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Streamlined Protocol for sgRNA validation (post-design), Popsuj et al. 2024

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Popsuj et al.[1]

[1]Georgia Institute of Technology

Stolfi Lab

Sabrina Hernandez
Georgia Institute of Technology

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We use this protocol and it's working

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Abstract

Compiled here is the wet lab protocol for CRISPR/Cas9 sgRNA validation by NGS, assuming already-designed sgRNAs and primers.

Guidelines

1) Prepare your sgRNA + Cas9 plasmid mix, one for each individual sgRNA plasmid to be tested: 25 ug Ef1a>Cas9; 75 ug U6>sgRNA to be tested; complete to 100 ul with water. (Will be mixed with 200 ul sea water + zygotes and 400 ul 0.96 M D-Mannitol for total electroporation volume of 700 ul.)

2) Follow standard electroporation protocol (Christiaen et al. 2009), raise embryos at room temperature to 16–20 hpf (larva stage).

3) Collect larvae in Eppendorf tube, let settle and remove as much sea water as possible, freeze at -80°C.

4) Extract the gDNA from the electroporated embryos using a QIAamp DNA micro kit (Qiagen cat. 56304):
- Add 180 ul **Buffer ATL** to each tube full of frozen larvae.
- Add 20 ul of Proteinase K (comes with the kit).
- Mix by flicking tube.
- Incubate in 55°C waterbath for 10 minutes to lyse the cells.
- Add 200 ul **Buffer AL** and mix by vortexing tubes for 15 seconds.
- Add 200 ul of 100% ethanol, mix by vortexing, then leave at room temperature for 15 minutes.
- Take individually wrapped QIAamp columns from fridge where they should be stored.
- Spin each sample in a column (label properly to not mix them up) at 6000 g (8000 rpm) for 1 minute.
- Discard flow-through. Wash with 500 ul **Buffer AW1**. Spin 1 minute at 6000 g.
- Discard flow-through. Wash with 500 ul **Buffer AW2**. Spin 1 minute at 6000 g.
- Discard flow-through. Spin empty for 3 minutes at max speed to completely dry the column.
- Place each column in a new labeled Eppendorf tube. Add 20 ul nuclease-free water to the center of the column filter and spin at max speed for 1 minute to elute the gDNA.

5) PCR from genomic DNA for the NGS assay. For every sample, prepare a 50 ul reaction:
- 46 ul AccuPrime Pfx "SUPERMIX" (contains water, buffer, dNTPs, and enzyme)
- 1.5 ul NGS forward primer at 20 uM
- 1.5 ul NGS reverse primer at 20 uM
- 1 ul genomic DNA from above (0.2 ug/ul)

Use program: "GENOMIC":
- 1x: 94°C for 2'

- 5x:
  - 94°C for 30"
  - 60°C for 30"
  - 68°C for 1'

- 5x:
  - 94°C for 30"
  - 58°C for 30"

- 68°C for 1'

- 25x:
  - 94°C for 30"
  - 55°C for 30"
  - 68°C for 1'

- 1x: 68°C for 5'

6) Check 2 ul of each PCR on a gel when done; include DNA ladder. If you see a single band, you may proceed. If you have zero or multiple bands, consider redesigning primers.

7) Purify the remaining ~48 ul of the PCR reaction using the QIAquick PCR purification kit. Label tubes with sgRNA used + primers used to track negative controls, etc. Elute in 50 ul of water unless the band on the gel appeared weak; if weak, elute in 25 ul.

8) Measure concentration of purified PCR reactions. For Azenta's amplicon sequencing service, submit at least 25 ul of purified product at 20 ng/ul.

Materials

- sgRNA + Cas9 plasmid mix (prepare one for each individual sgRNA plasmid to be tested): 25 ug Ef1a>Cas9; 75 ug U6>sgRNA to be tested; complete to 100 ul with water. (This will be mixed with 200 ul of sea water + zygotes and 400 ul of 0.96 M D-Mannitol for a total electroporation volume of 700 ul.)
- Standard electroporation setup (see Christiaen et al. 2009 for details)
- Eppendorf tubes
- QIAamp DNA Micro Kit (Qiagen, cat. 56304)
- **Buffer ATL**
- Proteinase K (comes with the QIAamp kit)
- **Buffer AL**
- 100% ethanol
- QIAamp spin columns (individually wrapped, stored refrigerated)
- **Buffer AW1**
- **Buffer AW2**
- Nuclease-free water
- AccuPrime Pfx "SUPERMIX" (contains water, buffer, dNTPs, and enzyme)
- NGS forward primer (20 uM)
- NGS reverse primer (20 uM)
- Genomic DNA (from electroporated embryos)
- DNA ladder and gel electrophoresis supplies
- QIAquick PCR Purification kit (for purifying PCR product)
- Thermal cycler capable of the specified PCR program
- Microcentrifuge capable of 6000 g (8000 rpm) and higher (for "max speed" spins)
- Instruments/consumables for measuring DNA concentration (e.g., Nanodrop / Qubit)
- Shipping / sequencing provider requirements (e.g., Azenta: minimum 25 ul at 20 ng/ul for amplicon sequencing)

Safety warnings

- Label columns and tubes carefully to avoid mixing samples.
- Spin empty columns at max speed for 3 minutes to completely dry the membrane before elution.
- If gel shows zero or multiple bands after PCR, you may need to redesign primers (do not proceed to sequencing with ambiguous bands).
- Follow kit manufacturers' instructions for handling reagents (e.g., Proteinase K, buffers, ethanol) and standard lab safety practices.

Before start

- Assumes already-designed sgRNAs and primers are available.
- Prepare plasmid mixes (see Materials) so each sgRNA + Cas9 mix is 100 ul before combining with seawater/zygotes and D-mannitol for electroporation.
- Ensure QIAamp columns are stored refrigerated and individually labeled prior to use.

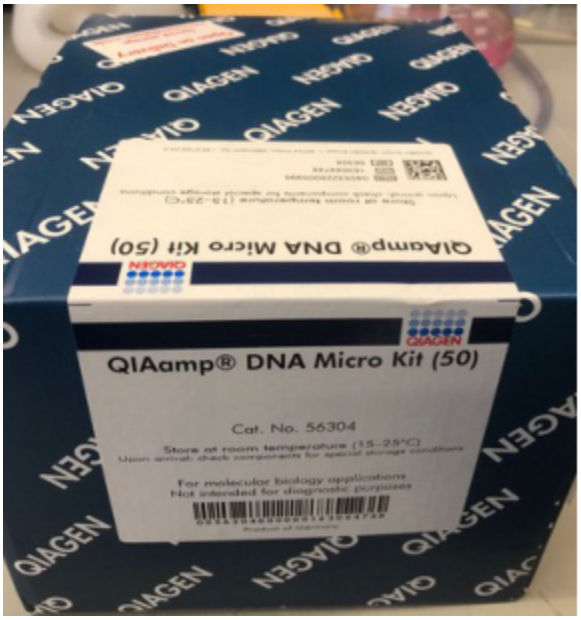
Validating sgRNAs by next-generation sequencing (NGS)

1 Prepare your sgRNA + Cas9 plasmid mix, one for each individual sgRNA plasmid to be tested:
25 ug Ef1a>Cas9
75 ug U6>sgRNA to be tested
Complete to 100 ul with water
(This will be mixed with 200 ul of sea water + zygotes and 400 ul of 0.96 M D-Mannitol for a total electroporation volume of 700 ul)

2 Follow standard electroporation protocol (Christiaen et al. 2009), raise embryos at room temperature to 16–20 hpf (larva st.).

3 Collect larvae in Eppendorf tube, let settle and remove as much sea water as possible, freeze at -80°C.

4 Extract the gDNA from the electroporated embryos using a QIAamp DNA micro kit (qiagen cat. 56304).



4.1 Add 180 ul **Buffer ATL** to each tube full of frozen larvae.

4.2 Add 20 ul of Proteinase K (comes with the kit).

4.3 Mix by flicking tube.

4.4 Leave in 55°C waterbath for 10 minutes to lyse the cells.

4.5 Add 200 ul **Buffer AL** and mix by vortexing tubes for 15 seconds.

4.6 Add 200 ul of 100% ethanol, mix by vortexing, then leave at room temperature for 15 minutes.

4.7 Take individually wrapped QIAamp columns from fridge where they should be stored.

4.8 

4.9 Spin each sample in a column (label properly to not mix them up) at 6000 g (8000 rpm) for 1 minute.

4.10 Discard flow-through. Wash with 500 ul **Buffer AW1**. Spin for 1 minute at 6000 g.

4.11 Discard flow-through. Wash with 500 ul **Buffer AW2**. Spin for 1 minute at 6000 g.

4.12 Discard flow-through. Spin empty for 3 minutes at max speed to completely dry the column.

4.13 Place each column in a new Eppendorf tube, labeled properly. Add 20 ul of nuclease-free water to the center of the column filter and spin at max speed for 1 minute to elute the gDNA; the eluted gDNA is ready for nanodrop measuring.

5 Do the PCR from genomic DNA for the NGS assay. For every sample, prepare a 50 ul reaction:
46 ul AccuPrime Pfx "SUPERMIX" (contains water, buffer, dNTPs, and enzyme)
1.5 ul NGS forward primer at 20 uM

1.5 ul NGS reverse primer at 20 uM
1 ul genomic DNA from above (0.2 ug/ul)
Use program: "GENOMIC":
1x: 94°C for 2'
5x: 94°C for 30"; 60°C for 30"; 68°C for 1'
5x: 94°C for 30"; 58°C for 30"; 68°C for 1'
25x: 94°C for 30"; 55°C for 30"; 68°C for 1'
1x: 68°C for 5'

6 Check 2 ul of each PCR on a gel when done; include a DNA ladder and take an image of the gel. If you see a single band, you may proceed. If you have zero or multiple bands, consider redesigning primers and do not proceed to sequencing.

7 When done, purify the remaining ~48 ul of the PCR reaction using the QIAquick PCR purification kit. Label tubes with sgRNA used + primers used so you know which ones to use as negative controls, etc.
Elute in 50 ul of water unless the band on the gel appeared weak; if weak, elute in 25 ul.

8 Measure the concentration of the purified PCR reactions. For Azenta's amplicon sequencing service, submit at least 25 ul of purified product at 20 ng/ul (or follow your sequencing provider's minimums).

Protocol references

- Christiaen, L., Wagner, E., Shi, W., & Levine, M. (2009). Isolation of sea squirt (Ciona) gametes, fertilization, dechorionation, and development. Cold Spring Harbor Protocols, 2009(12), pdb-prot5344.
- Christiaen, L., Wagner, E., Shi, W., & Levine, M. (2009). Electroporation of transgenic DNAs in the sea squirt Ciona. Cold Spring Harbor Protocols, 2009(12), pdb-prot5345.
- QIAamp DNA Micro Kit (Qiagen, cat. 56304) product information
- AccuPrime Pfx SUPERMIX product information
- QIAquick PCR Purification kit product information

Electroporation references:

Christiaen, L., Wagner, E., Shi, W., & Levine, M. (2009). Isolation of sea squirt (Ciona) gametes, fertilization, dechorionation, and development. Cold Spring Harbor Protocols, 2009(12), pdb-prot5344.

Christiaen, L., Wagner, E., Shi, W., & Levine, M. (2009). Electroporation of transgenic DNAs in the sea squirt Ciona. Cold Spring Harbor Protocols, 2009(12), pdb-prot5345.