Genomic Tagging in mammalian cells

(C-terminal constructs)

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Introduction

Repair of DNA double-strand breaks can occur via homologous recombination (normally with the replicated sister chromatid in mitotically dividing cells). This phenomenon can be exploited to manipulate the genome sequence experimentally with high precision. To this end, an artificial donor DNA molecule is provided. With a certain frequency, cells will use this homologous donor instead of the sister chromatid for repair. The specific introduction of a DNA double-strand break greatly stimulates the efficiency of HR-directed insertion of the desired DNA element. To this end, designer-nucleases (Zn-finger, TALEN) have been used with good success, but recently the CRISPR/cas systems have gained a lot of interest. The *cas9*-CRISPR system from *Streptococcus pyogenes* is particularly convenient since its sequence specificity can be programmed via an RNA subunit (derived from the CRISPR locus in *S. pyogenes,* see Fig. 1) [1-4]. This protocol describes a variation of our recently published approach for genome editing with PCR-based HR donor constructs in *Drosophila* cells [5].

The RNA subunit responsible for specificity can be expressed *in vivo* by creating a fusion gene between the U6 snRNA promoter (RNA polymerase III) and a DNA fragment encoding the CRISPR RNA. For the latter, we rely on the optimized sgRNA scaffold version from the Weissman lab [6].The construct can be designed at the computer (see Fig. 1) and after oligonucleotide synthesis everything can be assembled in a single PCR via overlap extension (Figure 2). To reduce undesired amplification products, a touch-down cycling strategy has proven effective. For human cells, we have only tried this approach so far (no transfection of *in vitro* transcribed sgRNA).

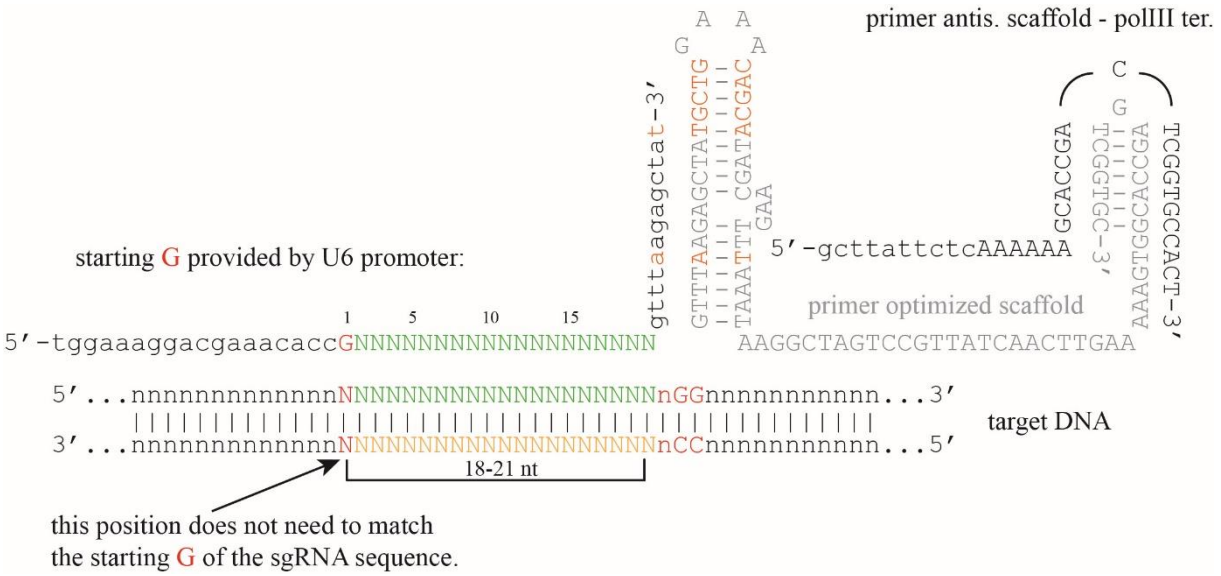

Figure 1:
Assembly of a DNA segment coding for the programming CRISPR RNA. The green sequence confers specificity for a target DNA locus. This sequence must be flanked by an NGG sequence (the protospacer associated motif = PAM) at the target locus in the genome; if that is the case then both strands of the target DNA will be cleaved by the *cas9* nuclease. 3' of the programming "spacer" sequence, the scaffold RNA "repeat" is introduced via a synthetic oligonucleotide template. Optimizations by Chen et al [6] are indicated in orange. Although the sequence depicted above is DNA, the resulting RNA secondary structure is indicated. 5' of the programming sequence, the sequence immediately upstream of the U6 snRNA TSS is included for overlap-extension PCR.

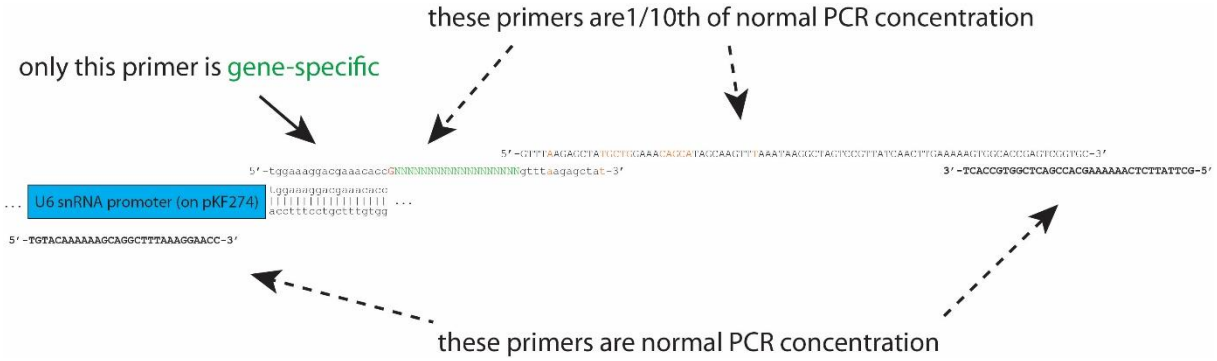

Figure 2:
Overlap extension PCR for *hs*-U6-promoter CRISPR RNA fusions; the human U6 snRNA promoter (~320 nt) has been cloned on plasmid pKF274, the sequence just upstream of the U6-promoter TSS is added to the programming oligonucleotide, the antisense-primer also harbors an RNA polymerase III termination signal. 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.

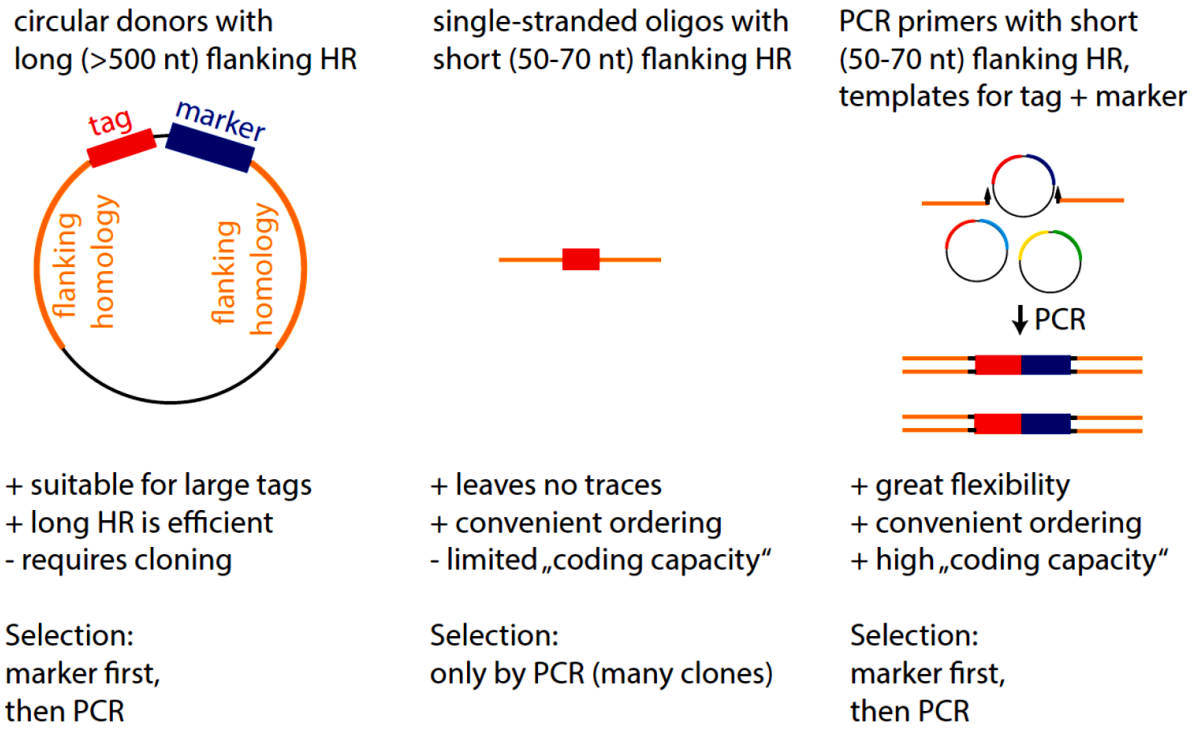

Figure 3:
Design principles of homologous recombination (HR) donor constructs. PCR selection refers to the test-PCR for integration of the construct at the desired genomic locus.

The synthesis of the HR donor constructs by PCR and design of the primers is described in figures 4, 5 and 6. Although the *copia* promoter is from Drosophila, it shows sufficient activity in HEK293T cells to allow selection with low doses of Blasticidin. This protocol is for C-terminal addition of a protein tag.

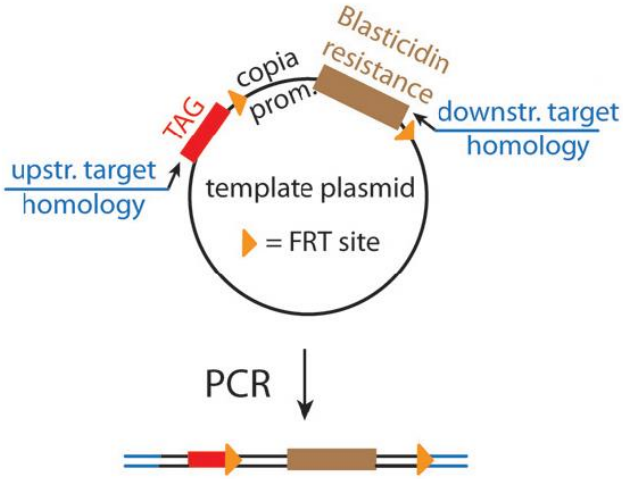

Figure 4:
Principle of HR donor generation for C-terminal tagging via PCR.

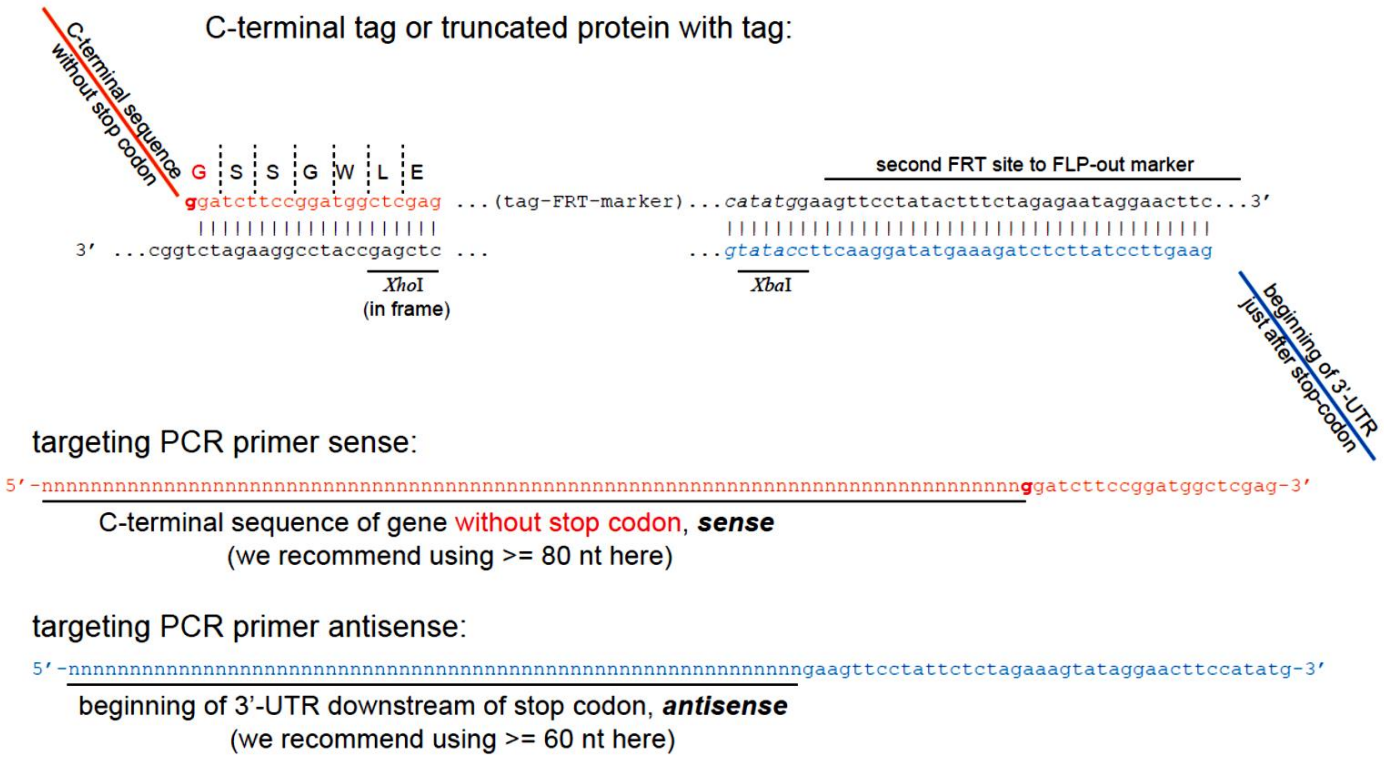

Figure 5:

Details of primer sequences, the resulting short linker and selection of homologous sequences on the primers.

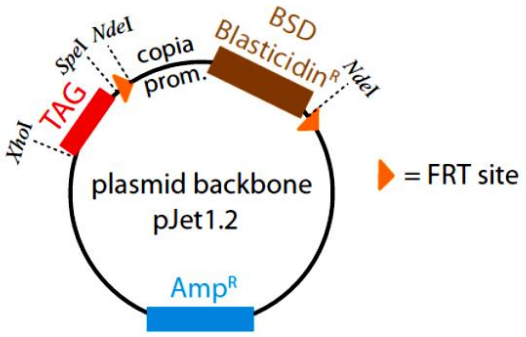

Figure 6:

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: pMH3 = #52528, pMH4 = #52529, pIW1 = #52530;

Ideally, the HR donor construct integrates via homologous recombination at the desired locus; however, end-joining activities can result in integration at other sites. A small molecule inhibitor developed for the human Lig4 enzyme (required for NHEJ) appears to improve targeting efficiency in HEK293T cells (see protocol for details).

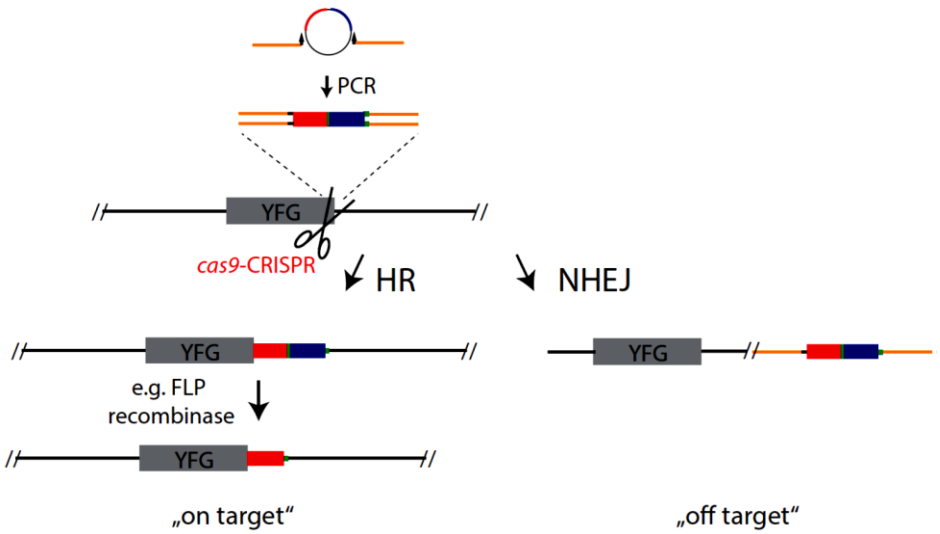

Figure 7:
Non-homologous end-joining competes with targeted integration; it can be reduced by RNAi or inhibition of Lig4.

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 you 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 hope that this mixture may display a somewhat reduced error rate during PCR, but we have not tested this specifically. As mentioned above, the Pfu polymerase does not give satisfactory results when used alone. Thus, its contribution to the final product is likely limited.
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 good efficiencies. However, the yield in PCR is somewhat lower and you may have to “fiddle around” more in order to get the reaction to work in your lab.

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 yes, 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 µl of a 1 µM 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] primer serving as template during PCR (1 µM concentration):

5'-GTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG
CACCGAGTCGGTGC-3'

- U6 promoter sense primer for PCR (10 µM concentration)

5'-TGTACAAAAAAGCAGGCTTTAAAGGAACC-3'

- sgRNA antisense primer for PCR (10 µM concentration)

5'-GCTTATTCTCAAAAAAGCACCGACTCGGTGCCACT-3'

- Sense primer for PCR containing the specific sequence for programming of the cas9 nuclease (1 µM concentration, specific sequence in upper case letters)

For overlap-extension PCR the sequence immediately upstream of the transcription start site of the U6-snRNA promoter is added:

5'-tggaaaggacgaaacaccGNNNNNNNNNNNNNNNNNNNgtttaagagctatgctg-3'

We have not systematically tested length variations of the CRISPR-programming sequence – it is reasonable to try between 18 and 21 nt here. The first G does not need to match the genome.

PCR mix for sgRNA template:

2	µl	1 uM oligo sgRNA scaffold
2	µl	1 uM primer CRISPR
2	µl	10 ng/µl plasmid pKF274 = human U6-promoter fused to T7 promoter (our stock #368)
2	µl	10 uM primer U6-promoter sense
2	µl	10 uM primer sgRNA antisense
10	µl	10x PCR buffer without detergent (10x conc.: 500 mM KCl, 100 mM Tris-HCl pH 8.3)
16	µl	25 mM $MgCl_2$
2	µl	10 mM (each) dNTP
60	µl	H_2O
2	µl	Taq/Pfu polymerase mix (3:1)

PCR program:

1:	94°C	2min.
2:	94°C	30 sec.
3:	53°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 (likely gives higher yield), we recommend using a column-based PCR cleanup kit before transfection.

Quality control: Load 3 µl of the reaction on a 1.2% agarose gel. The band should run at ~420 nt.

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

Material:

- general PCR reagents
- template plasmids for tagging (100 pg/µl dilution), e.g. pMH3 (GFP -Addgene #52528), pMH4 (Flag$_2$ - Addgene #52529), pIW1 (Strep$_2$ - Addgene #52530); best: pKS1 (Ag Förstemann #383) for C-tern GFP with thymidine kinase promoter for expression of Blasticidin resistance
- sense and antisense primers containing the desired homology regions (10 µM concentration); the length of the homology region can be varied according to the specification of your oligonucleotide synthesis provider. We recommend using at least 80 nt on the sense primer and at least 60 nt on the antisense primer.

e.g. sense:

```
5'-NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNggatcttccggatggctcgag-3'
```

e.g. antisense:

```
5'- NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNgaagttcctatactttctagagaataggaacttcCATATG-3'
```

<u>PCR mix for HR-donor tagging PCR:</u>

2	µl	100 pg/µl template plasmid
2	µl	10 uM primer sense
2	µl	10 uM primer antisense
10	µl	10x PCR buffer without detergent (10x conc.: 500 mM KCl, 100 mM Tris-HCl pH 8.3)
8	µl	25 mM $MgCl_2$
2	µl	10 mM (each) dNTP
72	µl	H_2O
2	µl	Taq/Pfu polymerase mix (1:1)

<u>PCR program:</u>

1:	94°C	2min.
2:	94°C	30 sec.
3:	53°C	30 sec.
4:	72°C	2 min.
5:	goto	step 2, 34 repetitions (=35 cycles in total)
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 (likely gives 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.

Quality control: Load 3 µl of the purified product on a 0.8% agarose gel. The final size of the product depends on the particular tag(s) amplified: GFP ~1850 nt, Flag$_2$ ~1150 nt, Strep2 ~1200 nt.

B: Cell culture and transfection

B-1. Use of the Lig-4 inhibitor SCR7

Sathees C. Raghavan and colleagues have developed an inhibitor (called SCR7) for the human Lig4 enzyme [7]. It seems that this also has a beneficial effect in our tagging-experiments in HEK-cells but we have not quantified the effect. More recent work suggests that this inhibitor may not be appropriate:
DOI: 10.1016/j.dnarep.2016.04.004

For the time being, the usefulness of SCR7 in the tagging experiments should therefore be regarded as "experimental" – the approach clearly works well even without the inhibitor, but the proportion of correctly modified sites in the population of drug-resistant cells may be somewhat higher with the inhibitor present.
So far, we have not attempted a knock-down of Lig4 by siRNAs.

The SCR7 inhibitor can be purchased from the company Xcessbio:
http://xcessbio.com/index.php/new-products-15/scr7.html

B2. Transfection

One day before transfection, split HEK293T cells to a density of 2 x 10^5 cells / ml (corresponds to something like 5-10% confluency), then plate 500 µl of cells per well of a 24-well plate. Let grow over night, then add the SCR7 inhibitor of Lig4 to the required concentration shortly before transfection.

Prepare the transfection mix (amount given per well of a 24-well plate). We use Fugene-HD as transfection reagent because it works well for Drosophila cells and reasonably good with HEK293T cells. Other reagents may work better, we suggest using whatever works best with your cells.

50 µl DMEM without FBS
100 ng of U6-sgRNA fusion PCR (step A-1)
25 ng of hcas9 expression plasmid from [1] (Addgene #41815)
100 ng of HR template PCR product (step A-2)
Mix, then add 2 µl Fugene-HD

Controls: It is a good idea to carry along a positive control (e.g. Actin-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 Blasticidin-selection later on.

Let the transfection mix stand for 30 minutes, then add the entire volume to one well of a 24-well plate.

B-3. Blasticidin selection of transfected cells

Material:

- DMEM with 10% FBS
- Blasticidin-S solution 10 mg/ml (e.g. Life Technologies A11139-03)

Day 5 post transfection: replace the transfection medium with DMEM containing 2 µg/ml of Blasticidin (10 µl of the stock solution per 50 ml of medium); the cells will proliferate rather slowly in the beginning. If you chose to add the SCR7 inhibitor, include it in this split as well (some of the non-incorporated HR donor molecules may still be present at this point).

Day 8 post transfection: Split the cells 1:5 into 2 µg/ml Blasticidin containing medium. It may be that the cells are not dense enough for this split yet. In this case, simply replace the Blasticidin-containing DMEM and continue growing the original plate.

Continue splitting the cells into Blasticidin-containing medium as required.

Note: Potentially the selection is slow because of the low transcriptional activity provided by the copia-promoter. If the cells are easy to clone (e.g.HEK293 cells), then diluting them out directly after the transfection followed by PCR-screening of the resulting clones may be a viable option.

To FLP-out the marker cassette, a multitude of FLP recombinase expression plasmids are available (e.g. pCAG-FLPe, Addgene #13787). We observed that transfection of our FLP plasmid driven by the *Drosophila* ubiquitin promoter (pMH5, Addgene #52531) also results in intermediate levels of expression in HEK293T cells – but we never carried out the full procedure to FLP out the cassette and confirm this by PCR.

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.

Material:
- Gel-extraction kit (e.g. Qiagen)
- cultured cells (~50-100 µl)

Procedure:
1. Resuspend cells in culture vessel and transfer 100 µl to an Eppendorf tube
2. Add 300 µl 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 µl 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 µl 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 µl of DNA per 25 µl 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 PCR buffer with high pH (~9.0-9.5) [8] can overcome the inhibitory effect of resuspended cells, 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 HEK-293T-cells (step C-1)
- general PCR reagents
- primer copia-as (#231 in Fig. 8): 5′-GTAGGTTGAATAGTATATTCCAACAGCATATG-3′
- gene-specific upstream sense primer (recommended: 200-1000 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 $MgCl_2$
0.5	µl	10 mM (each) dNTP
16	µl	H_2O
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;

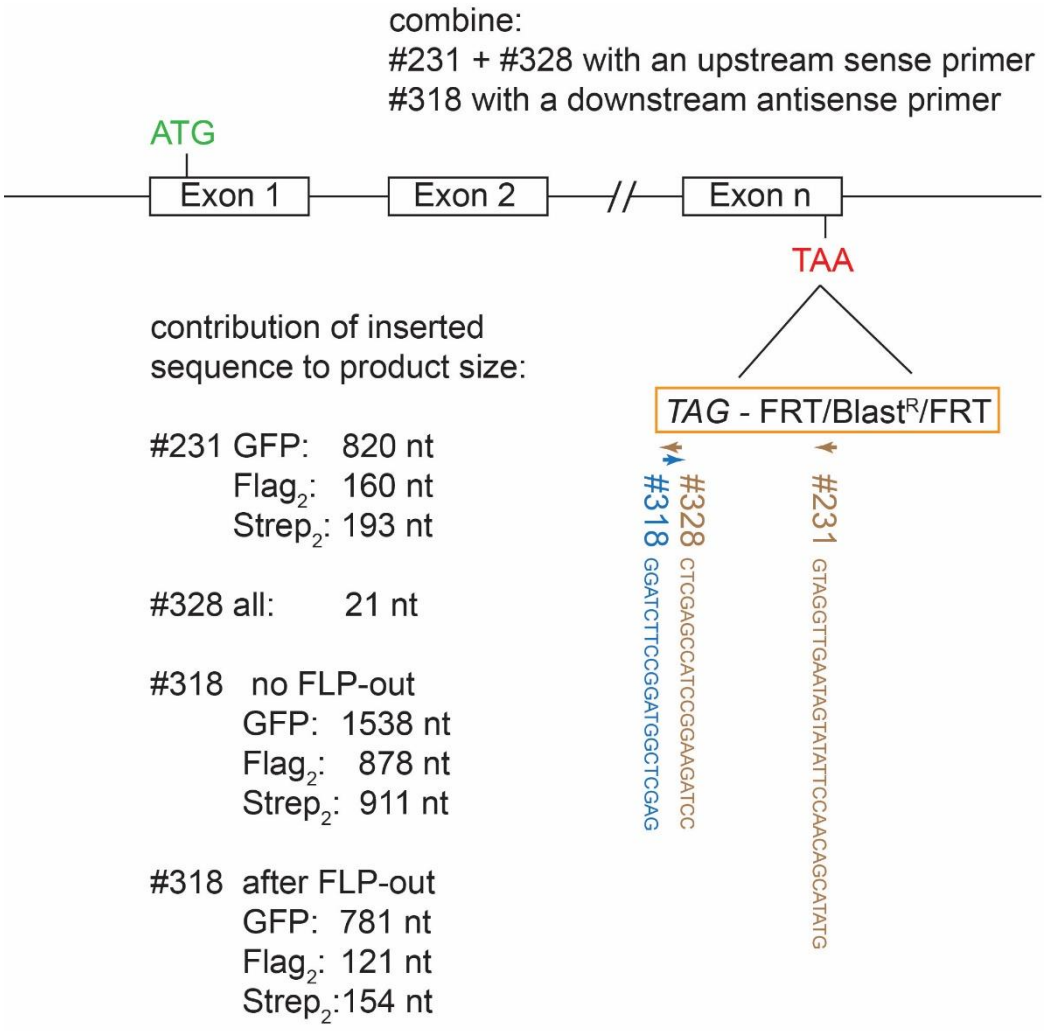

Figure 8:

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.

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

Material:
- purified DNA from selected HEK293T-cells (step C-1)
- general PCR reagents
- primer tags_common sense (# 318 in Fig. 8): `5'-ggatcttccggatggctcgag-3'`
- gene-specific downstream antisense primer (recommended: 200-1000 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 (we use Fermentas)
2	µl	25 mM $MgCl_2$
0.5	µl	10 mM (each) dNTP
16	µl	H_2O
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 (for C-2 and C-3) 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.

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 the content of one well in a 24-well plate, wash the cells twice with PBS and then extract them in PBS with 8M urea. This lyses the cells very efficiently and does not interfere with a Bradford assay. Boils the cells for 5 minutes, then centrifuge for 10 minutes at top speed. Take off the supernatant, make a Bradford assay (optinal) and mix the extract with SDS sample buffer. We then boil the extract again. To avoid distortions of gel due to the high urea concentrations, add an equivalent amount of PBS/urea to the MW marker as well. Prior to loading, the samples are centrifuged again 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.

References

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Appendix : Example primer sequences for C-terminal tagging

hs_actin_CRISPR	tggaaaggacgaaacaccGCCACCGCAAATGCTTCTAGgGTTTAAGAGCTATGCTG
hs_actin_ctag_s	GGCTCCATCCTGGCCTCGCTGTCCACCTTCCAGCAGATGTGGATCAGCAAGCAGGAGTATGACGAGTCCGGC CCCTCCATCGTCCACCGCAAATGCTTCggatcttccggatggctcgag
hs_actin_Ctag_as	gccatgccaatctcatcttgttttctgcgcaagttaggttttgtcaagaaagggtgtaacgcaactaagtca tagtccgcGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCCATATG
hs_Tubbeta_CRISPR	tggaaaggacgaaacaccGAGGCCGAAGAGGAGGCCTAGTTTAAGAGCTATGCTG
hs_Tubbeta_Ctag_s	GAGAGCAACATGAACGACCTCGTCTCTGAGTATCAGCAGTACCAGGATGCCACCGCAGAAGAGGAGGAGGAT TTCGGTGAGGAGGCCGAAGAGGAGGCCggatcttccggatggctcgag
hs_Tubbeta_Ctag_as	aaattctgagggagaggaaaggggcagttgagtaagacggctaagggaactgagaagcctgaggtgatgggg gctctgccGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCCATATG