

Step 1. Pre-reporter insertion with ssODN repair template

Component	Initial concentration		Final concentration		Volume
	μM	$\text{ng}/\mu\text{L}$	μM	$\text{ng}/\mu\text{L}$	μl
Cas9 (IDT)	61	10000	1.53	250.00	0.25
ALT-R tracrRNA	320	7098.34	6.40	141.97	0.20
ALT-R <i>dpy-10</i> crRNA	50	575.50	1.25	14.39	0.25
ALT-R <i>target gene</i> crRNA	100	~1180	5.00	~59.00	0.50
ssODN <i>dpy-10(cn64)</i> repair template	32.7	1000	0.92	28.00	0.28
ssODN FP 1-3	100	~5300	2.20	~116.60	0.22
Nuclease-free H_2O					8.30
Total volume					10.00

Step 2. FP insertion with PCR products

Component	Initial concentration		Final concentration		Volume
	μM	$\text{ng}/\mu\text{L}$	μM	$\text{ng}/\mu\text{L}$	μl
Cas9 (IDT)	61	10000	1.53	250.00	0.25
ALT-R tracrRNA	320	7098.34	10.25	227.15	0.32
ALT-R <i>dpy-10</i> crRNA	100	1150.99	2.00	23.02	0.20
ALT-R FP 1-3 crRNA	100	~1180	8.00	~94.40	0.80
ssODN <i>dpy-10(cn64)</i> repair template	32.7	1000	0.92	28.00	0.28
FP PCR product	~1.90	1000	1.10	570.00	5.70 ^a
Nuclease-free H_2O					2.45
Total volume					10.00

One-shot FP insertion

Component	Initial concentration		Final concentration		Volume
	μM	$\text{ng}/\mu\text{L}$	μM	$\text{ng}/\mu\text{L}$	μl
Cas9 (IDT)	61	10000	1.53	250.00	0.25
ALT-R tracrRNA	320	7098.34	16.00	354.92	0.50
ALT-R <i>dpy-10</i> crRNA	100	1150.99	2.00	23.02	0.20
ALT-R <i>target gene</i> crRNA	100	~1180	5.00	~59.00	0.50
ALT-R FP 1-3 crRNA	100	~1180	5.00	~59.00	0.50
ssODN FP 1-3	100	~5300	4.00	~212.00	0.40
ssODN <i>dpy-10(cn64)</i> repair template	32.7	1000	0.98	30.00	0.30
FP PCR product	~1.90	1000	1.10	570.00	5.70 ^a
Nuclease-free H_2O					1.65
Total volume					10.00

^a adjust the volume depending on the molecular weight of the PCR product to reach a final concentration of 1.10 μM .

Preparation of injection mix:

1. Pipette the required volume of nuclease-free H₂O.
2. To the nuclease-free H₂O, add the listed reagents in the following order: target gene crRNA, *dpy-10* crRNA, tracrRNA, and Cas9.
3. Incubate at 37° for 10 minutes.
4. Add ssODNs and/or PCR product repair templates.
5. Centrifuge at 13,200 rpm for 2 minutes.
6. Keep the mix over ice prior to loading capillary needles.
7. Excess mix can be stored at -20° and reused.

Figure S3. Standard composition of injection mixes for Nested CRISPR