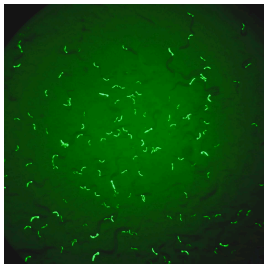
Jun 24, 2025 Version 1

FLInt 2.0 integration in *C. elegans* V.1



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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.

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: (5′-CTCCTCCGAGGACAACAACA-3′)

2 Incubate at 37ºC for 30 minutes

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

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

P0 worm preparation

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1. Culture tdTomato-expressing worms (see ‘Materials’) at 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

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1. Inject the mix into 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

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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

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 4 post-injection or during subsequent generations following the F1. The typical time on day 4 is chosen because F1 animals carrying extrachromosomal arrays and expressing tdTomato tend to be weaker at 25 ° C and require additional time to grow and develop resistance to hygromycin. Selection beyond day 4 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.

Observations from the developer

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1. ***FLInt 2.0 generates the small cutting site on tdTomato gene. It promotes small-sized array insertion.***
2. ***Co-injection with 2 plasmids mostly ends up with only one plasmid integrated. One plasmid integrated is recommended. In case of multiple-plasmids insertion, original FLInt method is recommended.***
3. ***Based on no.2, hygR plasmid might compete with your transgene plasmid even though it dramatically reduces the screening time. Having hygR gene constructed***

in the same plasmid is highly recommended.

Acknowledgements

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