![A close up of a logo

Description automatically generated]()

**Overview**

A screenshot of a cell phone

Description automatically generated

**Sequences**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Gene/guide | Target sequence  (SpyCas9 PAM=NGG) |  | Gene/guide | Target sequence  (SpyCas9 PAM=NGG) |
|  | cdkn1aEx2-g1 | ACTACTGTACCGCGCGTGTC |  | sox32Ex1-g1 | GGCTTAATGGGCCCGACGCG |
|  | cdkn1aEx2-g2 | GGCGGCGCTACGGCGCGATC |  | sox32Ex1-g2 | GCGTCTTACTCGAGTTTCCA |
|  | foxh1Ex2-g1 | CCCGTGATCACCGTAGGAGA |  | sox9bEx1-g1 | GCGGAGTCCTCGGAAAGACT |
|  | foxh1Ex2-g2 | TGGGCCTTGATTCGTCGCTT |  | sox9bEx1-g2 | GGACAGCGAGACCCCCCGCG |
|  | jak2aEx15-g1 | GTGCAGGAGTACGTGCGCTT |  | taEx6-g1 | CTGCCCAGTAACGGCCCCAT |
|  | jak2aEx15-g2 | GGTGGCTAAGCAGCTAGCAT |  | taEx6-g2 | GGGGCCCCATTGAACTGAGG |
|  | ndr2Ex1-g1 | GAACCAGACGCGCATACCCG |  | tbx5Ex5-g1 | TAAAGCCGTCCGGGCATCGC |
|  | ndr2Ex1-g2 | TAGAGATGCATCATGTACGT |  | tbx5Ex5-g2 | GGATGGACATAAAGCCGTCC |
|  | notch1aEx6-g1 | TCCGTGCGAGCAGGCAGCGC |  | tln1Ex29-g1 | GCGTGGCACCACCCAGGACC |
|  | notch1aEx6-g2 | GGAGGCGACGCGGTCGTGGC |  | tln1Ex29-g2 | GGCCTCGCGTGGCACCACCC |
|  | rbp1bEx2-g1 | TACGACATGGACTTTGTAGT |  | tln1Ex29-g3 | CGCGTGGCACCACCCAGGAC |
|  | rbp1bEx2-g2 | CATGGACTTTGTAGTTGGTC |  | tln1Ex29-g4 | CCGGGAAGTTTGGACAAGAC |
|  | sf3b1Ex7-g1 | TCACTACCTTTGGGACGGCC |  | tln2aEx45-g1 | GCCGTCACTGAGAAGGTACC |
|  | sf3b1Ex7-g2 | GGTCTCATCCCAACGACTGT |  | tln2aEx45-g2 | CGGCTGCATAGTGCTAGTTC |
|  | shhaEx1-g1 | GGGCAAGATAACGCGCAATT |  | tln2aEx45-g3 | ACAGACTCGTTCACCAAGAG |
|  | shhaEx1-g2 | TGTCGCGGAGAAGACCTTAG |  | tln2aEx45-g4 | TCACTGAGAAGGTACCAGGA |
|  |  |  |  | tpo-g1 | GTATTCTTCGTCCGTGGCAC |
|  |  |  |  | tpo-g2 | TGGTCGACTGGAAAGTCCGA |

|  |  |
| --- | --- |
| SpyCas9  tail oligo | AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC |

**PCR/genotyping primers for amplifying genomic target region**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Name | Forward primer | Reverse primer | Amplicon size |
|  | cdkn1aEx2 | acaagccacgcccactgttttc | ACTTCATGCGGGAGTGCAGCAC | 557 bp |
|  | foxh1Ex2 | ACCTGGAGCCAGCAGTCTGAGG | TGTTGGGGTAGAGCCACGCATC | 604 bp |
|  | jak2aEx15 | GAGCCAGCTCTCCCATAAAC | CGGGTCACCAGAACATTCC | 394 bp |
|  | ndr2Ex1 | CTGTCACGGCATGTCAATTTC | CGCCATTGAGCTTTATACACAC | 461 bp |
|  | notch1aEx6 | tgccttcaactgtacatggtactc | TTGAGTGCTTTGCGGACTACGg | 667 bp |
|  | rbp1bEx2 | acAATCGCCACTGGAAATTTGC | TGAAGGGAAAACGTTGGGTGGTG | 319 bp |
|  | sf3b1Ex7 | GGACCCTACGGGACCCATGTTC | TTTCAGCCCAGCCACTGCCATG | 580 bp |
|  | shhaEx1 | ACCCTGCTAGACAGACCGCTCG | GGGTGAGCAAGGAGGCAAGCAG | 473 bp |
|  | sox32Ex1 | TCTCGACCGGATGCTCCCTGAC | TGCTGAGGTCAGTGTTCTCCAGG | 299 bp |
|  | sox9bEx1 | AGCTGATCTGCGCGGTTTTCGG | TGACGTGCGGTTTGCTCTTCCC | 344 bp |
|  | taEx6 | CCCAGACCACAGCACTGACAACC | GCTGCGGTGGGAGTAATGGCTG | 295 bp |
|  | tln1Ex29 | TGCACTGAAGGACTGCATGGCC | gctgttagccgaagacgtctgcg | 567 bp |
|  | tln2aEx45 | AGGGTCGTCTGGCCTCTGCTAC | GTCCAGGTCAGCGATGATGCCG | 493 bp |
|  | tpo | CAGCATGCCCGGAGAATCAGCC | CACAAAAGGCCACGCTTTGCCC | 401 bp |

**Protocols**

**Single guide RNA (sgRNA) synthesis –**

**Step I: Annealing and extension of oligos for Spy Cas9 system:**

|  |  |  |  |
| --- | --- | --- | --- |
| Component |  |  |  |
| Target (gene) specific oligo (100 µM) | 1.5 | µl |  |
| Tail Oligo (100 µM) | 1.5 | µl |  |
| 2x Q5 Master Mix | 3.0 | µl |  |
| Total | 6.0 | µl |  |

Denature at 95 C for 3 minutes 🡪 ramp down to 72 C 🡪 incubate for 5 minutes 🡪 ramp down to 25 C

**Step II: In vitro transcription (IVT) using AmpliScribe T7-Flash kit from Lucigen**

|  |  |  |  |
| --- | --- | --- | --- |
| Extension product (from Step II) | 1.375 | μl |  |
| AmpliScribe T7-Flash 10X Reaction Buffer | 0.500 | μl |  |
| ATP | 0.500 | μl |  |
| GTP | 0.500 | μl |  |
| CTP | 0.500 | μl |  |
| UTP | 0.500 | μl |  |
| 100 mM DTT | 0.500 | μl |  |
| RiboGuard RNase Inhibitor | 0.125 | μl |  |
| AmpliScribe T7-Flash Enzyme Solution | 0.500 | μl |  |
| Total reaction volume | 5.000 | μl |  |
| Incubate at 37 C for 30 minutes to overnight (depending on class schedule) | | | |

**Step III: DNase I treatment (from T7 Ampliscribe-Flash kit)**

|  |  |  |
| --- | --- | --- |
| In vitro transcribed RNA (from Step III) | 5.00 | µl |
| DNaseI | 0.25 | µl |
| Incubate at 37 C for 30 minutes | | |

**Step IV: RNA purification**

* Add 15 µl of dH2O (to make up IVT reaction volume to 20 µl)
* Add 10 µl (1/2 x transcription reaction volume) 5 M ammonium acetate
* 60 µl (3 x transcription reaction volume) 100% ethanol.
* Mix well, incubate for 20 minutes at -80˚C until frozen (dry ice or liquid nitrogen will also work)
* Centrifuge for 15 minutes at 4˚C, maximum speed.
* Remove the supernatant (by decanting) and add 500 µl 70% ethanol.
* Centrifuge for 5 minutes at 4˚C, maximum speed.
* Remove supernatant (by decanting), dry at room temperature until pellet is dry
* Resuspend in 50 µl RNA storage solution + DTT (1 mM Sodium Citrate pH 6.4 + 20 mM DTT)
* Determine RNA concentration (UV Spectrophotometry)

**Step V: 6% Polyacrylamide Gels**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| # of gels | H2O | 10x TBE | 40% acrylamide solution (29:1) | 10% APS | TEMED |
|  | **mL** | **mL** | **mL** | **µL** | **µL** |
| 1 | 4.4 | 0.6 | 0.9 | 100 | 5 |
| 2 | 8.8 | 1.2 | 1.8 | 200 | 10 |
| 3 | 13.2 | 1.8 | 2.7 | 300 | 15 |
| 4 | 17.6 | 2.4 | 3.6 | 400 | 20 |
| 5 | 22.0 | 3.0 | 4.5 | 500 | 25 |
| 6 | 26.4 | 3.6 | 5.4 | 600 | 30 |
| 7 | 30.8 | 4.2 | 6.3 | 700 | 35 |
| 8 | 35.2 | 4.8 | 7.2 | 800 | 40 |
| 9 | 39.6 | 5.4 | 8.1 | 900 | 45 |

Run in 0.5x TBE buffer at 200 V for 25-30 minutes

**Preparation of genomic DNA (embryo lysis)**

Collect > 24 hpf embryo into a PCR tube

Add 20 µl embryo lysis solution (50 mM NaOH + 0.2 mM EDTA)

Incubate at 95 C for 10 minutes

Add 2 µl of 1 M Tris-HCl pH 8.0 to the tube (final concentration: 100 mM Tris-HCl)

Store at 4 C for a week or -20 C for long-term storage

**Genomic PCR**

|  |  |  |  |
| --- | --- | --- | --- |
| Genomic DNA (embryo lysis solution) | : | 0.50 | µl |
| Gene-specific FORWARD primer (10 µM) | : | 0.25 | µl |
| Gene-specific REVERSE primer (10 µM) | : | 0.25 | µl |
| 2x Q5 Polymerase Master Mix  (or other thermostable DNA polymerase) | : | 5.00 | µl\* |
| dH2O | : | 4.00 | µl |
| Total | : | 10.00 | µl |

Analyze by agarose or polyacrylamide gel electrophoresis

**In vitro sgRNA-Cas9 nuclease assay**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| CRISPR target/PCR product | : | 1.0 | µl | From genotyping PCR |
| sgRNA (from Step IV) | : | 0.5 | µl | (250-500 ng/µl) |
| NEBuffer 3.1 | : | 1.0 | µl |  |
| Spy Cas9 (100 ng/µl) | : | 0.5 | µl |  |
| dH2O | : | 7.0 | µl |  |
| Total | : | 10.0 | µl |  |

Incubate at 37 C for 30 minutes

Treat with RNase A (10 mg/ml) to degrade sgRNA (0.25 µl) 🡪 incubate at 37 C for 15 minutes

Treat with Proteinase K (20 mg/ml) to degrade Cas9 protein (0.25 µl) 🡪 incubate at 37 C for 15 mintues

Analyze reaction products by agarose or polyacrylamide gel electrophoresis

**sgRNA-Cas9 ribonucleoprotein (RNP) complex injection into zebrafish embryos**

|  |  |  |  |
| --- | --- | --- | --- |
|  | Stock conc.  ng/µl | Desired final  Concentration  ng/µl | ng/µl |
| Cas9 protein | 1000 | 25-50 |  |
| sgRNA |  | 10-50 |  |
| RNA storage solution |  |  |  |
| **Total volume** |  |  | 10.00 |

Inject 10-30 nL of injection solution into each 1-2 cell stage embryo