ImPaqT - A Golden Gate-based Immunological Toolkit for Zebrafish Transgenesis

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Summary statement

Transgenesis is a crucial tool for investigating cellular processes in zebrafish. We describe a new Golden Gate-based toolkit for the assembly of Tol2 transposon vectors for zebrafish immunology research.

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 immunological reagents to enable gene expression within major immune lineages, 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 these cell types. 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 power of this new system by generating a number of novel transgenic immune reporter lines. While our toolkit has 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.

38 **Introduction**

39 The zebrafish (*Danio rerio*) has emerged as a powerful model organism and is increasingly
40 used in various research fields and disciplines. Zebrafish share 70% of their genome and more
41 than 80% of disease genes with humans (Howe et al. 2013). This has led to the development
42 of many distinct zebrafish disease models (Santoriello and Zon 2012) including models of
43 hematopoietic and neurodegenerative diseases, cardiovascular diseases (Bowley et al. 2022)
44 such as atherosclerosis (Tang et al. 2021), cancer (Liu and Leach 2011; Russo et al. 2022),
45 and tuberculosis (Bouz and Al Hasawi 2018; Varela and Meijer 2022), among others.
46 Transgenic animals continue to play a key role for many experimental strategies, including
47 study of gene function, genetic screens, and development of disease models. Transgenic
48 zebrafish lines have been widely generated by applying the Tol2 transposon system with a
49 transgenesis rate of 50% (Kawakami et al. 2004). In this system, a transposon-donor plasmid
50 containing a DNA sequence of interest flanked by *Tol2* sites and synthetic mRNA encoding
51 the Tol2 transposase are introduced into the single cell of fertilized eggs by microinjection.
52 Upon translation, the transposase protein integrates the *Tol2* flanked gene fragment randomly
53 into the genome through a cut-and-paste mechanism without causing rearrangement or
54 modifications at the target site (Kawakami and Shima 1999; Kawakami, Shima, and Kawakami
55 2000; Kawakami et al. 2004; Kawakami 2007).

56 The generation of transgenic animals has become an invaluable tool for studying processes in
57 vivo at the cellular and molecular level. Zebrafish are increasingly chosen as model organism
58 because they are genetically tractable and optically transparent embryos enable live imaging
59 in real time without invasive procedures. While the use of zebrafish models has broadly been
60 increasing throughout science, one growing area of zebrafish research has been the use as
61 an immunological model. Zebrafish possess an immune system with close genetic similarity to
62 humans (Howe et al. 2013). Optically transparent zebrafish larvae have provided many new
63 discoveries on innate immune function through direct visualization of these cell populations in
64 live vertebrate animals. While larvae have been a particular focus of immunology studies, adult

65 zebrafish have also emerged as a useful immunological model – in contrast to larvae, which
66 only possess innate immunity, adult zebrafish have both innate and adaptive arms of immunity,
67 allowing modeling of the full spectrum of vertebrate immune response (Meeker and Trede
68 2008; Iwanami 2014). However, despite the increasing popularity of zebrafish in the field of
69 immunity, there are few resources for generating immunological tools and transgenic lines in
70 these animals.

71 We sought to generate immunological promoters as well as useful tools to facilitate the
72 development of new zebrafish transgenic lines. A number of approaches have previously been
73 used to generate Tol2 transgenesis constructs. Traditionally, this has been multi-step
74 restriction enzyme and ligase based subcloning. However, this has given way to many
75 advanced cloning approaches, including Gateway cloning (Hartley, Temple, and Brasch 2000;
76 Kwan et al. 2007; Katzen 2007), Gibson Assembly (Gibson et al. 2009), In-Fusion cloning (Zhu
77 et al. 2007; Sleight et al. 2010) and Golden Gate cloning, which have been used to speed up
78 and simplify the procedure of transgene assembly. Each of these approaches have different
79 advantages and disadvantages. Gateway cloning allows for the establishment and reuse of a
80 library of fragments (e.g. promoters, genes and fluorescent proteins) but requires the use of
81 lambda phage-derived *att* sequences to facilitate insertion of the desired sequences. These
82 *att* sequences result in the addition of ~20-25 bp of additional sequence in between each
83 element and expansion of the number of fragments to be assembled can require recloning of
84 the sequence with new *att* ends (Katzen 2007; Engler, Kandzia, and Marillonnet 2008; Sone
85 et al. 2008). By contrast, Gibson and In-Fusion cloning allow for joining of short (15-20 bp)
86 regions of homology, enabling the seamless cloning of many transgenes. However, the
87 requirement for homology for seamless cloning generally means that individual components
88 have to be adapted to upstream and downstream elements to generate new transgenes and
89 has an upper limit of approximately 5-6 fragments according to the manufacturers' protocols.
90 Golden Gate cloning utilizes type IIS restriction enzymes to cleave and join multiple fragments
91 in a one pot reaction (Padgett and Sorge 1996; Engler, Kandzia, and Marillonnet 2008). It

92 balances the tradeoffs of the previous cloning systems, requiring inclusion of only a short 4
93 base pair sequence, while enabling researchers to generate a library of vectors that can be
94 combinatorially assembled to generate distinct transgenes. Large numbers of DNA fragments
95 can also be assembled by Golden Gate with reports of assembly of up to 52 fragments in a
96 single reaction (Pryor et al. 2022). Despite the advantages of Golden Gate cloning, this
97 approach has not been widely applied in zebrafish transgene assembly. Only a few examples
98 of this approach have been described for Tol2 transgenesis including the recently described
99 GoldenFish toolkit which uses Golden Gate cloning to gain more flexibility in transgene design
100 (Jiang et al. 2023) and the Golden Gateway tool (Kirchmaier, Lust, and Wittbrodt 2013) which
101 combines the well-established cloning tools Gateway and Golden Gate cloning to simplify
102 creation of new components for Gateway assembly of transgenes.

103 We have developed ImPaqT, a new **Im**munological toolkit for **Paq**CI-based Golden Gate
104 Assembly of Tol2 **T**ransgenes, to facilitate the production of transgenic animals. ImPaqT
105 provides a collection of commonly used immunological promoters targeting distinct lineages of
106 vertebrate immunity, genetic tools and fluorophores supporting flexible generation of
107 transgenes for immunological research. This toolkit utilizes Golden Gate cloning due to the
108 advantages of this technique and incorporates a newly described type IIS restriction enzyme,
109 PaqCI, with a 7 base recognition sequence to reduce the frequency of off target cutting (from
110 1 in 4,096 bases (4^6) for a 6-base cutter to 1 in 16,384 bases (4^7), assuming random cutting).
111 We designed the kit to have consistent overhang sequences for each position in the backbone
112 (e.g. 5', middle, and 3' element) in order to make the toolkit modular and simplify the
113 construction of additional transgenes. As proof-of-concept, we show that this toolkit can
114 facilitate the production of expression constructs and we demonstrate the generation of
115 transgenic animals using the Tol2 transgenesis with these constructs. ImPaqT is modular,
116 enabling easy swapping of gene sequences, easily expandable, allowing the introduction of
117 multiple new genetic elements if needed, and relies only on the use of short (4 bp) overlaps
118 that can also be designed to be scarless if desired. To establish an immunological toolkit that

will be broadly useful to zebrafish researchers, we have generated a variety of immunological and non-immunological promoters, including *mfap4* (macrophages)*, irg1* (macrophages, induced by infection) *, lysC* (neutrophils), *lck* (T cells), *ubb* (ubiquitious) *and hsp70* (heat shock inducible), tools for genetic recombination (*iCre*), cell killing (*kid, nitroreductase)* and cell isolation tools (LNGFR), and an array of fluorescence proteins including m*Turquoise2*, *tdStayGold*, *mCitrine*, *mNeonGreen*, *dLanYFP* and td*Tomato* that can be used to rapidly develop new transgenes and new transgenic lines.

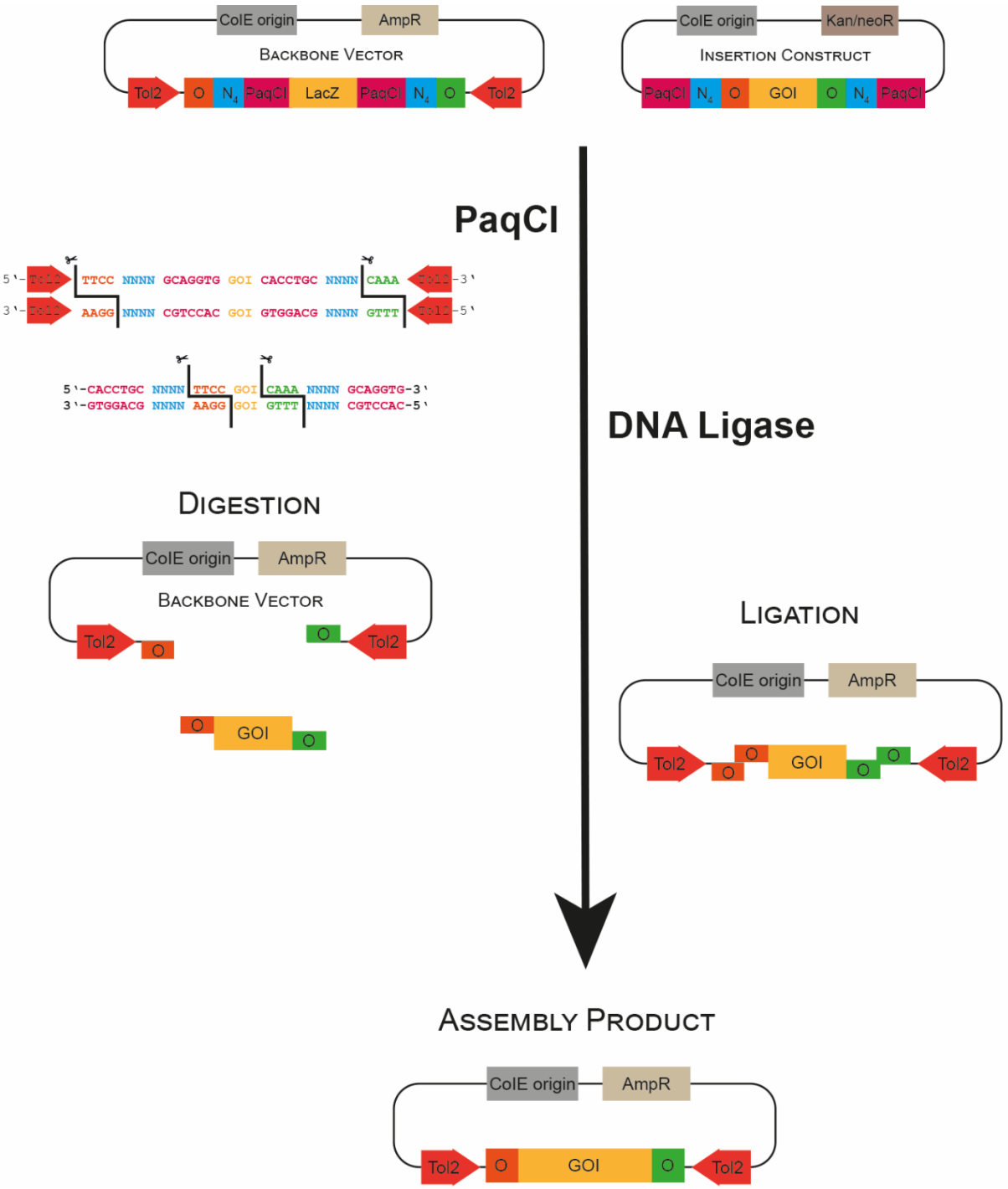
126 **Results**

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

128 We sought to create a toolkit for immune transgene construction for the zebrafish community.
129 Ultimately, we decided to design this toolkit to use Golden Gate cloning, given the short regions
130 of homology that this approach required, the ability to structure this toolkit in a modular fashion
131 and the expandability of the Golden Gate cloning approach to enable incorporation of
132 additional genetic elements as needed. Golden Gate assembly uses type IIS restriction
133 enzymes to generate a set of unique overhangs that are used to join fragments in a desired
134 order. To create a system for modular cloning of transgenes, we chose consistent 4-base
135 overhang sequences for each of the three elements such that DNA fragments amplified with
136 these overhangs can be cloned in the same position in the final construct, allowing these
137 elements to be used combinatorially. As zebrafish transgenes generally consist of a cell-type
138 specific promoter, a gene of interest, and a fluorescent marker, we structured our toolkit to
139 have three elements that would ultimately be joined within a backbone vector possessing Tol2
140 repeats for transgenesis. These elements were named based on their location within the final
141 construct - 5' element (5E) for the first position, a middle element (ME) and a 3' element (3E)
142 for the final position within the backbone.

143 To generate the individual components for Golden Gate assembly, genes and regions of
144 interest were generated by PCR amplification using primers containing the 7-base PaqCI
145 recognition sequence along with a spacer consisting of any four nucleotides that will be excised
146 from the final construct, and a 4-base overhang sequence specific to the location of the
147 sequence of interest in the final construct (Fig. S1). These PCR products were cloned into
148 vectors and sequence verified to generate a stable library of constructs for transgene
149 assembly. Cleavage with PaqCI leads to the creation of specific 4-base overhangs for each
150 position at their 5' and 3' end enabling proper assembly and single-step ligation into a
151 backbone vector for transgene generation. These backbone vectors possess inverted *Tol2*
152 repeats for transgenesis surrounding a PaqCI-flanked LacZ gene for blue-white screening to

153 identify backbone constructs containing transgene components. A simplified schematic for the
154 assembly using one insert is illustrated in Fig. 1. As we demonstrated below, we have found
155 that this approach allows for efficient, combinatorial transgene generation.



156

157 **Figure 1: Basic principles of Golden Gate cloning for Tol2 Transgenesis**
158 Schematic of a Golden Gate cloning reaction using one insert. The insertion construct and backbone
159 vector are assembled in a single-tube reaction using PaqCI and ligase. PaqCI is a type IIS restriction
160 enzyme. Its recognition site and cleavage are indicated. The font color corresponds to the color of the
161 respective feature indicated in the cartoon. The backbone vector consists of Tol2 sites, PaqCI
162 recognition sites (PaqCI) along with 4-base spacer sequences (N_4) and two overhangs (O). The insertion

163 construct includes the gene of interest (GOI) flanked by PaqCI, N_4 and the same two overhangs.
164 Assembly involves digestion of backbone and insertion constructs by PaqCI producing matching
165 overhangs and ligation through the DNA ligase forming the final assembly product.

166

167 ImPaqT facilitates the flexibility of transgenes design and the generation of transgenic
168 zebrafish

169 Having established this Golden Gate cloning-based technique for the assembly of Tol2
170 transposon vectors, we sought to create an array of constructs that would be beneficial to the
171 immunology community to generate transgenic animals. To these ends, we have generated
172 constructs for expression in commonly studied zebrafish immune populations, including T cells
173 (*lck*) (Langenau et al. 2004), neutrophils (*lysC)* (Hall et al. 2007) and macrophages (*mfap4* and
174 the Infection-inducible *irg1*) (Walton et al. 2015; Hall et al. 2013). To enable lineage tracing
175 approaches for labeling of hematopoietic derived cells, we also included the hematopoietic
176 stem cell (HSC)-specific promoter *runx1 +23* (Tamplin et al. 2015) as well as tools for broader
177 expression including the ubiquitious promoter *ubiquitin B* (*ubb*) (Mosimann et al. 2011), and
178 the temperature inducible heat shock protein 70-like promoter (*hsp70*) (Shoji, Yee, and
179 Kuwada 1998; Halloran et al. 2000; Kwan et al. 2007). We have also integrated a number of
180 tools to allow for manipulation of these immune populations, including tools for genetic
181 manipulation (*iCre* (Shimshek et al. 2002)), for rapid purification of cell populations (truncated
182 human *LNGFR* (Wattrus and Zon 2020)) and tools for cell-specific ablation (*nitroreductase*
183 (Curado et al. 2007; Curado, Stainier, and Anderson 2008) and *kid* (Labbaf et al. 2022)). To
184 enable the visualization of labeled cell populations, we provide a diverse array of best-in-class
185 fluorescent proteins in a range of colors to facilitate simultaneous imaging of distinct cell types.
186 These include *tdStayGold* (Hirano et al. 2022), m*Turquoise2* (Goedhart et al. 2012), *tdTomato*
187 (Shaner et al. 2004), m*Citrine* (Zacharias et al. 2002), *mNeonGreen* (Shaner et al. 2013) and
188 *dLanYFP* (Shaner et al. 2013). A summary of all available constructs is shown in Table 1.
189 ImPaqT is also designed to be easily expandable through simple cloning approaches.

190 **Table 1: Library of insertion constructs.**
191 5' element (5E), middle element (ME), 3' element (3E).

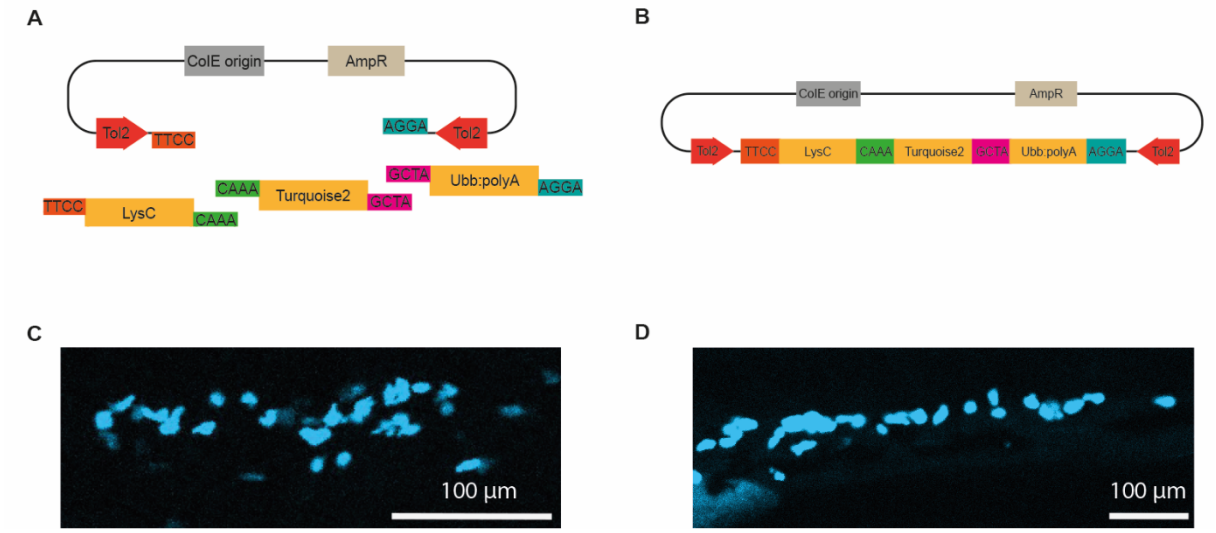
Insertion Elements	Position	Function	Reference
Irg1	5E	Infection-inducible macrophage-specific promoter	(Hall et al. 2013)
Mfap4	5E	Macrophage-specific promoter	(Walton et al. 2015)
LysC	5E	Neutrophil-specific promoter	(Hall et al. 2007)
Ubiquitin B (Ubb)	5E	Promoter for ubiquitious expression	(Mosimann 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, Yee, and Kuwada 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; Curado, Stainier, and Anderson 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)

192

193 Generation of new transgenic zebrafish lines using ImPaqT

194 To test our PaqCI-based Golden Gate assembly toolkit as well as generate new immunological
195 zebrafish lines for the community, we used ImPaqT to assemble a number of new transgenic
196 lines. Using the neutrophil-specific promoter *lysC*, we generated a *Tg(lysC:mTurquoise2)*^{ber2}
197 construct through Golden Gate assembly of 5E *lysC*, ME m*Turquoise2* and 3E *ubb:polyA* to
198 create the *lysC:*m*Turquoise2* transgene construct (Fig. 2A-B). The construct was coinjected
199 with *Tol2* transposase mRNA into single cell of fertilized eggs and at 2 dpi we observed strong
200 blue fluorescence signal within likely neutrophil populations in the CHT (Fig. 2C). Positive
201 animals were raised to adulthood and screening of F1 progeny of these animals revealed
202 robust expression of mTurquoise2 within putative neutrophil populations throughout the animal
203 (Fig. 2D). Crossing to already established neutrophil reporter *Tg(lyz:EGFP)*^{nz117} (Hall et al.
204 2007) fish confirmed specific expression of our transgene within neutrophils (Fig. S2).

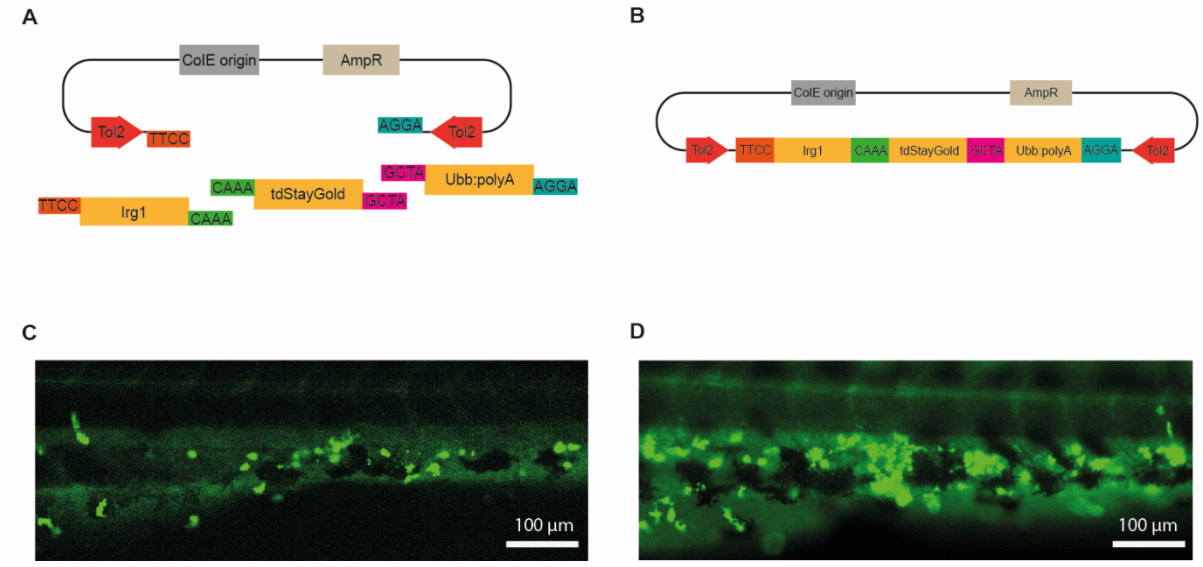


205

206 **Figure 2: Generation of *Tg(lysC:mTurquoise2)* zebrafish**
207 A: Schematic of the Golden Gate assembly of *Tg(lysC:mTurquoise2)*. The backbone vector contains
208 overhang sequences O1 (TTCC) and O4 (AGGA). *LysC* is a neutrophil-specific promoter containing
209 overhang sequences O1 and O2 (CAAA). *mTurquoise2* encodes a bright cyan fluorescent protein with
210 overhangs O2 and O3 (GCTA). *Ubb:polyA* is assembled at the final position within the backbone with
211 O3 and O4.B: Schematic of the final assembly product of *Tg(lysC:mTurquoise2)*. C-D: Images of a
212 *Tg(lysC:mTurquoise2)* F0 (C) and F1 (D) larva showing neutrophil response in cyan at 2 days post
213 fertilization.

214

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



226

227 **Figure 3: Generation of *Tg(irg1:tdStayGold)* zebrafish**
228 A: Schematic of the Golden Gate assembly of *Tg(irg1:tdStayGold)*. The backbone vector contains
229 overhang sequences O1 (TTCC) and O4 (AGGA). *Irg1* is a macrophage-specific promoter containing
230 overhang sequences O1 and O2 (CAAA). *tdStayGold* encodes a bright green fluorescent protein with
231 overhangs O2 and O3 (GCTA). *Ubb:polyA* is assembled at the final position within the backbone with
232 O3 and O4. B: Illustration of the final assembly product of *Tg(irg1:tdStayGold)*. C-D: Images of a
233 *Tg(irg1:tdStayGold)* F0 (C) and F1 (D) larva showing macrophage-specific tdStayGold expression.

234

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

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

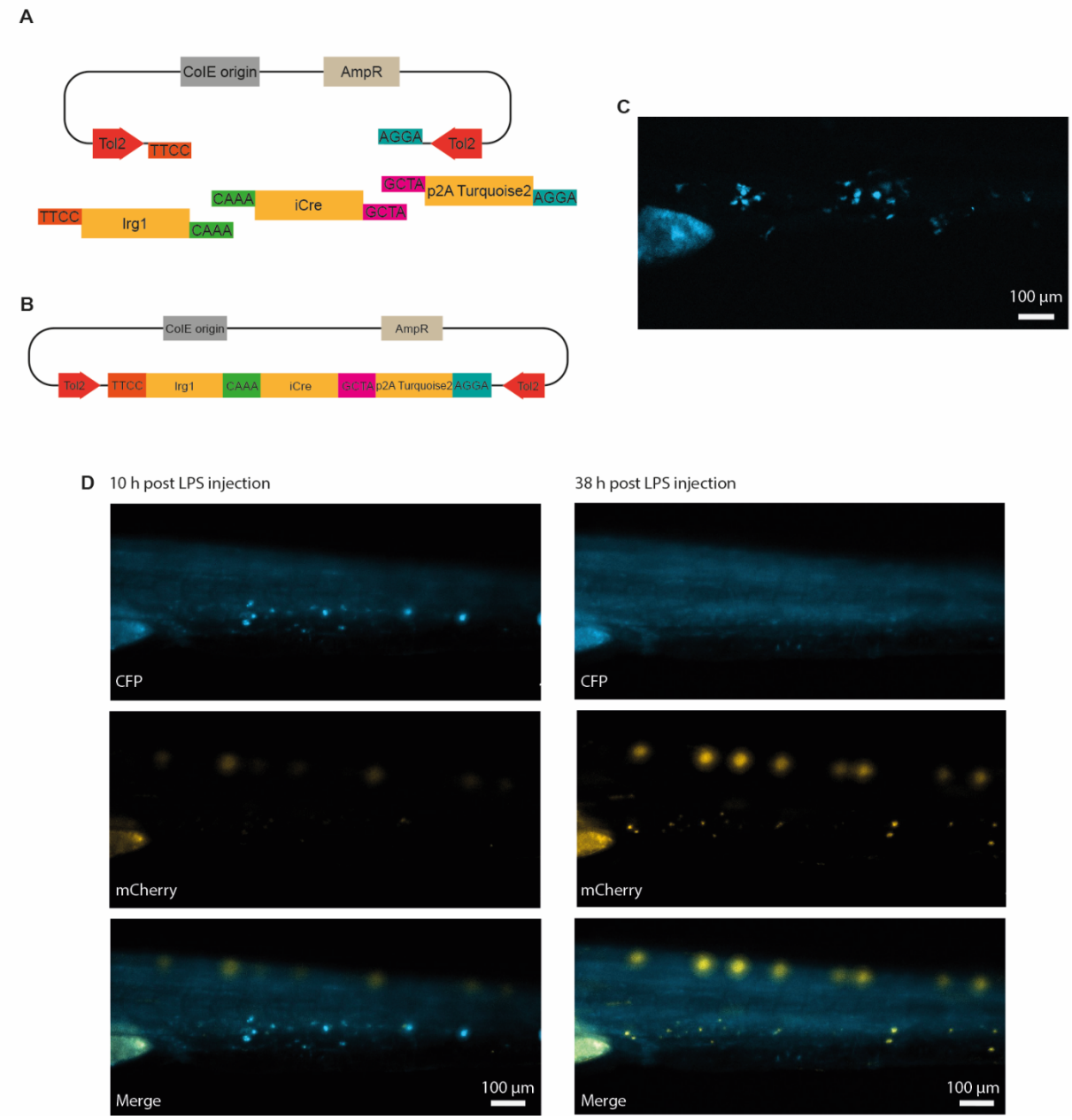


Figure 4: Generation of *Tg(irg1:iCre-p2A mTurquoise)* zebrafish for lineage tracing of individual macrophages

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 larvae showing macrophage-specific mTurquoise2 signal after stimulation with LPS. D: Images in cyan, mCherry and merge of a *Tg(-3.5ubb:LOXP-EGFP-LOXP-mCherry)* larvae 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. 10 h post LPS injection) and a macrophage-specific mCherry signal (e.g. 38 h post LPS injection).

<u>ImPaqT is expandable through the introduction of new overhang sequences</u>

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

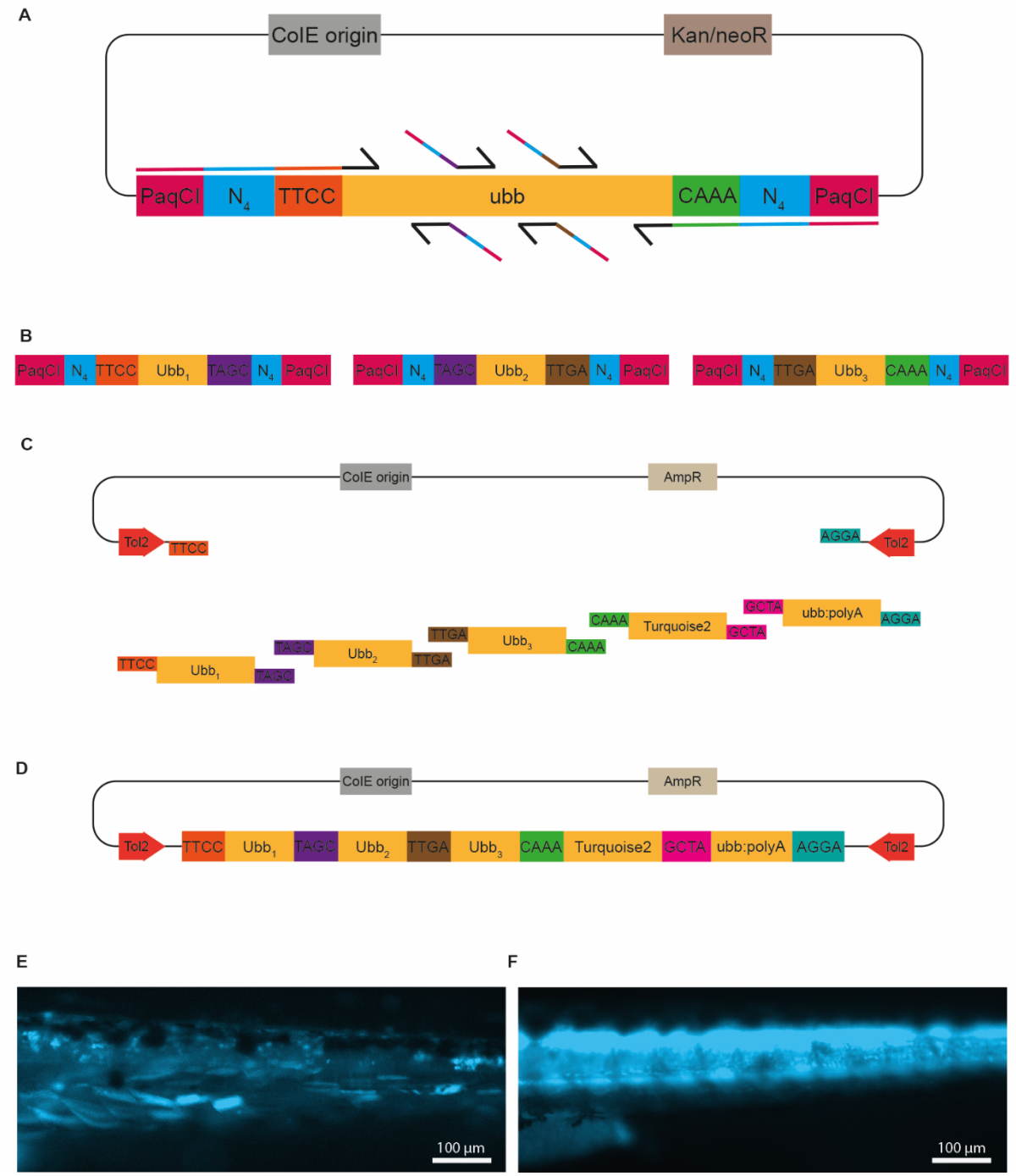


Figure 5: ImpaqT allows for expandability of individual positions while retaining compatibility with already established transposon components.
A: Schematic of the 5E *ubiquitinB* (*ubb*) promoter construct containing PaqCI recognition sites (PaqCI) along with 4-base spacer sequences (N_4) and two overhangs (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* for the middle position 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.

303 **Discussion**

304 Transgenic animals remain essential for many research questions. They enable the
305 visualization of cells and tissues, molecular pathways, immune responses and gene function
306 that drive the critical pathologies that scientists study. Use of the zebrafish as a model
307 organism has been steadily increasing in many research disciplines due to the unique
308 advantages of this model. This is true in immunology and host-pathogen interactions, where
309 the optical transparency afforded by the zebrafish model have led to many research
310 discoveries, but where, as a new immunology model, there are limited transgenic tools. Here,
311 we demonstrate a new cloning toolkit for the generation of immunological transgenes that is
312 based on Golden Gate cloning. The Golden Gate approach results in shorter overlap regions
313 (4 bp) than recombination-based techniques like Gateway cloning, while compared to
314 seamless, homology based cloning methods such as Gibson cloning, it allows for
315 straightforward modular cloning approaches. The Golden Gate approach also takes advantage
316 of off-the-shelf restriction enzymes and ligase, compared to other cloning approaches which
317 use proprietary enzymes for cloning.

318 We have termed this toolkit ImPaqT - **Im**munological toolkit for **Paq**CI-based Golden Gate
319 Assembly of Tol2 **T**ransgenes. It includes a range of immunologically focused promoters along
320 with genetic tools and fluorescent proteins that can be assembled using PaqCI-based Golden
321 Gate cloning. These tools will enable the flexible assembly of transgenes to drive expression
322 and to genetically manipulate, purify and ablate immune cell populations of interest. While
323 zebrafish possess the same immune lineages of other vertebrates, as a relatively new model
324 for immunology studies, the zebrafish has a more limited array of cell type-specific promoters
325 than mouse models. For instance, despite the identification of potential NK- and ILC-specific
326 (Hernandez et al. 2018) and mast cell-specific (Dobson et al. 2008) genes, promoters specific
327 for these cell populations have not been described. Our toolkit contains promoters for the most
328 commonly studied immune lineages in the zebrafish, enabling the generation of transgenes
329 for these diverse populations.

330 While Golden Gate cloning enables the use of shorter homology stretches and the
331 combinatorial use of constructs for the assembly of diverse transgenes, as a restriction
332 enzyme-based system, a limitation of Golden Gate systems, compared to other assembly
333 approaches, has been the risk of off-target cutting within the cloned sequences. This off-target
334 cutting requires mutation of insert sequences to eliminate these sites (termed domestication),
335 (Marillonnet and Grutzner 2020; Bird, Marles-Wright, and Giachino 2022; Grützner and
336 Marillonnet 2020), which can potentially alter function of domesticated promoters and genes.
337 While domestication can result in changes in the sequence that may alter behavior of the
338 genetic element, these in principle can be managed for genes of interest using silent mutations
339 in critical bases. While promoters cannot be managed through the same approach, promoter
340 fragments used for zebrafish are frequently large (commonly 2-6 kb) and it is unlikely that the
341 mutation of a single base pair will inadvertently ablate the promoter activity. Consistent with
342 this, we have not seen any changes in promoter activity or localization in the two promoters
343 that we domesticated for the ImPaqT toolkit, although further cloning of additional promoters
344 will more completely test this idea. Our choice of PaqCI seeks to address these limitations, as
345 PaqCI uses a 7 base recognition site. In contrast to the 6 base-cutters that have been
346 described in previous zebrafish Golden Gate approaches (Kirchmaier, Lust, and Wittbrodt
347 2013; Jiang et al. 2023), the rare cutting of PaqCI (if random, 1 in 16,384 bases) makes this
348 enzyme better suited for the large DNA fragments that are frequently used for zebrafish
349 promoters.

350 A limitation of existing systems has been the use of a fixed number of constructs for assembly
351 of the transgene. One benefit of the Golden Gate directed system is that it can be expanded
352 for cloning of complex transgenes, with some Golden Gate assemblies encompassing 20 or
353 even more than 50 fragments (Kucera 2021; Pryor et al. 2022). While we have designed the
354 system to incorporate 4 distinct DNA segments (3 inserts and a backbone vector) the flexibility
355 of Golden Gate cloning means that our system can be readily expanded and contracted while
356 maintaining compatibility with the other components of the kit. As proof-of-concept, we were
357 able to break a single 5E encoding the *ubb* promoter into 3 distinct plasmids that we could

358 subsequently assemble together with existing ME, 3E and backbone elements to generate a
359 functional *ubb* driven transgene. The flexibility of this system will simplify the construction of
360 large and complex transgenes. Potential applications would include breaking up larger
361 promoter fragments into smaller and easier to clone elements, insertion of recombination sites
362 for enzymes like Cre or Flp for rearrangement, assembly of multipromoter constructs encoding
363 genes such as Cas9 and CRISPR gRNAs for cell type-specific knockout, or the inclusion of
364 epitope or localization tags.

365 Lastly, while we have applied this Golden Gate method for the construction of Tol2 transgenes,
366 the advantages of the PaqCI-Golden Gate method will likely be useful for the editing of
367 zebrafish by other approaches as well. For instance, CRISPR insertion has been increasingly
368 used to generate knock-in zebrafish lines, via either homologous recombination, non-
369 homologous methods or by using short homology stretches. Knock-in approaches require the
370 construction of donor vectors which incorporate varied sequences such as homology regions,
371 fluorescent proteins and stretches of genomic DNA (Albadri, Del Bene, and Revenu 2017).
372 Similarly, many groups also use I-SceI insertion approaches to generate transgenic zebrafish
373 (Hoshijima, Jurynec, and Grunwald 2016). The PaqCI Golden Gate method could be adapted
374 to simplify the construction of transgenes and editing constructs for other zebrafish approaches
375 as well.

376 **Materials and Methods**

377 **Ethics statement**

378 Zebrafish husbandry and experiments were approved by the “Landesamt für Gesundheit und
379 Soziales” (LaGeSo) Berlin (Germany).

380 **Zebrafish husbandry and handling**

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

385 **Generation of transgenic zebrafish lines**

386 <u>Zebrafish strains</u>

387 All zebrafish strains, including *Tg(lysC:mTurquoise2)*^{ber2}*, Tg(irg1:tdStayGold)*^{ber3},*Tg(irg1:iCre-*
388 *p2A mTurquoise2)* and *Tg(ubb:mTurquoise2)*^{ber4}*,* were in the *AB wildtype background and
389 generated in this study by applying the *Tol2* transgenesis system as previously described
390 (Kawakami et al., 2004*). Tg(-3.5ubb:LOXP-EGFP-LOXP-mCherry)*^{cz1701} fish were obtained
391 from the European Zebrafish Resource Center (EZRC). *Tg(-3.5ubb:LOXP-EGFP-LOXP-*
392 *mCherry)*^{cz1701} (Mosimann et al. 2011) and *Tg(lysC:EGFP)*^{nz117} (Hall et al. 2007) have been
393 previously described.

394 <u>Preparation of the backbone vector and inserts</u>

395 The backbone vector containing the LacZ gene flanked by PaqCI sites and *Tol2* arms in a
396 vector backbone with a pMB1 origin and an AmpR gene for bacterial selection (pTWIST Amp
397 High Copy) was purchased from Twist Bioscience. The complete sequence is shown in Figure
398 S4. Primer sequences are shown in Table S1. Gene fragments for *runx1+23* (with the
399 endogenous PaqCI site mutated), *tdStayGold* and truncated human *LNGFR* (zebrafish codon
400 optimized and with endogenous PaqCI sites mutated) were purchased from Twist Bioscience

401 and TOPO cloned into pCRII TOPO (Thermofisher Zero Blunt TOPO). The complete
402 sequences are shown in Figure S5.

403 PaqCI recognition sites with 4-base spacer sequences and 4-base overhangs were added to
404 DNA sequences via PCR amplification. Primer were designed to add PaqCI sites such as
405 cleavage resulted in a gene fragment with a 4-base overhang that is compatible to the
406 overhang of another gene fragment that will be adjacent to it in the final assembly. Appropriate
407 4-base overhang sequences were confirmed using the online tool NEBridge Ligase Fidelity
408 Viewer™. PCR amplification was performed using Q5 polymerase and analyzed by gel
409 electrophoresis. The PCR product was cleaned up or, in case of multiple bands, the band of
410 correct size was excised under 70% UV light using a clean scalpel and DNA was extracted
411 using Nucleo Spin Gel and PCR Clean-up Kit (Macherey-Nagel). DNA sequences were
412 confirmed by sequencing. Gel purified gene fragments were subcloned into a Zero Blunt TOPO
413 vector (Invitrogen) and transformed into competent NEB 5α *E. coli* cells (C2987H). Cells were
414 cultivated in LB medium containing 50 µg/ml Kanamycin and plasmids were isolated using the
415 Nucleo Spin Plasmid Quick Pure protocol (Macherey-Nagel).

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

419 Golden Gate based assembly of expression constructs

420 Golden Gate assembly was performed according to the NEB protocol. In detail, 75 ng of the
421 backbone vector and each insertion construct was mixed with 10 U PaqCI, 0.5 µl PaqCI
422 activator (20 µM) and 400 U T4 DNA ligase in T4 DNA ligase buffer to a final volume of 20 µl.
423 Final assembly product was obtained after 60 Cycles of incubation at 37°C for 1 min and
424 incubation at 16°C for 1 min, followed by 5 min incubation at 37°C and 5 min incubation at
425 60°C using a thermocycler. The final product was transformed into competent NEB 5α *E. coli*
426 cells (C2987H) and cells were cultivated in LB medium containing 20 µg/ml Ticarcillin.
427 Plasmids were isolated using the Nucleo Spin Plasmid Quick Pure protocol (Macherey-Nagel)

428 and PCR purified using the Nucleo Spin Gel and PCR Clean-up Kit (Macherey-Nagel) before
429 microinjection.

430 Microinjections

431 The injection mixture contained 25 ng/µL polyadenylated mRNA encoding the *Tol2*
432 transposase, 50 ng/µL transposon donor plasmid and 10% phenol red in a final volume of 10
433 µl 1 X Tango Buffer (Thermo Fisher). The mMessage mMachine kit (Life Technologies) was
434 used to transcribe the mRNA from T3TS-Tol2 (Balciunas et al. 2006). A volume of ~1 nL was
435 injected into the single cell of fertilized eggs by using a microinjector.

436 Imaging

437 Injected larvae were screened for insertion of the transgene 2 days post injection (dpi) using a
438 Nikon ECLIPSE Ti2 microscope (Nikon) with a Dragonfly 200 confocal system (Oxford
439 Instruments). Animals that were positive for the transgene were raised to adulthood and
440 outcrossed to the *AB wildtype strain for selection of transgenic founder fish. Before screening
441 *Tg(irg1:iCre-p2A mTurquoise2)* and *Tg(irg1:tdStayGold)* embryos, the activity of the *irg1*
442 promoter was induced by injection of LPS. For that purpose, animals were anesthetized with
443 tricaine (MS-222) at a final concentration of 0.016% and injected into the caudal vein with LPS
444 (Sigma-Aldrich, F3665) (final: 0.8 mg/ml). Screening was performed at least 4 h after injection.
445 To reduce pigmentation, *Tg(-3.5ubb:LOXP-EGFP-LOXP-mCherry)* embryos were treated with
446 45 µg/ml 1-phenyl-2-thiourea (PTU) starting ca. 20 h post fertilization. Image analysis was
447 performed using the Fiji ImageJ software (National Institute of Health, USA).

448 Line and reagent availability

449 All new lines and plasmids are available from the corresponding author upon contact.

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455 **Competing interests**

456 No competing interests declared.

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459 **Data availability**

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

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