Efficient generation of *Rosa26* knock-in mice using CRISPR/Cas9 in C57BL/6 zygotes

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Additional file 2

Supplementary Methods

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Plasmid maps

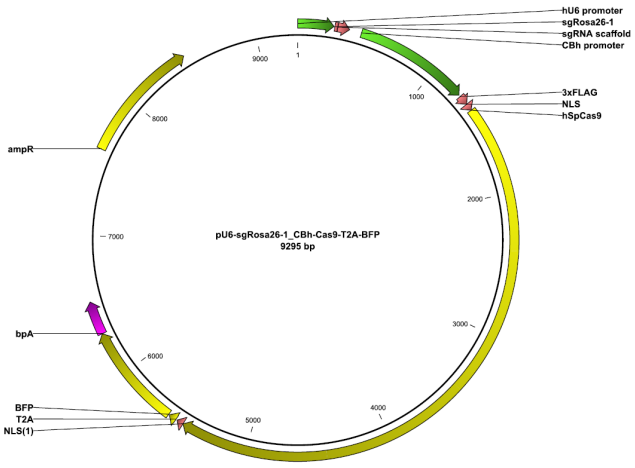
pCAG-Cas9-162A
pU6-sgRosa26-1_CBh-Cas9-T2A-BF

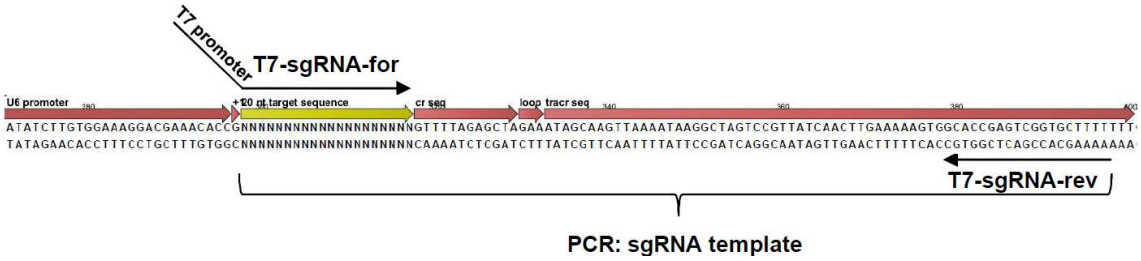
Supplementary Methods

1. Production of sgRNA

1.1. Generating a T7-PCR template for in vitro transcription ●TIMING 1 d

For sgRNA preparation amplify the sgRNA region from the sgRNA plasmid pU6-sgRosa26-1_CBh-Cas9-T2A-BFP (Addgene #64214) using primers T7-sgRosa26-1-for and sgRNA-rev:





Primer	Sequence (5′–3′)
T7-sgRosa26-for	**TTAATACGACTCACTATAGGACTCCAGTCTTTCTAGAAGAGT**
T7-sgRNA-rev	**AAAAGCACCGACTCGGTGCC**

Using these primers, set up 3-5 x 50ul PCR reactions:

Reagents	50µl PCR reaction	For 5 x 50µl PCR reactions
pU6-sgRosa26-1_CBh-Cas9-T2A-BFP	2 µl	10 µl
10x (5x) buffer	5 (10) µl	25 (50) µl
dNTPs (10 mM)	1 µl	5 µl
Primer T7-sgRosa26-for (50 pMol/µl)	1 µl	5 µl
Primer T7-sgRNA-rev (50 pMol/µl)	1 µl	5 µl
Herculase II	1 ul	5 ul
H_20	39 (34)µl	195 (170) µl
PCR program: 95°C 3min; 35 cycles of [95°C-20s; 54-58°C-20s; 72°C-20s]; 72°C-3 min		

• Run 5 ul of the PCR product on a 2% agarose gel to estimate its concentration and to verify that the product is unique and has the expected size of 100 bp. PCR should be optimized to yield a single specific band to avoid gel purification.

• Pool the 3-5 PCR reactions for purification using the Qiagen PCR purification kit. Elute with 30 µl RNAse free water. Quantify using a Biophotometer or Nanodrop; a concentration of ≥ 125 ng/ul is required for IVT (see below).

PAUSE POINT *samples can be stored at -20°C for 6 months*

1.2 In vitro Transcription (IVT) ●TIMING 1 d

The sgRNA PCR product is used as template for in-vitro transcription using the Ambion MEGA shortscript T7 Kit (AM1354) following the manual, for a 20 µl IVT reaction add:

- 1 µg of the sgRNA PCR template, in a volume up to 8 µl
- 2 µL 10X Reaction Buffer
- 2 µL ATP Solution (75 mM)
- 2 µL CTP Solution (75 mM)
- 2 µL GTP Solution (75 mM)
- 2 µL UTP Solution (75 mM)
- x µl water (Nuclease-free), to 18 µL final volume
- 2 µL T7 Enzyme Mix

➢ Mix, incubate 2-4 h at 37°C

1.3 sgRNA purification

Use the Ambion MEGAclear kit (AM1908) to purify the *in vitro* transcribed RNA following the manual:

To the 20 µl transcription reaction add:

- 80 µl **elution solution**
- 350 µl **binding buffer** (20 ml EtOH added to new bottle)
- 250 µl **Ethanol** (100%)

- Mix and apply to column in the collection tube
- Spin column for 1 min at 12.000 rpm
- Wash column 2x with 500 µl wash solution, spin 1 min, discard flowthrough
- Spin the empty column for 30 sec at 12.000 rpm
- Place column into a new collection tube
- Apply 50 µl elution solution, close the lid, place into an incubator at 65-70°C for 5-10 min (Alternatively, apply 50 ul of elution solution preheated to 95C)
- Spin for 1 min at 12.000 rpm
- Apply another 50 µl of elution solution, close the lid, place into an incubator at 65-70°C for 5-10 min (Alternatively, apply another 50 ul of elution solution preheated to 95C)
- Spin for 1 min at 12.000 rpm, save the eluate, discard the column

To the eluate (100 µl) add:

- 10 µl 5 M Ammoniumacetate (1/10 Vol)
- 275 µl Ethanol (2.5 Vol)

Incubate at -20°C for ≥ 30 min or **overnight**

PAUSE POINT *samples can be left overnight at -20°C for precipitation*

- Spin for 15 min at 15.000 g
- Wash the pellet with 500 µl of 70% Ethanol, spin 1 min, discard supernatant
- Spin the dry tube for 10 sec and take off last traces of Ethanol
- Air dry the open tube for 3-5 min

Resuspend the pellet in 30 µl $T_{10}E_{0.1}$ injection buffer, incubate 5-10 min at 37°C, mix

- use 1 ul to measure concentration by OD_{260} (expected yield: 0.5 - 1 µg/ul)
- run 1 ug RNA on a 2% agarose gel (RNAse-free) (sgRNA size: 100 nt)

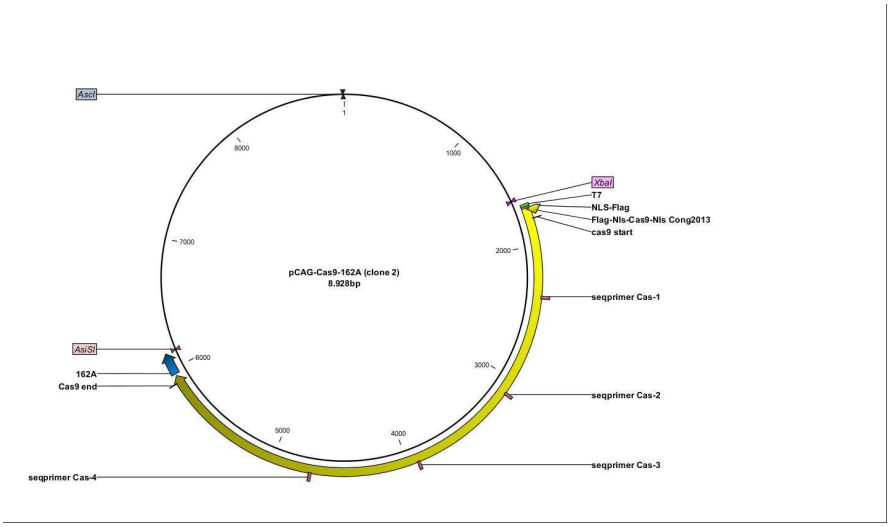
▲**CRITICAL STEP** Run 500 ng of denatured sgRNA (70^{0}C for 10 min) on a 2% agarose gel to confirm integrity before injection. Smeared bands indicate degradation and these samples should be discarded. Take care to use an RNAse free gel chamber and buffers !

PAUSE POINT *Store the samples at −80 ℃ for the preparation of injection aliquots (up to 2 months).*

2. Production of Cas9-162A mRNA

2.1 Isolation of the T7-Cas9 template for in vitro transcription ●TIMING 1 d

For IVT of Cas9 mRNA a 4.5 kb XbaI-AsiSI fragment including the T7 promoter, Cas9 coding region and a poly A tail (162A; plasmid coded), is isolated from the plasmid pCAG-Cas9-162A. AscI is used in addition to cut the plasmid´s backbone into smaller fragments. Map of pCAG-Cas9-162A:



1. Set up a restriction enzyme digestion as follows:

Reagent	In 100 µl
pCAG-Cas9-162pA plasmid DNA	10 µg
10x buffer NEB CutSmart	10 µl
XbaI (20 U/ul, NEB 0145)	2 µl
AsiSI (10 U/ul, NEB R0630)	4 µl
AscI (10 U/ul, NEB R0558)	4 µl
H_20	fill up to 100 µl

2. Incubate at 37^{o}C for 2h

3. Add 20 µl of 6x gel loading buffer to the digestion reaction and load 10 µl into the first lane of a 0.9 % agarose gel (12 x 12 cm), next to a size marker in the second lane. Keep three or more lanes free and load the remaining volume (110 µl) into the next 3-4 lanes. Run the gel at 120V for 1.5h and cut off the left 2 lanes and take an image of this gel segment under short wave UV light. There should be three fragments of 1.6, 2.8 and 4.5 kb visible.

4a. **Option A**: If the digestion looks ok, cut out the largest, 4.5 kb (XbaI-AsiSI) band (representing the T7-Cas9-162A IVT template) under **long wave UV light** (365 nm) and isolate the DNA using the Qiagen gel extraction kit with a final elution volume of 30 µl. (Alternatively, for higher concentrations, use the Qiagen Minelute gel extraction kit (Qiagen #28604) and 10 µl elution buffer)

4b. **Option B**: If the digestion looks ok, precipitate the DNA by adding 10 µl 3 MNaAc + 250 µl EtOH > 30 min/-20C. Spin 10 min, wash w. 70% EtOH, air dry & solve in 25 µl water (nuclease-free)

5. Determine the DNA concentration using a Biophotometer or Nanodrop, each IVT reaction needs 1 µg of the fragment in a volume of ≤ 6 ul, therefore a DNA concentration of ≥ 166 ng/µl is necessary.

2.2 Cas9 in vitro Transcription (IVT) ●TIMING 1 d

The Cas9-162pA template is in-vitro transcribed using the Ambion mMESSAGE mMACHINE®T7 Ultra Kit (AM 1345) following the manual, for a 20 µl IVT reaction add:

- 1 µg of the template DNA fragment, in a volume up to 6 µl
- 10 µl T7 2x NTP/ARCA
- 2 µl 10x buffer (at room temperature)
- x µl water (Nuclease-free), to a final volume of 18 µl
- 2 µl enzyme mix

➢ Mix, incubate for 2h at 37°C

2.3a Cas9 mRNA purification: Option 1 - MegaClearkit

Use the Ambion MEGAclear kit (AM1908) to purify the *in vitro* transcribed mRNA following the manual:

To the 20 µl transcription reaction add:

- - 80 µl **elution solution**
- - 350 µl **binding buffer** (20 ml EtOH added to new bottle)
- - 250 µl **Ethanol** (100%)
- Mix and apply to column in the collection tube
- Spin column for 1 min at 10-**15.000** g
- Wash column 2x with 500 µl wash solution, spin 1 min, discard flowthrough
- Spin the empty column for 30 sec at 10-15.000 g
- Place column into a new collection tube
- Apply 50 µl elution solution, close the lid, place into an incubator at 65-70°C for 5-10 min (Alternatively, apply 50 ul of elution solution, preheated to 95C)
- Spin for 1 min at 10-15.000 g

- Apply another 50 µl of elution solution, close the lid, place into an incubator at 65-70°C for 5-10 min (Alternatively, apply another 50 ul of elution solution, preheated to 95C)
- Spin for 1 min at 10-15.000 g, save the eluate, discard the column

Or: 2.3b Cas9 mRNA purification: Option 2 - Oligotex kit

Use the Qiagen mRNA mini kit (#70022) to purify the *in vitro* transcribed mRNA following the manual:

To the 20 µl transcription reaction add:

180 µl Water (nuclease-free)
200 µl buffer OBB (if precipitate present, warm at 37C)
20 µl Oligotex suspension (before warmed to 37C and vortexed)

- Mix and incubate at 70 C for 3 min
- place at room temperature for 10 min
- apply to column in the collection tube
- Spin the column for 1 min at 12.000 rpm
- Transfer column into a new collection tube
- Wash column with 400 µl buffer OW2, spin 1 min, discard flowthrough
- Wash column again with 400 µl buffer OW2, spin 1 min
- Transfer column into a new collection tube
- apply 25 µl of elution buffer OEB (preheated to 70C), pipette 3-4x to resuspend resin
- Spin for 1 min at 12.000 rpm
- Apply another 25 µl of buffer OEB (preheated to 70 C), pipette 3-4x to resuspend resin
- Spin for 1 min at 12.000 rpm, save the eluate, discard the column

2.4 Precipitation

To the eluate (100 µl) add:

- 5 µl 5 M Ammoniumacetate (1/10 Vol)
- 138 µl Ethanol (2.5 Vol)

Incubate at -20°C for ≥ 30 min or **overnight**

PAUSE POINT *samples can be left overnight at -20°C for precipitation*

- Spin for 15 min at 15.000 g
- Wash the pellet with 500 µl of 70% Ethanol, spin 1 min, discard supernatant
- Spin the dry tube for 10 sec and take off last traces of Ethanol
- Dry the pellet at air in the tube with open lid for 3-5 min

Resuspend the pellet in 30 µl $T_{10}E_{0.1}$ injection buffer, incubate 5-10 min at 37°C, mix

PAUSE POINT *Store the samples at −80 ℃ for the preparation of injection aliquots (up to 2 months).*

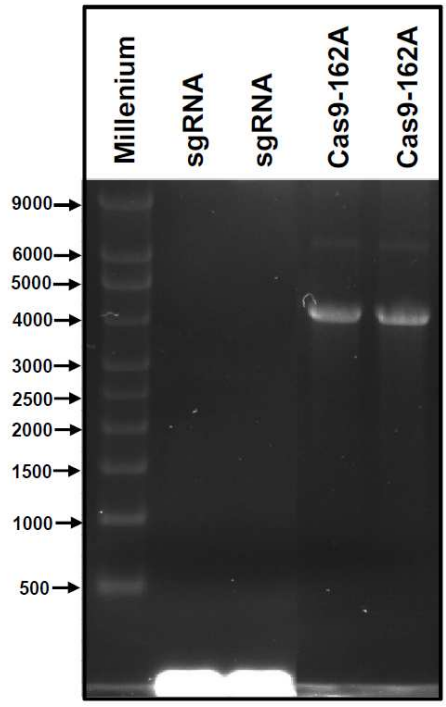
2.5 RNA analysis

- use 1 µl to measure concentration by OD_{260} (expected yield: 0.5 - 1 µg/ul)

Gel option A: run 1 µg RNA on a standard 0.9 % agarose gel (RNAse-free) (mRNA size: 4411 nt). Run 1 µg of heat denatured mRNA (70^{0}C for 10 min) on a 0.9% agarose gel (TAE/TBE) (1 µg/ml Ethidiumbromide) to confirm integrity before injection. Smeared bands indicate degradation and these samples should be discarded. Take care to use an RNAse free gel chamber and buffers !

Gel option B: For optimal results use denaturing (glyoxal) gel electrophoresis and Northern Max-Gly reagents from Ambion: Mix 1 ug RNA in 10 µl water (nuclease-free) with 10 ul NorthernMax®-Gly Sample Loading Dye (AM8551), incubate at 50C for 30 min. Load on a 0.9% agarose gel prepared with NorthernMax®-Gly 10x gel prep/running buffer (AM8678) and 1 µg/ml Ethidiumbromide. As size marker load 2 µg of RNA Millenium size marker (AM7150), treated as above with AM8551.

Example gel:



Gel option C: Run the RNA in the Agilent Bioanalyser using the Agilent RNA 6000 Nano kit (#5067-1511), following Agilents instruction manual: http://www.chem.agilent.com/Library/usermanuals/Public/G2938-90034_RNA6000Nano_KG.pdf

3. Preparation of targeting vector DNA ●TIMING 2h

1. Precipitate 15 µg of plasmid DNA (Qiagen Maxiprep) of the gene targeting vector by adding 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ethanol. Incubate the mixture for 30 min at RT and pellet the DNA by centrifugation at 16.000*g* for 10 min at RT.

2. Discard the supernatant and wash the pellet by the addition of 500 ul of 70% (vol/vol) ethanol (prepared with embryo-tested water, available from the TCF). Centrifuge the tube at

16.000*g* for 2 min at RT, discard the supernatant and repeat the washing step. Discard the supernatant and air-dry the pellet for 5 min.

3. Resuspend the DNA pellet in 30 µl of $T_{10}E_{0.1}$ microinjection buffer for 30 min at 37 ℃. Mix by pipetting and use 1 µl to determine the DNA concentration using a Biophotometer or Nanodrop. Store the DNA solution at −80 ℃ for the preparation of microinjection samples.

4. Preparation of microinjection buffer ($T_{10}E_{0.1}$)

$T_{10}E_{0.1}$ microinjection buffer contains 10 mM Tris and 0.1 mM EDTA (pH 7.4), made of reagents of the highest purity to avoid embryo toxicity.

Stock preparation:
- 1 M Tris-base stock solution is prepared by dissolving 3030 mg of Trizma base (Sigma ultra T6791, Mw=121,14) in 25 ml of embryo-tested water (Sigma W1503)

- 1 M Tris-acid stock solution is prepared by dissolving 3940 mg of Trizma hydrochloride (Sigma ultra T6666, Mw=157,6) in 25 ml of embryo-tested water.

- 5 mM EDTA stock solution is prepared by dissolving 93 mg of EDTA disodium salt dihydrate (Sigma E4884, Mw=372,24) in 50 ml embryo tested water.

For preparation of 25 ml $T_{10}E_{0.1}$ buffer combine in a 50 ml Falcon tube:

- 24.25 ml embryo-tested water

- 40.85 µl Tris-base stock (1 M)

- 209 µl TRIS-acid stock (1 M)

- 500 µl EDTA stock (5 mM)

Give this mixture into a 20-ml disposable syringe and filter through a Millex GV filter (Millipore, cat. no. SLGV033RS), discarding the first 5 ml of the filtrate. Use 100ul buffer to confirm that the pH value falls into the range of 7.0–7.5, by using pH test strips. Store $T_{10}E_{0.1}$ injection buffer in 1 ml aliquots at −80 ℃ for up to 2 years.

5. Preparation of aliquots for pronuclear microinjection ●TIMING 2 h

Thaw the sgRNA and Cas9 mRNA the DNA and a tube of $T_{10}E_{0.1}$ microinjection buffer. For each day of microinjection, a single-use aliquot of 30 µl of injection solution is used, in total a volume of 150 µl injection solution is needed for a complete pronucleus microinjection experiment of up to 5 microinjection days (5 x 10 superovulated female mice for zygote production). Standard concentrations are:

Cas9 mRNA:	**25 ng/µl**
sgRNA:	**12.5 ng/µl**
Targeting vector DNA:	**20 ng/µl**

To prepare 150 µl of a master mix, combine in a clean, dust-free 1.5-ml tube:

- 3750 ng of Cas9 mRNA
- 1875 ng of sgRNA (each)
- 3000 ng targeting vector
- adjust with $T_{10}E_{0.1}$ buffer to a final volume of 150 ul

For the removal of dust particles, the master mix is filtered by using a centrifugal filter (Ultrafree, PFTE, Millipore, cat. no. UFC30LG25, available from the TCF):

- load the master mix into the filter cartridge
- centrifuge at 12,000*g* for 1 min at RT.
- Pipette the filtrate in aliquots of 30 µl into five clean 1.5-ml tubes, labelled with the TCF project number and date of preparation.

Store the microinjection samples at −80 ℃ and them over to the TCF microinjection service. One tube is used for each day of embryo injection. Microinjection samples can be safely used for embryo injections up to 6 (-8) weeks after RNA production.

▲CRITICAL STEP Avoid any contamination of the microinjection samples with dust particles, which will block injection capillaries, by using 1.5-ml tubes taken from a freshly opened bag. Best use individually packed Eppendorf 1.5 ml `Biopure´ tubes (Cat No.: 0030 121.589).

6. Genotyping of blastocysts derived from microinjected zygotes

6.1 Blastocyst collection

Add 10 µl Quick DNA extraction solution (QuickExtract™ DNA Extraction Solution, QE09050, Epicentre) into each well of a 96 well PCR plate. A single blastocyst is mouth-pipetted into each well.

6.2. DNA Denaturation

PCR plates are briefly centrifuged and proceeded for DNA denaturation

68^0C	15 min
95^0C	15 min

6.3 1st PCR

performed nested PCR reaction. Use Herculase II Fusion DNA Polymerase.

Nested PCR for Rosa26 locus	R26F1	CCAAAGTCGCTCTGAGTTGTTATCAGT
	R26R1	GGAGCGGGAGAAATGGATATGAAG

1st PCR	In 25µl PCR
Denatured DNA	3 (Blastocyst) or 5 µl (Morula)
5x buffer	5
dNTP	0.25
Primer For	0.5
Primer Rev	0.5
DNA polymerase	0.3
H_20	Up to 25

98°C 3min; 35 cycles of [95°C-20s; 60°C-20s; 72°C-20s]; 72°C-3 min

6.4 2nd PCR

Nested PCR for Rosa26 locus	R26F2	GCCTCCTGGCTTCTGAGGACCG
	R26R2	TCTGTGGGAAGTCTTGTCCCTCC

2nd PCR	In 25µl PCR
1st PCR product	2
5x buffer	5
dNTP	0.25
Primer For	0.5
Primer Rev	0.5
DNA polymerase	0.3
H_20	Up to 25

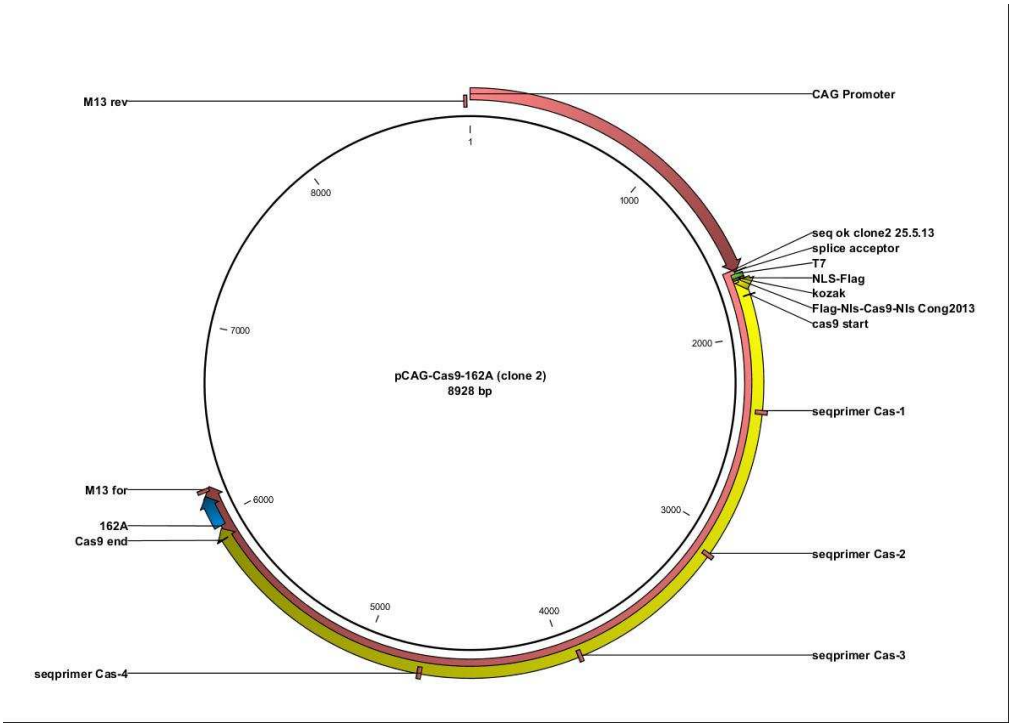
98°C 3min; 35 cycles of [95°C-20s; 60°C-20s; 72°C-20s]; 72°C-3 min

PCR products are purified using PCR extraction kit

Product = **196 bp** (XbaI digested: 115 + 85 bp)

III. Plasmid maps

Plasmid pCAG-Cas9-162A

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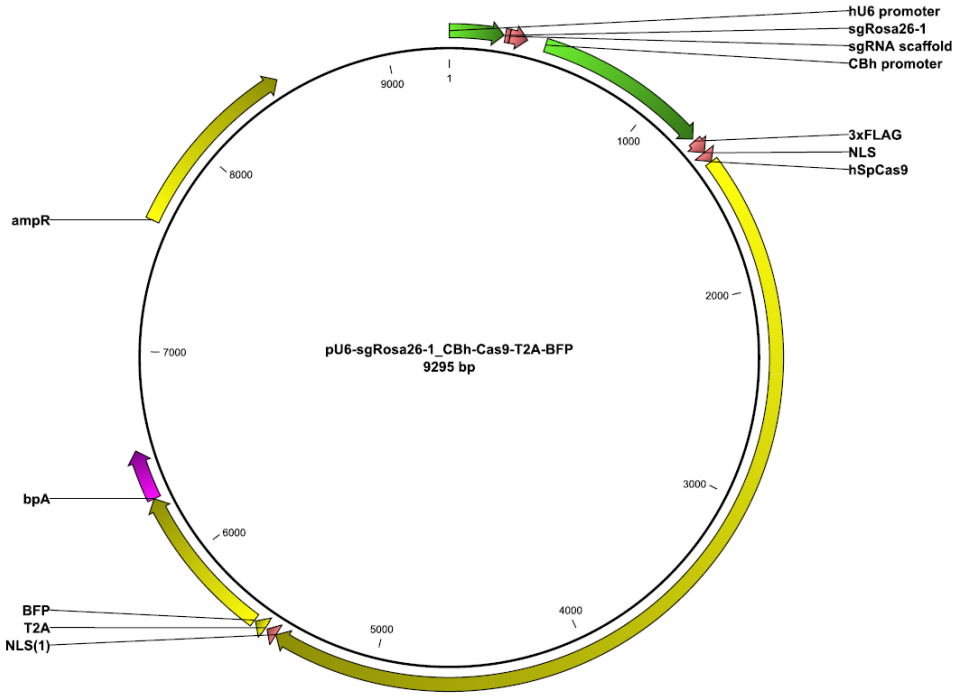


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7441 TTTTTATAGG TTAATGTCAT GATAATAATG GTTTCTTAGA CGTCAGGTGG CACTTTTCGG
7501 GGAAATGTGC GCGGAACCCC TATTTGTTTA TTTTTCTAAA TACATTCAAA TATGTATCCG
7561 CTCATGAGAC AATAACCCTG ATAAATGCTT CAATAATATT GAAAAAGGAA GAGTATGAGT
7621 ATTCAACATT TCCGTGTCGC CCTTATTCCC TTTTTTGCGG CATTTTGCCT TCCTGTTTTT
7681 GCTCACCCAG AAACGCTGGT GAAAGTAAAA GATGCTGAAG ATCAGTTGGG TGCACGAGTG
7741 GGTTACATCG AACTGGATCT CAACAGCGGT AAGATCCTTG AGAGTTTTCG CCCCGAAGAA
7801 CGTTTTCCAA TGATGAGCAC TTTTAAAGTT CTGCTATGTG GCGCGGTATT ATCCCGTATT
7861 GACGCCGGGC AAGAGCAACT CGGTCGCCGC ATACACTATT CTCAGAATGA CTTGGTTGAG
7921 TACTCACCAG TCACAGAAAA GCATCTTACG GATGGCATGA CAGTAAGAGA ATTATGCAGT

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7981 GCTGCCATAA CCATGAGTGA TAACACTGCG GCCAACTTAC TTCTGACAAC GATCGGAGGA
8041 CCGAAGGAGC TAACCGCTTT TTTGCACAAC ATGGGGGATC ATGTAACTCG CCTTGATCGT
8101 TGGGAACCGG AGCTGAATGA AGCCATACCA AACGACGAGC GTGACACCAC GATGCCTGTA
8161 GCAATGGCAA CAACGTTGCG CAAACTATTA ACTGGCGAAC TACTTACTCT AGCTTCCCGG
8221 CAACAATTAA TAGACTGGAT GGAGGCGGAT AAAGTTGCAG GACCACTTCT GCGCTCGGCC
8281 CTTCCGGCTG GCTGGTTTAT TGCTGATAAA TCTGGAGCCG GTGAGCGTGG AAGCCGCGGT
8341 ATCATTGCAG CACTGGGGCC AGATGGTAAG CCCTCCCGTA TCGTAGTTAT CTACACGACG
8401 GGGAGTCAGG CAACTATGGA TGAACGAAAT AGACAGATCG CTGAGATAGG TGCCTCACTG
8461 ATTAAGCATT GGTAACTGTC AGACCAAGTT TACTCATATA TACTTTAGAT TGATTTAAAA
8521 CTTCATTTTT AATTTAAAAG GATCTAGGTG AAGATCCTTT TTGATAATCT CATGACCAAA
8581 ATCCCTTAAC GTGAGTTTTC GTTCCACTGA GCGTCAGACC CCGTAGAAAA GATCAAAGGA
8641 TCTTCTTGAG ATCCTTTTTT TCTGCGCGTA ATCTGCTGCT TGCAAACAAA AAAACCACCG
8701 CTACCAGCGG TGGTTTGTTT GCCGGATCAA GAGCTACCAA CTCTTTTTCC GAAGGTAACT
8761 GGCTTCAGCA GAGCGCAGAT ACCAAATACT GTCCTTCTAG TGTAGCCGTA GTTAGGCCAC
8821 CACTTCAAGA ACTCTGTAGC ACCGCCTACA TACCTCGCTC TGCTAATCCT GTTACCAGTG
8881 GCTGCTGCCA GTGGCGATAA GTCGTGTCTT ACCGGGTTGG ACTCAAGACG ATAGTTACCG
8941 GATAAGGCGC AGCGGTCGGG CTGAACGGGG GGTTCGTGCA CACAGCCCAG CTTGGAGCGA
9001 ACGACCTACA CCGAACTGAG ATACCTACAG CGTGAGCTAT GAGAAAGCGC CACGCTTCCC
9061 GAAGGGAGAA AGGCGGACAG GTATCCGGTA AGCGGCAGGG TCGGAACAGG AGAGCGCACG
9121 AGGGAGCTTC CAGGGGGAAA CGCCTGGTAT CTTTATAGTC CTGTCGGGTT TCGCCACCTC
9181 TGACTTGAGC GTCGATTTTT GTGATGCTCG TCAGGGGGGC GGAGCCTATG GAAAAACGCC
9241 AGCAACGCGG CCTTTTTACG GTTCCTGGCC TTTTGCTGGC CTTTTGCTCA CATGT
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