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Transgenic Zebrafish Using Transposable Elements

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Abstract

DNA transposons are effective chromosomal engineering vehicles for making transgenic zebrafish. We describe both autonomous and non-autonomous transposable elements, and we compare and contrast popular transposon systems. The *Tol2* system is a robust gene transfer tool and has been selected as the primary transposon platform, facilitating the development of an array of reagents readily shared within the zebrafish community. We present common transposon and transposase vectors within the field based on the *Tol2* system. We describe methods with a high success rate of generating transgenic zebrafish using *Tol2* vectors, including key quality control steps during the transgenesis process. Together, this data should enable the ready generation of transgenic zebrafish for scientific inquiry.

I. Introduction

Stuart and colleagues reported the first germline transgenic zebrafish (*Danio rerio*) over twenty years ago (Stuart et al., 1988). Since then, increases in the efficiency of zebrafish genome engineering have dramatically amplified both the use and functionality of this vertebrate model organism. Beyond the revolution of fluorescent proteins, arguably no tool has aided zebrafish genome engineering more than active DNA transposons. Here we discuss how transposons work in general, the application of transposon systems to zebrafish, and share a method for routine transgenesis using the *Tol2* transposon system (Koga et al., 1996; Kawakami and Shima, 1999).

DNA or “cut and paste” transposons make up a significant portion of many genomes. When active, these “wild” transposons exist as both “autonomous” and “non-autonomous” versions. The autonomous version (Fig. 1A) encodes a complete and functional transposase that is capable of identifying, excising, and reinserting the DNA element defined by its inverted terminal repeats (ITR) or other elements with the same ITRs. Non-autonomous elements are DNA transposons that can still be moved by a functional transposase but are no longer able to produce their own transposase protein due to mutations or deletions within the coding region of the transposase (Fig. 1B). These elements are reliant on autonomous elements for mobilization. The ability of the transposon to work on non-autonomous elements easily permits the separation of transposase activity from the mobile element. Without this step, mobilization of the element could continue uncontrolled. This necessary step in the adaptation of wild transposons to “domesticated” genetic tools occurred rapidly once their mechanism was understood - the transposase coding sequence is simply replaced with an expression cassette of a gene of interest (Fig. 1C). As individual transposon elements were further understood, it was recognized that the inverted repeats could be modified to eliminate all unneeded sequences that might unknowingly affect transcription, splicing, polyadenylation, etc. Thus, current transposon tools are short– improving

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subcloning and transposition efficiencies (Fig 1D). The transposase can be supplied on a separate DNA expression cassette in *trans* (Fig 1E) or outside of the transposon cassette in *cis* (not shown). However, in zebrafish the most common way to supply the transposase is by co-injection of mRNA encoding the transposase (Fig. 1F). This insures that the transposase is only available transiently and that the transposon integrations will be stable following natural mRNA degradation.

The first DNA transposon widely adapted for genetic engineering was the P element in *Drosophila* (Rubin and Spradling, 1982; Spradling and Rubin, 1982). However, it would be years before a transposon system with significant activity in vertebrate cells was identified. In 1996, the first evidence of an active DNA transposon in the genome of the Medaka fish was reported (Koga et al., 1996). However, before the active *Tol2* transposase was characterized, another vertebrate DNA transposon, *Sleeping Beauty (SB)*, was reverse engineered from related, ancient, inactive transposons found in several fish species (Ivics et al., 1997). In a series of deliberate steps, the *Tol2* element was isolated from a mutational insertion in the Medaka *tyrosinase* locus and shown to be an autonomous native mobile element (Koga et al., 1996; Kawakami et al., 1998). This work permitted the identification of functional *Tol2* mRNA, leading to germline transgenesis of zebrafish following injection of a non-autonomous element and *Tol2* mRNA into zebrafish embryos (Kawakami and Shima, 1999; Kawakami et al., 2000). Similarly, development of the *SB* transposon system continued with demonstration of remobilization in zebrafish cells followed by germline transgenesis (Izsvák et al., 2000; Davidson et al., 2003). Since the initial documentation of *SB* and *Tol2* transposon activity in zebrafish, many subtle changes in design and use have been implemented. Significant efforts to engineer *SB* for improved function have led to improvement of the terminal repeats (Cui et al., 2002) and successive generations of "hyperactive" transposases (Zayed et al., 2004; Mátés et al., 2009; Geurts et al., 2003; Yant et al., 2004; Baus et al., 2005). Despite these advances in function, the *SB* transposon system failed to make a widespread splash within the zebrafish research community. Direct comparisons between *Tol2* and *SB* in zebrafish demonstrated that transposition of *Tol2* occurred more rapidly and with substantially higher overall activity in somatic tissues (Balciunas et al., 2006). The latter activity permitted efficient analysis of enhancer function in F0 (injected embryos) for the first time (Fisher et al., 2006). In addition, and unlike *SB*, *Tol2* transposition efficiency does not significantly drop with transposon size (up to 10kb) nor is it as sensitive to overexpression inhibition (Balciunas et al., 2006). Although other transposons have been subsequently used for germline transgenesis of zebrafish (Koga et al., 2008; Emelyanov et al., 2006), the best-characterized observations derive from the *Tol2* system. Collectively, these realizations have made *Tol2* the primary choice for generating transgenic zebrafish.

Since the initial characterization of *Tol2*, independent cloning of the *Tol2* element from Medaka (Parinov et al., 2004) and the subsequent production of several cloning vectors has facilitated the use of this element in zebrafish. Some have significantly reduced the length of the inverted terminal repeats, making expression vectors smaller and easier to handle while simultaneously removing potential undefined regulatory elements such as promoter, splicing, or polyadenylation signals within the terminal repeats (Balciunas et al., 2006; Urasaki et al., 2006). The availability of multiple sources of cloning vectors, including mini inverted repeat transposons and Tol2 transposase transcription vectors (Fig 1D,E), facilitated creation of the *Tol2* toolbox for zebrafish. Additional collections of *Tol2* transposon vectors made use of Gateway cloning vectors (Kwan et al., 2007; Villefranc et al., 2007). In particular, the distribution of the convenient "Tol2-kit" established a transformational series of versatile vectors for use by the scientific community (Kwan et al., 2007). The combination of multiple useful plasmids, the completeness of the kit for combining fluorescence reporters for promoter and fusion protein production, and an online 'user

community' (http://tol2kit.blogspot.com) helped to make the Tol2 transposon system a routine genetic engineering tool that is used widely by the zebrafish community.

Methods: State of the Art *Tol2* Transposon Tools for Zebrafish Transgenesis Figure 2 presents an overview of the basic process for production of a transgenic zebrafish using Tol2 transposase.

II. Requesting and assembling Tol2 system

A. Tol2 transposon DNA

The first step of the process involves cloning the desired expression cassette between Tol2 inverted terminal repeats (ITR). Many cloning vectors are available within the research community. Figure 3 documents some of the basic Tol2 cloning vectors and their sources. pTol2Dest2pA (Figure 3A) and pTol2Dest2pA2 (Figure 3B) are two minimal Gateway Cloning® multisite destination vectors that are compatible with many available entry vectors as well as other destination vectors available from the Lawson and Chien (Tol2kit) labs, respectively (Kwan et al., 2007; Villefranc et al., 2007). Both of these Gateway Destination vectors use ITRs that are shorter than the original Tol2 transposon vectors and are very similar, with the exception that the A2 version has removed about 2 kilobases of exogenous DNA from the *O. latipes tyrosinase* gene (the locus in which *Tol2* was original identified). Three conventional cloning vectors, pT2AL200R150G (Figure 3C), pminiTol2 (Figure 3D), and pKTol2-SE (Figure 3E) are available from the Kawakami, Ekker, and Clark labs, respectively (Urasaki et al., 2006; Balciunas et al., 2006). Each of these uses minimized ITRs, which help decrease the size of the transposon and plasmid. These *Tol2* cloning vectors have been used to produce transgenic zebrafish with good efficiency. The choice will come down to practicality and ease in generating the desired molecular biology cassette for use in zebrafish transgenesis experiments.

A key component of many zebrafish transgenesis vectors is the inclusion of a dominant reporter cassette for quality control during the transgenesis process and for downstream work with these lines. The role of the reporter is to provide an easy method of subsequent genotyping. Verification that transposition is occurring in the injected embryos is vital to successful transgenic fish production. This quality control check can be done quite easily when the expression cassette contains a dominant marker, like a fluorescent protein. If the primary transgene cassette will not produce a dominant marker directly in zebrafish larvae (i.e., there is no dominant marker, the marker is targeted for adult expression, or the marker is inducible), it is often beneficial to include a small selectable cassette, such as the gamma-crystalin (γ-cryst) promoter or the cardiac myosin light chain (cmlc) driving a fluorescent protein in the lens or heart, respectively (Huang et al., 2003; Davidson et al., 2003). Not only will these expression cassettes help in the production of the desired transgenic fish line, but they aid in husbandry by allowing easy selection of transgenic carriers as larvae.

B. Tol2 mRNA

Three widely available transcription vectors for making Tol2 transposase-encoding mRNA are shown in Figure 4: pCS-TP (Kawakami et al., 2004) (Figure 4A), pCS2-transposase (Kwan et al., 2007) (Figure 4B), and pT3TS-Tol2 (Balciunas et al., 2006) (Figure 4C). All three encode identical Tol2 transposase open reading frames. Both CS vectors, pCS2-TP and pCS-transposase, use the SP6 polymerase, share common linearization sites, and can potentially be used as an expression cassette with a CMV promoter and a functional SV40 poly(A) signal. However, the endogenous Tol2 UTRs (both 5' and 3') have been removed from pCS-transposase (Figure 4B). The pT3TS-Tol2 vector differs from the CS vectors by using the T3 polymerase and incorporating UTRs from the *Xenopus* beta globin gene,

sequences that have been shown to increase mRNA stability compared to CS-based vectors (Hyatt and Ekker, 1999). Ideally, synthetic, 5'-guanosine-capped mRNA should be produced using a kit such as Ambion's mMessage Machine® . *Tol2* transposition is robust and has a wide tolerance of transposon to transposase ratio. *Tol2* mRNA fidelity should be checked on an agarose gel (see Appendix C in Qiagen's mRNeasy kit for a convenient protocol, http://www.qiagen.com/hb/rneasymini), and care should be taken to avoid contamination with RNases. For these reasons, it is advisable to aliquot *Tol2* mRNA into single-use aliquots (for example 1μL at 200 ng/μL, stored at −80°C).

C. Positive control transposon

To assist in validating a newly constructed *Tol2* transposon, a functionally verified, positive-control transposon driving a fluorescent reporter can be requested from any of the sources of *Tol2* cloning vectors.

III. Microinjection

A. Injection setup

An example set-up for microinjection in zebrafish is fully described (Bill et al., 2009). Key components are the ability to visualize the procedure through the dissecting microscope, the micromanipulator control of the injection needle, and a volumetric regulator of the transposon DNA/transposase RNA solution during the injection process.

B. Final preparation of reagents

1. Transposon DNA: transposase mRNA injection mix
    a. On the morning of the injection, mix *Tol2* transposon DNA with an aliquot of *Tol2* mRNA. A recommended concentration is 12.5 ng/μL of both DNA and mRNA, diluted with RNase-free water as required. (It is important to use RNase-free reagents when isolating transposon plasmids initially and in the preparation of the DNA:RNA injection mix.)
    b. The DNA:RNA injection mix is complete and should be handled with gloves and kept on ice for the duration of the injection session.
2. DNA:H_2O control injection mix

    A second injection mix is prepared that lacks *Tol2* mRNA (DNA:H_2O) to serve as an injection control to permit visualization of transposase activity (see quality control below). Prepare a DNA only solution using the same concentration of DNA used in the DNA:RNA injection mix (12.5 ng/μL).

C. Injecting

1. Calibrate the injection volume (such as described inBill et al. (2009) or other suitable method) to inject 1–2 nL of DNA:RNA injection mix. Separately, calibrate and inject DNA:H_2O injection mix into different sibling embryos (keep separate for comparison).
2. Enhanced rates of transgenesis are seen in embryos injected at the one cell stage. Higher volumes are tolerated in the yolk; however, transgenesis injections should be targeted to the cell/yolk interface or directly into the cell body for the highest efficiencies.
3. Injected embryos are transferred to Petri dishes and stored between 28–30°C.
4. At the end of injection day, remove any dead or unhealthy embryos.

IV. Quality control

The initial quality control step comes from expression of the marker that has been introduced into the transposon or cassette. Detection of the marker confirms the success of injection. Second, comparison of the DNA:RNA injections to control (DNA:H_2O) injections is also indicative of injection success. When performing control injections (DNA:H_2O), the number of fluorescently tagged embryos as well as the distribution (mosaicism) of fluorescence within the embryos will be dramatically reduced relative to co-injection of DNA with functional *Tol2* transposase mRNA.

A. Quality control fails– troubleshooting

1. If no difference is noted between injections with or without transposase mRNA (i.e., both injections show low levels of fluorescence), then something has likely occurred to impair the transposition reaction. This is most often due to degradation of the *Tol2* mRNA, because it is the most sensitive component of the injection mix.
2. If marker expression is not observed in either injection condition (+/− *Tol2* mRNA), the integrity or identity of the transposon DNA is the likely first point for troubleshooting. The integrity of the DNA (or RNA) from the exact injection mix can be verified on an agarose gel if there is sufficient quantity left after the procedure. If the identity of the DNA transposon is in question, more thorough restriction digest analysis and/or sequencing of the transposon may be needed to determine the issue.

B. Quality control passed

1. Once *Tol2* mRNA-enhanced expression of the dominant marker is observed, the next step is to select and raise F0 embryos to produce transgenic fish.
2. Remove any embryos with obvious morphological defects as they are unlikely to produce healthy, fertile adults.

C. No dominant marker used

If the choice was to forgo including a dominant marker within the transposon, then check the quality of the *Tol2* mRNA by co-injecting with a functionally verified positive control transposon obtained from one of the *Tol2* source labs or a colleague. Subsequent transgenesis will need to be scored by PCR or some other detection method.

V. Select injected fish to raise as founder generation F0

A. Select 50–100 embryos that express the dominant marker in the appropriate context; e.g., heart expression from a cmlc promoter. If using a more ubiquitous promoter, most embryos will have some expression of a fluorescent protein. In this case, select the embryos with the most uniform (low mosaic) expression of the fluorescent protein, as they represent the embryos that have likely had early integration of a transposon. This increases the chances that there has been germline integration in these embryos.

B. Raise these selected embryos to produce F0 adults according to standard rearing protocols. Ideally, raise a minimum of 30 or so fish to adulthood to provide enough F0 adults to establish the desired transgenic fish line. However, while these fish are developing into adults, we recommend injecting another set within 2–4 weeks as backup in case something goes wrong with the first set (especially during rearing) or in case the injection was not as successful as first evaluated.

VI. Production of F1 generation

A. Outcross F0 fish

1. When F0 fish become sexually mature, usually between 2.5 and 4 months depending on rearing conditions, screen for germline transgenesis. Because the efficiency of transgenesis is generally high, it is desirable to outcross these fish with non-transgenic brood stock. Use fish that are easily distinguishable from F0 fish to prevent cross-contamination − leo^{tq270}/+ is one choice of a dominant visible marker with an altered pigmentation pattern in adult zebrafish (Watanabe et al., 2006), and there are many recessive pigment markers that can be used.
2. After obtaining eggs from an outcross of an F0, keep the adult fish separate for the week while determining whether or not it is transmitting the transposon through its germline.

B. Screen embryos for germline transmission

1. Examine the embryos for dominant marker expression at the suitable time in development. In general, germline transgenesis is mosaic, meaning that expression will not be Mendelian at this generation. If the F0 is transmitting the transposon through its germline, it is common to see about 10–15% of the embryos expressing the dominant marker. However, the actual transmittance can vary from a single embryo in a clutch to more than 50%; such higher expression frequencies are due to multiple transposon integrations in the founder fish germline.
2. Independent integration of transposons from one F0 to another often results in subtle (sometimes dramatic) differences in expression of the transgenic cassette used. Therefore, maintain expressing F1 embryos from different F0 fish as separate substrains. These siblings may represent a collection of multiple integration events within the mosaic F0 germline. The transposon integrations within these F1 embryos are now stable, and subsequent generations will inherit these in a Mendelian fashion. At this point, select a manageable number of substrains of the transposon (4–6) and trim this down in subsequent generations based on signal to noise and proper expression domains in the F2 embryos.
3. F0's that produce 40 or more viable embryos that do not show any expression of the dominant marker are considered negative and retired from the screen.

VII. Select the best substrain(s) based on observation of F2 generation

The F2 embryos from F1 adults will inherit stable transposons in a Mendelian fashion. This permits evaluation of many embryos for proper expression based on the promoter used within the injected transposon. In addition to spatio-temporal control there will likely be differences in signal to noise ratio of the transgene. Select one or more lines to maintain.

VIII. Discussion

The use of transposons in zebrafish makes this animal perhaps the most readily modifiable organism within a biological scientists' genetic toolbox. Most injected animals pass at least one transgenic chromosome to their offspring. The *Tol2* system is robust and has been selected as the primary transposon platform, facilitating the development of an array of reagents readily shared within the zebrafish community.

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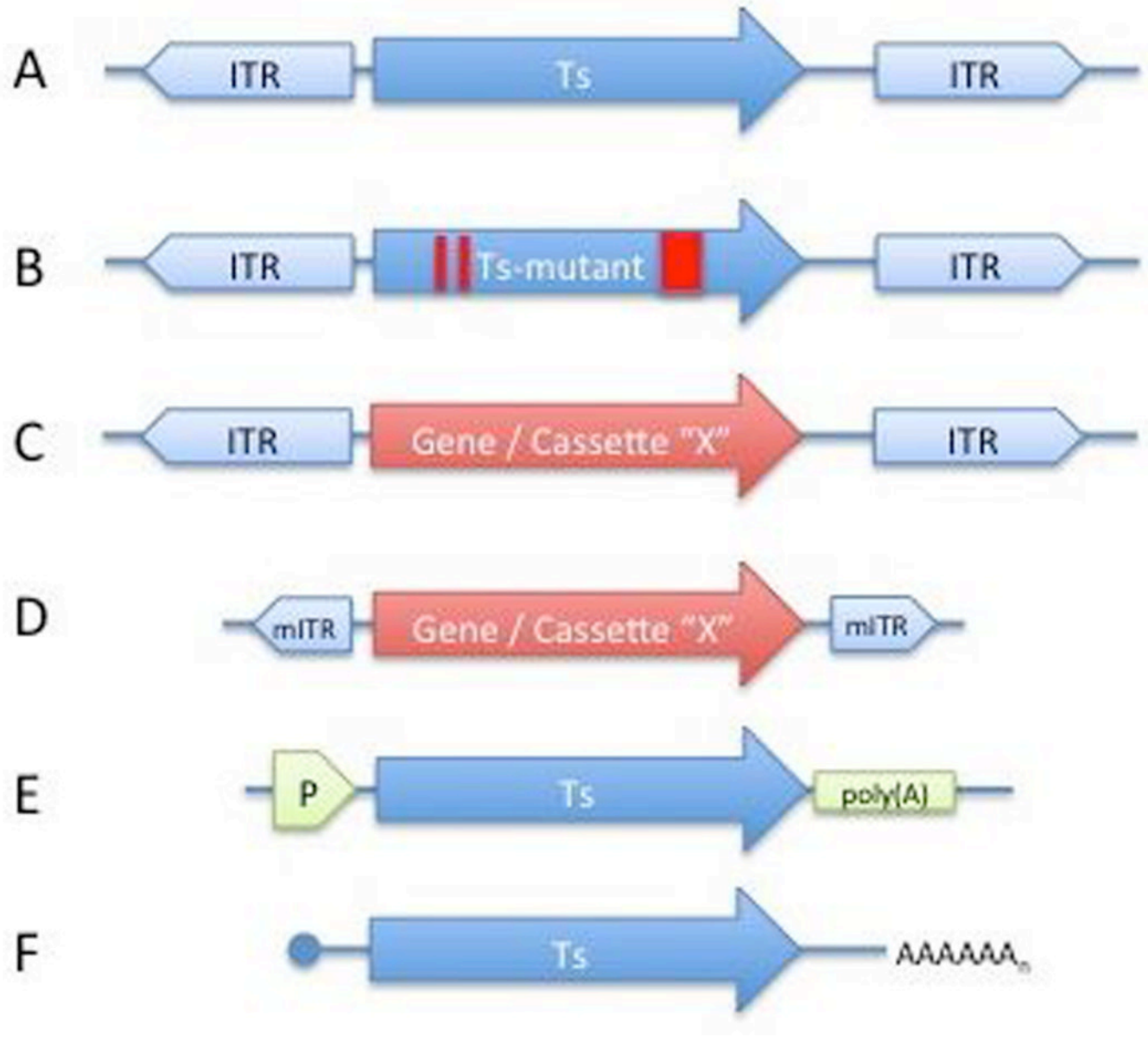

Figure 1. Transposon structures

A) An autonomous transposon found within the genome in its "wild" configuration contains full inverted terminal repeats (ITR) capable of driving expression of an active transposase located within the ITRs. The transposase is transcribed and processed including polyadenylation from signals located within the ITRs. The transposase mRNA is translated into protein that can recognize sequences at the distal ends of the transposon and "cut" it from the genomic DNA and "paste" it into a new location within the genome. B) Mutations (red areas) in the transposase cause the transposon to become non-autonomous, meaning the transposon has lost its ability to produce functional transposase protein. However, the ITRs of a non-autonomous transposon can be recognized if functional transposase is provided from another source (e.g. an autonomous element elsewhere in the genome). C) An engineered or "domesticated" transposon can be made by replacing the transposase coding region with a different gene or expression cassette. D) As the transposon sequences required for mobilization are understood, a transposon can be made using so-called "minimal" ITR

sequences (mITR). Doing so likely removes elements that are required for normal expression of the transposase (promoter, polyadneylation signals, etc.). E) A separate DNA expression cassette can be made by placing the transposase sequence between a promoter (P) and a polyadenylation signal (poly(A)). F) Alternatively, *in vitro* transcribed mRNA can be produced as a transient source of transposase. In zebrafish work, the most common genetic manipulations include the combination of a minimal transposon (D) and *in vitro* transcribed mRNA (F).

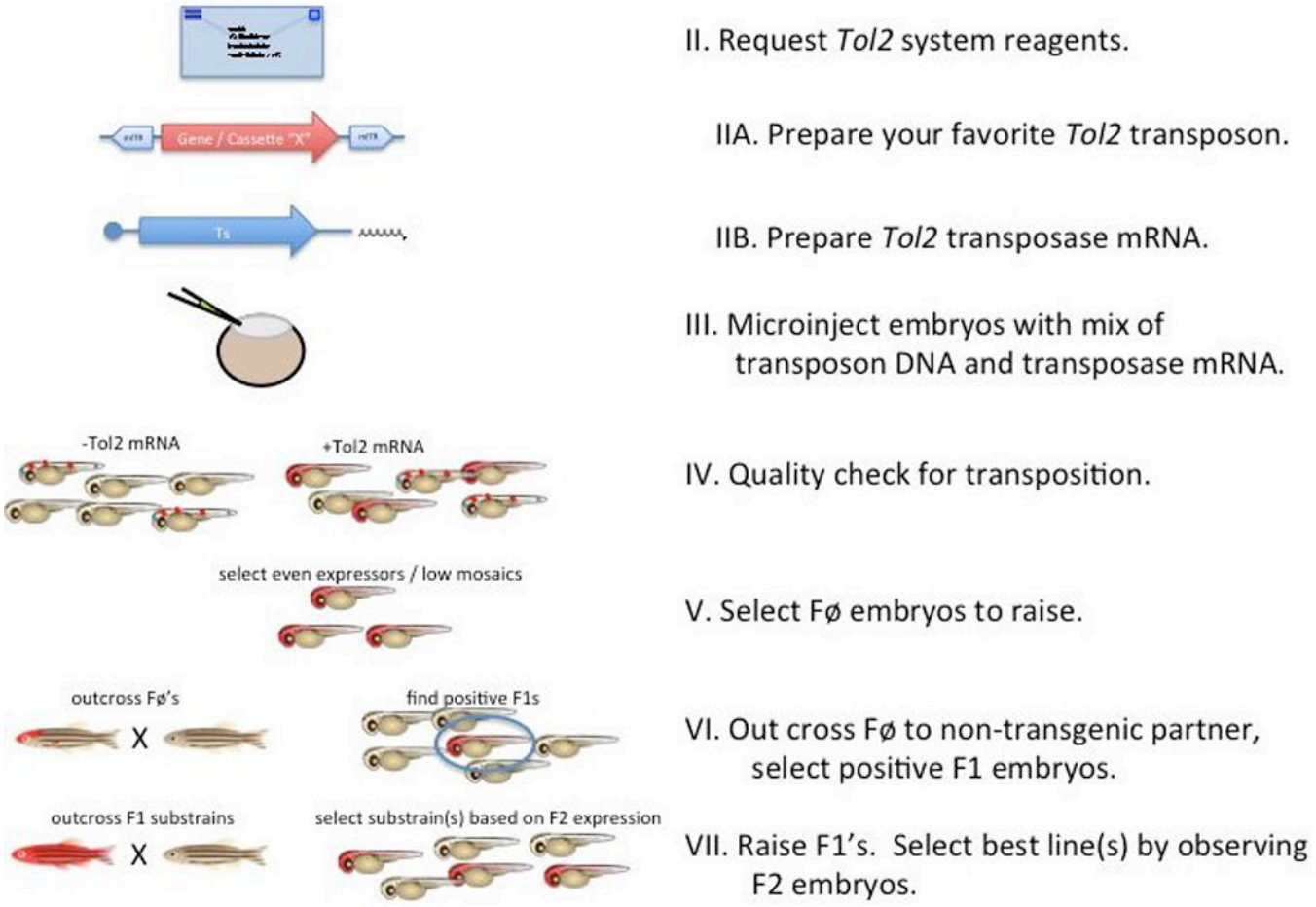

Figure 2. Overview of Tol2 transgenesis method

The basic approach to producing a transgenic zebrafish using the Tol2 transposon system is diagramed and listed. See text for details.

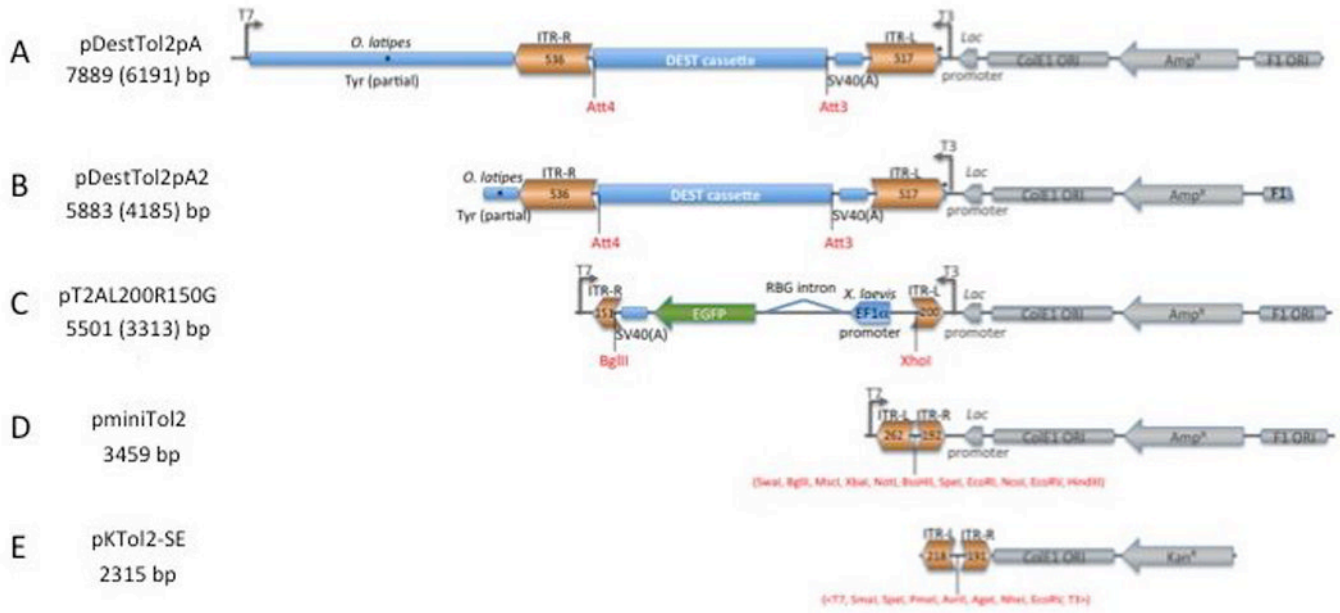

Figure 3. Available Tol2 transposon vectors

A and B) pDestTol2pA and pDestTol2pA2 are multisite gateway cloning vectors available from the Lawson lab (Villefranc et al., 2007) (http://lawsonlab.umassmed.edu/gateway.html) and the Chien lab (Kwan et al., 2007) (http://chien.neuro.utah.edu/tol2kitwiki/index.php/Main_Page), respectively. They are largely the same although the A2 version removes a large chunk of the *O. latipes tyrosinase* (tyr) gene that was cloned along with the original *Tol2* transposon ITRs. C) pT2AL200R150G is a minimal *Tol2* vector available from the Kawakami lab (Urasaki et al., 2006) (http://kawakami.lab.nig.ac.jp/). It includes a simple GFP expression cassette that is removed when cloning a gene between the *Bgl*II and *Xho*I restriction endonuclease sites. D) pminiTol2 is a minimal Tol2 transposon vector, available from the Ekker lab (Balciunas et al., 2006) (http://zfishbook.org), that shares the same multiple cloning sites as many SB vectors derived from pT/HB or pT/BH (Geurts et al., 2003). E) pKTol2-SE is a minimal Tol2 vector with a simplified vector backbone available from the Clark laboratory (http://zfishbook.org). pKTol2-SE shares a multiple cloning site with other vertebrate transposon cloning vectors: pKT2-SE, pPBT-SE, and pPPTn-SE for *Sleeping Beauty* (Clark et al., 2007; Ivics et al., 1997), *piggyBac* (Clark et al., 2007; Fraser et al., 1996), and *Passport* (Clark et al., 2009), respectively. The size of each plasmid (A–E) is noted. In cases where some of the plasmid is removed in the cloning process (A–C), the length of remaining elements is shown in parentheses. Abbreviations: ITR-R (Tol2 inverted terminal repeat right or 3'), ITR-L (Tol2 inverted terminal repeat left or 5'), DEST cassette (required for Gateway cloning, replaced with contents of entry vector), EF1α (elongation factor 1 alpha promoter), RBG intron (rabbit beta-globin intron), EGFP (enhanced green fluorescent protein), SV40(A) (SV40 polyadenylation signal), T7 (T7 polymerase binding site), T3 (T3 polymerase binding site), Lac Promoter (Lac operon promoter), ColE1 ORI (plasmid origin of replication), AmpR (ampicillin resistance gene), KanR (Kanamycin resistance gene), and F1 ORI (single-stranded phagemid origin).

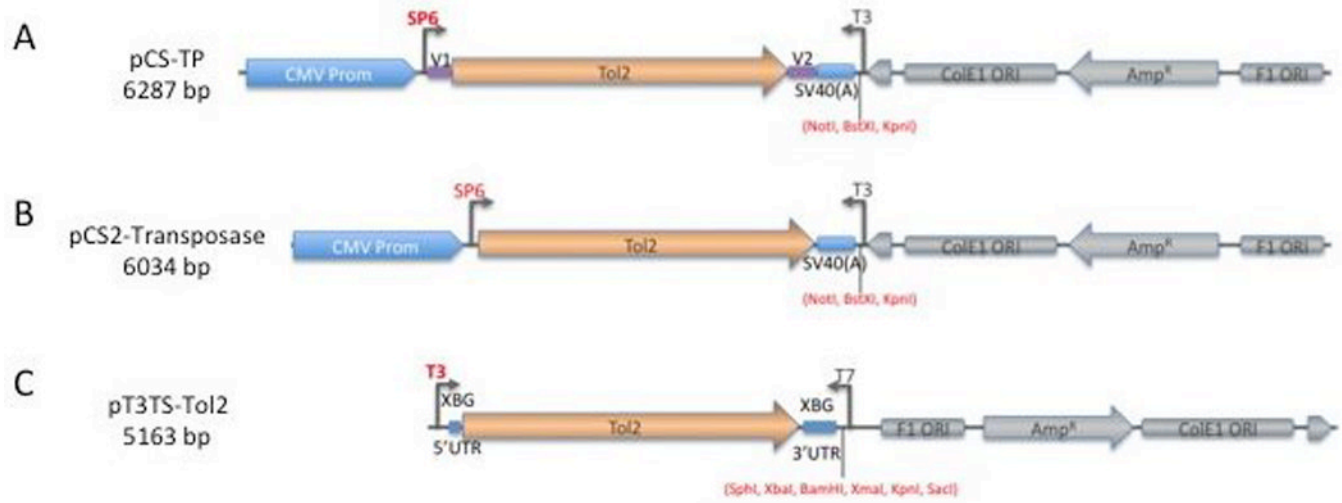

Figure 4. Available Tol2 transposase transcription vectors

A and B) pCS-TP and pCS2-Transposase are very similar transcription vectors available from the Kawakami lab or Chien lab, respectively. Both are based on a pCS vector backbone and use the bacteriophage SP6 polymerase. pCS2-transposase lacks the V1 and V2 regions that correspond to cDNA from the native Tol2 transposase untranslated regions. C) pT3TS-Tol2 is available from the Ekker lab. It uses T3 polymerase to produce mRNA and includes untranslated regions from the *Xenopus* beta-globin gene. The restriction sites available for linearization of the plasmid are shown in red.