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# **A medium-scale assay for enhancer validation in amniotes**

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**Key words:** conserved regulatory elements, electroporation, green fluorescent protein (GFP), otic placode, PCR, regulatory regions, reporter plasmid

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1 **Abstract**  
2

3 **Background:** Enhancers are key elements to control gene expression in time and space and thus  
4 orchestrate gene function during development, homeostasis and disease. Whole genome  
5 approaches and bioinformatic predictions have generated a tremendous pool of potential  
6 enhancers, however their spatiotemporal activity often remains to be validated *in vivo*. Despite  
7 recent progress in developing high throughput strategies for enhancer evaluation, these remain  
8 mainly restricted to invertebrates and *in vitro* cell culture.

9 **Results:** Here we design a medium-scale method to validate potential enhancers in an amniote  
10 embryo, the chick. Using a unique barcode for different reporter vectors allows us to detect the  
11 activity of nine separate enhancers in a single embryo by one-step RT-PCR. The assay is  
12 sufficiently sensitive to expand its capacity further by generating additional barcoded vectors.

13 **Conclusion:** As a rapid, sensitive and cost-effective way to assess enhancer activity in an amniote  
14 vertebrate, this method provides a major advance and a useful alternative to the generation of  
15 transgenic animals.  
16

## 1 **Introduction**

2 Enhancers are *cis*-regulatory DNA sequences, which increase the expression of their target genes.  
3 They are usually located distal to the transcription start site (TSS), but are also found within introns  
4 or downstream; they function irrespective of their orientation, distance and location with respect to  
5 the TSS. Enhancers harbour a high density of transcription factor binding sites, and their  
6 interacting factors are thought to enhance transcription by interacting with the general  
7 transcriptional machinery in the promoter region. The transcription of most genes is regulated by  
8 multiple enhancer elements. Their dynamic activity ensures accurate spatial-temporal expression  
9 of their targets during development, homeostasis and disease and other biological processes.  
10 Recent evidence suggests that enhancer regions are flanked by “active marks” on histone tails like  
11 H3K27ac (Creighton et al., 2010; Ernst and Kellis, 2010) and H3K4me1 (Cui et al., 2009;  
12 Heintzman et al., 2009; Ernst and Kellis, 2010), as well as being associated with the histone  
13 acetyltransferase P300 (Heintzman et al., 2007; Visel et al., 2009). In addition, enhancers are  
14 depleted of nucleosomes to provide access for interacting transcription factors (He et al., 2010;  
15 Andreu-Vieyra et al., 2011). Based on these features, chromatin immunoprecipitation using  
16 antibodies against H3K27ac, H3K4me1 and/or P300 followed by sequencing, DNase  
17 hypersensitivity assays, Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE)-seq or  
18 their combination have identified tens of thousands of potential enhancers in developing organs e.g.  
19 the heart (May et al., 2012), in embryonic stem cell derived neural crest cells (May et al., 2012;  
20 Rada-Iglesias et al., 2012), and in different human and murine cell human types. The Encode  
21 project has contributed a large collection of such enhancers (Consortium, 2012).

22  
23 Despite the large number of genome-wide results, not all enhancers have been identified and the  
24 tissue-specificity of many enhancers remains unknown, while others turn out to be false positive  
25 (Bonn et al., 2012). To validate a large number of candidate enhancers, low-cost and time-effective  
26 methods are necessary. Traditionally, enhancer activity is assayed using transgenic approaches in  
27 *Drosophila* (McCall et al., 1994), zebrafish (Parinov et al., 2004; Bessa et al., 2009) or mouse  
28 (Pennacchio et al., 2006) where reporter constructs are introduced, in which candidate enhancers  
29 are cloned upstream of a minimal promoter followed by a reporter like fluorescent proteins or  $\beta$ -  
30 galactosidase. For example, the activity of 1154 enhancers was validated in different organs during  
31 embryonic development and deposited in the VISTA Enhancer Browser (<http://enhancer.lbl.gov/>).  
32 Although this approach provides high spatiotemporal resolution of enhancer activity using e.g.  
33 fluorescent imaging, generation of transgenic animals is labour intensive and costly, and thus not  
34 ideally suited for large-scale enhancer validation. Luciferase reporter assays in cell culture  
35 (Nordeen, 1988) are more efficient, however may not recapitulate *in vivo* enhancer activity.

36  
37 Recently, several methods for large-scale enhancer validation emerged, but none was applied to  
38 vertebrates due to the bottleneck of generating transgenic animals. Massive parallel reporter

1 assays have recently been developed to allow simultaneous analysis of thousands of reporter  
2 plasmids (Patwardhan et al., 2012; Kheradpour et al., 2013). In this assay, the candidate enhancer  
3 is placed upstream of a minimal promoter with a unique DNA barcode downstream and a pool of  
4 reporter plasmids is introduced into cells. Barcode-containing transcripts are then sequenced and  
5 quantified by deep sequencing. This method provides a powerful tool to dissect the functional  
6 nucleotides or motifs within identified enhancers. However, the length of DNA fragments that can  
7 be analysed is limited, because the method relies on chemical synthesis to generate them. A  
8 similar strategy is employed in sea urchin, where many eggs can easily be injected with a pool of  
9 reporter constructs; embryos are screened for fluorescence and the expressed barcodes are  
10 quantified by NanoString and RT-qPCR (Nam and Davidson, 2012). Finally, a large-scale *in vivo*  
11 method was recently reported in *Drosophila melanogaster* (Gisselbrecht et al., 2013). Transgenic  
12 flies are generated with a pool of enhancer-EGFP constructs. Transgenic animals containing GFP  
13 expressing cells are then crossed with lines harbouring cell-type-specific markers. Double positive  
14 cells are selected by FACS and the genomic DNA isolated for deep sequencing to identify cell-  
15 type-specific enhancers. Despite these successes, it is difficult to apply these methods to  
16 vertebrate embryos due to the difficulty to generate transgenic animals with a pool of large number  
17 of different DNA molecules.

18  
19 Here we developed a customised method for rapid enhancer validation using the chick as an  
20 amniote model system. The chick embryo is easily accessible, cost efficient and lends itself to  
21 widespread electroporation for gene transfer and rapid analysis of reporter activity within a few  
22 hours after electroporation (Uchikawa et al., 2003; Barembaum and Bronner-Fraser, 2010; Sato et  
23 al., 2010; Betancur et al., 2011). Chick embryos can be grown *in ovo* as well as outside the egg,  
24 and are therefore adaptable for different experimental paradigms. Using a barcoding strategy, we  
25 developed vectors to test up to 9 putative enhancers in a few hundred cells in a single embryo. The  
26 method can easily be expanded to provide additional barcoded vectors if required. The entire  
27 procedure is time- and cost- effective, involving electroporation of plasmid DNA into the chick  
28 followed by one-step RT-PCR. Results can easily be obtained within two days without expensive  
29 reagents and equipment. Assuming that 5-10 different assays can be performed in parallel, this  
30 strategy makes the validation of hundreds of putative enhancers possible in amniotes in a relatively  
31 short time at low cost.

32

## 33 **Results**

### 34 **Modification of the pTK-EGFP vector**

35 In chick, gene transfer is easily achieved using a several square pulses of low voltage to  
36 electroporate plasmid DNA into target cells. Depending on size and shape of the electrodes  
37 electroporation leads to widespread expression of the transgene. Traditionally, detection of several  
38 enhancer constructs in a single embryo is limited by the availability of different fluorescent reporter

1 proteins and relatively high concentrations of plasmid DNA are required for detection by fluorescent  
2 microscopy. To increase the capacity, sensitivity and efficiency of enhancer validation, we modified  
3 one of the standard reporter vectors, the pTK-EGFP vector which is widely used in chick  
4 (Uchikawa et al., 2003). The original vector contains a minimal TK promoter, the first exon  
5 transcribing a 5'UTR, followed by an intron and a second exon which encodes EGFP (Fig. 1).  
6 Potential enhancers are inserted into the multiple cloning site (MCS) upstream of the minimal  
7 promoter. We introduced two important modifications in this vector. First, we introduced a barcode  
8 to generate 9 different vectors: we replaced 16 nucleotides (nt) upstream of the first exon-intron  
9 junction with 9 different 16 bp barcodes, thus generating 9 vectors each containing a unique  
10 barcode (Fig. 1 and Table 1).

11  
12 Second to facilitate the insertion of potential enhancers, we modified the MCS. Because potential  
13 enhancers are usually cloned by PCR using Taq DNA polymerase, which produces a 3' A overhang,  
14 we inserted two *XcmI* restriction sites into the MCS (Fig. 1). *XcmI* digestion linearises the vector  
15 and produces 3' T overhangs compatible with T/A cloning. This allows fast cloning of PCR products  
16 directly into the reporter vector. In addition, unique *EcoRV* and *SpeI* sites were also introduced,  
17 allowing more choice for cloning.

18

### 19 **Detection of enhancer activity *in vivo***

20 To assay enhancer activity after electroporation we designed an RT-PCR strategy that detects  
21 barcode-specific transcripts driven by the potential enhancer. Transcripts from each reporter vector  
22 are detected by a barcode-specific forward primer and a common reverse primer. To distinguish  
23 amplicons from RNA transcripts and plasmid DNA, the forward primers span the intron: each  
24 primer matches the barcodes plus an extra 4 nucleotides downstream of the intron-exon junction  
25 (Fig. 1; blue and green line). The same reverse primer located within the EGFP coding region is  
26 used in combination with each forward primer. Thus, only RNA transcripts targeted by barcode-  
27 specific and common reverse primers will be amplified to produce a 129 bp long product.

28

29 To test the method, we cloned various known otic enhancers: Sox10E (Betancur et al., 2011),  
30 Spalt4F14 (Barembaum and Bronner-Fraser, 2010) and mSix1-21 (1x, 2x, and 4x; Sato et al., 2010)  
31 into vectors containing different barcodes. These five enhancer-containing plasmids were mixed  
32 with four barcoded empty vectors as negative control, each at a final concentration of 0.2 µg/µl.  
33 The plasmid pool was electroporated together with a control plasmid, which expresses RFP driven  
34 by the ubiquitous β-actin promoter, into the chick head ectoderm at HH6. At HH10-12, RFP  
35 expression is observed in a large domain, while EGFP expression is confined to the otic placode  
36 driven by the known otic enhancers (Fig. 2). The otic placode was dissected (Fig. 2C, square),  
37 RNA isolated and 2.0 ng RNA were used for RT-PCR with unique barcode and common reverse  
38 primers. Gel electrophoresis reveals that known positive enhancers produce a clear PCR product

1 at around 129 bp (Fig. 3A, lane 1, 2, 3, 4 and 8), while the negative control plasmids do not (Fig.  
2 3A, lane 5, 6, 7, and 9). Weak unspecific bands above 500 bp are observed in both enhancer  
3 containing and negative control plasmids (Fig. 3A), however they can clearly be distinguished from  
4 expected product of ~129 bp.

5  
6 To test whether negative enhancers produce false positive signal in the assay, we cloned two  
7 neural tube-specific enhancers, Sox2-N2 and Sox2-N4 (Uchikawa et al., 2003) into vectors with  
8 different barcodes. Both plasmids were mixed with the five plasmids containing otic enhancers and  
9 two barcoded empty vectors. Otic placodes were dissected at HH10-12 and processed as  
10 described above. Consistently, known otic enhancers produced barcode-specific transcripts as  
11 detected by RT-PCR (Fig. 3B, lane 1-5), while non-otic enhancers did not produce any specific  
12 signal (Fig. 3B, lane 8 and 9).

13  
14 To test whether this assay is useful for dispersed cells and other tissues, we cloned neural crest  
15 (FoxD3-NC1/NC2, Sox10E (Simoes-Costa et al., 2012; Betancur et al., 2011) and neural tube  
16 enhancers (Sox2-N1/N2/N3; Uchikawa et al., 2003) into our barcoded vectors. These constructs  
17 were electroporated into the cranial ectoderm at HH6 together with Six1-21 1x/2x/4x (Sato et al.,  
18 2012). As expected, the expression of EGFP was observed in both the neural tube and the neural  
19 crest at HH10 (Fig. 4A-D) and in the otic placode where Sox10E and Six1-21 are active (Fig. 4A-D;  
20 (Sato et al., 2012). We then dissected the head region rostral to the hindbrain (Fig. 4e) and the otic  
21 placodes (Fig. 4f) and assessed enhancer activity by RT-PCR. In the head region,  
22 FoxD3\_NC1/NC2, Sox2-N2/N4, and Sox10E are positive (Fig. 4E, lanes 1-5) showing that  
23 enhancer activity can be detected even in dispersed cells. In addition, we observe a 129 bp band  
24 for Six1-21 2x/4x due to their weak activity in the olfactory placode (Fig. 4E, lanes 8-9; Sato et al.,  
25 2012) demonstrating that this method is sufficiently sensitive to detect activity in a small proportion  
26 of tissue. In contrast, Sox2-N3 is not active at this stage (Fig. 4E, lane 6) in accordance with  
27 published data (Uchikawa et al., 2003). When the otic placode was assayed, Sox10E and Six1-21  
28 (1x/2x/4x) were detected as positive (Fig. 4F, lanes 2, 8, 9; see also Fig. 3). In addition, FoxD3-  
29 NC1/NC2 also produced a weak signal (Fig. 4F, lanes 1 and 5) presumably due to the presence of  
30 few migrating neural crest cells surrounding the otic placode. Consistent with our previous results,  
31 Sox2-N2/N4 (see Fig. 3B, lanes 8 and 9) and Sox2-N3 are inactive in the otic placode (Fig. 4F,  
32 lanes 3, 4, 6; see Uchikawa et al., 2003) .

33  
34 In summary, the strategy described here allows the detection of enhancers that are active in small,  
35 dissected tissue samples (otic placode) and in a mixture of different tissues (neural tube, neural  
36 crest, head mesenchyme). It is sufficiently sensitive to capture enhancer activity in dispersed cells  
37 (neural crest cells) and in a small proportion of cells (olfactory placode).

38

## 1 **Sensitivity of enhancer detection *in vivo***

2 To assay many enhancer constructs in the same tissue it is essential to detect low amounts of  
3 transcripts. To assess the sensitivity of the assay we diluted the Sox10E plasmid to a final  
4 concentration of 2.0 µg/µl, 0.2 µg/µl and 0.02 µg/µl, and electroporated the plasmid into the otic  
5 placode as described above. *In vivo*, intense otic EGFP expression is observed using the two top  
6 concentrations of Sox10E plasmid, whereas plasmids at 0.02 µg/µl yields virtually no detectable  
7 EGFP (Fig. 5).

8

9 Next, we used RT-PCR to assess the sensitivity of our method. First, we isolated total RNA from  
10 the EGFP+ otic placode after electroporation of 2.0 µg/µl plasmid; 2 ng, 0.2 ng and 0.02 ng RNA  
11 were used for RT-PCR. A 129 bp band was detected with the lowest amount of RNA (Fig. 5). Using  
12 electroporation of 0.2 µg/µl plasmid required at least 0.2 ng RNA to yield a detectable signal (Fig.  
13 6). Consistent with the faint EGFP expression after electroporation of 0.02 µg/µl plasmid DNA, only  
14 a very faint band was observed after RT-PCR (Fig. 6). Based on these observations, we suggest  
15 that individual reporter plasmids for electroporation should at least have a concentration > 0.02  
16 µg/µl. In order to detect some weak enhancers, higher concentration such as 0.1~0.2 µg/µl will be  
17 required.

18

## 19 **Discussion**

20 Understanding the regulation of gene expression is central to understanding many biological  
21 processes including the mechanisms that control development, disease and tissue regeneration.  
22 Non-coding regions of the genome harbour enhancer elements, which are often conserved across  
23 species and provide key elements that regulate cell and tissue specific gene expression. Over the  
24 last decade, new bioinformatics approaches have become powerful tools to predict regulatory  
25 regions, while genome wide experimental approaches have led to the discovery of vast numbers of  
26 putative enhancer elements. These findings provide a good resource to uncover gene regulatory  
27 networks that underlie both development and disease, however verification of the *in vivo* activity of  
28 such enhancers still remains the bottleneck in amniote species. While large-scale *in vivo* enhancer  
29 validation has been very successful in invertebrates like *Drosophila* and sea urchin (Nam and  
30 Davidson, 2012; Gisselbrecht et al., 2013), progress has been slower in amniote species due to  
31 laborious process to generate transgenic animals. Recently, parallel sequencing was employed to  
32 detect the activity of thousands of short 100-200 bp DNA sequences (Patwardhan et al., 2012;  
33 Kheradpour et al., 2013). Despite of this impressive capacity, this approach is mainly applied to  
34 cultured cells *in vitro*.

35

36 Therefore, it is desirable to develop a rapid, cost effective medium throughput strategy to test  
37 enhancer activity in amniotes. The chick provides a good developmental system, because it is  
38 easily accessible at many stages, lends itself to *in vivo* manipulation including electroporation of

1 reporter constructs and results can be obtained within a few days. Traditionally, fluorescent  
2 proteins like eGFP, RFP, CFP or YFP are used as reporters, thus limiting the number of enhancers  
3 that can be tested in a single embryo to a few. Here, we have combined established  
4 electroporation approaches with a barcoding system that identifies transcripts specific to each  
5 enhancer. Specifically, to distinguish electroporated plasmid DNA from the enhancer-driven  
6 transcript the barcode sequence is separated from the GFP reporter sequence through one intron  
7 (see Fig. 1). This medium throughput strategy allows the detection of up to nine different  
8 enhancers in tissue collected from only one or two embryos, in this case from two otic placodes  
9 (approximately 2000–3000 cells yielding around 20 ng total RNA). It is reasonably sensitive given  
10 that transcripts can be detected from plasmids electroporated at a concentration of only 0.02 µg/µl,  
11 and importantly there is virtually no background for enhancers that are inactive. We expect that in  
12 future this approach can be scaled up further to detect the activity of 15-20 reporter constructs.

13  
14 Electroporation of plasmid DNA at very high concentration can cause malformations in the embryo.  
15 In our experience, the concentration should be less than 6 µg/µl, with an optimum of 2~3 µg/µl.  
16 Even under this condition, it is possible to increase the throughput further by increasing the number  
17 of electroporated cells. For example, compared to the small size of the otic placode used here, a  
18 much larger area can be targeted in the developing neural tube. In this case, if total RNA recovered  
19 is increased to about 500 ng, using ~10 ng RNA for each PCR reaction will allow testing about 50  
20 barcoded enhancer plasmids per assay. Since results can generally be obtained within two days  
21 after electroporation, this strategy provides a rapid, cost efficient and scalable method to test  
22 enhancer activity in the living embryo and quickly select enhancers for further investigation.

23  
24 The method described here is also useful to examine enhancer activity over time as different  
25 tissues can be harvested at different developmental stages. Plasmid-driven expression of a  
26 transgene is generally observed for about two days or more (Nakamura and Funahashi, 2001),  
27 allowing the examination of enhancers throughout this period. However, as cells divide plasmids  
28 are diluted. Therefore, to assay enhancer activity at late developmental stages stable integration of  
29 reporters using systems like Tol2 transposition (Sato et al., 2007) is required and our barcode  
30 strategy can be adapted using suitable vectors. In this case, transfection is carried out at early  
31 stages to target a large number of cells and embryos are cultured *in ovo* to test enhancers later.

32  
33 Here, we use electroporation for embryo transfection, a method widely used to target epithelial  
34 tissues like the early epiblast or ectoderm before or during gastrulation (EGX-XIV, HH2-8; Cui et al.,  
35 2006; Voiculescu et al., 2008), mesoderm and endoderm before ingression (HH2-5; Sweetman et  
36 al., 2008; Voiculescu et al., 2008), the neural tube (HH9 onwards; Sakamoto et al., 1998;  
37 Nakamura et al., 2000; Croteau and Kania, 2011) or somites (HH9-18; Scaal et al., 2004). Thus,  
38 enhancer activity in cells and tissues derived from these regions can be validated by our assay.

1 However, different tissues will require different transfection strategies including lipofection, which  
2 has been used for the hypoblast a pre-gastrulation stages (Albazerchi et al., 2007), or  
3 sonoporation, which has successfully been used for example for limb mesenchyme (Ohta et al.,  
4 2008). The vectors described here are suitable for these transfection approaches.

5  
6 In summary, we have developed a relatively quick and sensitive assay to detect the activity of  
7 predicted enhancers in an amniote model, the chick embryo. This allows efficient validation of  
8 enhancers acquired from bioinformatics predictions and genome-wide experiments such as  
9 CHIPseq.

## 11 **Experimental procedures**

### 12 **Plasmid modification**

13 The 16 bp DNA barcodes were inserted into the original pTK-EGFP vector (Uchikawa et al., 2003)  
14 by PCR to replace the original 16 nucleotides at the 3' end of exon1 (Fig. 1) with a 16 bp barcode  
15 following the method as described (Li et al., 2011). PCR primers are listed in Table 1. PCR reaction  
16 was set up containing 10 ng pTK-EGFP vector as template, 10 µl 5X Phusion HF buffer (NEB), 0.5  
17 µl phusion DNA polymerase (NEB), 5 µM primer pair, 200µM dNTPs and water up to 50 µl reaction  
18 volume. The cycling conditions were 98°C for 30 seconds followed by 20 cycles of 98°C for 10  
19 seconds, 60°C for 20 seconds, and 72°C for 100 seconds, and 72°C for 10 minutes. 1 µl DpnI was  
20 added to the PCR product and the reaction was incubated for 30 minutes at 37°C to digest the  
21 original vector. 3µl PCR product was transformed into the DH5α competent cells. To introduce the  
22 modified multiple cloning site, the original pTK-EGFP vector was digested with *KpnI* and *XhoI*, and  
23 annealed double oligonucleotides containing sequences of the new multiple cloning sites (sense  
24 strand: 5'- CCATGGATATCATGGCCAACTGACTAGTGGC-3', antisense strand: 5'-  
25 TCGAGCCACTAGTCAGTTGGCCATGATATCCATGGGTAC-3') were ligated to the linearized  
26 vector. All constructs were verified by sequencing.

### 28 **Electroporation and embryo culture**

29 Fertilized hens' eggs (Winter farm) were incubated at 38° until they had reached stage HH 6  
30 (Hamburger and Hamilton, 1951). Embryos were collected in Tyrode's saline on filter paper rings  
31 (Chapman et al., 2001) and electroporated using five 50 ms pulses of 4 V, at an interval of 750 ms  
32 using an OvoDyne electroporator (IntraCel). For electroporation, barcode-containing plasmids (final  
33 concentration of 0.2 µg/µl each) were mixed with control plasmid (pActB-RFP; 1.0 µg/µl) and 0.1%  
34 fast green. Embryos were cultured until HH9-10 for assaying the enhancers of cranial neural tube  
35 and neural crest, and HH10-12 for assaying enhancers of otic placode. For the cranial neural tube  
36 and neural crest, the entire head region rostral to the hindbrain was collected. For the otic placode,

1 fluorescent placodes were freed from underlying mesoderm and dissected from the ectoderm  
2 using steel needles..

3

#### 4 **One-step RT-PCR**

5 RNA was extracted with RNAqueous micro total RNA isolation kit (AM1931, Life Technologies)  
6 following the manufacturer's instruction, and eluted in 20 µL elution buffer. One-step RT-PCR was  
7 performed using a Qiagen one-step RT-PCR kit (210212, Qiagen) in a total volume of 10 µl with  
8 primers listed in Table 2. PCR was performed with 35 cycles following the manufacturer's protocol.  
9 RT-PCR was set up using 2µl total RNA, 2 µl 5x RT-PCR buffer, 0.2µM dNTPs, 0.6 µM primer pair,  
10 0.4 µl enzyme mix, 5U Recombinant RNasin® Ribonuclease Inhibitor; water was added up to 10 µl  
11 reaction volume. The cycling conditions were as follows: 50°C for 30 minutes, 95°C for 15 minutes,  
12 and 35 cycles of 94°C for 30 seconds, 65°C for seconds, 72°C for 30 seconds, and 72°C for 10  
13 minutes. PCR products were analysed on 1.5% agarose gels and imaged.

14

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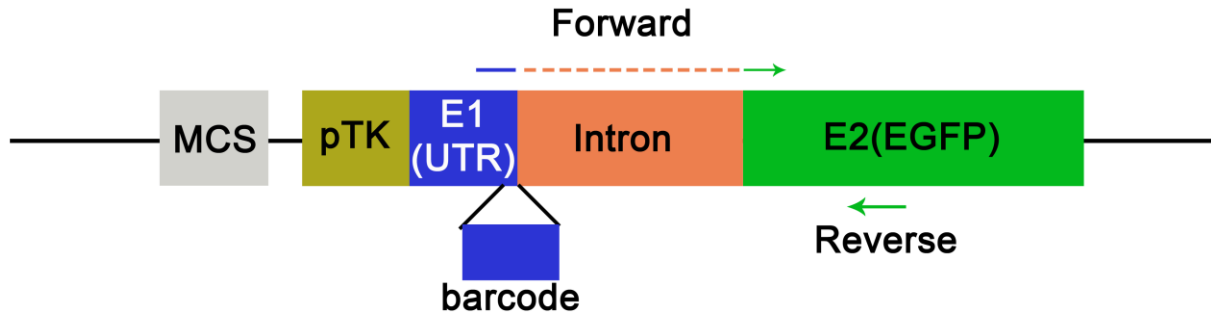
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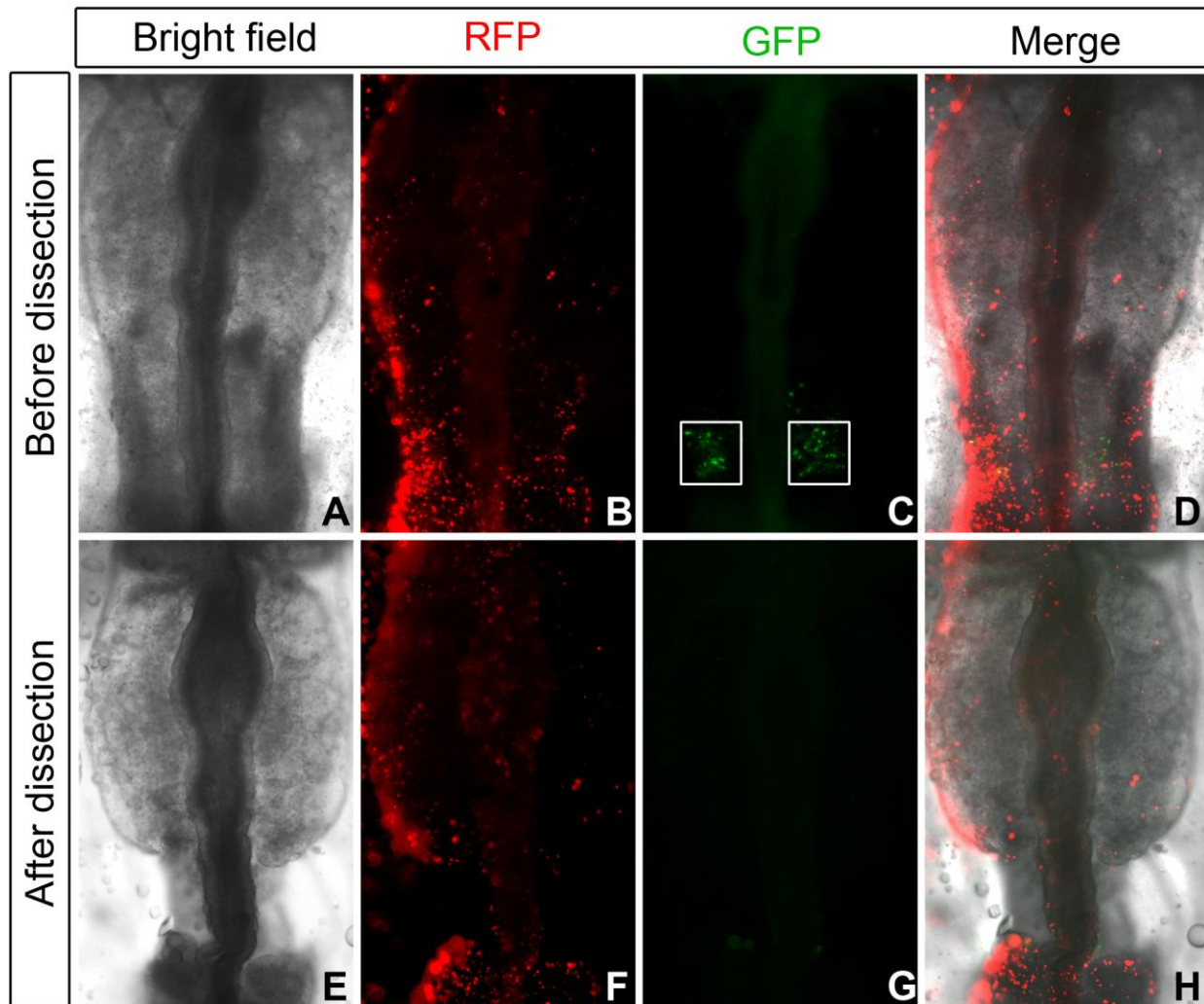
1 **Figures**



**MCS**

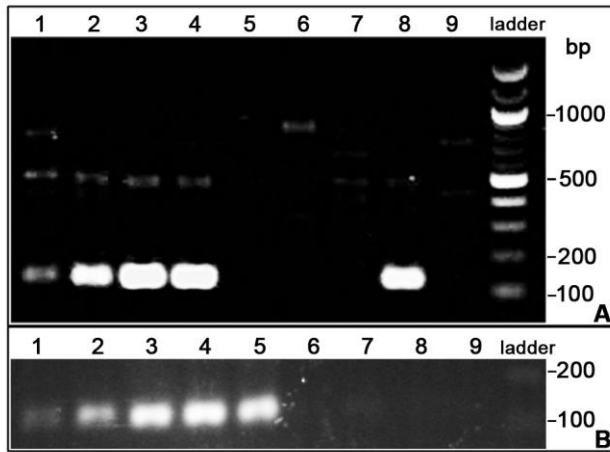


2  
 3 **Figure 1. Diagram showing the modified pTK-EGFP vector.** 16 bp DNA barcodes are inserted  
 4 at the 3' end of the first exon (E1), separated by an intron from the EGFP coding sequence in exon  
 5 2 (E2). The forward primers for RT-PCR span the first and second exon consisting of the 16 bp  
 6 barcodes and 4 bp of the 5' end of E2. The common reverse primer is located within the EGFP  
 7 coding sequence. The modified multiple cloning site contains two extra unique restriction sites,  
 8 *EcoRV* and *SpeI*, and two *XcmI* sites which produce a 3' T overhang after *XcmI* digestion.



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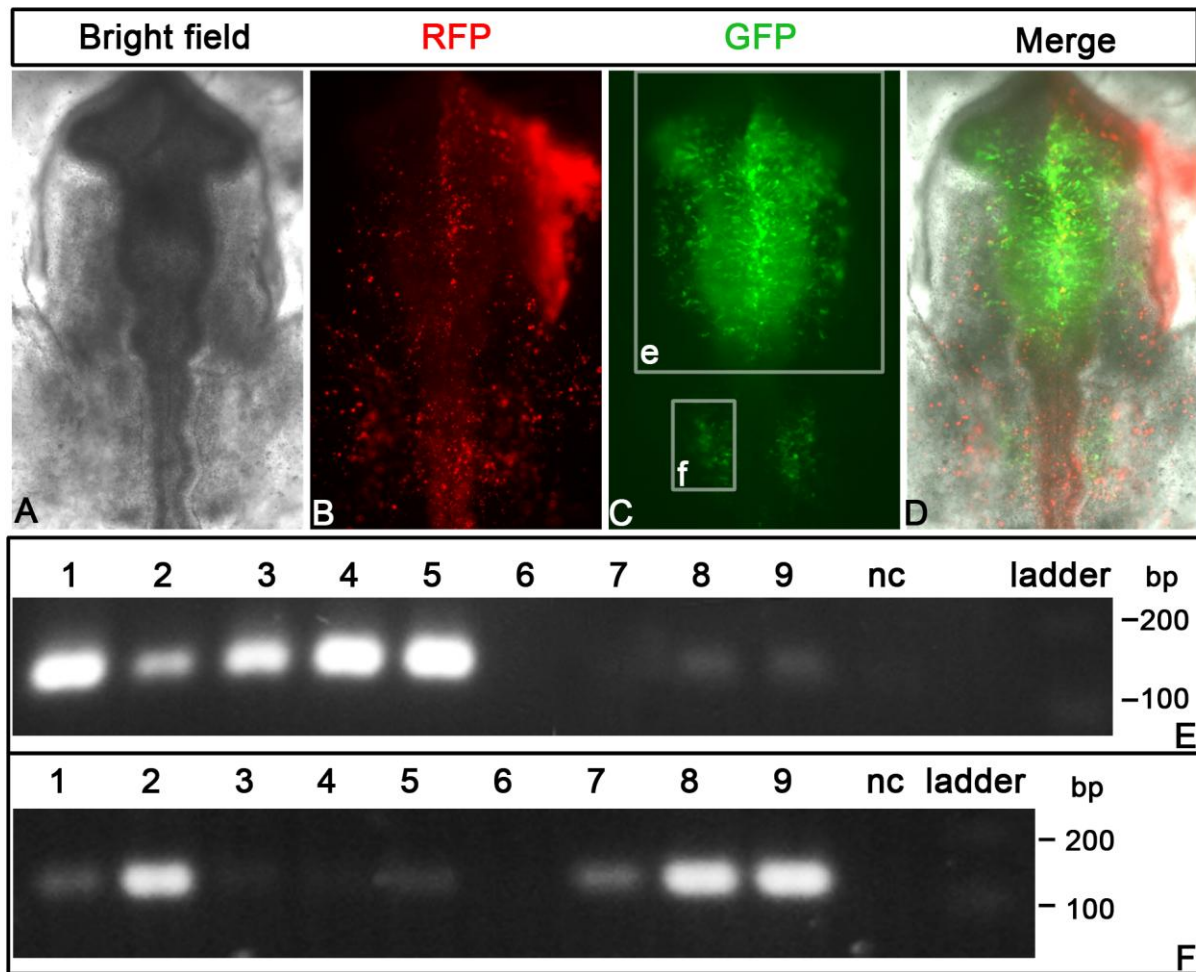
**Figure 2. Dissection of electroporated embryos.** RFP is expressed from the control plasmid using a  $\beta$ actin promoter and indicates the location of successful electroporation, while Sox10E-driven EGFP is detected only in the otic placode (A-D). The otic placode demarcated by the white rectangle (C) is dissected for RT-PCR analysis (E-H).



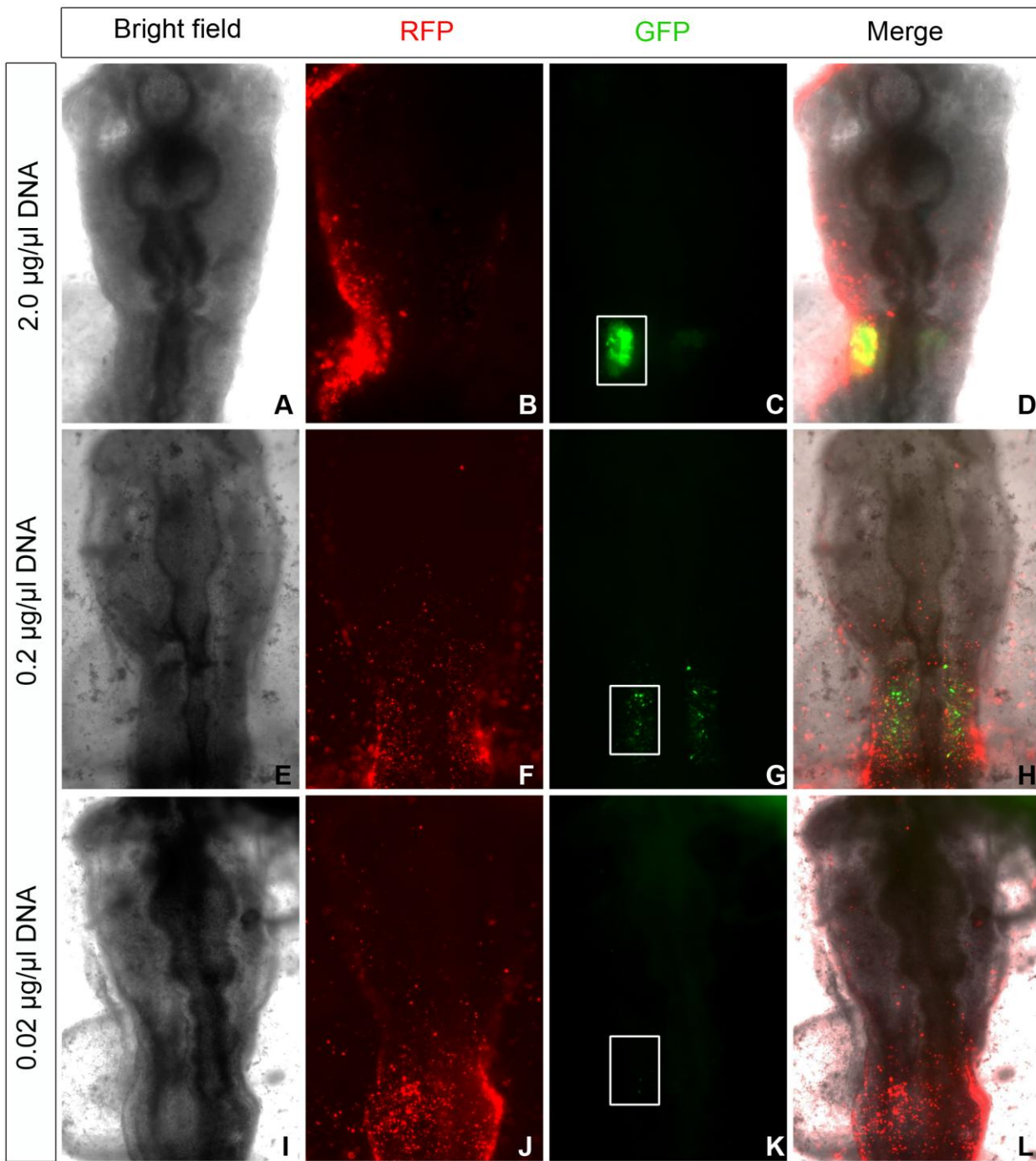
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2 **Figure 3. Otic enhancer detection by RT-PCR. (A) The activity of otic enhancers.** Spalt4F14  
 3 (Barembaum and Bronner-Fraser, 2010), mSix1-21 1x, mSix1-21 2x, mSix1-21 4x (Sato et al.,  
 4 2010) and Sox10E (Betancur et al., 2011) activity is detected by the ~129 bp amplicons, as shown  
 5 in lanes 1, 2, 3, 4 and 8, respectively. Negative control plasmids do not show any band (lane 5, 6, 7,  
 6 and 9). Unspecific bands higher than 129 bp are also present, but can clearly be distinguished  
 7 from the positive signal. **(B) Non-otic enhancers are not detected.** While the otic enhancers  
 8 Spalt4F14, mSix1-21 1x, mSix1-21 2x, mSix1-21 4x and Sox10E produce a specific signal of ~129  
 9 bp (lanes 1-5), the non-otic enhancers Sox2-N2 and Sox2-N4 (Uchikawa et al., 2003) produce no  
 10 signal (lanes 8 and 9), nor do the empty vectors (lanes 6 and 7).

11



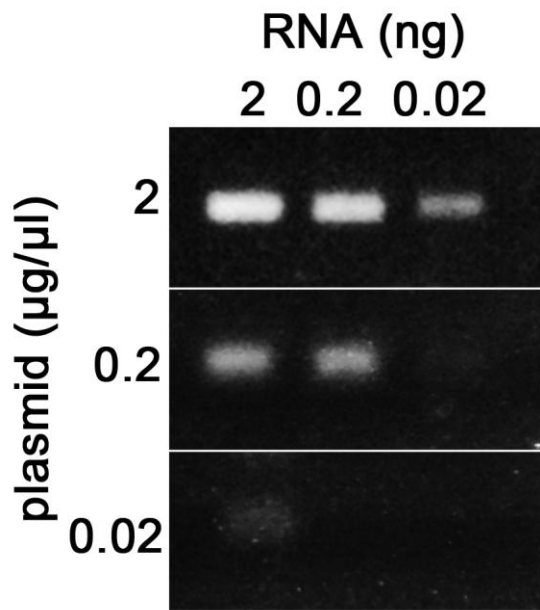
1  
2 **Figure 4. Detection of enhancers in neural tube and neural crest.** (A-D) embryos were  
3 electroporated with a mixture of barcoded plasmids containing FoxD3-NC1 and -NC2, Sox10E,  
4 Sox2-N2, Sox2-N4, mSix1-21 1x/2x/4x enhancers, empty vector and  $\beta$ -actin promoter-driven RFP  
5 at HH6/7. At HH10 enhancer activity (EGFP) is seen in the neural tube, the neural crest and otic  
6 placode (C, D), while RFP expression is widespread. The white rectangles e and f indicate the  
7 regions dissected for assaying enhancers. (E) positive enhancers in the head region are detected  
8 by the 129 bp bands (FoxD3-NC1: lane 1, Sox10E: lane 2, Sox2-N2: lane 3, Sox2-N4: lane 4,  
9 FoxD3-NC2: lane 5, Six1-21-2x: lane 8, Six1-21-4x: lane 9). Lane 6, 7 and 10 show Sox2-N3, Six1-  
10 21-1x and the empty vector, respectively. (F) otic enhancers are captured by the assay. Sox10E  
11 (lane 2), Six1-21-1x/2x/4x (lanes 7-9) produce the specific band around 129 bp. Lane1 (FoxD3-  
12 NC1) and lane 5 (FoxD3-NC2) show weak signal probably resulting from neural crest  
13 contamination. The neural tube enhancers (Sox2-N2: lane 3, Sox2-N4: lane 4, Sox2-N3: lane 6)  
14 are negative in the otic placode.  
15



1

2 **Figure 5. EGFP reporter detection in vivo.** The Sox10E enhancer construct (Betancur et al.,  
 3 2011) was electroporated into the otic region at a concentration of 2  $\mu\text{g}/\mu\text{l}$ , 0.2  $\mu\text{g}/\mu\text{l}$  and 0.02  $\mu\text{g}/\mu\text{l}$ ,  
 4 respectively. RFP expression driven by the ubiquitous chick  $\beta$ -actin promoter (B, F, J) indicates  
 5 widespread electroporation, while enhancer activity is observed only in the otic placode (C, G).  
 6 White box in C, G and K indicates the otic region.

7



1  
 2 **Figure 6. Assay sensitivity.** When 2 µg/µl Sox10E reporter plasmid is used for electroporation,  
 3 positive signals are detected with RNA amounts as low as 0.02 ng. When 0.2 µg/µl Sox10E  
 4 reporter plasmid is used, a band is only detected with 0.2 ng RNA, while only a very faint signal is  
 5 produced with 0.02 µg/µl plasmid and 2 ng RNA.  
 6

1 Table 1. Primers used to generate barcoded vectors.  
2

	<b>Primers</b>	<b>Comments</b>
>T1F1	5'- CAGTTTTCAAGCCGGAgtgtagatcaaggttacaagacag -3'	For vector with barcode 1
>T1R1	5'- TCCGGCTTGAAAAGTgacgaccaacttctgcagtaag -3'	
>T2F1	5'- TGATACACCGAGTCGTgtagatcaaggttacaagacag -3'	For vector with barcode 2
>T2R1	5'- ACGACTCGGTGTATCAacgaccaacttctgcagtaag -3'	
>T3F1	5'- AGCTCTTCGCAAAGTGtagatcaaggttacaagacag -3'	For vector with barcode 3
>T3R1	5'- CACTTTCGGAAGAGCTacgaccaacttctgcagtaag	
>T4F1	5'- CAGCTTACTCGTAAGGtagtagatcaaggttacaagacag -3'	For vector with barcode 4
>T4R1	5'- CCTTACGAGTAAGCTgacgaccaacttctgcagtaag -3'	
>T5F1	5'- ACGATGAAGCCTTGTGtagtagatcaaggttacaagacag -3'	For vector with barcode 5
>T5R1	5'- GACAAGGCTTCATCGtaggaccaacttctgcagtaag -3'	
>T6F1	5'- TGCCTGCATAGATACGtagtagatcaaggttacaagacag -3'	For vector with barcode 6
>T6R1	5'- CGTATCTATGCAGGCAacgaccaacttctgcagtaag -3'	
>T7F1	5'- GAAGTATCCGGTCATCtagtagatcaaggttacaagacag -3'	For vector with barcode 7
>T7R1	5'- GATGACCGGATACTTCacgaccaacttctgcagtaag -3'	
>T8F1	5'- TCCAAGGAAGGCTTCTgtagatcaaggttacaagacag -3'	For vector with barcode 8
>T8R1	5'- AGAAGCCTTCCTTGGaagaccaacttctgcagtaag -3'	
>T9F1	5'- AGTTTATACGCCGCTAgtagatcaaggttacaagacag -3'	For vector with barcode 9
>T9R1	5'- TAGCGGCGTATAACCTacgaccaacttctgcagtaag -3'	

3 Note: nucleotides in capital case are sequences corresponding to the barcode sequences. Nucleotides with  
4 small case are sequences matching the vector for primer pairing.  
5  
6  
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8

9 **Table 2. Primers for RT-PCR**  
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	<b>Primers</b>	<b>Comments</b>
>1F	5'-CAGTTTTCAAGCCGGAgtgt- 3'	For vector with barcode 1
>2F	5'- TGATACACCGAGTCGTgtgt- 3'	For vector with barcode 2
>3F	5'- AGCTCTTCGCAAAGTGgtgt- 3'	For vector with barcode 3
>4F	5'- CAGCTTACTCGTAAGGgtgt- 3'	For vector with barcode 4
>5F	5'- ACGATGAAGCCTTGTGgtgt- 3'	For vector with barcode 5
>6F	5'- TGCCTGCATAGATACGgtgt- 3'	For vector with barcode 6
>7F	5'- GAAGTATCCGGTCATCgtgt- 3'	For vector with barcode 7
>8F	5'- TCCAAGGAAGGCTTCTgtgt- 3'	For vector with barcode 8
>9F	5'- AGTTTATACGCCGCTAggtgt- 3'	For vector with barcode 9
>Rev	5'- GTCCAGCTCGACCAGGATG- 3'	Universal reverse primer

11 Note: for forward primers, nucleotides in capital case are sequences corresponding to the  
12 barcode sequences.