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CRISPR/Cas9 knock-in methodology for the sea urchin embryo

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CRISPR technology has revolutionized the biological research world, making animals heretofore recalcitrant to genetic manipulation, accessible to analysis of specific gene functions. Building upon the demonstration of targeted gene mutations in the sea urchin (CRISPR knock-out) (Fleming et al., 2021; Lin et al., 2019; Lin & Su, 2016; Liu et al., 2019; Vyas et al., 2022), investigators may now be able to insert exogenous DNA into specific locations in the genome (CRISPR knock-in). Such Cas9-mediated knock-ins will reveal sites of gene expression, and function. By judicious selection of exogenously encoded tags *e.g.* a fluorescent reporter, an investigator may then follow specific gene activities and cell lineages throughout development in live embryos. This tag can also be used for protein pull-down without requiring an antibody for the targeted protein. Here we describe a procedure for CRISPR-based knock-in DNA in the sea urchin *Strongylocentrotus purpuratus*.

Sea urchin larvae produce echinochrome pigments that require several gene functions including the enzyme polyketide synthase 1 (PKS) (Barsi et al., 2015; Calestani et al., 2003; Calestani & Wessel, 2018; Perillo et al., 2020; Wessel et al., 2020). *Sp* PKS1 expression is restricted to a small population of ~50 cells of the Veg2 lineage of the animal (Calestani et al., 2003; Barsi et al., 2015). We realized that using PKS1 to evaluate CRISPR knock-in success was highly stringent since the insertion must occur within that small lineage, and be expressed by yet a smaller population of the lineage. Mutations of the gene encoding PKS1 by CRISPR knock-out resulted in albino larvae, an easy phenotype to assess with simple brightfield microscopy (Oulhen & Wessel, 2016a). A single gRNA was previously shown to mutate PKS1 by Cas9 activity, nearly 100% of the time in embryos from *S. purpuratus* and *Hemicentrotus pulcherrimus* (Liu et al., 2019; Oulhen et al., 2022; Oulhen & Wessel, 2016a). We took advantage of this highly efficient gRNA to test and to optimize Cas9-mediated methodology in the sea urchin *Strongylocentrotus purpuratus*.

We tested three different donor templates for their efficacy in selectively knocking-in exogenous DNA encoding a fluorescent protein: plasmid DNA, linear double stranded DNA,

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single stranded DNA. The key for this test is a highly efficient gRNA against the target gene, and a DNA repair template that contains homologous regions to the target sequence for homology directed repair (SFigure.1).

Investigators have previously injected linear DNA into sea urchin eggs/early embryos, which results in rapid and extensive concatenation (McMahon et al., 1985) that appears to be detrimental to high-fidelity insertion (data not shown). To counter this concern, we tested circular plasmid – based strategies. Here the DNA repair template targeting the cleaved genomic locus was contained within a plasmid and was accessible for insertion before or following CRISPR-Cas9 cutting of the same flanking sequence in the plasmid as in the targeted genomic locus. This strategy resulted in GFP insertions into PKS1, but was inconsistent for reasons not yet clear (SFigures 2 and 3).

We obtained the best results using a different strategy based on a double strand PCR product as the DNA repair template whose termini were blocked from concatenation (Gutierrez-Triana et al., 2018; Kimura et al., 2014; Paix et al., 2017; Paix et al., 2019; Seleit et al., 2021; Winkler et al., 1991). We provide a detailed protocol in the Supplemental document explaining how to insert a Neon fluorescent tag in the gene *Sp* PKS1 using this strategy. This approach relies on a double-stranded DNA donor (single stranded DNA did not yield insertions, SFigure 1) containing short homology arms from the *Sp* PKS1 gene, flanking a fluorescent protein reporter sequence (Figures 1 and 2). The fluorescent protein Neon was selected for this task because of its intense fluorescence properties (Addgene 98877). Forward and reverse primers were designed that contain 30 to 40 bp of the targeted gene (longer homologous arms e.g. 200 bp were not successful), followed by 18 to 21 bp of the fluorescent protein sequence for annealing with the plasmid template. The 5’ end of the forward and reverse primers were biotinylated to prevent concatenation of the DNA once it had been amplified and injected into the embryo. The first five bases of the primers were also modified (phosphorothioate) to reduce exo-DNA degradation (Figure. 2). Development of the method presented here was guided by a similarly successful protocol for use in the medaka (Seleit et al., 2021). This same method has been used successfully in the teleost Medaka and the sea anemone, *Nematostella*. The percentage of success depends on the gene targeted. For example, in Medaka, 11% (for mapre1b) to 59% (for g3bp1) of embryos expressed the fluorescent reporter (Seleit et al., 2021). In *Nematostella* a recent study reported that between 2.2% (for lamin) and 37.7% (for cdh1) of injected embryos were successfully fluorescent (Paix et al., 2023).

We obtained 2% of successful and consistent CRISPR PKS1 knock-in larvae presenting fluorescent pigment cells. All of these successful knock-in larvae were mosaic (not every cell of these larvae contained the insert). A low frequency of visual neon insertion actually underestimates the efficiency of the method since PKS1 is expressed in a small population of cells. By genotyping the knock-in locus using PCR and sequencing of the resulting amplicon, we found actually that 10% of the injected larvae contained the correct insert (SFigures 4,5 and 6). The resulting fluorescent larvae were albino, indicating that the Neon insertion into the ketosynthase domain of PKS1, resulted in a nonfunctional PKS1 enzyme not based on sequence but based on structure (Li et al., 2022). Moving forward, we will test other domains in the large PKS1 protein for their essential functionality, and to

broaden the utility of the procedure by testing the targeting of ubiquitously expressed genes. We will also test smaller, non-fluorescent tags that may enhance efficiency of insertion. Additional protocol variations will be tested in the future to attempt to achieve a higher knock-in efficiency. It is possible that homologous recombination is not highly efficient in the sea urchin. Some protocols rely instead of non-homologous end joining (NHEJ) (He et al., 2016). In zebrafish, due to the inefficiency of homologous recombination and the error-prone nature of the integrations in this animal, researchers have developed a creative approach to insert their PCR donors by taking advantages of the non-coding regions of the targeted genes (Levic et al., 2021).

CRISPR/Cas9 knock-in will enable researchers to follow the expression of their favorite genes in live cells and embryos, even over multiple generations (Vyas et al., 2022) if the insertion is transmitted to the germline. Fundamental processes such as the biology of the pigment cells (using *Sp* PKS1 gene) and the biology of the germ cells (using *Sp* Nanos2 (Oulhen & Wessel, 2016b)), can now be explored in live embryos with this method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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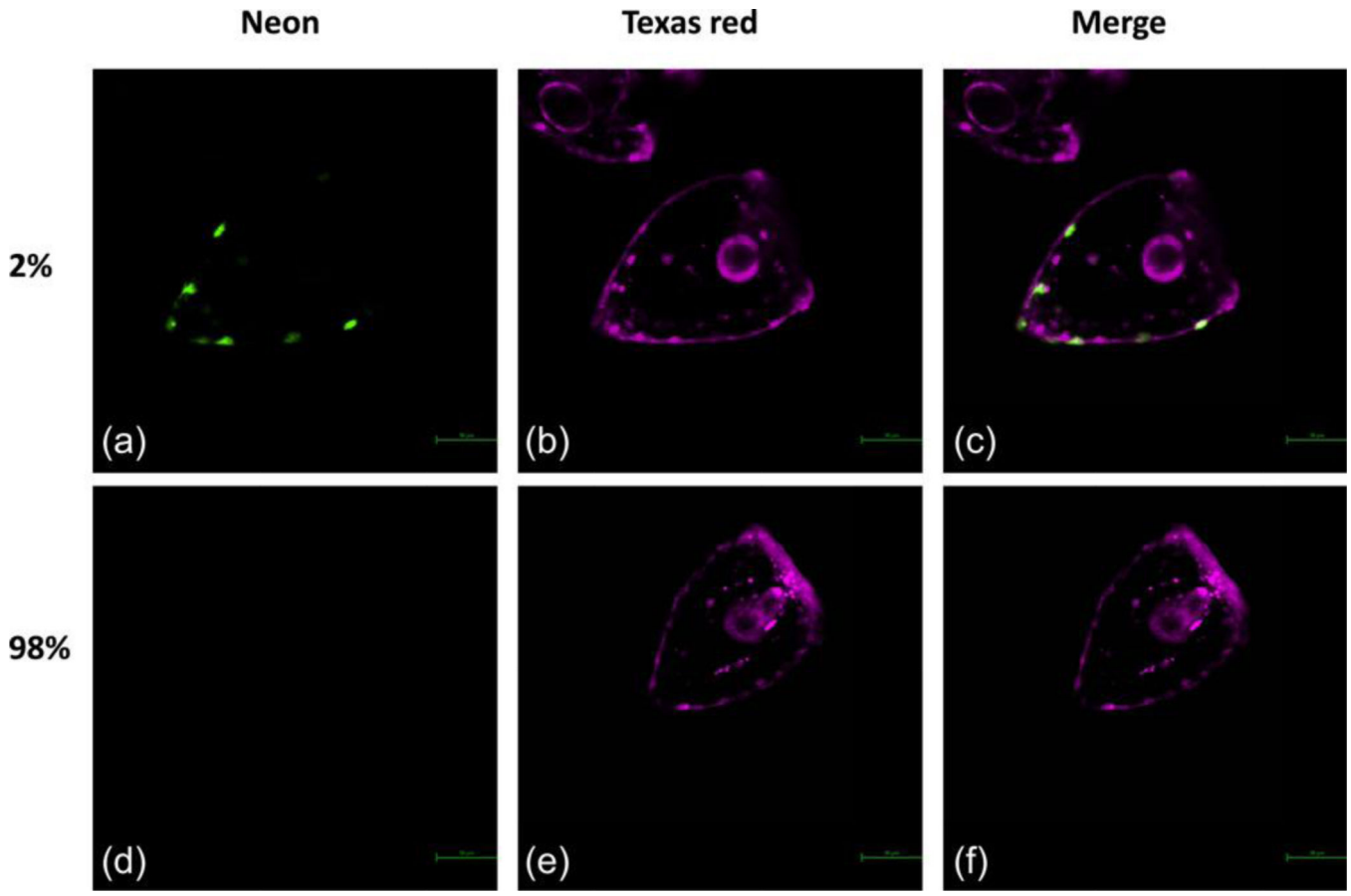

Figure 1: Sp PKS1 Crispr knock-in.

Two percent of embryos express *Sp* PKS1 Neon in their pigment cells (A). Ninety-eight percent of embryos didn't show any detectable expression of *Sp* PKS1 Neon (D). The Texas red dye was co-injected in the zygotes with the knock-in components. This fluorescent dye is used to visualize and sort embryos after injections.

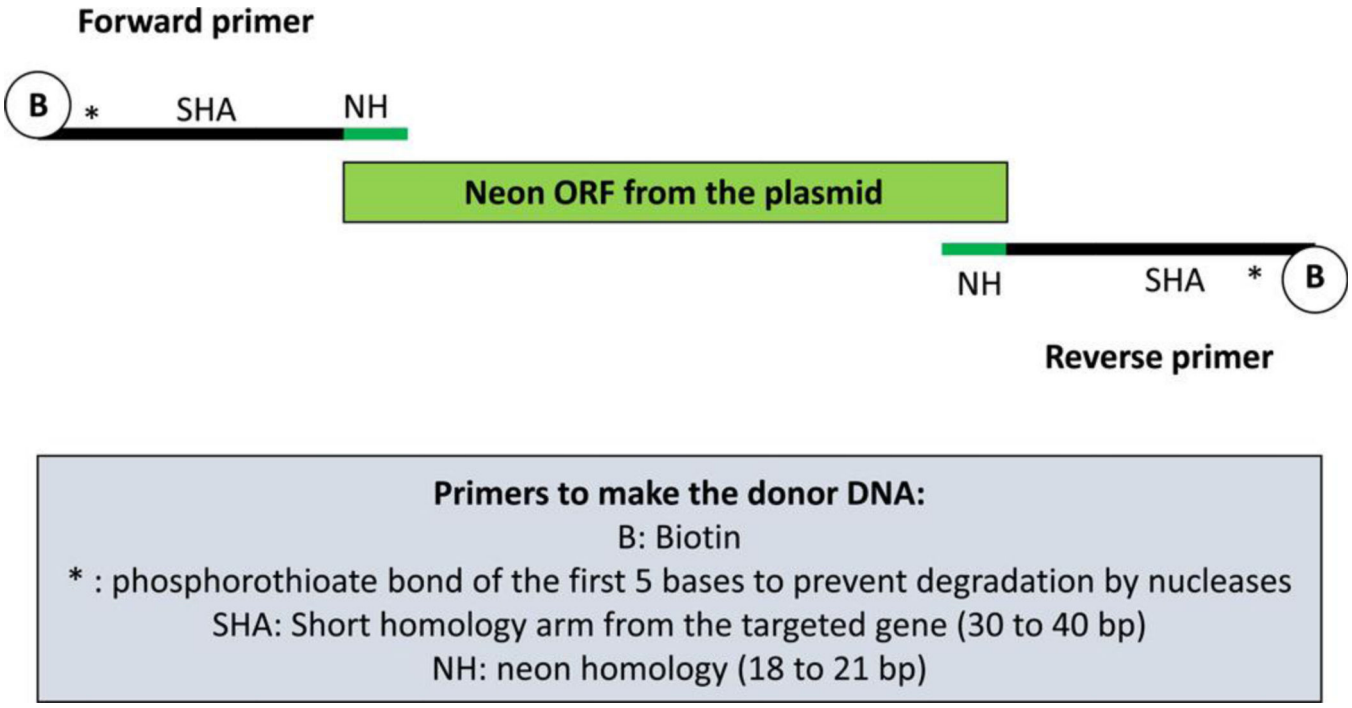

Figure 2:
Description of the method used to design the primers for successful CRISPR/Cas9 knock-in (double stranded DNA, 40 bp of homologous arms from the *Sp* PKS1 gene).