

Genomic Tagging in *Drosophila* cells

(C-terminal constructs)

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Version: Apr. 06, 2016

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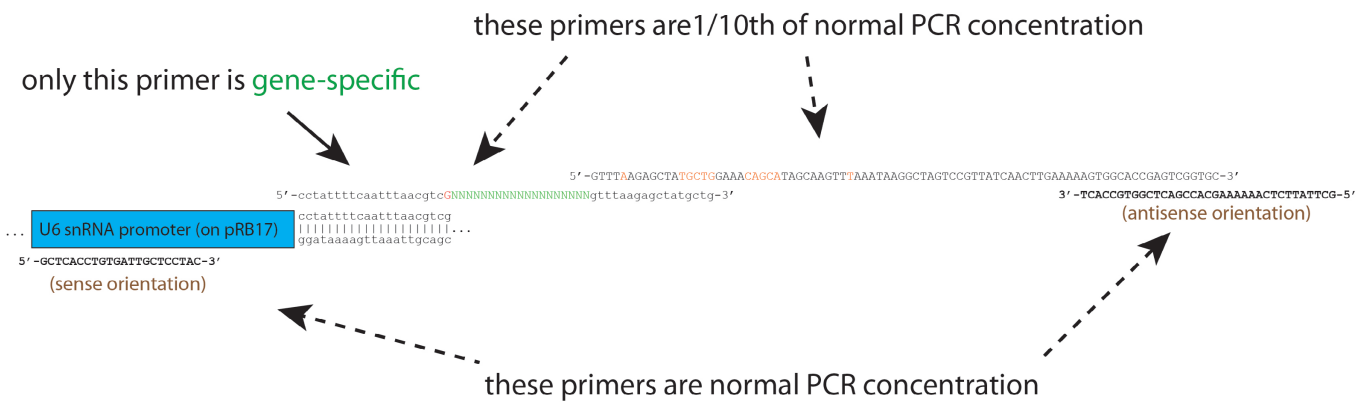
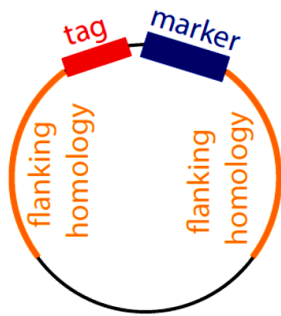


Figure 2: Overlap extension PCR for U6-promoter CRISPR RNA fusions; the U6 snRNA promoter (~400 nt) has been cloned on plasmid pRB17 (with the sequence of the T7 RNA-polymerase promoter appended after the transcription start site, but we no longer use this feature). The sequence just upstream of the U6-promoter TSS is added to the oligonucleotide that provides the cleavage specificity sequence. (see detailed protocol). Assembly of the desired fusion gene occurs during PCR via the overlapping sequences. Only the outside primers are introduced at normal concentrations; the other oligonucleotides are more dilute, since their role is more the one of a PCR template rather than a primer.

To introduce a desired genomic modification at this locus, a template for homology-directed repair must be provided. This is referred to as the homologous recombination (HR) donor construct. To facilitate recovery of the desired events, a selection marker is introduced as well; it can later be removed by FLP-mediated site-specific recombination. The flanking homology regions needed in the HR donor construct can be introduced via cloning or simply added as extensions to PCR primers. For very short tags or point mutations, a synthetic single-stranded oligonucleotide can also serve as HR donor (not part of this protocol). Figure 3 illustrates the principles of each strategy.

Ideally, we choose a *cas9*/CRISPR target site that is disrupted upon integration of the tagging cassette. If a corresponding PAM sequence cannot be identified, the target site will also be present in one of the homology arms of the HR donor PCR product. In this case it is important to introduce a silent point mutation to prevent *cas9*/CRISPR mediated cleavage of the HR donor or the modified locus after integration.

circular donors with long (>500 nt) flanking HR



+ suitable for large tags
+ long HR is efficient
- requires cloning

Selection:
marker first,
then PCR

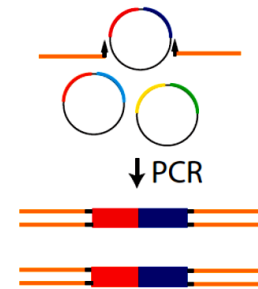
single-stranded oligos with short (50-70 nt) flanking HR



+ leaves no traces
+ convenient ordering
- limited „coding capacity“

Selection:
only by PCR (many clones)

PCR primers with short (50-70 nt) flanking HR, templates for tag + marker



+ great flexibility
+ convenient ordering
+ high „coding capacity“

Selection:
marker first,
then PCR

Figure 3:

Design principles of homologous recombination (HR) donor constructs. PCR selection refers to the test-PCR for integration of the construct at the desired genomic locus.

The synthesis of the HR donor constructs by PCR and design of the primers is described in figures 4, 5 and 6. This protocol is for C-terminal addition of a protein tag. Addition of a tag at the N-terminus is conceptually analogous but certain details change. To avoid confusions, we created a separate protocol for N-terminal tagging.

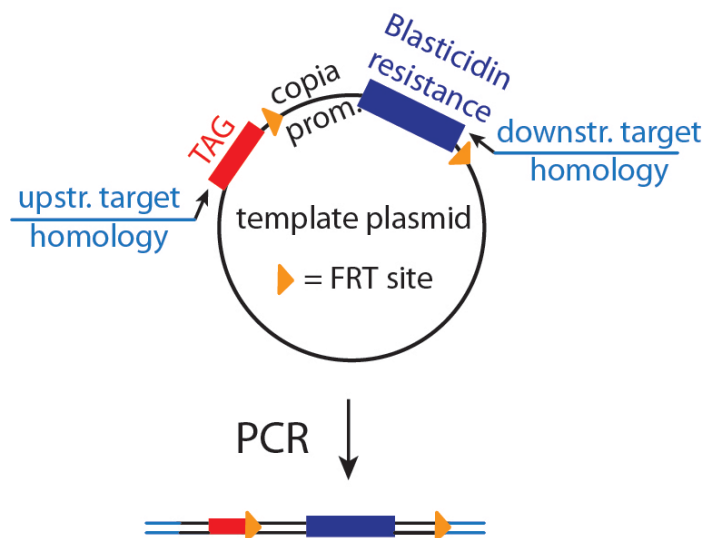
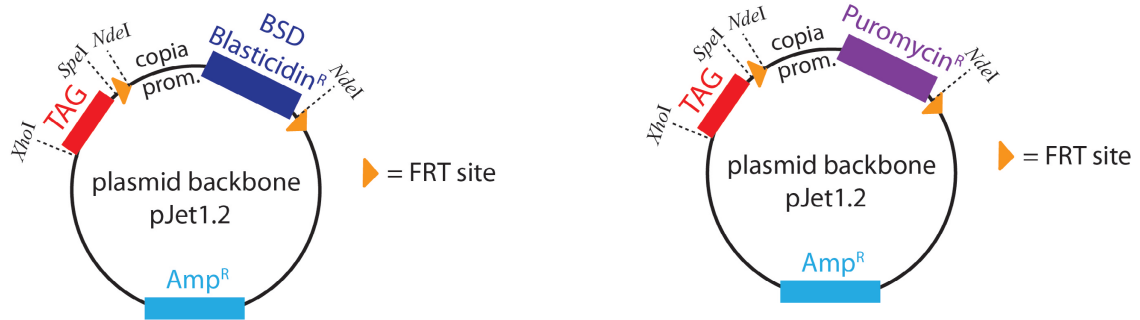


Figure 4:

Principle of HR donor generation for C-terminal tagging via PCR; In addition to Blasticidin, Puromycin-resistance templates are now also available.



pMH4 / pSK25:

```

G S S G W L E D Y K D D D D K S G A D Y K D D D D K *
...ggatcttcgggatggctcgagGATTTATAAAGATGATGATGATAAAAtcggagccGATTTATAAAGATGATGATGATAAAAGAACTAGTGAAGTTCCCTATACTTTCTAGAGAAATAGGAACTTCaataaacatagCTGTTG...
XhoI                               2x FLAG-tag                               SpeI                               FRT site                               NdeI

```

pKF296 / pKF297:

```

TEV cleavage
G S S G W L E E N L Y F Q G G A S I P N P L L G L D S P A A P S I P N P L L G L D G S *
...ggatcttcgggatggctcgagGAGAACCTGTACTCCAGGGCGGTGCCAGCATCCCAACCCCGCTGTTGGCCCTGGATAGCCAGCAGCTCCCTAGCATCCCAACCCACTGCTGGGTCTGGATGGCAGCTGAactagt
XhoI                               2x V5 (short)-tag                               SpeI
GAAGTTCCCTATACTTTCTAGAGAAATAGGAACTTCaataaacatagCTGTTG...
FRT site                               NdeI

```

pSK32:

```

TEV cleavage
G S S G W L E E N L Y F Q G G S G S G K P I P N P L L G L D S T *
...ggatcttcgggatggctcgagGAAAACCTGTATTTTCAGGGCGGTCCGGATCTGGCAAGCCGATCCCGAATCCCGTGTAGCCCTGGATAGCACCTGAactagtGAAGTTCCCTATACTTTCTAGAGAAATAGGAACTTCaataaacatagCTGTTG...
XhoI                               V5 (long)-tag                               SpeI                               FRT site                               NdeI

```

pIW1 / pSK24:

```

twin STREP-tag
G S S G W L E S A W S H P Q F E K G G S G G S G G G S W S H P Q F E K *
...ggatcttcgggatggctcgagAGCGCTTGAGGCCACCCGCACTTCGAGAAAggtggaggttcggaggtggatcgTGGAGCCACCCGCACTTCGAAAAATGAactagtGAAGTTCCCTATACTTTCTAGAGAAATAGGAACTTCaataaacatagCTGTTG...
XhoI                               twin STREP-tag                               SpeI                               FRT site                               NdeI

```

pMH3 / pSK23:

```

G S S G W L E M V ... (EGFP) ... Y K *
...ggatcttcgggatggctcgagATCCGAG... (720 nt EGFP CDS) ...TACAACATAactagtCAAGTTCCCTATACTTTCTAGAGAAATAGGAACTTCaataaacatagCTGTTG...
XhoI                               SpeI                               FRT site                               NdeI

```

pSK15 / pSK41:

```

G S S G W L E P Q L D *
...ggatcttcgggatggctcgagCCCCAACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGGactagtGAAGTTCCCTATACTTTCTAGAGAAATAGGAACTTCaataaacatagCTGTTG...
XhoI                               attP site                               SpeI                               FRT site                               NdeI

```

Tag	Blasticidin-resistance		Puromycin-resistance	
	<i>name</i>	<i>Addgene-#</i>	<i>name</i>	<i>Addgene-#</i>
eGFP	<i>pMH3</i>	52528	<i>pSK23</i>	72851
Twin-Strep	<i>pIW1</i>	52530	<i>pSK24</i>	72852
2xFlag	<i>pMH4</i>	52529	<i>pSK25</i>	72853
TEV-V5(long)-tag			<i>pSK32</i>	72854
TEV-2xV5(short) tag	<i>pKF296</i>	74773	<i>pKF297</i>	74774
PhiC31 attP site	<i>pSK15</i>	72855	<i>pSK41</i>	74886

Figure 6:

With a single set of target homology containing primers, a series of HR donor products can be generated by exchanging the template plasmid. The template plasmids (and others) are available at Addgene.

Ideally, the HR donor construct integrates via homologous recombination at the desired locus. Alternatively, non-homologous end joining (NHEJ) can also lead to genomic integration of the donor construct; in this case the integration site is not directed but random. To reduce these unwanted NHEJ-events, transient depletion of the essential NHEJ-factor Lig4 and in addition the

microhomology-mediated end joining (MMEJ) factor Mus308 via RNAi has proven effective (see Fig. 7). In addition, a small molecule inhibitor developed for the human Lig4 enzyme appears to also inhibit *Drosophila* Lig4 (see protocol for details).

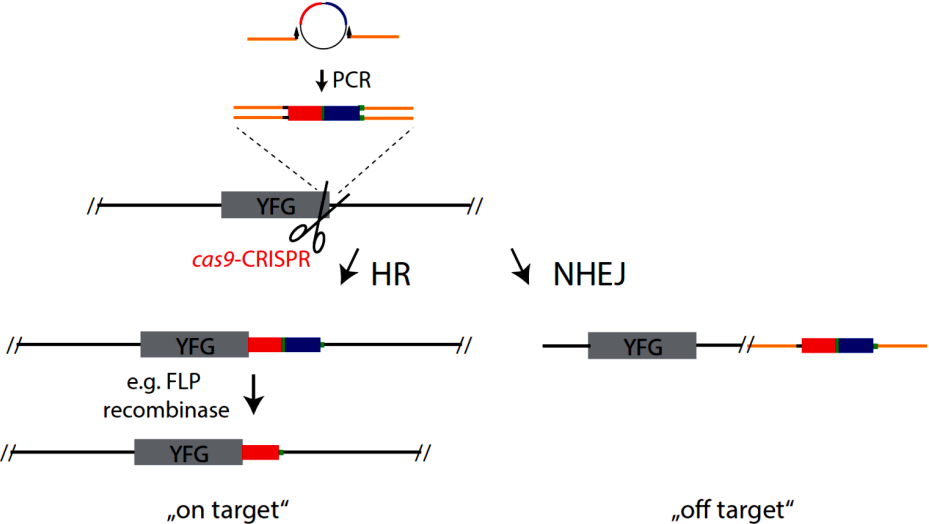


Figure 7: Non-homologous end-joining competes with targeted integration; it can be reduced by RNAi or inhibition of *lig4* and *mus308* (the latter is essential for microhomology-mediated end joining MMEJ).

General considerations

1. PCR conditions

The PCR programs (cycling protocols) given in this protocol have been established in our lab on our thermal cyclers (Eppendorf Mastercycler Gradient, Sensoquest Labcycler Gradient). Depending on your model of cycler and PCR tubes, it is possible that you may have to adapt/optimize the conditions in order to get enough product.

2. Thermostable polymerase

None of the proofreading PCR polymerases that we tried so far (Pfu, Phusion, Q5) have yielded sufficient amounts of the desired product when used on their own. Standard Taq polymerase, however, is a reliable partner for the reactions needed. In our experience, a 1:1 mix of Taq and Pfu polymerase also works well. We expect that this mixture may display a somewhat reduced error rate during PCR, but we have not quantified this specifically.

Expression plasmids are available at Addgene for Taq (pAKTaq, #25712) and Pfu (pET16B.Pfu, #12509). The enzymes are quite easy to prepare.

3. PCR buffers

Many commercially provided PCR buffers contain some detergent to stabilize the polymerase protein. We found that this has a negative effect on transfection efficiency, thus we recommend purification of the PCR product (e.g. via the Qiagen PCR purification kit) in this case. Alternatively, it is easy to make a detergent-free 10x PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3 at room temperature). In our experience, a PCR that was performed in this buffer can be directly transfected with excellent efficiencies.

4. Oligonucleotide synthesis

The longer the oligonucleotide, the more expensive it is - this logic is of particular importance for the HR donor PCR primers. We have thus tried to use shorter homology-containing regions and found that in principle it is possible to make the primers shorter, but it does reduce the efficiency of HR [5]. In other words, you will recover a lower proportion of drug-resistant cells that express the tag of interest. We buy our oligonucleotides at Eurofins/MWG (<http://www.eurofinsgenomics.eu/>), they offer the synthesis of primers with a maximal length of 120 nt. Other suppliers can probably provide equally suited reagents, we recommend running a 15% Urea/PAGE gel (standard mini-gel size as for Western blots is sufficient) to compare the quality of long primers between suppliers. It can be stained with Sybr Gold or ethidium bromide, loading 1 μ l of a 1 μ M solution is amply sufficient. We order the primers without any additional purification (i.e. no HPLC or PAGE purification, despite the website recommendation) and find that this works well for tagging while keeping the price in a very reasonable range. However, we have not made a quantitative comparison between standard and purified primers.

A: Generation of Materials

A-1. Generation of a U6-sgRNA template for transfection by overlap-extension PCR

Material:

- general PCR reagents
- sgRNA optimized scaffold [1, 6] primer serving as template during PCR (1 μ M concentration):
5' -GTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGC-3'
- U6 promoter sense primer for PCR (10 μ M concentration)
5' -GCTCACCTGTGATTGCTCCTAC-3'
- sgRNA antisense primer for PCR with RNA pol-III termination signal (10 μ M concentration)
5' - gcttattctcAAAAAAGCACCGACTCGGTGCCACT-3'
- Sense primer for PCR containing the specific sequence for programming of the cas9 nuclease (1 μ M concentration, specific sequence is underlined and in upper case letters – use 18 or 19 nt)
e.g. to target Actin5C gene:
5' -cctatcttcaatttaacgctcgACCGCAAGTGCTTCTAAGAgtttaagagctatgctg-3'

PCR mix for sgRNA template:

- 2 μ l 1 uM oligo sgRNA scaffold
- 2 μ l 1 uM primer CRISPR
- 2 μ l 10 ng/ μ l plasmid pRB17 = U6-promoter fused to T7 promoter (Addgene #52527)
- 2 μ l 10 uM primer U6-promoter sense
- 2 μ l 10 uM primer sgRNA antisense
- 10 μ l 10x PCR buffer without detergent (10x conc.: 500 mM KCl, 100 mM Tris-HCl pH 8.3)
- 16 μ l 25 mM MgSO₄
- 2 μ l 10 mM (each) dNTP
- 60 μ l H₂O
- 2 μ l Taq/Pfu polymerase mix (1:1)

PCR program:

- 1: 94°C 2min.
- 2: 94°C 30 sec.
- 3: 50°C 30 sec.
- 4: 72°C 30 sec.
- 5: goto step 2, 34 repetitions
- 6: 4°C pause

The use of a PCR buffer without detergent makes it possible to directly transfect the PCR product without any purification. If you use a PCR buffer with detergent (may give higher yield), we recommend using a column-based PCR cleanup kit before transfection.

Quality control: Load 3 μ l of the reaction on a 1.2% agarose gel. The band should run at ~600 nt.

A-2. Generation of homologous recombination template for tagging by PCR

Material:

- general PCR reagents
- template plasmids for tagging (100 pg/ μ l dilution), e.g. pMH3 (GFP -Addgene #52528), pMH4 (Flag₂ - Addgene #52529), pIW1 (Strep₂ - Addgene #52530)
- sense and antisense primers containing the desired homology regions (10 μ M concentration); the length of the homology region can be varied according to the specification of your oligonucleotide synthesis provider. We recommend using 60 nt on both the sense primer and the antisense primer.

e.g. sense to target *actin5C* gene (specific sequence underlined):

5' - TGGATCTCCAAGCAGGAGTACGACGAGTCCGGCCCCCTCCATTGTGCACCGCAAGTGCTTCggatcttcgga
tggtcgag-3'

e.g. antisense to target *actin5C* gene (specific sequence underlined):

5' - CCTCCAGCAGAATCAAGACCATCCCGATCCTGATCCTCTTGCCCAGACAAGCGATCCTTCgaagttcctatt
ctctagaaagtataggaacttcCATATG-3'

PCR mix for HR-donor tagging PCR:

- 6 μ l 100 pg/ μ l template plasmid
- 2 μ l 10 uM primer sense
- 2 μ l 10 uM primer antisense
- 10 μ l 10x PCR buffer without detergent (10x conc.: 500 mM KCl, 100 mM Tris-HCl pH 8.3)
- 6 μ l 25 mM MgSO₄
- 2 μ l 10 mM (each) dNTP
- 70 μ l H₂O
- 2 μ l Taq/Pfu polymerase mix (1:1)

PCR program:

- 1: 94°C 2min.
- 2: 94°C 30 sec.
- 3: 50°C 30 sec.
- 4: 72°C 2 min.
- 5: goto step 2, 24 repetitions (=25 cycles in total)
- 6: 4°C pause

The use of a PCR buffer without detergent (recipe below) makes it possible to directly transfect the PCR product without any purification. If you use a PCR buffer with detergent (may give higher yield), we recommend using a column-based PCR cleanup kit before transfection.

A reduced number of PCR cycles may lead to lower error rates; this potentially increases the recovery of correctly modified genomes, but we have not systematically tested this yet. However, this also reduces product yield. If more material is needed, you may either scale up the reaction volume or increase the cycle number to 30.

Hint: The U6-sgRNA fusion PCR (A-1) and the HR donor PCR (A-2) can also be performed in parallel in the same block. In this case, the HR donor cycling protocol should be used and 30 cycles are preferable.

Quality control: Load 3 μ l of the purified product on a 0.8% agarose gel. The final size of the product depends on the particular tag(s) amplified: GFP \sim 1850 nt, Flag₂ \sim 1150 nt, Strep2 \sim 1200 nt for Blasticidin-resistance, add \sim 200 nt for Puromycin-resistance.

A-3. Generation of dsRNA to deplete *lig4* and *mus308*

Material:

- general PCR reagents
- Primers with T7 extension (sense and antisense, 10 μ M concentration) for *lig4*
sense: 5' -taatacgactcactatagggCCCAATGATCCAAAGTGTTTTTGCA-3'
antisense: 5' -taatacgactcactatagGGAAGTAGGATGCCTTCGCGA-3'
- Primers with T7 extension (sense and antisense, 10 μ M concentration) for *mus308*
sense: 5' -taatacgactcactatagggGCTGGGACTCCACCGAAAAG-3'
antisense: 5' -taatacgactcactatagggTACCGTCGCCGTCCAGTAATG-3'
- Drosophila cDNA as template for PCR
- 10x IVT buffer as described below
- 100 mM solution of each NTP These can either be purchased ready-made or prepared from the powder, which is significantly cheaper. You need to adjust the pH to ~7 with NaOH (sticks are fine and help to avoid RNase contamination) after dissolving the salts!
- 1M DTT, RNase inhibitor, T7 RNA polymerase, RNase-free DNase-I

PCR mix for dsRNA template (make separate reactions for *lig4* and *mus308*):

- 1 μ l *Drosophila* cDNA
- 1 μ l 10 uM primer sense
- 1 μ l 10 uM primer antisense
- 5 μ l 10x PCR buffer (we use Fermentas)
- 4 μ l 25 mM MgCl₂
- 1 μ l 10 mM (each) dNTP
- 36.5 μ l H₂O
- 0.5 μ l Taq polymerase

PCR program:

- 1: 94°C 2min.
- 2: 94°C 30 sec.
- 3: 50°C 30 sec.
- 4: 72°C 30 sec.
- 5: goto step 2, 34 repetitions (=35 cycles in total)
- 6: 4°C pause

Use this product directly as template for *in vitro* transcription.

10x T7 transcription buffer:

- 500 mM Tris-HCl pH7.9
- 25 mM spermidine
- 260 mM MgCl₂ (really 260 mM)
- 0.1% Triton-X100

IVT recipe:

25 µl PCR product from above
10 µl 10 x T7 transcription buffer
4 µl ATP (100 mM)
4 µl GTP (100 mM)
4 µl UTP (100 mM)
4 µl CTP (100 mM)
0.6 µl 1 M DTT
42.4 µl H₂O (RNase-free)
4 µl T7 RNA polymerase

IVT incubation

37°C >= 3 hours, best is over night; we use our incubator for bacterial plates for this as it will prevent condensation of water under the lid

add 1 µl DNase-I

37°C 30 min.

95°C 10 min.

65°C 20 min.

Cool to RT, spin to remove the precipitate of Magnesium-pyrophosphate that may have formed. Make several aliquots of the supernatant and determine the concentration as described below. This dsRNA can be added directly to the cell culture medium to induce RNAi by soaking, provided it is sterile (the 5 min. 95°C incubation described above is normally sufficient to sterilize the preparation).

Quality control: Load 1 µl and 1 µl of a 1:10 dilution on a 1% agarose gel. The band should run at ~600 nt, sometimes we see a certain extent of "laddering" (=multimer sized fragments). Ideally, the 1:10 dilution should still give a well visible band that can be used to estimate the concentration in comparison with the marker bands. Spectrophotometric quantification is impossible due to the presence of high amounts of free nucleotides.

B: Cell culture and transfection

B-1. Knock-down of *lig4* and *mus308*

Material:

- reagents prepared in section A of this protocol
- Fugene HD transfection reagent (e.g. Promega #E2311)
- Schneider's medium (we use Bio&Sell # BS 2.43G02J)
- Fetal bovine serum (FBS) and, if desired, Penicillin/Streptomycin as a cell culture additives; we prepare our medium with 10% FBS and 1x Pen/Strep.

Day 1: Split a dense culture of S2-cells to a density of 1×10^6 cells/ml in Schneider's medium with 10% FBS; one well of a 24-well plate (= 500 μ l of medium) is usually sufficient for about 10-15 editing transfections later. Add dsRNA for induction of *lig4* and *mus308* knock-down to a final concentration of 1 μ g/ml.

Note: The combined knock-down of the two end-joining pathways is synthetically sick/lethal in S2-cells. If you observe that the cells are sick after the knock-down, reduce the dsRNA dose until the procedure is well tolerated.

Day 4: Count the cells and adjust them to a density of 1.5×10^6 cells/ml in Schneider's medium with 10% FBS. Dispense 80 μ l of this cell suspension per well of a 96-well plate. The cells are now ready for transfection. Alternatively, you can assemble the transfection mix in the plate first, then add the cell suspension to the mix (= reverse transfection).

Note: If you prefer a larger format for your transfections, scaling up the volumes according to the total culture volume is a good start. For example, in a 24-well dish you may use a total culture volume of 500 μ l; you should thus plate 400 μ l of cell suspension and use a total volume of 100 μ l for the transfection mix.

For maximum convenience and efficiency, use cells with stable *cas9* expression such as our S2-cell clones 9-4 (neomycin-resistant) or 5-3 (hygromycin-resistant) with myc-tagged *cas9*.

B2. Transfection

Prepare the transfection mix (amount given per well) according to the type of experiment you are doing (transient or stable *cas9* expression). We find that the procedure is most efficient with stable *cas9* expression; transient co-transfection of a *cas9* expression plasmid leads to a slightly lower proportion of tag-expressing cells after selection and growth recovery of the culture during selection is slower. The amounts of Fugene-HD and nucleic acids may need to be adjusted according to your cells and culture conditions. We recommend using the Act5C-GFP tagging approach for optimization (see section A-2).

A) Transient expression of *cas9*

10 μ l Schneider's medium without FBS (or the required amount to reach a final volume of 20 μ l)
50 ng of U6-sgRNA fusion PCR (step A-1)
50 ng of pRB14 (or any other plasmid that permits expression of a *cas9* enzyme in *Drosophila* cells)
50 ng of HR template PCR product (step A-2)
Mix, then add 1 μ l Fugene-HD directly from the glass vial

This approach will likely work in any cultured and transfectable *Drosophila* cell.

B) Stable expression of *cas9*

10 μ l Schneider's medium without FBS (or the required amount to reach a final volume of 20 μ l)
75 ng of U6-sgRNA fusion PCR (step A-1)
75 ng of HR template PCR product (step A-2)
Mix, then add 1 μ l Fugene-HD directly from the glass vial

Use cells with stable *cas9* expression, e.g. our S2-cell clones 9-4 (neomycin-resistant) or 5-3 (hygromycin-resistant) with myc-tagged *cas9*.

Controls: It is a good idea to carry along a positive control (e.g. Act5C-GFP) and a negative control for your targeting construct. One suggestion for a negative control is to leave out the sgRNA component. Another negative control should be to leave out the HR donor PCR; this is at the same time a positive control for the Blastidin- or Puromycin-selection later on.

Let the transfection mix stand for 60 minutes, then add the entire volume (20 μ l) to the cell culture well (or prepare the mix in the wells, then add the cells). For larger transfections, scaling up the amounts of the reagents according to total culture volume is a good start.

B-3. Blasticidin or Puromycin selection of transfected cells

Material:

- Schneider's medium with 10% FBS
- Blasticidin-S solution 10 mg/ml (e.g. Life Technologies A11139-03)
- Puromycin solution 10 mg/ml (e.g. Life Technologies A11138-03)

Day 4 post transfection: Split the cells 1:5 into medium containing 10 µg/ml Blasticidin (=50 µl of 10mg/ml stock for 50 ml of medium) or 0.5 µg/ml Puromycin (= 2.5 µl of 10mg/ml stock for 50 ml of medium); the cells will proliferate rather slowly in the beginning.

Day 11 post transfection: Split the cells again 1:5 into 10 µg/ml Blasticidin or 0.5 µg/ml Puromycin containing medium. This time, growth in selective medium should resume almost normally. Sometimes the cells have not proliferated enough by day 11; in this case, wait a few more days until you perform the second split.

Note: In our hands, selection is complete after this split. Molecular analysis (PCR, western Blot) can already be performed after one round of selection. Since this requires some more material, you may find it convenient to directly split the cells up from 96-well to 24-well format plates on day 4 post transfection.

Marker removal:

If you wish to remove the selection marker from the cells' genomes, you can transfect them with a FLP-recombinase expression plasmid (e.g. pKF295, Addgene #71872). This will delete the *copia*-Blasticidin or puromycin resistance cassette and leave a single FRT-sequence as the editing "scar" (in addition to the tag-sequence, of course). Since FLP-out and transfection efficiencies are not 100%, you need to perform clonal selection after this step.

C: Molecular Analysis

C-1. Molecular Analysis: Small-scale isolation of genomic DNA

Unfortunately, one cannot directly use resuspended cells as templates for PCR (= the equivalent of a colony-PCR in microbiology) because of inhibitory effects (either in the medium or from the cells). Thus, it is best to make a small-scale DNA isolation if you have only a limited number of samples. See below for a direct-PCR recommendation if higher throughput is required.

Material:

- Gel-extraction kit (e.g. Qiagen)
- cultured cells (~50-100 μ l)

Procedure:

1. Resuspend cells in culture vessel and transfer 100 μ l (50 μ l can suffice if the amount is limiting) to an Eppendorf tube
2. Add 300 μ l of Qiagen buffer QG to the cells in medium, vortex thoroughly
3. Apply the entire sample to a Qiagen spin column from the gel extraction kit and discard the flow-through
4. Wash the column with 700 μ l of buffer PE and discard the wash
5. Centrifuge the empty column 1 min. at full speed to completely dry the matrix
6. Transfer column to fresh Eppendorf cup; apply 50 μ l of buffer EB to the column, let stand for 1 min., then centrifuge for 1 min. at full speed to recover the eluate

This DNA preparation works well as template in PCR.

Use 2 μ l of DNA per 25 μ l reaction.

Although this procedure is reasonably fast and convenient, it is labor-intensive and costly if a large number of e.g. cell clones needs to be analyzed. In this case, the use of a self-made PCR buffer with high pH (~9.0-9.5) [7] can overcome the inhibitory effect of resuspended cells and medium, albeit at the cost of an overall reduction in PCR efficiency. We have made good experience using this approach for screening cultures, but you should limit it to short amplicons (e.g. use primer #328 from Fig. 8). The PCR conditions may need to be further optimized depending on the gene specific primer. Thorough genotyping of selected clones is best done with purified genomic DNA.

C-2. Molecular Analysis: PCR to check for integration

Material:

- purified DNA from selected S2-cells (step C-1)
- general PCR reagents
- integration construct specific primer (e.g. #646 in Fig. 8):
5' -GGTATTCTCTTACAATATGTTTTATGGCATAAAAAGG-3'
- gene-specific upstream sense primer (recommended: 150-500 nt upstream of integration site, sense orientation)

Procedure:

1. Assemble components of the PCR on ice except the Taq polymerase, use 2.5 µl template
2. Start thermocycler and set it to pause with the block at 94°C
3. Add Taq polymerase to PCR and close tube
4. Place tube from ice directly into the hot block of the thermal cycler, run PCR program
5. Analyze 10 µl of the PCR on a 1% agarose gel. See Fig. 8 for information on how to calculate the expected band size.

PCR recipe:

- 2 µl isolated genomic DNA (or resuspended cells)
- 1 µl 10 uM primer copia as
- 1 µl 10 uM primer gene specific sense
- 2.5 µl 10x PCR buffer (standard pH with purified DNA template, high pH with resuspended cells)
- 3 µl 25 mM MgCl₂
- 0.5 µl 10 mM (each) dNTP
- 16 µl H₂O
- 0.5 µl Taq polymerase

PCR program:

- | | | |
|----|------|--|
| 1: | 94°C | 2min. |
| 2: | 94°C | 20 sec. |
| 3: | 55°C | 20 sec. |
| 4: | 72°C | 90 sec. |
| 5: | goto | step 2, 34 repetitions (=35 cycles in total) |
| 6: | 4°C | pause |

Possible modifications:

- use a hot-start enzyme if you do not wish to preheat the thermocycler;

combine:
 #646 + #328 with an upstream sense primer
 #318 with a downstream antisense primer

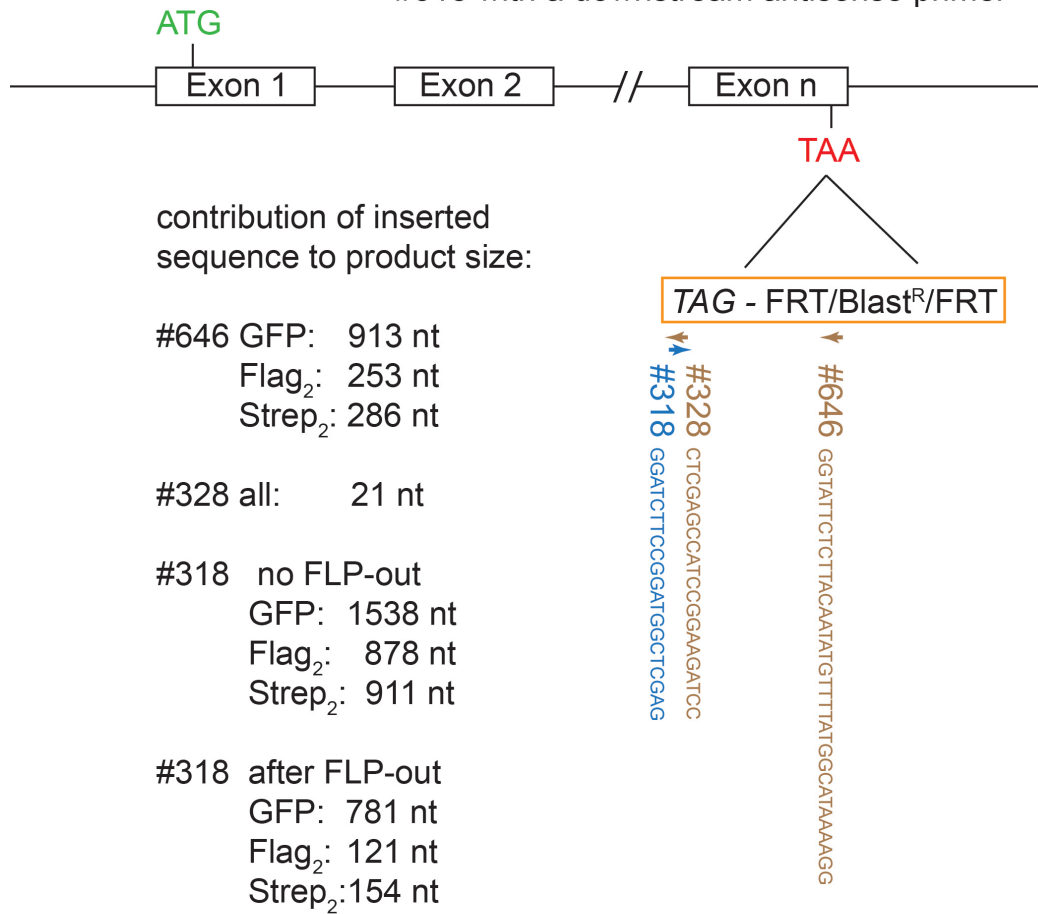


Figure 8:

This is a schematic drawing of the target locus and the integrated cassette. The oligonucleotide numbers refer to our lab's list; the sequences are given 5' to 3'. The size of the expected PCR product is calculated by adding the distance between the gene-specific primer and the integration site to the appropriate length as indicated in this figure. The primers will work for both, Blastidicin and Puromycin resistance; the sizes of the PCR products are identical for both markers.

C-3. Molecular Analysis: PCR to check for FLP-out of marker

Material:

- purified DNA from selected S2-cells (step C-1)
- general PCR reagents
- primer tags_common sense (# 318 in Fig. 8): 5' -ggatcttccggatggctcgag-3'
- gene-specific downstream antisense primer (recommended: 100-500 nt downstream of integration site, antisense orientation)

Procedure:

1. Assemble components of the PCR on ice except the Taq polymerase, use 2.5 µl template
2. Start thermocycler and set it to pause with the block at 94°C
3. Add Taq polymerase to PCR and close tube
4. Place tube from ice directly into the hot block of the thermal cycler, run PCR program
5. Analyze 10 µl of the PCR on a 1% agarose gel. See Fig. 8 for information on how to calculate the expected band size.

PCR recipe:

- 2 µl isolated genomic DNA
- 1 µl 10 uM primer tags_common sense
- 1 µl 10 uM primer gene specific antisense
- 2.5 µl 10x PCR buffer
- 2 µl 25 mM MgCl₂
- 0.5 µl 10 mM (each) dNTP
- 16 µl H₂O
- 0.5 µl Taq polymerase

PCR program:

- 1: 94°C 2min.
- 2: 94°C 20 sec.
- 3: 55°C 20 sec.
- 4: 72°C 90 sec.
- 5: goto step 2, 34 repetitions (=35 cycles in total)
- 6: 4°C pause

Possible modifications:

- use a hot-start enzyme if you do not wish to preheat the thermocycler;
- the combination of gene-specific sense and antisense primers can also be used to check if all chromosomal alleles have been tagged. Since the unmodified locus will always produce a small PCR product, and is thus in a competitive advantage during PCR, any cell clone that produces only a product corresponding to the modified locus is very likely a clone with modification of all available alleles. This PCR only makes sense after clonal selection.

C-4. Molecular Analysis: Western Blot

Numerous Western blot protocols are available and whatever system is up-and-running in your lab will likely do the job. Therefore, this section is more a collection of items that you may find useful as additions or alternatives to established procedures.

1) Sample preparations: For a quick check it is not necessary to make high-quality protein extracts; we harvest 50-100 μ l of cells by centrifugation, aspirate the supernatant as much as possible to remove the serum-containing medium (consider using a suction device) and then resuspend & boil the cell pellet in 50 μ l of 1x SDS sample buffer. Prior to loading, the samples are centrifuged for 5 minutes at top speed to pellet any non-solubilized material. This procedure generates quite reproducible and sufficiently concentrated extracts. Target proteins expressed at very low expression levels may nonetheless require some sort of specific extraction and concentration protocols prior to Western blot detection.

2) Transfer type: We routinely use a wet-transfer with Towbin buffer containing 10% Ethanol (instead of the 20% methanol) in a Bio-Rad chamber with electric tension set to 100 V. This gives good transfer results in 60 minutes, for proteins >100 kDa we increase transfer time to 75 or 90 minutes (this may require some optimization for each target protein).

3) Incubation with primary antibody: We dilute the primary antibodies (Flag: M2 / Sigma, GFP: B-2 / Santa Cruz, Strep: Strep-Mab HRP / IBA) 1:5000 in TBS with Tween-20 and 5% milk. For the anti-Flag M2, blocking/incubating/washing with 0.02% Tween gives excellent results, while for the GFP and Strep-Mab antibodies 0.1% Tween is preferable to avoid higher background staining. We find that incubation with the primary antibody overnight in a cold room (e.g. in a 50 ml conical tube on a roller) **significantly** increases signal strength compared with 120 minutes incubation at room temperature. The anti-Flag M2 monoclonal antibody also weakly recognizes one ~130 kDa and ~70 kDa endogenous *Drosophila* protein. Therefore, a control lane with untreated S2-cells is essential (and for the other tags a good idea, too).

4) Just a reminder if you are using the cell lines with stable cas9 expression: This construct has a myc-tag, therefore we do not recommend the use of myc-tag cassettes for other proteins in conjunction with our cell lines or vector for cas9-expression.

D: Cloning of cells expressing tagged proteins

Cell cloning can be done either with the Blasticidin-resistant population (add the antibiotic to the culture medium in this case) or after the marker cassette has been FLP'ed out upon transient transfection of the pMH5 vector (in this case the medium must not contain Blasticidin).

1. Add 100 μ l of selection media per well to a 96 well plate, leaving the first three columns empty. Including 20% of conditioned medium (= "old", sterile-filtered cell culture medium) usually improves the yield.
2. Count the cells that you are cloning and adjust the density to 8000 ($=8 \times 10^3$) cells / ml. Dispense 125 μ l of this into each well of the first three columns of the 96 well plate. This corresponds to plating 1000 cells per well.
3. Using an eight channel multipipetter, transfer 25 μ l from column 1 to column 4, from 2 to 5 and from 3 to 6. Then repeat this scheme for the rest of the plate. In the end, you have made three successive steps of 5-fold dilutions, with three columns (24 wells) per dilution step.
4. Wrap a strip of parafilm around the plate and incubate at 25°C for at least two weeks. (*Note:* Continue culturing the pool of selected cells in parallel in case no colonies develop.)
5. Check wells to see if colonies are developing. You may have to wait up to three weeks to easily see colonies by eye. If none have developed after three weeks, repeat selection with the pool of selected cells, starting with 5000 cells per well.
6. Note the position of isolated cell clumps with a waterproof pen on the bottom of the dish. This helps to find it again under the cell culture hood and to remove each colony in about 10 μ l of medium with a P20 pipette. First, transfer it to a fresh well of a 96-well plate containing 100 μ l of medium.
7. When clonal cell in the 96 well plate are getting dense, dilute them into 24 well plates. When these wells reach high density, split cells again and incubate the new plate at 25°C as before. The old plate can then be used for molecular analysis as described in section C.
8. Amplify the clones of interest further in 6-well plates and freeze aliquots. To be really sure that the population is clonal (in cases where this is essential), we suggest performing two rounds of dilution & selection.

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