ImPaqT - A Golden Gate-based Toolkit for Zebrafish Transgenesis

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Abstract

Transgenic animals continue to play an essential role in many aspects of zebrafish research, including the development of disease models. The most widely used system for zebrafish transgenesis is the Tol2 transposon system. Here, we have developed ImPaqT (**Im**munological toolkit for **Paq**CI-based Golden Gate Assembly of Tol2 **T**ransgenes), a new Tol2-based transgenesis system that utilizes Golden Gate assembly to facilitate the production of transgenic zebrafish lines. This system allows for rapid assembly of multiple fragments into a single transgene, facile swapping of individual sequences to generate new transgenes and an easy cloning workflow to incorporate new genetic elements into the existing kit. Within this toolkit framework, we have generated a number of reagents to enable gene expression within immune and non-immune cell types, an array of best-in-class fluorescent proteins to visualize cell populations and transgenes as well as tools to simplify genetic manipulation, purification and ablation of targeted cells. Unlike recombination-based systems, the Golden Gate approach is also expandable, allowing the incorporation of complex designs such as multi-fragment promoters within the established modular framework of ImPaqT. Here, we demonstrate the function of this new system by generating a number of novel transgenic immune reporter lines. While our toolkit is focused on the immune system as an emerging area of study within zebrafish research, the ImPaqT approach can be broadly adapted to the construction of almost any zebrafish transgene, offering new tools for the generation of transgenes within the zebrafish community.

Introduction

The zebrafish (*Danio rerio*) has emerged as a powerful model organism and is increasingly used in various research fields and disciplines. Zebrafish share 70% of their genome and more than 80% of disease genes with humans (Howe et al. 2013). This has led to the development of many distinct zebrafish disease models (Santoriello and Zon 2012) including models of hematopoietic and neurodegenerative diseases, cardiovascular diseases (Bowley et al. 2022) such as atherosclerosis (Tang et al. 2021), cancer (Liu and Leach 2011; Russo et al. 2022), and tuberculosis (Bouz and Al Hasawi 2018; Varela and Meijer 2022), among others. Transgenic animals continue to play a key role for many experimental strategies, including study of gene function, genetic screens, and development of disease models. Transgenic zebrafish lines have been widely generated by applying the Tol2 transposon system with a transgenesis rate of 50% (Kawakami et al. 2004). In this system, a transposon-donor plasmid containing a DNA sequence of interest flanked by *Tol2* sites and synthetic mRNA encoding the Tol2 transposase are introduced into the single cell of fertilized eggs by microinjection. Upon translation, the transposase protein integrates the *Tol2* flanked gene fragment randomly into the genome through a cut-and-paste mechanism without causing rearrangement or modifications at the target site (Kawakami and Shima 1999; Kawakami et al. 2000, 2004; Kawakami 2007).

A number of approaches have previously been used to generate Tol2 transgenesis constructs. Traditionally, multi-step restriction enzyme and ligase based subcloning was used. However, this has given way to many advanced cloning approaches, including Gateway cloning (Hartley et al. 2000; Kwan et al. 2007; Katzen 2007), Gibson Assembly (Gibson et al. 2009), In-Fusion cloning (Zhu et al. 2007; Sleight et al. 2010) and Golden Gate cloning, which have been used to speed up and simplify the procedure of transgene assembly. Each of these approaches have different advantages and disadvantages. Gateway cloning allows for the establishment and reuse of a library of fragments (e.g. promoters, genes and fluorescent proteins) but requires the use of lambda phage-derived *att* sequences to facilitate insertion of the desired

60 sequences. These *att* sequences result in the addition of ~20-25 bp of additional sequence in
61 between each element and expansion of the number of fragments to be assembled can require
62 recloning of the sequence with new *att* ends (Katzen 2007; Engler et al. 2008; Sone et al.
63 2008). By contrast, Gibson and In-Fusion cloning allow for joining of short (15-20 bp) regions
64 of homology, enabling the seamless cloning of many transgenes. However, the requirement
65 for homology for seamless cloning generally means that individual components have to be
66 adapted to upstream and downstream elements to generate new transgenes and have an
67 upper limit of approximately 5-6 fragments with currently available kits. Golden Gate cloning
68 utilizes type IIS restriction enzymes to cleave and join multiple fragments in a one pot reaction
69 (Padgett and Sorge 1996; Engler et al. 2008). It balances the tradeoffs of the previous cloning
70 systems, requiring inclusion of only a short 4 base pair sequence, while enabling researchers
71 to generate a library of vectors that can be combinatorially assembled to generate distinct
72 transgenes. Large numbers of DNA fragments can also be assembled by Golden Gate with
73 reports of assembly of up to 52 fragments in a single reaction (Pryor et al. 2022). Despite the
74 advantages of Golden Gate cloning, this approach has not been widely applied in zebrafish
75 transgene assembly. Only a few examples of this approach have been described for Tol2
76 transgenesis including the recently described GoldenFish toolkit which uses Golden Gate
77 cloning to gain more flexibility in transgene design (Jiang et al. 2022) and the Golden Gateway
78 tool (Kirchmaier et al. 2013) which combines the well-established cloning tools Gateway and
79 Golden Gate cloning to simplify creation of new components for Gateway assembly of
80 transgenes.

81 Here, we have sought to apply Golden Gate cloning approaches to create a toolkit for zebrafish
82 transgenesis. We have developed ImPaqT, a new **Im**munological toolkit for **Paq**CI-based
83 Golden Gate Assembly of Tol2 **T**ransgenes, to facilitate the production of transgenic animals.
84 Studies of immune function have been of growing interest in the zebrafish community due to
85 the close genetic similarity of the immune systems of humans and zebrafish (Howe et al. 2013),
86 but there are few compiled resources for these cell types. ImPaqT provides a collection of

87 commonly used immunological promoters targeting distinct lineages of vertebrate immunity,
88 genetic tools and fluorophores supporting flexible generation of transgenes for immunological
89 research. This toolkit utilizes Golden Gate cloning due to the advantages of this technique and
90 incorporates a newly described type IIS restriction enzyme, PaqCI, with a 7 base recognition
91 sequence to reduce the frequency of off target cutting (from 1 in 4,096 bases (4^6) for a 6-base
92 cutter to 1 in 16,384 bases (4^7), assuming random cutting). We designed the kit to have
93 consistent overhang sequences for each position in the backbone (e.g. 5', middle, and 3'
94 element) in order to make the toolkit modular and simplify the construction of additional
95 transgenes. As proof-of-concept, we show that this toolkit can facilitate the production of
96 expression constructs and we demonstrate the generation of transgenic animals using the Tol2
97 transgenesis with these constructs. ImPaqT is modular, enabling easy swapping of gene
98 sequences, easily expandable, allowing the introduction of multiple new genetic elements if
99 needed, and relies only on the use of short (4 bp) overlaps that can also be designed to be
100 scarless if desired. To establish an immunological toolkit that will be broadly useful to zebrafish
101 researchers, we have generated a variety of immunological and non-immunological promoters,
102 including *mfap4* and *mpeg1.1* (macrophages)*, irg1* (macrophages, induced by infection)*, lyz*
103 (neutrophils), *lck* (T cells), *ubb* (ubiquitious) and *hsp70* (heat shock inducible), tools for genetic
104 recombination (*icre*), cell killing (*kid, nitroreductase)* and cell isolation tools (*LNGFR*), and an
105 array of fluorescence proteins including *mTurquoise2*, *tdStayGold*, *mCitrine*, *mNeonGreen*,
106 *dLanYFP* and *tdTomato* that can be used to rapidly develop new transgenes and new
107 transgenic lines.

108 **Results**

109 PaqCI-based Golden Gate cloning enables the construction of Tol2 expression vectors

110 We sought to create a toolkit for immune transgene construction for the zebrafish community.
111 Ultimately, we decided to design this toolkit to use Golden Gate cloning, given the short regions
112 of homology that this approach required, the ability to structure this toolkit in a modular fashion
113 and the expandability of the Golden Gate cloning approach, enabling incorporation of
114 additional genetic elements as needed. Golden Gate assembly uses type IIS restriction
115 enzymes to generate a set of unique overhangs that are used to join fragments in a desired
116 order. To create a system for modular cloning of transgenes, we chose consistent 4-base
117 overhang sequences for each of the three elements such that DNA fragments amplified with
118 these overhangs can be cloned in the same position in the final construct, allowing these
119 elements to be used combinatorially. The individual overhang sequences were chosen using
120 NEBridge Ligase Fidelity Viewer (https://ligasefidelity.neb.com/viewset/run.cgi), which enables
121 the selection of efficient joining ends with minimal off-target ligation based on high throughput,
122 combinatorial tests of all 4-base overhangs (Pryor et al. 2020; Potapov et al. 2018). Using this
123 tool, we selected ends predicted to have 1% or less off-target ligation with the other ends used
124 in the toolkit. As zebrafish transgenes generally consist of a cell-type specific promoter, a gene
125 of interest, and a fluorescent marker, we structured our toolkit to have three elements that
126 would ultimately be joined within a backbone vector possessing Tol2 repeats for transgenesis.
127 These elements were named based on their location within the final construct - 5' element (5E)
128 for the first position, a middle element (ME) and a 3' element (3E) for the final position within
129 the backbone.

130 To generate the overhangs required to join individual components for Golden Gate assembly,
131 we chose the 7-base cutter PaqCI to limit off-target cutting of inserts used in assembly.
132 Searches of the zebrafish genome revealed that PaqCI cuts once every ~17 kb on average
133 (~82600 sites in the GRCz11 zebrafish genome assembly), while the 6-base cutters BsaI and
134 BsmBI, commonly used for Golden Gate, cut every ~9 kb (~151800 sites) or ~13 kb (~107200

135 sites). Genes and regions of interest were generated by PCR amplification using primers
136 containing the 7-base PaqCI recognition sequence along with a spacer consisting of any four
137 nucleotides that is excised from the final construct, and a 4-base overhang sequence defined
138 by the location of the sequence of interest in the final construct (Fig. S1). These PCR products
139 were cloned into vectors and sequence verified to generate a stable library of constructs for
140 transgene assembly. Cleavage with PaqCI leads to the creation of specific 4-base overhangs
141 for each position at their 5' and 3' end enabling proper assembly and single-step ligation into
142 a backbone vector for transgene generation. These backbone vectors possess inverted *Tol2*
143 repeats for transgenesis surrounding a PaqCI-flanked LacZ gene for blue-white screening to
144 identify backbone constructs containing transgene components. A simplified schematic for the
145 assembly using one insert is illustrated in Fig. 1. As we demonstrated below, we have found
146 that this approach allows for efficient, combinatorial transgene generation.

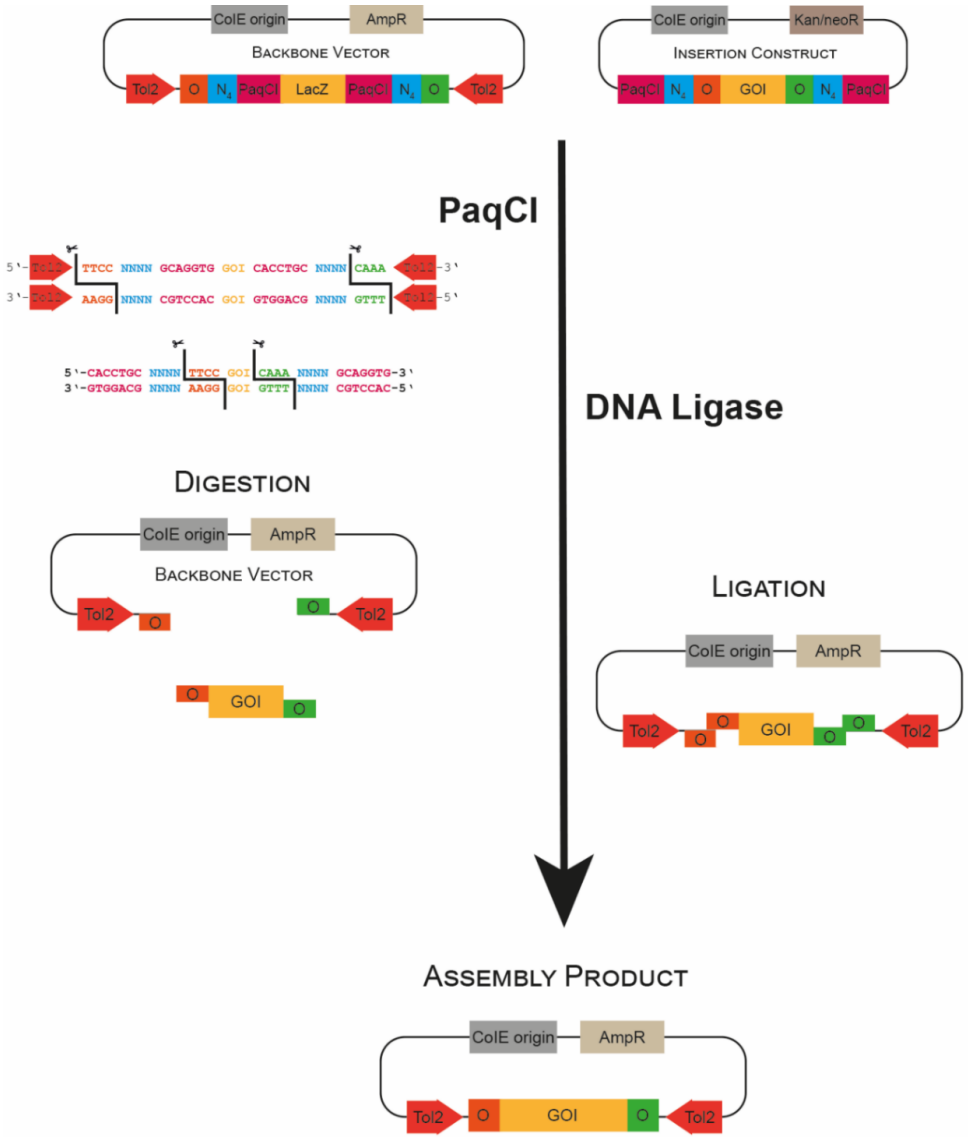


Figure 1: Basic principles of Golden Gate cloning for Tol2 Transgenesis
Schematic of a Golden Gate cloning reaction using one insert. The insertion construct and backbone vector are assembled in a single-tube reaction using PaqCI and ligase. PaqCI is a type IIS restriction enzyme. Its recognition site and cleavage are indicated. The font color corresponds to the color of the respective feature indicated in the cartoon. The backbone vector consists of Tol2 sites, PaqCI recognition sites (PaqCI) along with 4-base spacer sequences (N_4) and two overhangs (O). The insertion construct includes the gene of interest (GOI) flanked by PaqCI, N_4 and the same two overhangs. Assembly involves digestion of backbone and insertion constructs by PaqCI producing matching overhangs and ligation through the DNA ligase forming the final assembly product.

<u>ImPaqT facilitates the flexibility of transgenes design and the generation of transgenic zebrafish</u>

Having established this Golden Gate cloning-based technique for the assembly of Tol2 transposon vectors, we sought to create an array of constructs that would be beneficial to the immunology community to generate transgenic animals. To these ends, we have generated

163 constructs for expression in commonly studied zebrafish immune populations, including T cells
164 (*lck*) (Langenau et al. 2004), neutrophils (*lyz)* (C. Hall et al. 2007) and macrophages (*mfap4*,
165 *mpeg1.1* and the infection-inducible *irg1*) (Walton et al. 2015; Ellett et al. 2011; C. J. Hall et al.
166 2013). To enable lineage tracing approaches for labeling of hematopoietic derived cells, we
167 also included the hematopoietic stem cell (HSC)-specific promoter *runx1 +23* (Tamplin et al.
168 2015) as well as tools for broader expression including the ubiquitious promoter *ubiquitin B*
169 (*ubb*) (Mosimann et al. 2011), and the temperature inducible heat shock protein 70-like
170 promoter (*hsp70*) (Shoji et al. 1998; Halloran et al. 2000; Kwan et al. 2007). We have also
171 integrated a number of tools to allow for manipulation of these immune populations, including
172 tools for genetic manipulation (*icre* (Shimshek et al. 2002*)*), for rapid purification of cell
173 populations (truncated human *LNGFR* (Wattrus and Zon 2020)), expression CRISPR gRNAs
174 and other small RNAs (zebrafish U6 promoter (Zhou et al. 2018)) and cell-specific ablation
175 (*nitroreductase* (Curado et al. 2007, 2008) and *kid* (Labbaf et al. 2022)). To visualize labeled
176 cell populations and genes, we provide a diverse array of best-in-class fluorescent proteins in
177 a range of colors to facilitate simultaneous imaging of distinct cell types. These include
178 *tdStayGold* (Hirano et al. 2022), *mTurquoise2* (Goedhart et al. 2012), *tdTomato* (Shaner et al.
179 2004), m*Citrine* (Zacharias et al. 2002)*, mNeonGreen* (Shaner et al. 2013) and *dLanYFP*
180 (Shaner et al. 2013). A summary of all available constructs is shown in Table 1. ImPaqT is also
181 designed to be easily expandable through simple cloning approaches.

182 **Table 1: Library of insertion constructs.**
183 5' element (5E), middle element (ME), 3' element (3E).

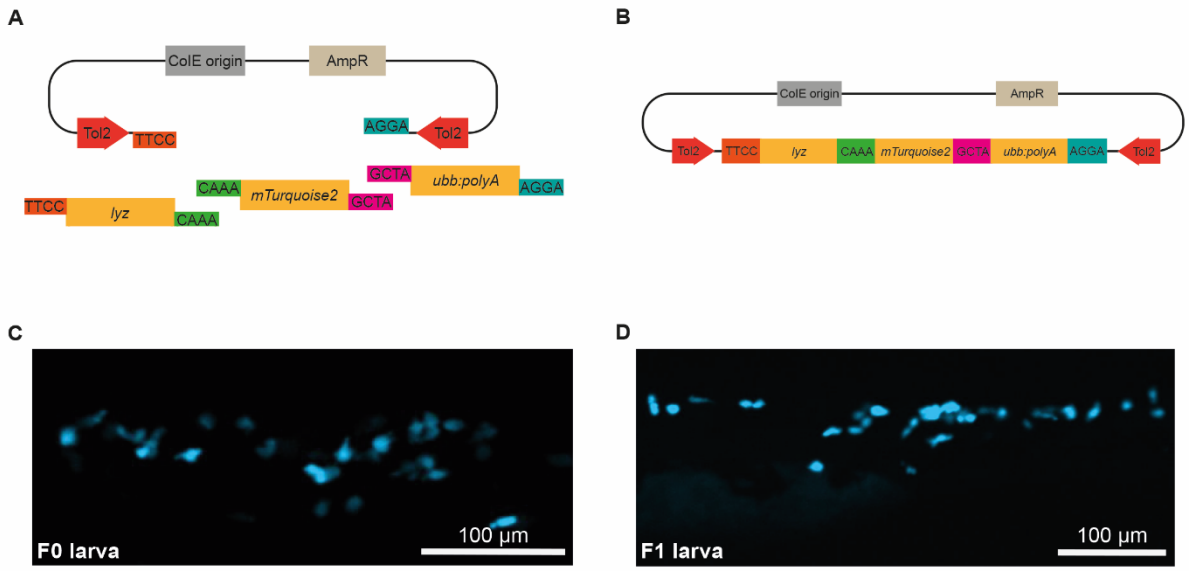
Insertion Elements	Position	Function	Reference
irg1	5E	Infection-inducible macrophage-specific promoter	(C. J. Hall et al. 2013)
mfap4	5E	Macrophage-specific promoter	(Walton et al. 2015)
mpeg1.1	5E	Macrophage-specific promoter	(Ellett et al. 2011)
lyz	5E	Neutrophil-specific promoter	(C. Hall et al. 2007)
ubiquitin B (*ubb*)	5E	Promoter for ubiquitious expression	(Mosimann et al. 2011)
ubb:loxP GFP loxP	5E	Promoter for ubiquitious expression of floxed GFP for use in combination with Cre	(Mosimann et al. 2011)
hsp70	5E	heat shock protein 70-like promoter for inducible gene expression	(Shoji et al. 1998; Halloran et al. 2000; Kwan et al. 2007)
lck	5E	T cell-specific promoter	(Langenau et al. 2004)
runx1 + 23	5E	Promoter for expression in hematopoietic stem cells	(Tamplin et al. 2015)
icre	ME	Cre recombinase for inducible recombination of DNA between loxP sites	(Shimshek et al. 2002)
(p2A) dLanYFP	3E	Yellow fluorescent protein	(Shaner et al. 2013)
mNeonGreen	ME, 3E	Green/ yellow fluorescent protein	(Shaner et al. 2013)

LNGFR	ME	Low affinity nerve growth factor receptor for rapid cell enrichment	(Wattrus and Zon 2020)
nitroreductase	ME	Inducible ablation of specific cell lineages	(Curado et al. 2007, 2008)
kid	ME	Ablation of specific cell lineages	(Labbaf et al. 2022; de la Cueva-Mendez et al. 2003)
(p2A) tdTomato	ME, 3E	Red fluorescent protein	(Shaner et al. 2004)
tdTomato CAAX	ME	Red fluorescent protein with membrane localization protein	(Walton et al. 2015)
(p2A) mTurquoise2	ME, 3E	Cyan fluorescent protein	(Goedhart et al. 2012)
mCitrine	ME	Yellow fluorescent protein	(Zacharias et al. 2002)
tdStayGold	ME	Green fluorescent protein	(Hirano et al. 2022)
ubb:polyA	3E	Ubiquitious expression of a SV40 poly A	(Walton et al. 2015)
polyA-gRNA (Control):U6	3E	gRNA targeting control site under control of U6 promoter	(Zhou et al. 2018)
polyA-gRNA (GFP):U6	3E	gRNA targeting GFP under control of U6 promoter	(Zhou et al. 2018)

184

185 Generation of new transgenic zebrafish lines using ImPaqT

186 To test our PaqCI-based Golden Gate assembly toolkit as well as generate new immunological
187 zebrafish lines for the community, we used ImPaqT to assemble a number of new transgenic
188 lines. Using the neutrophil-specific promoter *lyz*, we generated a T*g(lyz:mTurquoise2)*^{ber2}
189 construct through Golden Gate assembly of 5E *lyz*, ME *mTurquoise2* and 3E *ubb:polyA* to
190 create the *lyz:*m*Turquoise2* transgene construct (Fig. 2A-B). The construct was coinjected with
191 *Tol2* transposase mRNA into single cell of fertilized eggs and at 2 dpi we observed strong blue
192 fluorescence signal within likely neutrophil populations in the CHT (Fig. 2C). Positive animals
193 were raised to adulthood and screening of F1 progeny of these animals revealed robust
194 expression of mTurquoise2 within putative neutrophil populations throughout the animal (Fig.
195 2D). Crossing to the already established neutrophil reporter *Tg(lyz:EGFP)*^{nz117} (C. Hall et al.
196 2007) zebrafish confirmed specific expression of our transgene within neutrophils (Fig. S2).

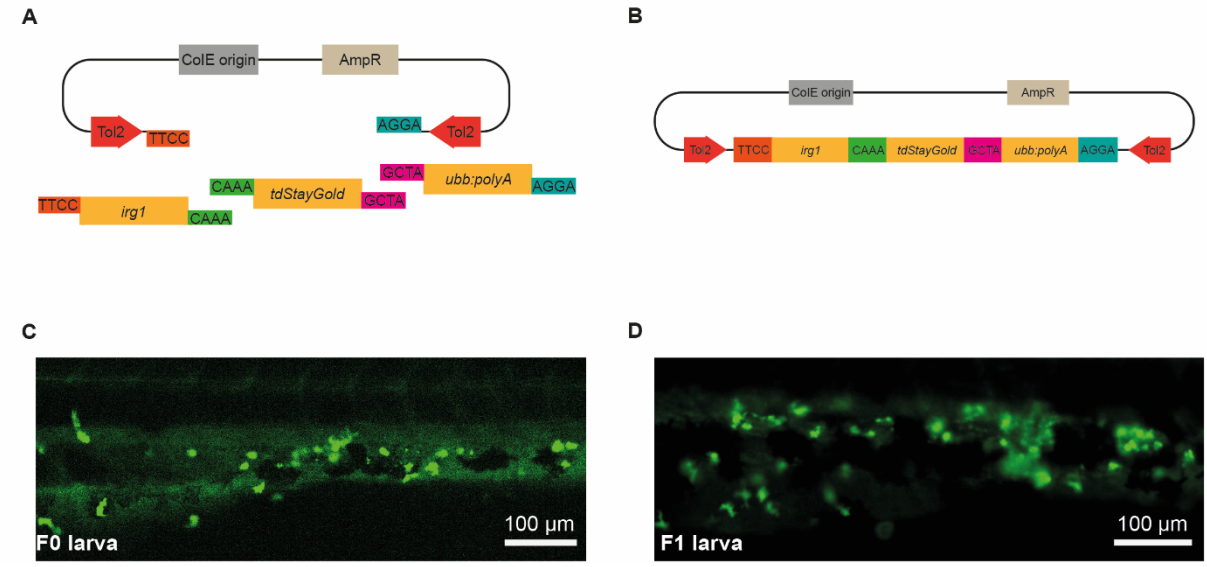


197

198 **Figure 2: Generation of *Tg(lyz:mTurquoise2)* zebrafish**
199 A: Schematic of the Golden Gate assembly of *Tg(lyz:mTurquoise2)*. The backbone vector contains
200 overhang sequences O1 (TTCC) and O4 (AGGA). *lyz* is a neutrophil-specific promoter containing
201 overhang sequences O1 and O2 (CAAA). *mTurquoise2* encodes a bright cyan fluorescent protein with
202 overhangs O2 and O3 (GCTA). *ubb:polyA* is assembled at the final position within the backbone with
203 O3 and O4.B: Schematic of the final assembly product of *Tg(lyz:mTurquoise2)*. C-D: Images of a
204 *Tg(lyz:mTurquoise2)* F0 (C) and F1 (D) larva showing neutrophil response in cyan at 2 days post
205 fertilization.

206

207 Similarly, we used the ImPaqT toolkit to generate a new macrophage-specific transgene using
208 the infection-inducible macrophage-specific promoter *irg1* and the recently described bright
209 green fluorescent protein *tdStayGold* (Hirano et al. 2022). To generate the
210 *Tg(irg1:tdStayGold)*^{ber3} construct, the 5E *irg1,* ME *tdStayGold* and 3E *ubb:polyA* elements were
211 combined into the backbone vector (Fig. 3 A-B). We injected zebrafish with this construct and
212 as *irg1* is an inflammation responsive promoter that is only weakly expressed in the absence
213 of immune stimulation, we injected larvae with LPS before screening. After LPS injection, we
214 were able to identify a number of F0 founders with strong green fluorescence within
215 subpopulations of their macrophages (Fig. 3C). These positive F0 larvae were raised to
216 adulthood and screening of F1 progeny revealed expression of tdStayGold throughout
217 macrophage populations upon stimulation with LPS (Fig. 3 D).



218

219 **Figure 3: Generation of *Tg(irg1:tdStayGold)* zebrafish**
220 A: Schematic of the Golden Gate assembly of *Tg(irg1:tdStayGold)*. The backbone vector contains
221 overhang sequences O1 (TTCC) and O4 (AGGA). *irg1* is a macrophage-specific promoter containing
222 overhang sequences O1 and O2 (CAAA). *tdStayGold* encodes a bright green fluorescent protein with
223 overhangs O2 and O3 (GCTA). *ubb:polyA* is assembled at the final position within the backbone with
224 O3 and O4. B: Illustration of the final assembly product of *Tg(irg1:tdStayGold)*. C-D: Images of a
225 *Tg(irg1:tdStayGold)* F0 (C) and F1 (D) larva showing macrophage-specific tdStayGold expression.

226

227 We also aimed to test the utility of the tools that we have designed within our kit. To these
228 ends, we created an infection-inducible, macrophage specific *icre* transgene construct,
229 *Tg(irg1:icre-p2A mTurquoise2)*. This construct was generated by combining the 5E *irg1,*

230 ME *icre* and 3E *p2A mTurquoise2* (Fig. 4A and 4B). Animals injected with this transgene
231 showed macrophage-specific mTurquoise2 signal after injection with LPS (Fig. 4C). To further
232 confirm that this construct was able to drive rearrangement of floxed reporters, we injected
233 *Tg(-3.5ubb:LOXP-EGFP-LOXP-mCherry)*^{cz1701} (Mosimann et al. 2011*)* zebrafish with this
234 construct. These animals were injected with LPS and imaged for 48 h to visualize
235 rearrangement of the floxed construct. LPS treatment drove the induction of *icre* and the co-
236 translationally cleaved *p2a mTurquoise2* transgene within macrophages starting ca. 4 h post
237 LPS injection. While the induction of the *irg1:icre-p2a mTurquoise2* transgene was transitory,
238 this short-term induction of Cre could induce permanent rearrangement of the *loxP-EGFP-*
239 *loxP-mCherry* construct as seen by induction of red fluorescent mCherry within macrophage
240 populations (Fig. 4D, S3, Movie S1). Control animals without the *icre*-construct neither showed
241 mTurquoise2 nor mCherry signal in macrophages (Fig. S3, Movie S 2). This experiment
242 showed not only the functionality of the individual insertion constructs but also the range of
243 assembly constructs that can be generated with them.

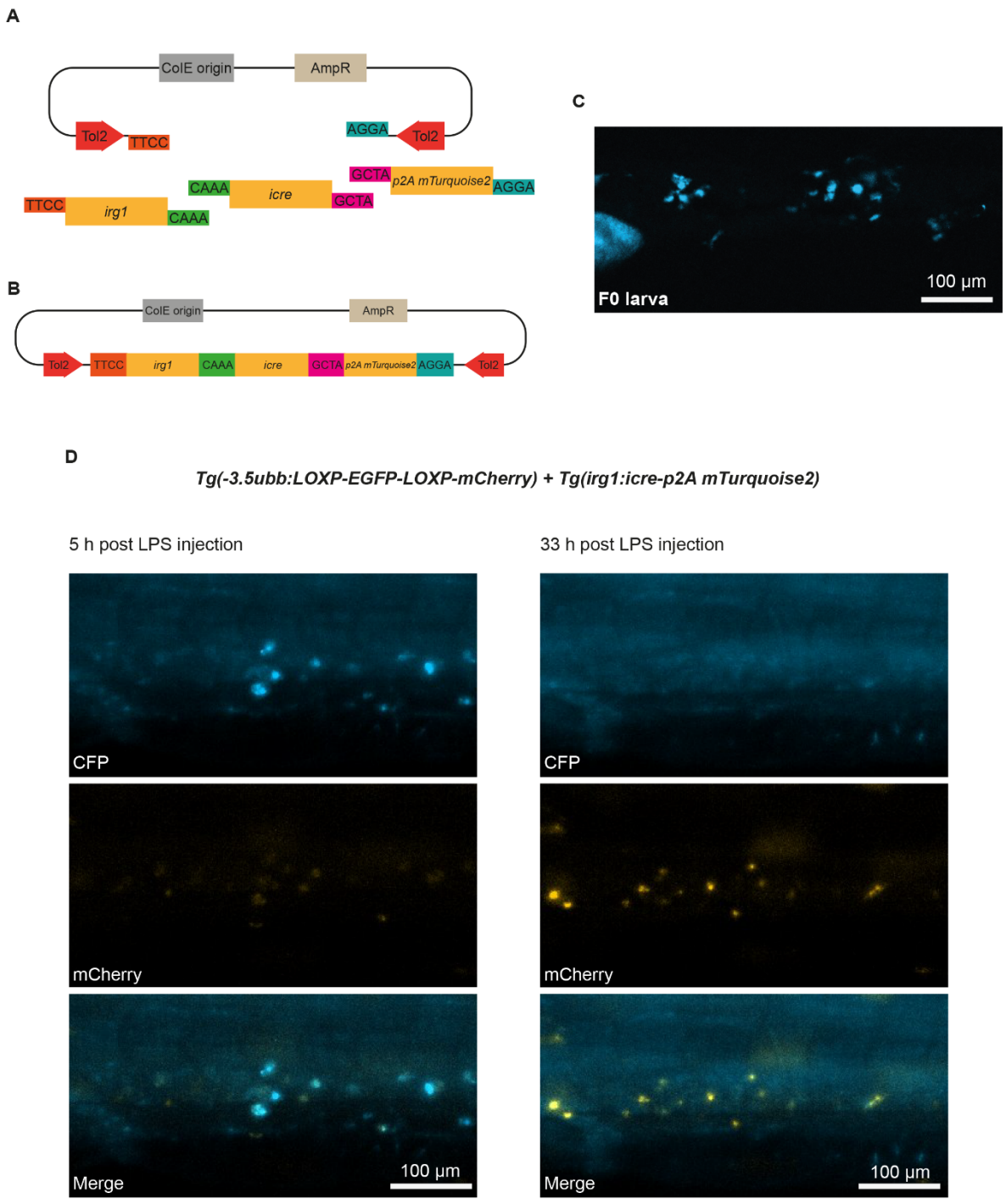


Figure 4: Generation of *Tg(irg1:icre-p2A mTurquoise2)* zebrafish for lineage tracing of individual macrophages

A: Schematic of the Golden Gate assembly of *Tg(irg1:icre-p2A mTurquoise2)*. The backbone vector contains overhang sequences O1 (TTCC) and O4 (AGGA). The macrophage promoter *irg1* contains overhang sequences O1 and O2 (CAAA). *icre* contains overhangs O2 and O3 (GCTA) for the middle position. *p2A mTurquoise2* is assembled at the final position within the backbone with O3 and O4. B: Illustration of the final assembly product of *Tg(irg1:icre-p2A mTurquoise2)*. C: Image of a *Tg(irg1:icre-p2A mTurquoise2)* F0 larva showing macrophage-specific mTurquoise2 signal after stimulation with LPS. D: Images of a *Tg(-3.5ubb:LOXP-EGFP-LOXP-mCherry)* larva that was introduced to the *Tg(irg1:icre-p2A mTurquoise2)* construct at single cell stage and was treated with PTU and LPS. The images show a macrophage-specific mTurquoise2 signal (e.g. 5 h post LPS injection) and a macrophage-specific mCherry signal (e.g. 33 h post LPS injection).

257 ImPaqT is expandable through the introduction of new overhang sequences

258 A limitation of many existing systems for transgenesis is that these systems have a fixed
259 number of elements that can be combined to generate transgenes. Golden Gate methods
260 however, are expandable through the addition of new overhangs and have been used to join
261 10-24 or even more than 50 fragments of DNA in a single reaction (Kucera 2021; Pryor et al.
262 2022). The ability to expand this kit to include larger numbers of constructs would simplify the
263 generation of complex transgenes including multifragment promoters, loxP sites, localization
264 tags (e.g NLS or prenylation signal) or multigene constructs for example. To provide proof of
265 concept for the expandability of ImPaqT, vectors were designed to enable the construction of
266 a *ubb* promoter-driven construct from three similar sized *ubb* promoter fragments, ubb_1, ubb_2
267 and ubb_3. These fragments were constructed such that the first fragment (ubb_1) and the last
268 fragment (ubb_3) use the same O1 and O2 overhangs used by 5E constructs so that the array
269 of promoter fragments can be cloned into the existing position of 5E elements and used
270 alongside already existing ME and 3E constructs (Fig. 5A-B). New internal overhangs were
271 designed for the ubb_1, ubb_2 and ubb_3 fragments to combine these distinct fragments (Fig. 5A-
272 D). Individual *ubb* fragments were used for Golden Gate assembly in combination with
273 ME *mTurquoise2* and 3E *ubb:polyA* (Fig. 5C) which led to the expected transgenesis construct
274 *Tg(ubb:mTurquoise2)*^{ber4} (Fig. 5D) that was injected into embryos at single cell stage.
275 Screening of injected animals confirmed that transgenic embryos established with this
276 construct had the expected expression pattern (Fig. 5E). We also found robust expression in
277 the F1 progeny (Fig. 5F) after raising positive F0 animals, confirming that injected animals are
278 viable and able to pass on the transgene to the next generation. Together, these findings
279 demonstrated proof-of-concept for the expansion of this transgene assembly system enabling
280 users to add fragments as needed through the addition of novel unique 4-base overhangs
281 while maintaining compatibility with existing ImPaqT constructs.

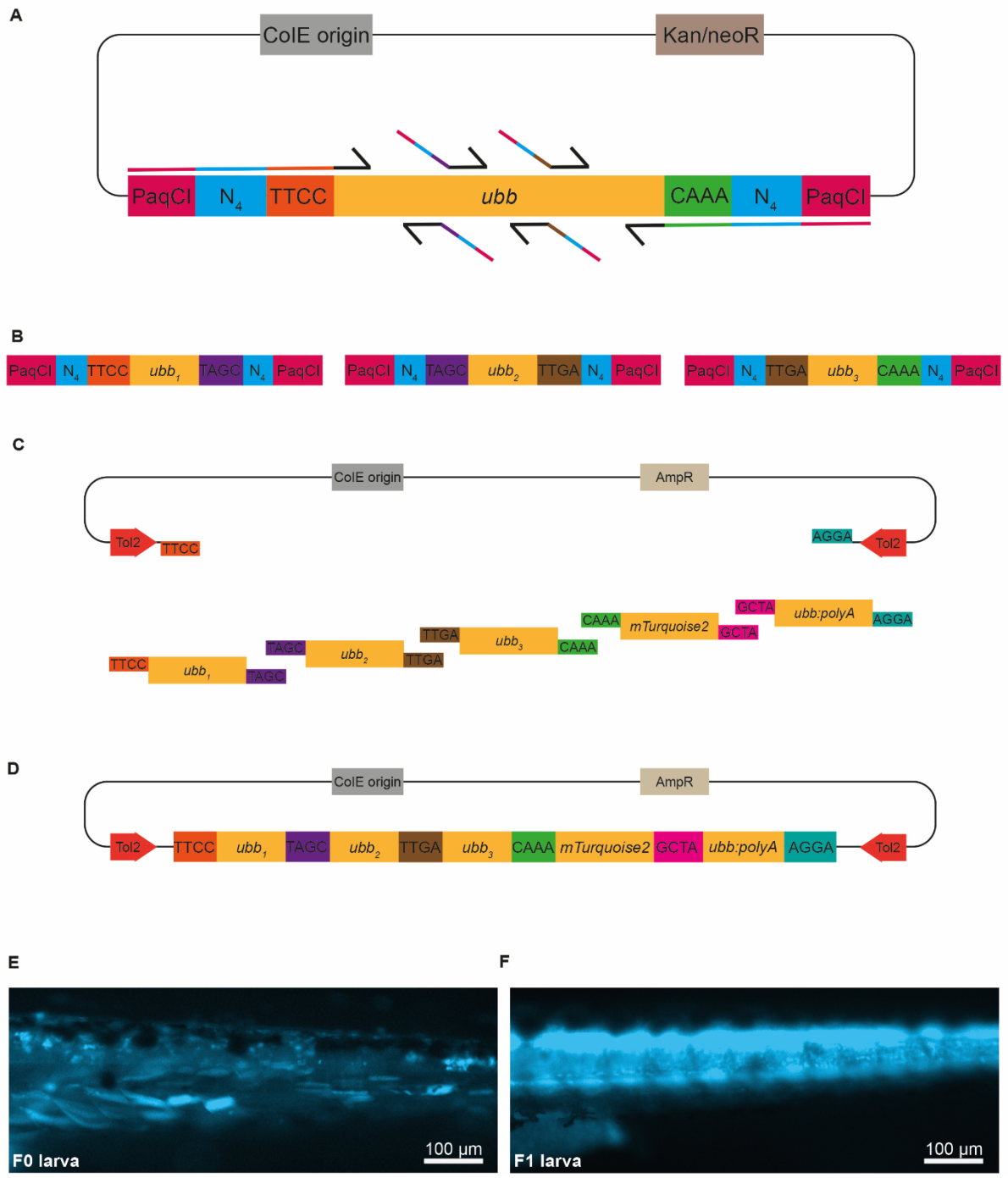


Figure 5: ImPaqT allows for expandability of individual positions while retaining compatibility with already established transposon components.
A: Schematic of the 5E *ubiquitin B* (*ubb*) promoter construct containing PaqCI recognition sites (PaqCI) along with 4-base spacer sequences (N_4) and two overhangs (O1: TTCC, O2: CAAA) for positioning at the 5' end of the assembly construct. Primers were designed to break up *ubb* into three fragments with new overhang sequences. B: Schematic of PCR amplified *ubb* fragments $ubb_{1\text{-}3}$ with PaqCI, N_4 and O1 and O1A (TAGC), O1A and O1B (TTGA), and O1B and O2, respectively. C: Schematic of the Golden Gate assembly of 5E $ubb_{1\text{-}3}$, ME *mTurquoise2* and 3E *ubb:polyA* into the backbone vector. D: Illustration of the final assembly product of *Tg*($ubb_{1\text{-}3}$*:mTurquoise2)*. E-F: Images of a *Tg(ubb:mTurquoise2)* F0 (E) and F1 (F) larva showing ubiquitous mTurquoise2 signal.

294 ImPaqT allows for rapid transgene generation through cloning from PCR

295 Within the framework of ImPaqT, insertion elements are PCR amplified for the addition of
296 PaqCI sites, spacer sequences and overhangs that are required for the Golden Gate assembly
297 reaction, and subsequently subcloned into vectors prior to the assembly of the transgene. This
298 subcloning step not only ensures an error-free and stable storage of these constructs but also
299 allows for the generation of a stable library of elements that can be easily combined, re-used
300 and shared within the community. However, the additional cloning step is also more time
301 consuming and, in rare cases where the construct is toxic to bacteria, can result in alteration
302 of parts of the construct. In individual experimental strategies, e.g. for testing new elements or
303 for the single use of individual elements, it may be redundant to subclone and store the PCR
304 product. Instead, the Golden Gate assembly can be performed directly with PCR products,
305 resulting in faster and more flexible transgene generation. To demonstrate, that ImPaqT also
306 allows for rapid creation of functional transgenes by cloning directly from PCR, we assembled
307 *Tg(mpeg1.1:tdTomato-p2A-rac2^{WT})* and *Tg(mpeg1.1:tdTomato-p2A-rac2^{D57N})* by using PCR
308 products directly for the Golden Gate assembly reaction without subcloning into vectors. Rac2
309 is a small GTPase involved in immune cell migration (Salas-Vidal et al. 2005) and dominant-
310 negative mutations of Rac2 including the patient-derived D57N mutation are linked with
311 migration arrest in immune cells (Deng et al. 2011). Here, we used the macrophage-specific
312 *mpeg1.1* promoter to drive tdTomato followed by a self-cleaving peptide and then either Rac2
313 (Rac2^{WT}) or a dominant-negative mutant form of Rac2, Rac2^{D57N}. N-terminal tagging of Rac2
314 is necessary as Rac2 undergoes a C-terminal prenylation event which is required for its
315 membrane localization and activity. As this approach required us to invert our usual N-terminal
316 gene, C-terminal fluorescent protein order, the flexibility afforded by PCR cloning simplified the
317 construction of this swapped gene order. To generate these two transgenes, PCR products
318 encoding the macrophage-specific promoter *mpeg1.1* with overhangs O1 and O2, *tdTomato*
319 with overhang O2 and O3, and p2A with overhang O3 and O3B (CTCG) were combined
320 together with a dsDNA fragment (IDT gBlock) encoding either wildtype or dominant-negative
321 *rac2* with overhangs O3B and O4 (Fig. 6A, S5D-E). These fragments were cloned together

with the already existing backbone vector to generate *Tg(mpeg1.1:tdTomato-p2A-rac2^{WT})* and *Tg(mpeg1.1:tdTomato-p2A-rac2^{D57N})* (Fig. 6A-C). The final transgene construct was injected into embryos at single cell stage. To observe macrophage migration, at 2 dpf embryos were anesthetized and the tip of the tail fin was cut with a scalpel to create a wound. Subsequent imaging demonstrated that while in *Tg(mpeg1.1:tdTomato-p2A-rac2^{WT})* embryos macrophages readily migrated to the wound in the tail fin 2 h post wounding (Fig. 6D), in *Tg(mpeg1.1:tdTomato-p2A-rac2^{D57N})* animals, macrophages were frequently observed throughout the body but were not seen migrating to the tailwound throughout imaging (Fig. 6E). Together, this data provided proof of concept that ImPaqT can be used for rapid generation of functional transgenes and also offered new insights about the utility of this toolkit for immunological research.

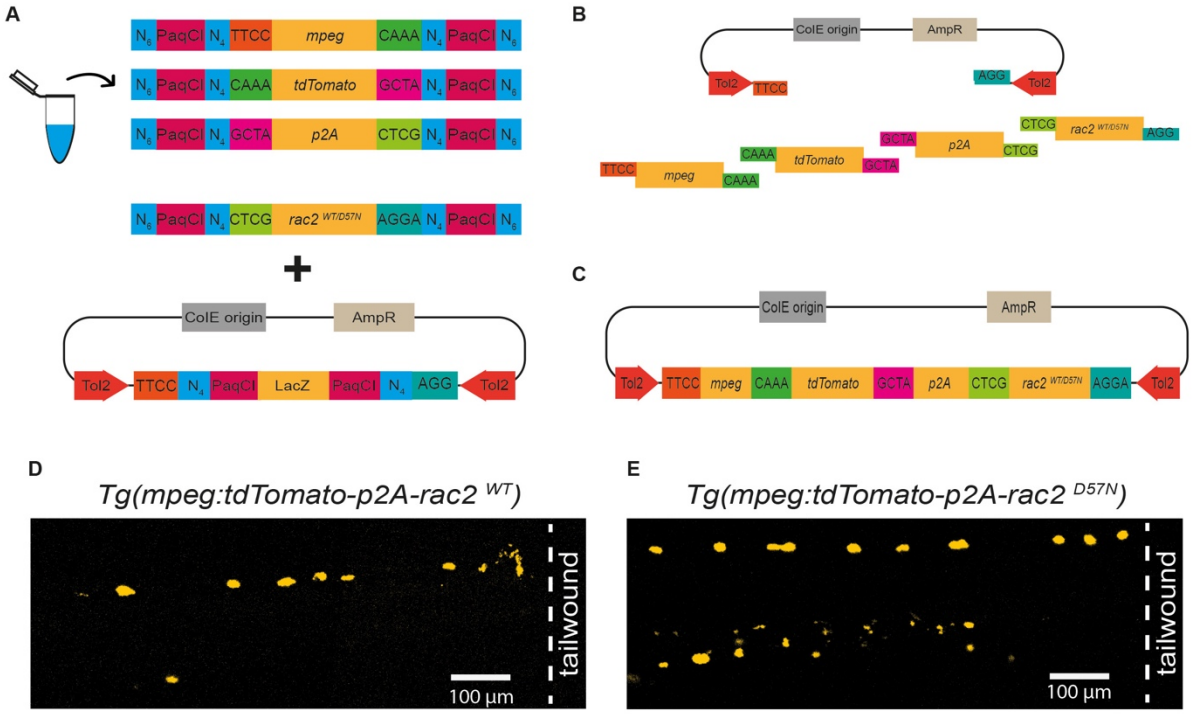


Figure 6: ImPaqT allows for rapid transgene generation by Golden Gate assembly from PCR. A: Schematic of PCR amplified fragments *mpeg1.1*, *tdTomato*, and *p2A* containing PaqCI, N_4 and overhangs O1 (TTCC), O2 (CAAA), O3 (GCTA) and O3B (CTCG) and dsDNA fragment encoding *rac2$^{WT/D57N}$* containing O3B and O4 (AGGA). B: Schematic of the Golden Gate assembly of *mpeg1.1, tdTomato, p2A and rac2$^{WT/D57N}$* into the backbone vector. C: Illustration of the final assembly products *Tg(mpeg1.1.tdTomato-p2A-rac2$^{WT/D57N}$)*. D-E: Images of a *Tg(mpeg1.1.tdTomato-p2A-rac2^{WT})* F0 (D) and *Tg(mpeg1.1.tdTomato-p2A-rac2^{D57N})* F0 (E) larva 2 h post tail wounding showing macrophages.

341 **Discussion**

342 Transgenic animals remain essential for many research questions. They enable the
343 visualization of cells and tissues, molecular pathways, immune responses and the perturbation
344 of these pathways to identify the molecular mechanisms driving pathology. Use of the zebrafish
345 as a model organism has been steadily increasing in many research disciplines due to the
346 unique advantages of this model. This is true in immunology and host-pathogen interactions,
347 where the optical transparency afforded by the zebrafish model have led to many research
348 discoveries, but where, as a new immunology model, there are limited transgenic tools. Here,
349 we demonstrate a new cloning toolkit for the generation of immunological transgenes that is
350 based on Golden Gate cloning. The Golden Gate approach results in shorter overlap regions
351 (4 bp) than recombination-based techniques like Gateway cloning, while compared to
352 seamless, homology based cloning methods such as Gibson cloning, it allows for
353 straightforward modular cloning approaches. The Golden Gate approach also takes advantage
354 of off-the-shelf restriction enzymes and ligase, compared to other cloning approaches which
355 use proprietary enzymes for cloning.

356 We have termed this toolkit ImPaqT - **Im**munological toolkit for **Paq**CI-based Golden Gate
357 Assembly of Tol2 **T**ransgenes. It includes a range of immunologically focused promoters along
358 with genetic tools and fluorescent proteins that can be assembled using PaqCI-based Golden
359 Gate cloning. These tools will enable the flexible assembly of transgenes to drive expression
360 and to genetically manipulate, purify and ablate immune cell populations of interest. While
361 zebrafish possess the same immune lineages of other vertebrates, as a relatively new model
362 for immunology studies, the zebrafish has a more limited array of cell type-specific promoters
363 than mouse models. For instance, despite the identification of potential NK- and ILC-specific
364 (Hernández et al. 2018) as well as mast cell-specific (Dobson et al. 2008) genes, promoters
365 specific for these cell populations have not been described. As new immune-specific promoters
366 are identified, these can be readily added to this toolkit. While we have targeted the immune

367 system as a particular area of need, this Golden Gate approach can also be adapted to any
368 other tissue or cell lineages within the zebrafish.

369 While Golden Gate cloning enables the use of shorter homology stretches and the
370 combinatorial use of constructs for the assembly of diverse transgenes, as a restriction
371 enzyme-based system, a limitation of Golden Gate systems, compared to other assembly
372 approaches, has been the risk of off-target cutting within the cloned sequences. This off-target
373 cutting requires mutation of insert sequences to eliminate these sites (termed domestication)
374 (Marillonnet and Grützner 2020; Bird et al. 2022; Grützner and Marillonnet 2020), which can
375 potentially alter function of domesticated promoters and genes. While domestication can result
376 in changes in the sequence that may alter behavior of the genetic element, these in principle
377 can be managed for genes of interest using silent mutations in critical bases. Commonly,
378 endogenous PaqCI sites can be mutated by PCR amplification of the element using primers
379 designed to incorporate a silent mutation in the PaqCI site (e.g. CACCT**G**C is changed to
380 CACCT**C**C) (Fig. S6A), followed by In-Fusion cloning to reconstitute the construct (Fig. S6B-
381 C). Alternatively, domestication can be performed in other ways, for example by using overlap
382 extension PCR or QuickChange. While promoters cannot be managed through the same
383 approach, promoter fragments used for zebrafish are frequently large (commonly 2-6 kb) and
384 it is unlikely that the mutation of a single base pair will inadvertently ablate the promoter activity.
385 Consistent with this, we have not seen any changes in promoter activity or localization in the
386 two promoters that we domesticated for the ImPaqT toolkit, although further cloning of
387 additional promoters will more completely test this idea. Our choice of PaqCI itself seeks to
388 address the limitations of domestication, as PaqCI uses a 7 base recognition site. In contrast
389 to the 6 base-cutters that have been described in previous zebrafish Golden Gate approaches
390 (Kirchmaier et al. 2013; Jiang et al. 2022), the rare cutting of PaqCI (if random, 1 in 16,384
391 bases) makes this enzyme better suited for the large DNA fragments that are frequently used
392 for zebrafish promoters.

393 As a Golden Gate-based toolkit, ImPaqT introduces only short (4-base) overlap regions
394 between individual elements, in contrast to recombination-based systems like Gateway that
395 require the addition of long (20-25 bp) sequences. However, it must be considered that the
396 addition of 4 extra bases could still potentially lead to side effects on the endogenous functions
397 of promoters or proteins. While it is unlikely that the addition of 4 bases for promoters or 2
398 codons for proteins has detrimental effects, for some proteins that require free C- or N-termini,
399 these methods could be disruptive and other methods of targeting, including internal tagging
400 or transgenesis markers, need to be used. In critical cases, overhang sequences can be
401 designed to match the sequence of the final product, ensuring that there is ultimately no
402 sequence change from the endogenous sequence (Bird et al. 2022) at the cost of reduced
403 flexibility. Similarly in the case of promoters, the 4 bp addition could rarely result in the creation
404 of novel transcription factor binding sites, given the short sequences that are recognized by
405 these proteins. This might be magnified at the transcriptional start site, given the density of
406 regulatory elements here. Importantly, consistent with the relative rarity of these risks to both
407 proteins and promoters, we have not observed any side effects on endogenous functions of
408 promoters or proteins in our study.

409 A limitation of existing systems has been the use of a fixed number of constructs for assembly
410 of the transgene. One benefit of the Golden Gate directed system is that it can be expanded
411 for cloning of complex transgenes, with some Golden Gate assemblies encompassing 20 or
412 even more than 50 fragments (Kucera 2021; Pryor et al. 2022). While we have designed the
413 system to incorporate 4 distinct DNA segments (3 inserts and a backbone vector) the flexibility
414 of Golden Gate cloning means that our system can be readily expanded and contracted while
415 maintaining compatibility with the other components of the kit. As proof-of-concept, we were
416 able to break a single 5E encoding the *ubb* promoter into 3 distinct plasmids that we could
417 subsequently assemble together with existing ME, 3E and backbone elements to generate a
418 functional *ubb* driven transgene. The flexibility of this system will simplify the construction of
419 large and complex transgenes. Potential applications would include breaking up larger
420 promoter fragments into smaller and easier to clone elements, insertion of recombination sites

421 for enzymes like Cre or Flp for rearrangement, assembly of multipromoter constructs encoding
422 genes such as Cas9 and CRISPR gRNAs for cell type-specific knockout, or the inclusion of
423 epitope or localization tags. Our largest reaction comprised 6 fragments (Fig. 5), although in
424 theory, ImPaqT allows for the assembly of even more elements than investigated here. While
425 larger constructs are likely possible, in our experience 6 fragments is a sufficient level of
426 complexity for most transgenes.

427 In addition, we have also showed, that ImPaqT can be used to rapidly generate functional
428 transgenes by Golden Gate assembly of PCR products and larger dsDNA fragments that can
429 be readily ordered from suppliers without the need to subclone insertion elements into vectors
430 prior to the assembly. The Golden Gate assembly of PCR products saves time and can be
431 useful for rapid testing of new elements or for the single use of individual constructs, when
432 long-term storage, multiple uses or sharing is not planned.

433 Lastly, while we have applied this Golden Gate method for the construction of Tol2 transgenes,
434 the advantages of the PaqCI-Golden Gate method will likely be useful for the editing of
435 zebrafish by other approaches as well. For instance, CRISPR insertion has been increasingly
436 used to generate knock-in zebrafish lines, via either homologous recombination, non-
437 homologous methods or by using short homology stretches. Knock-in approaches require the
438 construction of donor vectors which incorporate varied sequences such as homology regions,
439 fluorescent proteins and stretches of genomic DNA (Albadri et al. 2017). For the application of
440 ImPaqT for CRISPR/Cas9-mediated gene editing, we also incorporated the element polyA-
441 gRNA:U6 with a gRNA targeting either a control site (Zhou et al. 2018) or GFP that can be
442 used as either a non-targeting gRNA or to assess the efficiency of gene disruption in GFP-
443 expressing populations with both under the control of the U6 promoter. The construct is
444 included in the library as a 3' element but with overhang sequences CGTA (O3C) and O4 to
445 enable the more complex constructions that are frequently seen in these constructs (e.g. a
446 tissue-specific promoter, cas9, a fluorescent protein and the U6-promoter-gRNA cassette).
447 The gRNA sequences of 3E polyA-gRNA:U6 can easily be exchanged by homology-directed

448 cloning to address new targets. Similarly, many groups also use I-SceI insertion approaches
449 to generate transgenic zebrafish (Hoshijima et al. 2016). The PaqCI Golden Gate method
450 could be adapted to simplify the construction of transgenes and editing constructs for these
451 other approaches as well. Although the Golden Gate approach is similarly efficient to other
452 methods to generate transgenesis constructs, the minimal additional sequence, and the ability
453 of the Golden Gate method to expand and contract as needed provide flexibility that is
454 beneficial for all of these editing and transgenesis methods. Our efforts here have been
455 focused on immune populations, but the toolkit can also be broadened for the use beyond the
456 immune system. In fact, with the addition of new cell-type specific promoters, the tools in
457 ImPaqT can be used in other fields, such as developmental biology, regeneration and
458 neuroscience, in which the zebrafish has been a popular model organism.

459 **Materials and Methods**

460 **Ethics statement**

461 Zebrafish husbandry and experiments were approved by the "Landesamt für Gesundheit und
462 Soziales" (LaGeSo) Berlin (Germany).

463 **Zebrafish husbandry and handling**

464 Zebrafish (*Danio rerio*) embryos were raised in petri dishes containing E3 medium at 28°C for
465 up to five days. Animals older than 5 days were maintained in a circulating system with
466 constant water conditions of 28°C, pH 7.0 - 7.3, and a conductivity of 600-700 µS on a 12-hour
467 light/ 12-hour dark cycle. They were fed twice a day with artemia and once a day with dry food.

468 **Generation of transgenic zebrafish lines**

469 <u>Zebrafish strains</u>

470 All zebrafish strains, including *Tg(lyz:mTurquoise2)*^{ber2}, *Tg(irg1:tdStayGold)*^{ber3}, and
471 *Tg(ubb:mTurquoise2)*^{ber4} were in the *AB wildtype background and generated in this study by
472 applying the Tol2 transgenesis system as previously described (Kawakami et al. 2004).
473 Transient injection of *Tg(irg1:icre-p2A-mTurquoise2)* and *Tg(mpeg1.1:tdTomato-p2A-*

474 *rac2*$^{WT/D57N}$) were also done in *AB background. *Tg(-3.5ubb:LOXP-EGFP-LOXP-mCherry)*^{cz1701}
475 zebrafish were obtained from the European Zebrafish Resource Center (EZRC). *Tg(-*
476 *3.5ubb:LOXP-EGFP-LOXP-mCherry)*^{cz1701} (Mosimann et al. 2011) and *Tg(lyz:EGFP)*^{nz117} (C.
477 Hall et al. 2007) have been previously described.

478 Preparation of the backbone vector and inserts

479 The backbone vector containing the LacZ gene flanked by PaqCI sites and *Tol2* arms in a
480 vector backbone with a pMB1 origin and an AmpR gene for bacterial selection (pTWIST Amp
481 High Copy) was purchased from Twist Bioscience. The complete sequence is shown in Figure
482 S4. Primer sequences are shown in Table S1. Gene fragments for *runx1+23* (with the
483 endogenous PaqCI site mutated), *tdStayGold* and truncated human *LNGFR* (zebrafish codon
484 optimized and with endogenous PaqCI sites mutated) were purchased from Twist Bioscience
485 and TOPO cloned into pCRII TOPO (Thermofisher Zero Blunt TOPO). The sequence for
486 *rac2*^{WT} and *rac2*^{D57N} containing PaqCI sites, spacer sequence (N_4), overhangs O3B and O4 as
487 well as 6-8 additional bases at the 5' and 3' ends (N_{6-8}) and the sequence for the
488 element polyA-gRNA(Control):U6 and polyA-gRNA(GFP):U6 (Addgene Plasmid #107599)
489 containing PaqCI sites, N_4, overhangs O3C and O4 were purchased as gBlocks from IDT. The
490 complete sequences are shown in Figure S5.

491 PaqCI recognition sites with 4-base spacer sequences and 4-base overhangs were added to
492 DNA sequences via PCR amplification. Primer were designed to add PaqCI sites such as
493 cleavage resulted in a gene fragment with a 4-base overhang that is compatible to the
494 overhang of another gene fragment that will be adjacent to it in the final assembly. Appropriate
495 4-base overhang sequences were confirmed using the online tool NEBridge Ligase Fidelity
496 Viewer™. PCR amplification was performed using Q5 polymerase and analyzed by gel
497 electrophoresis. The PCR product was cleaned up or, in case of multiple bands, the band of
498 correct size was excised under 70% UV light using a clean scalpel and DNA was extracted
499 using Nucleo Spin Gel and PCR Clean-up Kit (Macherey-Nagel). DNA sequences were
500 confirmed by sequencing. Gel purified gene fragments were subcloned into a Zero Blunt TOPO

501 vector (Invitrogen) and transformed into competent NEB 5α *E. coli* cells (C2987H). Cells were
502 cultivated in LB medium containing 50 µg/ml Kanamycin and plasmids were isolated using the
503 Nucleo Spin Plasmid Quick Pure protocol (Macherey-Nagel). For the Golden Gate assembly
504 of transgenes from PCR, inserts were PCR amplified with an additional 6-8 bases added to
505 the 5' and 3' end for efficient restriction digestion and Golden Gate assembly directly from
506 PCR.

507 Both the *ubb* and *lck* promoters had endogenous PaqCI cleavage sites in the promoter
508 sequence. To domesticate these promoters, we mutated these PaqCI sites with the indicated
509 PCR primers (Table S1) and assembled them with In-Fusion cloning (Takara Bio).

510

511 Golden Gate based assembly of expression constructs

512 Golden Gate assembly was performed according to the NEB protocol. In detail, 75 ng of the
513 backbone vector and each insertion construct was mixed with 10 U PaqCI, 0.5 µl PaqCI
514 activator (20 µM) and 400 U T4 DNA ligase in T4 DNA ligase buffer to a final volume of 20 µl.
515 Final assembly product was obtained after 60 Cycles of incubation at 37°C for 1 min and
516 incubation at 16°C for 1 min, followed by 5 min incubation at 37°C and 5 min incubation at
517 60°C using a thermocycler. The final product was transformed into competent NEB 5α *E. coli*
518 cells (C2987H) and cells were cultivated in LB medium containing 20 µg/ml Ticarcillin.
519 Plasmids were isolated using the Nucleo Spin Plasmid Quick Pure protocol (Macherey-Nagel)
520 and PCR purified using the Nucleo Spin Gel and PCR Clean-up Kit (Macherey-Nagel) before
521 microinjection.

522 Microinjections

523 The injection mixture contained 25 ng/µL polyadenylated mRNA encoding the Tol2
524 transposase, 50 ng/µL transposon donor plasmid and 10% phenol red in a final volume of 10 µl
525 1 X Tango Buffer (Thermo Fisher). The mMessage mMachine kit (Life Technologies) was used

526 to transcribe the mRNA from T3TS-Tol2 (Balciunas et al. 2006). A volume of ~1 nL was injected
527 into the single cell of fertilized eggs by using a microinjector.

528 Imaging

529 Injected larvae were screened for insertion of the transgene 2 days post injection (dpi) using a
530 Nikon ECLIPSE Ti2 microscope (Nikon) with a Dragonfly 200 confocal system (Oxford
531 Instruments). Animals that were positive for the transgene were raised to adulthood and
532 outcrossed to the *AB wildtype strain for selection of transgenic founder zebrafish. Before
533 screening *Tg(irg1:icre-p2A mTurquoise2)* and *Tg(irg1:tdStayGold)* embryos, the activity of the
534 *irg1* promoter was induced by injection of LPS. For that purpose, animals were anesthetized
535 with tricaine (MS-222) at a final concentration of 0.016% and injected into the caudal vein with
536 LPS (Sigma-Aldrich, F3665) (final: 0.8 mg/ml). Screening was performed at least 4 h after
537 injection. To reduce pigmentation, *Tg(-3.5ubb:LOXP-EGFP-LOXP-mCherry)* embryos were
538 treated with 45 µg/ml 1-phenyl-2-thiourea (PTU) starting ca. 20 h post fertilization. To visualize
539 macrophage migration in *Tg(mpeg:tdTomato-p2A-rac2*$^{WT/D57N}$*)* embryos, animals at 2 dpf were
540 anesthetized with 0.016% tricaine and a piece of the tail fin was cut with a scalpel, followed by
541 imaging 2 h post transection. Image analysis was performed using the Fiji ImageJ software
542 (National Institute of Health, USA).

543 Line and reagent availability

544 All new lines and plasmids are available from the corresponding author upon contact and will
545 also be deposited to AddGene.

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Competing interests

No competing interests declared.

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Data availability

All relevant data can be found within the article and its supplementary information.

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