

Generation of cell lines for the usage of Intron-encoded Scarless Programmable Extranuclear Cistronic Transcripts (INSPECT)

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Abstract

Despite their pivotal role in evaluating (patho)physiological cell states, traditional gene reporters can follow gene expression but leave marks on proteins or significantly modify mature mRNA. Multi-time point readouts of non-coding RNAs are, to date, not applicable without changing their nucleotide sequence, which may affect their natural function, half-life, and localization. We thus created INSPECT (Intron-encoded Scarless Programmable Extranuclear Cistronic Transcripts) as a minimally invasive transcriptional reporter nested within an intron of a gene of interest. Post-transcriptional excision of INSPECT results in a mature RNA with no sequence changes and an additional synthetic transcript that leaves the nucleus by hijacking the nuclear host export machinery for cytosolic translation into a reporter protein. Here, we first illustrate the cloning of such an INSPECT DNA donor construct and an auxiliary CRISPR/Cas9 plasmid to insert INSPECT into a gene of interest and, secondly, exemplary for *NEAT1*_long, we showed the generation of such a cell line.

Introduction

Most of the general workflow and cloning procedure for the CRISPR/Cas9-assisted insertion of INSPECT into the host genome is similar to the procedure which we previously published for insertion of exon-specific isoform expression reporter system (EXSISERS) (doi:10.1038/s41556-021-00678-x) with slight adaptations.

In this protocol, we describe how to clone the genetic constructs for CRISPR/Cas9-mediated integration of the INSPECT reporter system into a given GOI. The plasmids can be generated in a week, and the respective INSPECT reporter cell line can be conveniently generated within 8 weeks via double selection using puromycin and ganciclovir.

Reagents

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

Alternative: Platinum™ SuperFi II PCR Master Mix (12368010, Thermo Fisher Scientific)

BbsI-HF® (R3539S, New England Biolabs)

Esp3I® (R0734S, New England Biolabs)

CutSmart® Buffer (usually provided together with the restriction enzymes, 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)

Platinum™ SuperFi II PCR Master Mix (12368010, Thermo Fisher Scientific)

Alternative: 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)

jetOPTIMUS DNA transfection reagent (101000051, Polyplus Transfection)

Alternative: 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)

Optional: AZD7648 (HY-111783, MedChemExpress)

Ganciclovir (G2536, Sigma-Aldrich)

Puromycin Dihydrochloride (A1113803, Thermo Fisher Scientific)

Plasmids:

pMasterBlaster_Cas9:P2A:i53

pINSPECTv1_PuroR-HSV-Tk-**SigP**-NLuc

Primers for spacer cloning:

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

Example: To insert pINSPECTv1_PuroR-HSV-Tk-**SigP**-NLuc_HsNEAT1_total into *NEAT1*, following deoxynucleotides were used to insert the spacer into the CRISPR/Cas9 plasmid: CACCGCATCTGAAAACCTTTACCCC and AAACGGGGTAAAGGTTTTTCAGATGC. After ligation of the heterodimerized deoxynucleotides into the BbsI digested pMasterBlaster_Cas9:P2A:i53 backbone, the spacer will be G + CATCTGAAAACCTTTACCCC (G+20) followed by the *S. pyogenes* sgRNA scaffold. You can replace the first nucleotide of a 20 bp spacer with a G (G+19) or add an additional G in front of the 20 bp spacer (G+20) 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.

Important:

It is critical to pay close attention to the selection of the INSPECT insertion site. Generally, the insertion site within the target GOI must follow the consensus NAG[^]GWW. This insertion site leads to a functional splice donor and splice acceptor sequence in combination with the inserted INSPECT construct. We recommend to validate the insertion *in silico* by using a splice prediction software (e.g. NetGene2) (doi:10.1016/0022-2836(91)90380-0).

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 INSPECT's 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/s41587-020-0604-2, doi:10.1126/science.aba88).

Equipment

Standard lab equipment.

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 μ l.

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' (G+19 spacers), or alternatively 5'-CACCGN20-3' and 5'-AAACn20-3' (G+20 spacers)) to a final concentration of 100 μ M 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 μ l 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 min

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 it 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 into the reaction tube and 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 $^{\circ}\text{C}$, to the cells without mixing it by pipetting.

Shake at 37 $^{\circ}\text{C}$ 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 $^{\circ}\text{C}$.

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 $\mu\text{g}/\text{ml}$ (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 $^{\circ}\text{C}$ 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 $^{\circ}\text{C}$ 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 μ l 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/ μ l, 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 μ g/ml carbenicillin (100 μ l 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 pINSPECTv1_PuroR-HSV-Tk-SigP-NLuc

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

2 µg of pINSPECTv1_PuroR-HSV-Tk-SigP-NLuc

5 µl CutSmart

1 µl Esp3I

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 2881 (INSPECT plasmid backbone) and 6232 bp (INSPECTv1_PuroR-HSV-Tk-**SigP**-NLuc 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 μ l.

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 *NEAT1*_total.

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 MgSO₄) 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 min
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 *NEAT1*_total homology arms, a T_m of 72 °C and an elongation time of 1 min was used to PCR-amplify the 1.5 kbp 5'-homology arm, and a T_m of 68 °C and an elongation time of 2 min was used to PCR-amplify the 2.8 kbp 3'-homology arm.

Primers used to PCR-amplify 5'-HA for *NEAT1*_total (underlined: 5'-overlap for DNA assembly, **bold**: primer binding site):

GGCGAATTGGAGCTCGTCTCGGGAGTTAGCGACAGGGAGGGATG

CTCATTAAGTTGTGCTGTAAAAAGGATACTT**ACCTTTACCCCAGGAAAGGAGGG**

Primers used to PCR-amplify 3'-HA for *NEAT1*_total:

CTCTCCCCTCTCCTCTTTTCTTTTCTGCAGGTTTTTCAGATGCTGCATCTTCTAAATTG

GAACAAAAGCTGGGTACCGTCTCC**CACAGCCTCTGCTTGCTTACTG**

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 μ l of DNA Gel Loading Dye (6x) to each of the 50 μ l PCR reactions and mix well by pipetting up and down

Load this mixture in the respective well, including a reference well with 10 μ l 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 *NEAT1*_total 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 be 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 μ l.

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 INSPECTv1_PuroR-HSV-Tk-**SigP**-NLuc into *NEAT1*_total:

x μ l (50 ng) INSPECT plasmid backbone from **step II.A)** (2881 bp)

x μ l (1:1 molar ratio) INSPECTv1_PuroR-HSV-Tk-**SigP**-NLuc insert from **step II.A)** (6232 bp)

x μ l (1:1 molar ratio) 5'-HA for *NEAT1*_total from **step II.C)** (1573 bp)

x μ l (1:1 molar ratio) 3'-HA for *NEAT1*_total from **step II.C)** (2832 bp)

fill up to 10 μ l with nuclease-free water

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

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

Place it into a PCR thermocycler and incubate the 20 μ l 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 also be used but expect less DNA).

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

G) DNA Sanger sequencing of INSPECT 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:

M13-40FOR: GTTTTCCCAGTCACGAC

loxP_rv: GATCCAAGCATCACCATCGAC

INSPECT_3'_fw: GGCCGCTCTATACTCGAC

M13-48REV: CGGATAACAATTTACACAG

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 μ l 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/ μ l.

III. Step-by-step protocol for generating an INSPECT 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 INSPECTv1_PuroR-HSV-Tk-**SigP**-NLuc into *NEAT1*_total 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 μ g/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 INSPECT 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 pINSPECT 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

INSPECT donor/targeting plasmid 1.2 µg

jetOPTIMUS buffer fill up to 240 µl

Repeat for 6 tubes; mix thoroughly.

jetOPTIMUS buffer and reagent should be stored at -20 °C; place it at room temperature roughly 10 minutes before usage.

Vortex the reagent tube for 5 s before use.

Add 2.4 µl of jetOPTIMUS reagent 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 10 minutes at room temperature.

Optional: During the incubation, add 0.5 µM AZD7648 to the cells to inhibit DNA-PKcs, a key player of NHEJ, to enhance INSPECT insertion efficiency.

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% CO₂ atmosphere and H₂O saturated atmosphere.

C) Puromycin selection

Culture cells 3–7 days after transfection to establish a puromycin resistance (optionally with 0.5 AZD7648), 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 *NEAT1*_total 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 INSPECT 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 2.4 µg

jetOPTIMUS buffer fill up to 240 µl

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

jetOPTIMUS buffer and reagent should be stored at -20 °C; place it at room temperature roughly 10 minutes before usage.

Vortex the reagent tube for 5 s before use.

Add 2.4 µl of jetOPTIMUS reagent 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 10 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 INSPECT 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 INSPECT 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 Platinum™ SuperFi II PCR Master Mix to room temperature and invert it gently until any remaining white flakes (normally MgSO₄) 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 Platinum™ SuperFi II PCR Master Mix

fill up to 50 µl with nuclease-free water

*Example: For *NEAT1*_total, following genotyping sequencing primers were used:

geno_HsNEAT1_total_fw: ACTATAGTGTTCCCTCATGGCGAGCAG

geno_HsNEAT1_total_rv: GTGTACCCACCATTCCCTTCTCCTAG

Program a PCR cyclor with the following parameters:

STEP TEMPERATURE TIME

1. Initial Denaturation 98 °C 2 min
2. Denaturation 98 °C 10 s
3. Annealing 60 °C 10 s
4. Elongation 72 °C x min (1 min per kilobase*)
5. go back to step 2 and repeat 34x, then continue with 6.
6. Final extension 72 °C 5 min
6. Hold 4 °C Hold

* Normally SuperFi II is able to synthesize 1 kbp in 30 s but some complex genomic DNA might require longer extension, thus we suggest always 1 min per kilobase. Especially for heterozygous clones, prolonged extension time allows the equivalent efficient amplification of WT (short) and transgenic (long) amplicon.

Place the PCR reaction tube into the cyclor 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 3942 bp (3133 bp from INSPECTv1_PuroR-HSV-Tk-**SigP**-NLuc + 809 bp *NEAT1*_total genomic sequence) is expected for the INSPECT insertion within *NEAT1*_total; 7045 bp would indicate that the selection cassette still is not removed. The size of the WT allele without INSPECT insertion is expected to be 809 bp. All clones containing the 7045 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 3942 bp band (indicating homozygous integration) or clones that are positive for only two bands, one at 3942 bp and one at 809 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 μ l.

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/ μ l, 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 close to the INSPECT splice donor and acceptor sites 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 *NEAT1*_total, following primers were used for sequencing of the transgenic allele; in addition, the first two primers were used for the WT allele:

geno_HsNEAT1_total_fw: ACTATAGTGTTCCCTCATGGCGAGCAG

geno_HsNEAT1_total_rv: GTGTACCCACCATTCCCTTCTCCTAG

loxP_rv: GATCCAAGCATCACCATCGAC

INSPECT_3'_fw: GGCCGCTCTATACTCGAC

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

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 pINSPECTv1_PuroR-HSV-Tk-**SigP**-NLuc: 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 INSPECT 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 INSPECT 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

overall: roughly 6 weeks

Figures

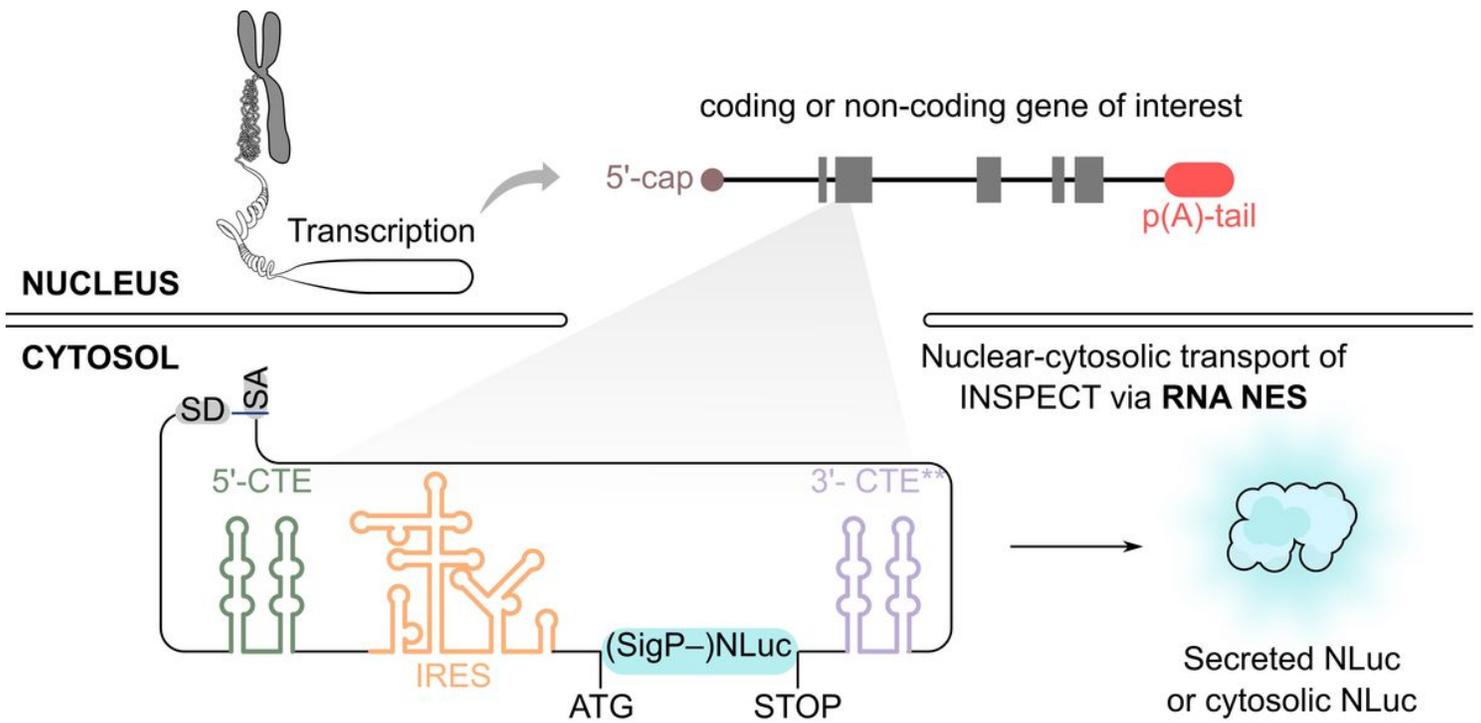


Figure 1

Principle and mechanism of the INSPECT.

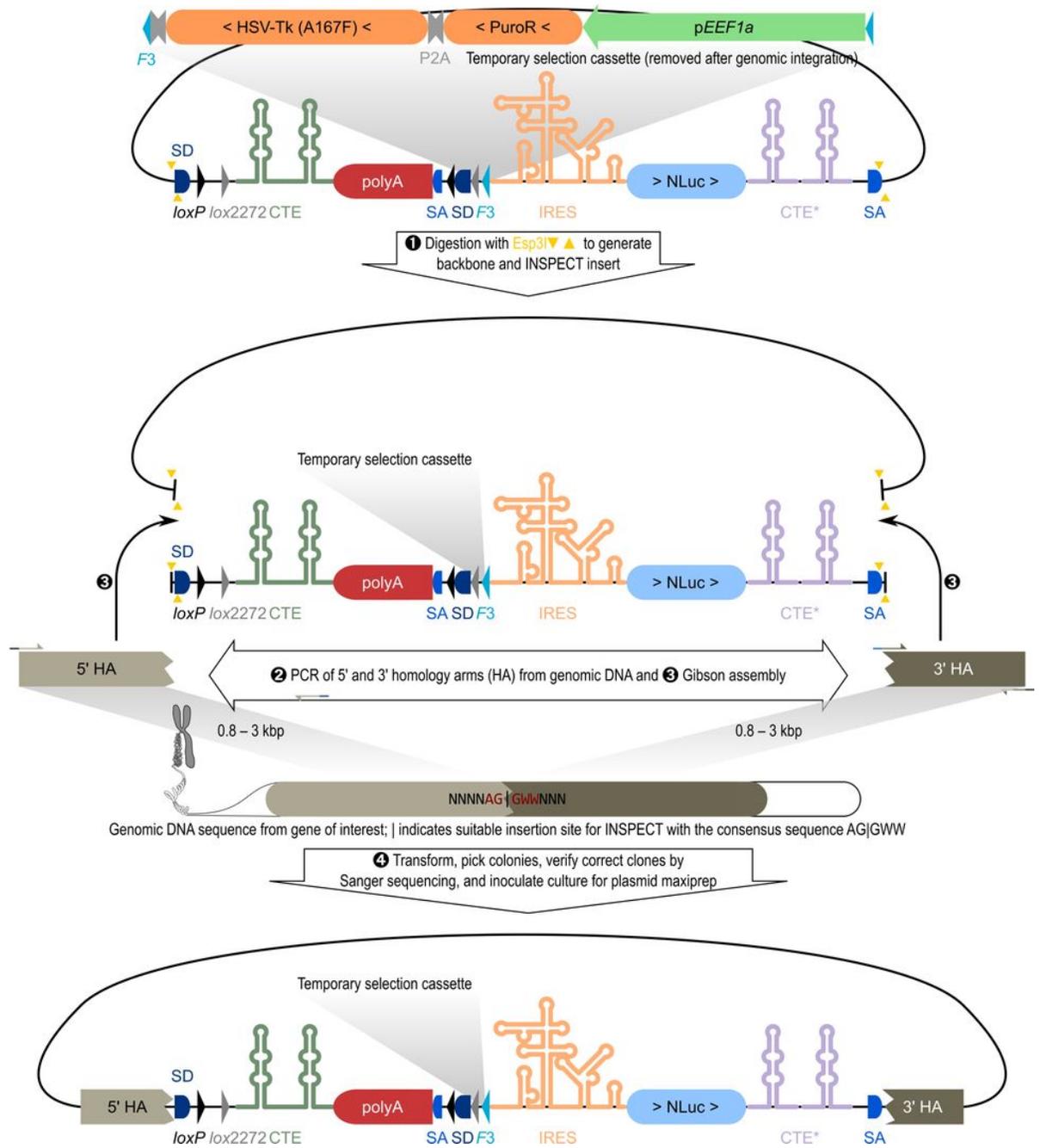


Figure 3

One-step protocol for generating the INSPECT donor plasmid.

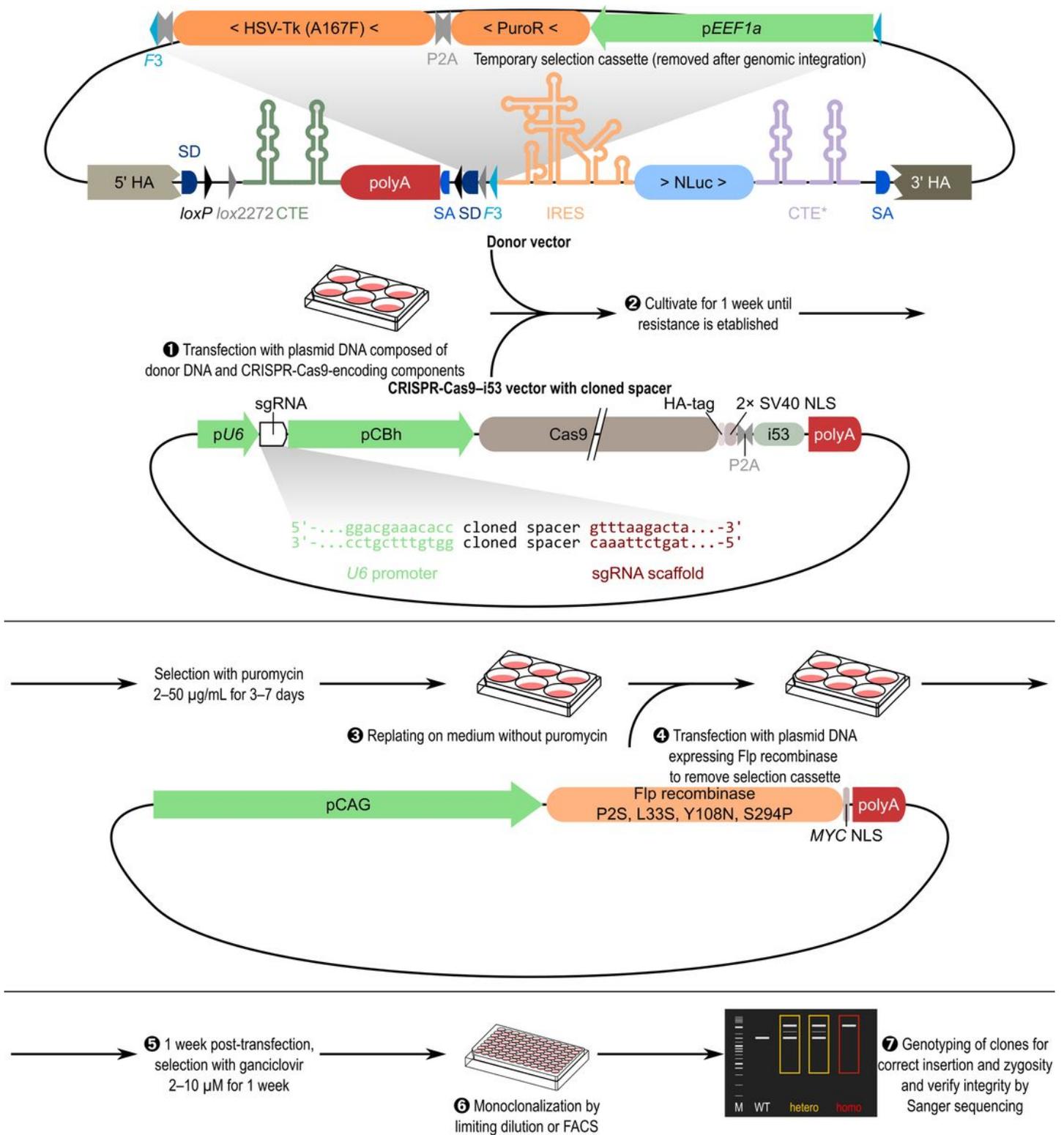


Figure 4

One-step protocol for generating the INSPECT donor plasmid.