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FLInt 2.0 integration in *C. elegans (updated)* V.2

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Protocol status: Working
We use this protocol and it's working

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Abstract

This protocol is modified from the original FLInt method (Malaiwong et al., 2023) to integrate multi-copy transgenic array in *C. elegans* chromosome. This new version of FLInt promotes the highly-selected features of transgenic worms to distinguish the true integrants from the false positives (Malaiwong et al., 2026). The protocol relies on a single designated CRISPR–Cas9 cutting site within the H2B region.

Materials

Worm strains for FLInt integration

	A	B	C	D
	Strains	landing locus	Genotypes	Available from
	EG7837	Chr I	*unc-119(ed3) III; oxTi712 [eft-3p::tdTomato::H2B::unc-54 3'UTR + Cbr-unc-119(+)] I*	CGC
	EG7866	Chr II	*unc-119(ed3) III; oxTi564 [eft-3p::tdTomato::H2B::unc-54 3'UTR + Cbr-unc-119(+)] II*	CGC
	EG7898	Chr III	*unc-119(ed3) III; oxTi619 [eft-3p::tdTomato::H2B::unc-54 3'UTR + Cbr-unc-119(+)] III*	CGC
	EG7917	Chr IV	*unc-119(ed3) III; oxTi616 [eft-3p::tdTomato::H2B::unc-54 3'UTR + Cbr-unc-119(+)] IV*	CGC
	EG7944	Chr V	*unc-119(ed3) III; oxTi553 [eft-3p::tdTomato::H2B::unc-54 3'UTR + Cbr-unc-119(+)] V*	CGC
	EG7826	Chr X	*unc-119(ed3) III; oxTi308 [eft-3p::tdTomato::H2B::unc-54 3'UTR + Cbr-unc-119(+)] X*	CGC

FLInt 2.0 mix preparation

1 Prepare the FLInt 2.0 ribonucleoprotein (RNP) (10µL)

1. 1µL of 100µM crRNA(FLInt2.0) (IDT) >>> final concentration of crRNA = 10µM
2. 0.5µL of 200µM tracrRNA (IDT) >>> final concentration of tracrRNA = 10µM
3. 1µL of Cas9 endonuclease (IDT) >>> final concentration of Cas9 = 6.2µM
4. 7.5µL of nuclease-free water

crRNA sequence for FLInt 2.0 (H2B site): (5′-ATGCTAACTAGTTTACTTGC-3′)

NOTE: The concentration of the crRNA could be adjusted for better efficiency

2 Incubate at 37ºC for 30 minutes

3 Aliquot 2µL of FLInt mix into PCR tube and store at -20ºC for the further use

NOTE: The volume of the FLInt mix can be higher than 2µL for better efficiency

Injection mix preparation

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1. Prepare the injection mix according to the standard protocol (Plasmid of interest: x ng/µL, Co-injection marker: y ng/µL, 1kb Plus DNA ladder (Invitrogen), as needed to bring the total DNA concentration to 100ng/µL)
2. Add nuclease-free water to bring the volume to 8µL
3. Add 2µL of the FLInt2.0 RNP mix to reach a final volume of 10µL
4. Centrifuge the mixture at 12,000rpm for 5–10 minutes before injection

NOTE: The volume of the FLInt mix can be higher than 2µL for better efficiency

P0 worm preparation

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1. Culture tdTomato-expressing worms (see ‘Materials’) at 16°C or 20°C to obtain healthy young adults
2. Select well-fed, young adult worms using a paintbrush under a dissecting microscope
3. Mount the worms onto 2% dried agarose pad on coverslip

Microinjection

6 1. Inject the mix into single or both gonads of P0 worms
2. After injection, transfer 10–15 P0 worms to a 5-cm NGM plate seeded with OP50, incubate
the plates at 25°C

!!! Good injection is the most critical step to obtain the integrants.
Even though the selection method helps eliminate the false positives from the actual candidates, the good injection increases the chance of the integrated events

Hygromycin selection

7 **(In case of hygR gene is applied)**

Three days post-injection, apply 500µL of 5 mg/mL hygromycin B (Thermo Fisher Scientific) to each plate, allow the plates to dry on the benchtop for approximately 30 minutes, then return them to the 25°C incubator

Selection of candidate worms

8 1. On day 6 or 7 post-injection, identify healthy, non-tdTomato, co-injection marker-positive adult worms
2. Transfer these candidate worms to individual small NGM/OP50 plates and culture them at 25°C

!!! Without hygromycin selection, worm chucking might be required to maintain the growing population

!!! Some worms might carry dimmed red signals. Please make sure you pick the complete dark worms

!!! The successful integration yields a good number of dark worms (20-40 candidates). A few candidates on the plate might not be the actual integrants.

Screen for integrated lines

9 On day 9 post-injection, identify plates with 100% co-injection marker expression in the progeny. These lines are potential integrants.

!!! Some plates might produce Unc worms. This phenotype tended to be non-integrated

Verification of transgene integration

10 Lines with uniform co-injection marker expression are considered candidate integrated lines. Confirm expression of the gene of interest via fluorescence or other relevant assays. If gene expression is not visually detectable, perform genotyping via PCR

Designation of the integrated lines

11 Integrated lines derived from the same P0 plate are considered identical due to the inability to trace individual lineages. However, separating P0 worms after injection enables the isolation of multiple distinct integrated lines.

Alternative procedures

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1. Hygromycin selection can be performed at any time after day 3 post-injection or during subsequent generations following the F1. Selection beyond day 3 is also feasible, as integrants can still remain within tdTomato-expressing populations. Researchers may choose an alternative time for hygromycin treatment based on the suitable time schedule. However, food supplementation might be required for animals recovering after drug selection.
2. OP50 seeding can be adjusted based on the timing of screening and the expected number of worms. Since P0 worms produce both F1 and F2 progenies on the same plate—and homozygous integrants typically emerge in the F2 generation—it is essential to seed concentrated OP50 prior to the experiment to ensure a sufficient food source through F2 development. Additionally, 10-cm Petri dishes can be used to accommodate larger worm populations when necessary.
3. Separating P0 worms onto individual plates after injection helps in accurately identifying integrated lines. Since multi-copy integrants on the same plate may originate from different P0 worms and thus represent distinct insertion sequences, tracking progeny may be crucial. Without such tracking, only one integrated line per plate should be registered to avoid confusion. Researchers should carefully consider whether obtaining multiple integrated lines from the same experiment is necessary based on their experimental goals.
4. Culturing injected P0 worms at 25 °C is not essential, as incubation at 20 °C may promote better fitness in tdTomato-expressing strains. However, previous reports suggest that higher temperatures enhance integration efficiency (Frøkjær-Jensen et al. 2014) , increasing the chance of obtaining integrants from each injection round. By using the FLInt2.0 method, a small number of integrants can be easily identified on each plate following drug selection.
5. Based on Mello et al., 1991, adding single-stranded oligos might help integration efficiency. However, the role of oligos in integration process is still unclear.

Observations from the developer

13 ***1. Co-injection of multiple plasmids typically requires a DNA ladder to promote extrachromosomal array formation and complexity; however, large insertions of ladder-derived DNA can lead to non-array integration events, increasing false positives.***

2. Reducing stuffer DNA (ladder) potentially helps promoting better selection. Combining the hygR cassette directly into the target plasmid and injecting without a DNA ladder minimizes false positives and facilitates identification of true integrants, as lower total DNA concentrations fail to generate heritable extrachromosomal arrays. However, this strategy is recommended for single-plasmid integration.

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