**Step by step protocol for gene knock out by CRISPR/Cas9 in *P. pacificus***
(Modified from Witte et al. 2015 Dev Genes Evol 225:55-62)

Material required:

Cas9 protein lyophilized or freshly prepared
sgRNA recognizing the desired target

Protein storage buffer: 20 mM HEPES-KOH pH 7.5, 150 mM KCl, 1 mM DTT

TE: 10 mM Tris-HCL, 1mM EDTA, pH 8.0

Basic set up for *P. pacificus* maintenance

Protocol:

Rehydrate or dilute the Cas9 protein with water to a concentration of 5 µg/µl.

Add one volume of protein storage buffer (resulting in 2.5 µg/µl Cas9 in 0.5 X protein storage buffer) and make 2 µl aliquots. Freeze the aliquots not used immediately.

Rehydrate or dilute the sgRNA with protein storage buffer to a concentration of 3µg/µl.

Add 0.5 µl of sgRNA to a 2-µl Cas9 aliqot.

Incubate at 37°C for 10 minutes.

Dilute 10 times in TE (not protein storage buffer, this appears to be important).

Centrifuge at 4°C for 10 minutes and inject following the protocol for transgene injection (Cinkornpumin and Hong, 2011).

Place the injected (P0) worms individually onto plates.

After 16-18 hours remove the P0 worms (Make sure you have about 10 surviving, fertile injected worms. How many you need to inject in order to reach this depends on your injection skills).

Once the F1 progeny are young adults, pick 20 F1s per P0 individually onto plates.

Let the F1s lay eggs for 24 - 48 hours.

Test the F1s for the presence of mutations around the sgRNA target site using appropriate single worm genotyping protocols based on High Resolution Melting analysis and/or PCR-sequencing **[Cross-Reference to the corresponding protocols]**.

Recover the mutation from the progeny of the F1 worm (F2).