/ml puromycin for selection; for other locus or cells, try with different concentrations.

Observe cells daily and replace the medium when the color of the medium turns yellowish, including the same amount of puromycin.

Puromycin is a fast-acting eukaryotic antibiotic and the cells should die within 24 hours on the control plate without transfection; if not, the used puromycin concentration is not optimal, and the next higher concentration should be considered for subcultivation.

After one week of selection, the two most stringent conditions, where significant growth of cells can be observed should be chosen for further processing (cells should have reached 10%–50% confluency by now).

Aspirate the medium and briefly rinse it gently with room temperature DPBS (1–2 ml per well).

Add 1 ml of Accutase and incubate for 10 min for cell detachment.

During this 10 min incubation, prepare 2x T75 flasks, each with 10 ml pre-warmed (37 °C) medium containing the two highest puromycin selected.

Gently pipette the cells up and down and transfer the cells from the two selected conditions from 6-well plate to the T75 flasks.

Place the cell culture plate back to the 37 °C incubator with a 5% CO2 atmosphere and H2O saturated atmosphere.

Change the medium any other day, when the medium color is yellowish, including puromycin, until the cells reach a 50–90% confluency.

D) Puromycin N-acetyltransferase and HSV thymidine kinase cassette removal using a site-specific recombinase

Aspirate medium from the selected polyclonal HEK293T EXSISERS cells growing in T75 flasks with a minimal 50% confluency and a maximal confluency of 90%.

Briefly rinse with PBS to remove residual medium containing FBS and aspirate again.

Add 3 ml Accutase detachment solution onto the cells and incubate for 10 min while gently swirling it every 2 minutes.

Count the cell number per ml and prepare a cell solution in warm medium without puromycin (room temperature to 37 °C) with 200,000 cells/ml for a total volume of 40 ml in a 50 ml Falcon tube. Accutase should not exceed 10% (<= 4 ml) of the total volume.

If the cell concentration is not high enough, transfer the cell Accutase solution in a 15 ml Falcon tube and centrifuge for 5 min at 500 rcf, room temperature. Aspirate the supernatant, resuspend the cell pellet gently in 3 ml medium without puromycin, count the cells and go back to **step III.D)4.**

Seed the selected cells on a 6-well plate (600,000 cells/well in 3 ml) without puromycin. For each selection condition, seed at least 4 separate wells. If two selection conditions are selected, a total of 8 wells are seeded.

24 hours after seeding, prepare the transfection mix in 4x 1.5 ml Eppendorf reaction:

Transfection mixture per tube:

pCAG_iFlpe-NLS/pCAG_Cre-NLS 2.4 µg

OptiMEM fill up to 240 µl

Example: pEXSISERSgp41-1-BSD-PuroR-HSV-Tk and pEXSISERSgp41-1-NLuc-PuroR-HSV-Tk, both requires pCAG_iFlpe-NLS to remove the selection cassette. pEXSISERSNrdJ-1:FLuc-PuroR-HSV-Tk requires pCAG_Cre-NLS to remove the selection cassette.

Repeat for 4 tubes (if one has 8 wells seeded); mix thoroughly.

X-tremeGENE HP or 360 should be stored at -20 °C; place it at room temperature roughly 10 minutes before usage.

Vortex the glass bottle briefly before usage.

Add 7.2 µl of X-tremeGENE HP or 360 to the transfection mixture per tube and tap it gently but thoroughly with the fingers for 10 seconds, while avoiding unnecessary drops on the plastic walls of the Eppendorf reaction tube to ensure maximum transfection efficiency.

Let the transfection mix incubate for 20 minutes at room temperature.

Under the cell culture hood, add each transfection mix in a dropwise manner to one single well of a 6-well plate with the polyclonal EXSISERS HEK293T cells seeded 24 hours before and gently shake the plate back and forth. Two wells for each selection condition are transfected, the two remaining wells are not transfected and will be used later as controls during ganciclovir counterselection.

Place the cell culture plate back to the 37 °C incubator with 5% CO2 atmosphere, and H2O saturated atmosphere.

Change the medium any other day, when medium color is yellowish, without puromycin until the cells reach a confluency of 90% confluency.

Cultivate the cells for at least 1 week and split if they reach a confluency of 90% before proceeding with the next step to ensure that the cassette is removed and the HSV-Tk is degraded.

E) Ganciclovir counter selection

When the cells reach 90% after a week (earliest point), aspirate the medium and rinse 1x with room temperature DPBS (1–2 ml)

Add 1 ml Accutase per well and incubate for 10 min at room temperature.

Count the cells and seed 200,000 cells in 3 ml back in the same well.

Add ganciclovir (GCV) to a final concentration of 2 µM to one recombinase-transfected and one non-transfected well for each of the two conditions, originally selected with two different puromycin concentrations. Repeat with 10 µM ganciclovir.

GCV counterselection requires more time to induce cell death since cells have to be in the S-phase to induce toxicity.

Change medium every two days with ganciclovir, independent of the medium color, to avoid toxic accumulation and thus bystander effect of activated toxic GCV (GCV-triphosphate) products from cells still containing the cassette.

Cultivate the cells with ganciclovir for 1–2 weeks and never let the cells reach more than 80% confluency to avoid toxic bystander effects.

Split when necessary and cultivate the cells until they have a replication time similar to the unmodified parental cell line.

The surviving cell population is monoclonalized via limiting dilution or using FACS machine into a 96 well plate containing 100 µl culture media per well 100 µl conditioned medium, harvested and sterile-filtered from confluent cultured cells).

Optional: After reaching a proper colony size, cells are duplicated using 50 µl Accutase per well for detachment, and half of the volume (25 µl) is transferred onto a second 96-well plate supplemented with 200 µl fresh medium containing 1 µg/ml puromycin. Cells that underwent successful cassette excision should not survive puromycin treatment indicating that the original clone does not contain the puromycin-N-acetyltransferase cassette anymore. Clones that survive ganciclovir integration and the puromycin test are discarded since they had either a partial integration of EXSISERS or an additional integration event containing only the puromycin N-acetyltransferase gene without the HSV thymidine kinase gene.

Expand the clones to 48-well plate (500 µl culture medium).

After cells have reached 90% confluency in a 48-well format, continue with **step III.F)**.

F) Genotyping:

Detach cells using 200 µl of Accutase solution per well. Use half of the cell mass for subsequent isolation of genomic DNA using Wizard® Genomic DNA Purification Kit, Maintain the other half is in culture by expanding it to 6-well/T25/T75 flasks with fresh medium.

Isolate the genomic DNA using the Wizard® SV Genomic DNA Purification System according to the manufacturer's protocol and elute DNA from the spin column with 100 µl 65 °C hot nuclease-free water.

Resuspend the four deoxynucleotide primers for genotyping (see primer section) in a suitable liquid to a final concentration of 100 µM, *e.g.*, in nuclease-free water or buffer EB.

Incubate for 10 min at room temperature or 37 °C to fully solubilize the deoxynucleotides. Vortex gently after incubation. The primers should have a concentration of 100 µM.

Prepare a 10 µM solution of each primer by distributing 9 µl of nuclease-free water into each of four separate 1.5 ml Eppendorf tubes and add 1 µl of the 100 µM primer solution.

Thaw the LongAmp Hot Start Taq 2X Master Mix to room temperature and invert it gently until any remaining white flakes (normally $MgSO_4$) are fully dissolved.

Pipette the following reaction mixtures into a 0.2 ml Eppendorf PCR reaction tube to PCR-amplify the region of knock-in; preferentially at least one primer should bind outside of the homology arm to excluded false-positive PCR results by randomly integrated plasmids.

Reaction:

x µl (50–500 ng) genomic DNA from **step III.F)2.**

2.5 µl genotyping forward primer (10 µM)*

2.5 µl genotyping reverse primer (10 µM)*

25 µl LongAmp Hot Start Taq 2X Master Mix

fill up to 50 µl with nuclease-free water

*Example: For *FOXP1* exon 18b, following genotyping sequencing primers were used:
geno_HsFOXP1cd18b_1_fw: GAACCTATTTTGGGCTTGTATGC

geno_HsMmFOXP1cd18b_1_rv: AGAGGATGGAAATTATGATACTGCTG

Program a PCR cycler with the following parameters:

STEP TEMPERATURE TIME

1. Initial Denaturation 94 °C 30 s

2. Denaturation 94 °C 10–30 s

3. Annealing x °C 15–60 s

4. Elongation 65 °C x min (1 min per kilobase*)

5. go back to step 2 and repeat 34x, then continue with 6.

6. Final extension 65 °C 10 min

6. Hold 4 °C Hold

* Normally LongAmp is able to synthesize 1 kbp in 50 s but some complex genomic DNA might require longer extension, thus we suggest always 1 min per kilobase.

Place the PCR reaction tube into the cycler and start the program.

Pour a 1% agarose gel with a suitable comb in 1x TAE buffer by cooking, *e.g.*, 0.8 g agarose with 80 ml 1x TAE buffer in a suitable boil-proof Erlenmeyer flask until all agarose is fully dissolved (no agarose clots should be visible).

Let it cool to ~50 °C.

Under a safety hood, put 1:10,000 of SYBR Safe DNA gel stain stock solution into the still liquid 1% agarose TAE buffer mixture.

Under a safety hood, mix gently.

Under a safety hood, pour the hot solution into a suitable gel electrophoresis vessel and let it solidify at room temperature for 30 min.

Add 10 µl of DNA Gel Loading Dye (6x) to each of the 50 µl PCR reaction and mix well by pipetting up and down

Load this mixture in the respective well including a reference well with 10 µl of the DNA ladder and run at 90 V for 30 min (exact voltage and running time depends on the gel size and the chambers used; please see manufacturer's instructions for more details.

Use a suitable blue light illuminator to visualize DNA gel stains to check the gel.

A band of 3635 bp (2489 bp from EXSISERSgp41-1:BSD + 2489 bp *FOXP1* exon 18b genomic sequence) is expected for the EXSISERS insertion within exon 18b of *FOXP1*; 5931 bp would indicate that the selection cassette still is not removed. The size of the WT allele without EXSISERS insertion is expected to be 2489 bp. All clones containing the 5931 bp should be discarded. Also, clones with additional bands not corresponding to any of the bands also should be discarded. All clones with only WT bands should be discarded.

Only consider clones that are positive for only the 3635 bp band (indicating homozygous integration) or clones that are positive for only two bands, one at 3635 bp and one at 2489 bp (indicating heterozygous insertion with one WT allele) for further expansion.

Note: Homozygous clones are not necessarily required in general. For most applications, heterozygous insertions are sufficient. If heterozygous clones are chosen, the WT band must be sent for Sanger sequencing to exclude NHEJ/MMEJ/SSA-mediated substitutions/insertions/deletions in the WT allele

Cut out all the bands for Sanger sequencing analysis with the gel cutter and use the DNA Gel Extraction Kit to extract the DNA after the manufacturer's protocol using a final elution volume of 25 µl.

Measure the DNA concentration on a suitable DNA quantification machine, *e.g.*, NanoDrop™ One/OneC spectrophotometer.

G) DNA Sanger sequencing of cut out linear DNA fragments from genotyping PCR

The gel-extracted linear DNA of the transgene (and WT allele, if homozygous clones are chosen) should have a concentration ranging from 10–50 ng/µl, which is suitable for most Sanger sequencing services. Please ask the respective companies for detailed instructions.

Use the two genotyping primers and the two Sanger sequencing primers which bind to the EXSISERS N- and C-intein moieties to verify that insertion has occurred as intended without mutations for the PCR band that corresponds to transgenic allele; use only the first two primers for the Sanger sequencing of the WT band.

Example: For *FOXP1* exon 18b, following primers were used for sequencing of the transgenic allele; in addition, the first two primers were used for the WT allele:

geno_HsFOXP1cd18b_1_fw: GAACCTATTTTGGGCTTGTATGC

geno_HsMmFOXP1cd18b_1_rv: AGAGGATGGAAATTATGATACTGCTG

seq-5-HA-rv-gp41-1: GCCGGTGTTGCTCAGCAC

seq-3-HA-fw-gp41-1: GAAGATCCTGAAGATCGAAGAACTG

Download the Sanger sequencing results as *.ab1 files and map it to the designed plasmid reference file, using appropriate software, *e.g.*, Geneious, Benchling, or SnapGene.

Identify correct clones and expand them; discard the clones carrying mutations.

Use at least 3 clones for experiments to exclude clonal artifacts.

Troubleshooting

Please see the individual paragraphs provided in each respective section.

Time Taken

I. Cloning of a spacer sequence into pMasterBlaster_Cas9:P2A:i53

Day 1

Digestion of pMasterBlaster_Cas9:P2A:i53: 3 h

Heterodimerization of the deoxynucleotides: 45 min

Ligation and transformation of chemically competent cells: 3 h

Day 2

Picking colonies and plasmid miniprep: 8 h

DNA Sanger sequencing of pMasterBlaster_Cas9:P2A:i53 with cloned spacer overnight

Day 3

Inoculation of a sequence-verified clone: 15 min

Day 4

Plasmid maxiprep: 6 h

II. Cloning of the CRISPR/Cas9 donor plasmid for homologous recombination

Day 1

Digestion of pEXSISERSgp41-1-BSD-PuroR-HSV-Tk : 3 h

Isolation of genomic DNA from mammalian cells and PCR-amplification of homology arms: 4 h

Gibson isothermal DNA assembly and transformation of chemically competent cells: 3 h

Day 2

Picking colonies and plasmid miniprep

DNA Sanger sequencing of EXSISERS donor plasmid with cloned homology arms overnight

Day 3

Inoculation of a sequence-verified clone for maxiprep

Day 4

Plasmid isolation of the overnight 100 ml culture

III. Step-by-step protocol for generating an EXSISERS knock-in cell line.

Day 1

seeding of HEK293T: 1.5 h

Day 2

Transfection for CRISPR/Cas9-mediated knock-in: 2 h

Day 3

Puromycin selection

About one week

removal of the selection cassette via transfection of a site-specific recombinase

At least one week

Ganciclovir counter selection

roughly 4 weeks

Monoclonalization and culturing until genotyping

overall: roughly 6 weeks

Anticipated Results

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Figures

Figure 1

Principle and mechanism of the exon-specific isoform expression reporter system (EXSISERS). a, Schematic depiction of mRNA biogenesis, maturation, and subcellular trafficking in mammalian cells. b, Knock-in of EXSISERS into exons of interest (EOIs) results in a post-translational intein-mediated, scarless self-excision of modular, exon-specific reporters. c, Dysregulation of alternative splicing (AS) results in aberrant isoform ratios implicated in several neurodegenerative diseases. EXSISERS enables d, longitudinal monitoring of isoforms at the protein level with e, cellular resolution such that the distribution of exon-specific isoform expression can be measured across cell populations.

Figure 2

One-step protocol for generating the EXSISERS donor/targeting plasmid and the CRISPR/Cas9 plasmid. a, The donor/targeting plasmid is digested with the type IIS restriction enzyme BbsI. 5'- and 3'-homology arms are generated via PCR from the genomic DNA flanking the genomic insertion site. The insertion site must be upstream of the amino acid cysteine, serine, or threonine. The resulting fragments are all assembled via Gibson isothermal assembly. The assembled product is transformed into competent E. coli NEB stable. Colonies are inoculated on a small scale (2 ml) for a plasmid miniprep. The plasmid preparations from the clones are sequenced using a standard Sanger protocol on miniprep plasmid DNA. Correct clones are re-inoculated in a 50-100 ml culture overnight for a plasmid maxiprep culture at 37 °C. If the cell line of choice is sensitive towards endotoxins, an endotoxin-free plasmid midi/maxiprep kit should be used in the following to isolate the plasmid DNA. b, The CRISPR/Cas9 cloning plasmid is digested with BbsI. The linearized plasmid backbone is ligated with a deoxynucleotide dimer encoding the CRISPR spacer.

Figure 3

General protocol for generating an EXSISERS cell line. Both, the DNA donor and the CRISPR/Cas9 plasmids, are delivered to the cell line of choice via electroporation (nucleofection) or transfection. A few days later, the cells are selected with puromycin for 3-7 days. The concentration for each cell line has to be determined with a classical kill curve, usually ranging from 0.5-50 µg/ml. The cells are replated without puromycin, and the cells are transfected/electroporated with a plasmid expressing a suitable site-specific recombinase (Flp or Cre) to remove the auxiliary selection cassette. After at least a week, the cells are counter-selected with ganciclovir (typically 2-10 µM) for at least another week. The medium has to be changed at least every 2 days to prevent the accumulation of toxic ganciclovir triphosphate. Cells that still contain the selection cassette will be eradicated during this selection and only cells carrying EXSISERS but without the cassette will survive. The selected population is monoclonalized via limiting dilution or FACS, and clones are then genotyped via PCR for their zygosity. If heterozygous clones are chosen, the WT band must also be sent for Sanger sequencing to exclude NHEJ/MMEJ/SSA-mediated insertions/deletions in the WT allele. Additional small mutations can be co-introduced efficiently to the genomic site of interest (piggybacked on the EXSISERS donor construct) if the mutation is located within 100 nt of the CRISPR/Cas9-mediated double-strand break.