Generation and validation of homozygous fluorescent knock-in cells using genome editing

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Gene tagging with fluorescent proteins (FPs) is essential to investigate the dynamic properties of cellular proteins. CRISPR/Cas9 technology is a powerful tool for inserting fluorescent markers into all alleles of the gene of interest and permits functionality and physiological expression of the fusion protein. It is essential to evaluate such genome-edited cell lines carefully in order to preclude off-target effects caused by either (i) incorrect insertion of the FP, (ii) perturbation of the fusion protein by the FP or (iii) non-specific genomic DNA damage by CRISPR/Cas9. In this protocol, we provide a step-by-step description of our systematic pipeline to generate and validate homozygous fluorescent knock-in cell lines.

We have used the paired Cas9D10A nickase approach to efficiently insert tags into specific genomic loci *via* homology-directed repair (HDR) with minimal off-target effects. Since it is neither time nor cost-effective to perform whole genome sequencing of each cell clone, we have developed an efficient validation pipeline consisting of junction PCR, Southern Blot analysis, Sanger sequencing, microscopy, Western blot analysis and live cell imaging for cell cycle dynamics. Using this pipeline, 70% of the targeted genes could be tagged homozygously with FPs and resulted in physiological levels and phenotypically functional expression of the fusion proteins.

INTRODUCTION

To study protein dynamics and functions within a cell, proteins are commonly tagged with fluorescent markers. Mahen *et al.*[1] revealed that tagged proteins at the endogenous genomic locus is the best choice to study quantitative properties of the fusion protein by live cell imaging and biophysical methods. For instance, with genome-edited cell lines it is now possible to systematically and quantitatively map the cellular proteome in four dimensions (4D) using Fluorescence Correlation Spectroscopy (FCS)-calibrated 4D live cell imaging [2,19]. However, it is essential to validate genome-edited cell lines carefully in order to control off-target effects which can be caused by insertion of the FP tag elsewhere in the genome, perturbation of the fusion protein by the FP, or non-specific DNA damage by unspecific binding of guide RNAs (gRNAs) leading to double strand DNA (dsDNA) breaks and genome rearrangements. We

have developed a validation pipeline for endogenously tagged cells which is described in this protocol.

Paired Cas9D10A nickase approach

All nucleases used for genome editing in mammalian cells (e.g. ZFNs, TALENs, Cas9) trigger a dsDNA break at a specific genomic locus which can be repaired by two major DNA repair pathways: non-homologous end joining (NHEJ) or homology directed repair (HDR). To generate knock-in cell lines, HDR is essential to insert the tag of interest which is done in the presence of an exogenously introduced repair template during the dsDNA break [3-12]. CRISPR/Cas9 systems consist of either wildtype Cas9 or mutant Cas9 which perform either dsDNA breaks or nicking of single strand DNA, respectively, guided by small gRNAs. The paired Cas9D10Anickase approach is the most suitable method for generating endogenously tagged cell lines, as it avoids off-target effects while providing effective DNA repair for generating endogenously tagged cell lines [12-17] (Figure 1).

Efficiency of homozygous genome modification

To reflect the physiological concentration of a protein within single cells and ensure that the tagged protein fully replaces the function of the endogenous protein, it is essential to tag all alleles with the fluorescent marker. This consequently results in expression of homozygous fluorescently tagged proteins of interest. Additionally, live cell proteomics using Fluorescence Cross-Correlation Spectroscopy (FCCS) is dependent on homozygous fluorescently tagged cell lines to characterize protein complexes in single cells in a time-resolved manner[19]. We have routinely generated homozygous knock-in cell lines using the paired Cas9D10A nickase approach, which resulted in 70% of homozygous knock-in cell lines compared to 46% when using ZFNs. This data confirms that the paired Cas9D10A nickase approach is more susceptible to HDR[16, 17], resulting in increased efficiency of homozygosity.

There are several reports where inhibition of the NHEJ repair pathway (e.g. Scr7 inhibitor, depletion of LIG4 /XRCC4) has been used to increase HDR and subsequently homozygosity [20-24]. However, we did not observe a significant improvement of homozygosity by depleting LIG4 /XRCC4 using RNAi or by applying Scr7 to HeLa cells (unpublished data). Moreover, Greco et al [25] showed that Scr7 is not a very potent inhibitor of the NHEJ repair pathway.

There are as well several studies investigating DNA repair by NHEJ and HDR during the cell cycle in human cells [26-28]. Homologous recombination is nearly absent in G1 phase, but most active in S phase during the cell cycle [28]. Consequently, we serum-deprive cells 24-48h prior to transfection, thereby synchronizing the cells partially in G1 phase. We then release cells into S phase during the targeted double strand DNA break to elevate the chance of HDR in the presence of the repair template. This approach lead to an increase of up to 2% of homozygosity for some gene targets.

Homozygous tagging is not feasible for some essential proteins when structure and size of the FP tag perturb the function of the fusion protein. Therefore, some genes can only be heterozygously tagged. In our experience, and based on data in systematic cDNA tagging efforts, approximately 25% of human genes cannot be functionally tagged with FPs [40].

Validation of genome-edited cell lines

Careful validation of genome-edited cell lines is essential to ensure no disturbance due to the FP tag or off-target effects caused by CRISPR/Cas9. The generation of many tagged cell lines is often necessary to study pathways or protein complexes and networks within a cell. Unfortunately, whole genome sequencing is not cost- and time-effective for a large number of modified clones (up to 50 cell clones). Therefore, we have established a validation pipeline for genome-edited cell lines performing effective quality control at every level of the workflow (Figure 2).

To ensure high quality and reliable data, several homo- and heterozygous cell clones of each protein of interest are kept and used for further experiments. If only heterozygously tagged clones have been produced, a second round of genome editing of these clones is performed to achieve homozygosity. In addition, heterozygously tagged clones can have loss of function mutations in the untagged allele(s) due to dsDNA breaks without recombination, which can lead to pseudo-homozygous cell clones, expressing only the tagged fusion protein from the recombined allele(s). Details of each step within the workflow is discussed in the next section.

Experimental design

Design and delivery of gRNAs and donor plasmids

The design of gRNAs and donors depend on where the protein of interest should be tagged, typically either at the N- or C-terminus. It is prudent to perform an extended literature search for localization data, functionality studies of tagged versions of the protein of interest and expression data in other species such as yeast or mouse to provide insight as to which end of the protein of interest can be tagged while maintaining its functionality intact and localization correct.

Sequence variations (SNPs), which are different from the reference genome for the species the cell line of choice is derived from, can interfere with CRISPR/Cas9 targeting. Therefore and since single point mutations can inhibit gRNA binding, it is advisable to sequence the target locus. The gRNAs should bind directly to the target site, such as to the start or stop codon of the open reading frame. The probability of HDR is best within 100 bp of the double strand DNA break site. Beyond that the efficiency of HDR drops by four fold [5]. Two gRNAs, one for the antisense and one for the sense DNA strand, are necessary when using the Cas9D10A nickase. Appropriately designed gRNAs [5,13-15] are inserted as annealed oligonucleotides into Cas9/gRNA expression plasmids containing two expression cassettes, a human codon-optimized Cas9D10 nickase and the guide RNA.

The donor plasmid contains the template for homologous recombination with the fluorescent marker gene (e.g. mEGFP or mCherry) flanked by 500-800 bp homology arms complementary to the target site of the gene of interest. The fluorescent marker replaces either the start or the stop codon and is in-frame with the gene of interest. By default, there should be a short flexible linker incorporated between the tag and the gene to improve functionality of the tagged protein. Neither an exogenous promoter nor a mammalian selection marker should be part of the donor plasmid to prevent expression of the marker gene without recombination with the target gene.

The delivery method of the plasmids depends on the cell line of choice and should be optimized. HeLa cells are transfected very well using lipofection which is used for the generation of endogenously tagged cell lines within this protocol.

Fluorescence activated cell sorting (FACS) of FP positive cell clones

Donor plasmids are used as DNA repair templates. BacORI is essential to replicate plasmids within *E.coli*, but contains cryptic promoter regions that can cause some background expression in transfected cells prior to genomic integration for as long as the plasmid is

maintained [29]. Therefore, single cell sorting to select fluorescent cells is performed 7-10 days post transfection reducing the number of false positive cell clones during FACS. Depending on the survival rate of single cells of the cell line of choice after FACS, several hundreds of single cell clones should be sorted for downstream characterization. Often, the best growth condition for single cell clones has to be optimized for each parental cell line. The conditions in this protocol have been optimized for widely used HeLa cells.

The fluorescent signal of endogenously tagged proteins depends on its physiological expression level and is often relatively dim compared to commonly used overexpression systems and high sensitivity FACS sorters need to be used. For cell cycle regulated proteins, it might additionally be necessary to synchronize cells in a specific cell cycle stage to enrich cells with high signal for FACS. The efficiency of positively tagged cells 7-10 days after plasmid transfection ranges between 0.05-10% (e.g. Figure 3), but is typically around 0.5%.

Junction PCR

Correct integration of the fluorescent marker into the expected locus can be tested by PCR, using primers binding outside of the homology arms in the genomic locus and within the recombined fluorescent tag (Figure 4A). To determine if all alleles are tagged with the fluorescent marker (= homozygosity), primers located outside of the right and left homology arm are used. The resulting one or two PCR products indicate homozygosity or heterozygosity, respectively. This is not a quantitative PCR, therefore the tagged-gene in heterozygous clones might not be detected due to competition between the two PCR products (Figure 4B). Consequently, it is advisable to repeat the junction PCR with primers within the homology arms resulting in shorter PCR products (Figure 4C) improving amplification of both PCR products. However, homozygously tagged genes will always result in only one PCR product when using primers for the gene of interest at the target site (Figure 4, clone #97).

Southern blot analysis

Southern blot analysis is essential to show homozygosity and to exclude off-target integration of the tag. A probe against the tag should result in only one expected product on the Southern Blot (Figure 5, EGFP probe, mEGFP-NUP358). However in some cases additional DNA fragments are detected indicating either random integration of the donor plasmid or other off-target effects due to genome rearrangement after double strand DNA breaks (Figure 5, GFP probe, green asterisks). Insertion of more than one mEGFP-tag is also possible resulting in a shift of the tagged DNA fragment as demonstrated in clones #1 and #18 (Figure 5, EGFP and

NUP358 blot, clone #1 and #18, green asterisks). The expression of randomly inserted tags should be investigated thoroughly using Western blot analysis, since extra FP can interfere with the outcome of experiments such as quantitative live cell imaging (see Nature Protocols manuscript Politi *et al.*[2]).

Probes binding to the endogenous locus of interest outside of the homology arms detect hetero- and homozygosity (Figure 5, NUP358 probe, Nup358 and mEGFP-NUP358). Rearrangements within the endogenous locus of interest can occur and are detected by extra unexpected bands (Figure 5, NUP358 probe, red asterisks).

Clones that show no extra unexpected DNA fragments are used for further experiments.

Sanger sequencing

PCR performed with primers around the target region followed by Sanger sequencing is used to detect mutations within the insertion site of the tag at the genomic locus. Sequences of parental wildtype cells and the donor plasmid are used as reference. A sequencing example followed by alignment analysis is shown in Figure 6. Two different HeLa cell clones either expressing mEGFP-NUP358 homozygously (Figure 6, #97 tagged) or heterozygously (Figure 6, #118 tagged and #118 untagged) are aligned to the mEGFP-NUP358 donor plasmid and HeLa wt sequences. HeLa wt and #118 untagged do not contain mEGFP as expected, but there is a deletion (63nt) within the #118 untagged NUP358 gene causing a deletion of 6 amino acids in the first exon of the untagged NUP358 (Figure 6, #118 untagged). However, the mEGFP-NUP358 of the same clone (Figure 6, #118 tagged) is correct and mEGFP is integrated without any mutations at the insertion sites (Figure 6, #118 tagged) as expected. The homozygote clone #97 contains only the tagged mEGFP-NUP358 whereby the mEGFP is inserted correctly into the target site (Figure 6, #97 tagged). Consequently, the mEGFP was correctly inserted at the N-terminus of NUP358 and this homozygously tagged clone can be used for further experiments.

Additionally, if there are only heterozygous cell clones after the first round of genome editing, all untagged alleles of heterozygous cell clones are sequenced to detect possible mutations within the target region. This is essential since point mutations could hinder gRNA binding in the second round of genome-editing required to generate homozygous cell clones. Some proteins cannot be homozygously tagged due to the structure and size of the FPs which can disturb the function of the protein.

Western blot analysis

Expression of the fluorescently tagged protein is further characterized by Western blot analysis (Figure 7). An antibody against the FP, such as anti-GFP, detects specific expression of the tagged proteins of interest as a specific band at the expected size (Figure 7, upper panel anti-GFP, mEGFP-NUP358 = 385 kD). Random integration of the donor plasmid can cause expression of free FP when it is inserted in euchromatin regions, which is detectable as a 27kDa protein band. HeLa mEGFP-NUP358 clones #26 and #122 seem to express low amounts of free mEGFP, as a 27kDa is detectable with GFP antibody (Figure 7, upper panel, anti-GFP). Expression of free FP can interfere with protein characterization experiments such as quantitative live cell imaging. Consequently, clones expressing free FP are not used for further experiments. A specific antibody against the protein of interest, if available, is very useful to distinguish homo- and heterozygous clones as shown in Mahen *et al.* [1]. However, in the case of mEGFP-NUP358 this is not feasible since the differences between the 358kD untagged NUP358 and 385kD tagged mEGFP-NUP358 cannot be detected by routine Western blot analysis due to the large size of the protein.

Microscopy of tagged cell clones

To detect correct localization of the tagged protein, live cell imaging or immuno-staining in fixed cells can be performed. Live cell imaging has the advantage of fully preserving the FP fluorescence and being able to follow a cell throughout the cell cycle and investigate if the tagged protein localizes as expected at different cell cycle stages.

NUP358 is a nuclear pore complex protein which localizes to the nuclear envelope during interphase. HeLa mEGFP-NUP358 cell clones were analyzed using live cell imaging and only clones which showed correct localization to the NE without additional non-specific GFP localization were selected for further experiments (Figure 8).

Cell cycle analysis

The cell cycle can be influenced by the tag or off-target effects after the genome editing process. A major interest in our group is the characterization of proteins involved in cell division and in cell cycle control. We therefore established an automated workflow consisting of automated time-lapse microscopy and its analysis to measure the cell cycle kinetics of many cells at once (Figure 9). Similar assays for other cellular functions of interest can be established using high-throughput microscopy with appropriate markers [41].

First, the nuclei of the cells are stained with SiR-DNA[42] to determine the cell cycle status of each individual cell. Subsequently automated live cell imaging is performed every 5 min for 24h (Figure 9A). The images are automatically analyzed to track single cells, detect interphase and mitotic cells, and measure mitotic timing (Figure 9B and C). For instance, the mitotic time from the start of prophase to the onset of anaphase is measured (Figure 9C) and statistically evaluated (Figure 9D). The duration of mitosis is compared to wildtype cells. Cell clones with significantly extended (Figure 9D, mEGFP-CDC20 #140) or shortened mitotic time (Figure 9D, mEGFP-Bub3 #74) are discarded.

The mitotic time of all cell clones per tagged gene are analyzed. For example, all clones of HeLa mEGFP-NUP358 cells behave as HeLa wt cells during mitosis (Figure 10), validating their use in future experiments, such as FCS-calibrated 4D live cell imaging [2, 34].

Outlook and future development

CRISPR/Cas9 technology is developing rapidly. One of the major issues are off-target effects, which can be significantly decreased using the paired Cas9D10A nickase approach. In addition, transfection of pre-formed Cas9 gRNA complexes (Cas9 ribonucleoprotein = Cas9RNP) has been reported to increase the efficiency of gene knock-out with less off-target effects [30-32]. However, in our experience using Cas9RNP or mRNA rather than plasmids to express Cas9 protein does not improve the efficiency of homozygous knock-ins in human cell lines (unpublished data) but rather results largely in heterozygously tagged genes. Given that our application of quantitative FCS-calibrated live cell imaging (see Nature Protocols Manuscript Politi *et al.*) [2] is dependent on homozygous fluorescent tagged proteins, the Cas9RNP approach is not suitable for this pipeline at the moment.

Paix *et al*. [33] has demonstrated that linear DNAs with short homology arms used as DNA repair template can be applied for genome-editing knock-in approaches. We were indeed able to use double stranded linear DNA templates consisting of mEGFP flanked by short 50 bp homology arms, but could only generate heterozygous knock-in cell clines, without the production of homozygotic knock-ins.

In summary, the paired Cas9D10 nickase plasmid based approach described in this protocol remains the optimal procedure for the generation of human cell lines with homozygous fluorescent gene knock-ins.

MATERIALS

REAGENTS

gRNA cloning

- pX335-U6-Chimeric_BB-CBh-hSpCas9n(D10A) (Addgene plasmid ID#42335)
- Oligos for gRNA construction (see Table 1)
- NEB® 5-alpha Competent *E. coli* (High Efficiency) (New England Biolabs, cat. no. C2987H)
- QIAquick gel extraction kit (QIAGEN, cat. no. 28704)
- QIAprep spin miniprep kit (QIAGEN, cat. no. 27106)
- SYBR Safe DNA stain, 10,000× (Life Technologies, cat. no. S33102)
- FastDigest BbsI (BpiI) (Thermo Scientific, cat. no. FD1014)
- Roche Rapid Ligation Kit (Roche, cat. 11635379001)
- LB medium (see reagent set up)
- LB agar medium (see reagent set up)
- Ampicillin (Sigma, cat. no. A9518)
- Agarose (Sigma, cat. no. A9539)
- TBE (see reagent set up)
- Gene™Ruler DNA Ladder Mix (Thermo Scientific, cat. no.SM0331)
- Loading Dye (6x) (Thermo Scientific, cat. no. R0611)

T7E1 assay

- HotStar HighFidelity PCR kit (QIAGEN, cat. no. 202605)
- Oligos (see Table 1)
- T7 Endonuclease I (New England BioLabs, cat. no.M0302S)
- NEB buffer 2 (New England BioLabs, cat. no. B7002S)

Mammalian cell culture

- HeLa Kyoto cells
- High Glucose Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, cat. no. 41965-039)
- Fetal Bovine Serum (FBS, Life Technologies, cat. no.10270106),
- 10000 units/ml Penicillin – Streptomycin (Life Technologies, cat. no. 15140122)
- 200 mM L-Glutamine (Life Technologies, cat. no. 25030081)
- PBS (see reagent set up)
- 0.05% Trypsin (Life Technologies, cat. no. 25300054)

Transfection

- Donor plasmids synthesized from Geneart (Thermo Scientific) or Genewiz (Sigma Aldrich)
- jetPrime (Polyplus Transfection, cat.no. 114-07)
- Opti-MEM® I Reduced Serum Medium, GlutaMAX™ Supplement (Life Technologies, cat.no. 51985-026)

FACS

- FACS buffer (see reagent set up)

Junction PCR

- HotStar HighFidelity PCR kit (QIAGEN, cat. no. 202605)
- DNA lysis buffer (see reagent set up)

Southern blot analysis

- PCR DIG probe Synthesis kit (Roche, cat. no. 11 636 090 910)
- Blocking agent (Roche, cat. no. 1109617600)
- Epicentre MasterPure DNA purification kit (cat. no. MCD85201)
- DIG Easy Hyb Buffer (Roche, cat. no. 11603558001)
- anti-digoxigenin-Alkaline Phosphatase Fab fragments (Roche, cat. no.11093274910)
- CDPstar chemiluminescent substrate (Sigma, cat. no. C0712)
- High performance autoradiography film (GE Healthcare, cat. no. 28906845)
- SSC (see reagent set up)
- SDS (see reagent set up)
- NaCl (see reagent set up)
- Trizma® base (Sigma, cat.no. T1503)
- Neutralisation solution (see reagent set up)
- Denaturation Solution (see reagent set up)
- DIG1 (see reagent set up)
- DIG1 wash buffer (see reagent set up)
- DIG block (see reagent set up)
- DIG3 (see reagent set up)
- Stripping solution (see reagent set up)
- Zero Blunt ®TOPO®PCR Cloning Kit (Life Technology, cat. no. K2800-20)

Sanger Sequencing

- Oligos for amplification of target sequence and sequencing (see Table 1)
- HotStar HighFidelity PCR kit (QIAGEN, cat. no. 202605)
- MinElute Gel Extraction Kit (QIAGEN, cat. no. 28604)

Western blot analysis

- Cas9-HRP antibody (abcam, cat. no. ab202580)
- GFP antibody (Roche, cat. no. 11814460001)
- RFP antibody [3F5] (ChromoTek, cat. no. 3f5)
- Non-Fat Dry Milk powder
- TWEEN® 20 ® 20 ® 20 (Sigma, cat. no. P9416)
- PBS (see reagent set up)
- DTT (see reagent set up)
- cOmplete EDTA-free Protease Inhibitor Tablets (Roche, cat. no.11873580001)
- PhosStop Tablets(Roche, cat.no. 04906845001)
- Cell lysis buffer (see reagent set up)
- Bio-Rad Protein Assay (Bio-Rad, cat no. 500-0006)
- NuPAGE® 4-12%Bis-Tris Gels (Thermo Scientific, cat. no. NP0323BOX)
- Western LIGHTNING™ *plus*- (Perkin Elmer, cat. no. NEL105001EA) or Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, cat. no. RPN2232)
- NuPAGE MOPS SDS Running Buffer, 20X (Thermo Scientific, cat. no. NP0001)
- NuPAGE LDS sample buffer, 4X (Thermo Scientific, cat. no. NP0008)
- PageRuler Plus Prestained Protein Ladder 10-250 kDA (Thermo Scientific, cat no. 26619)

- Transfer buffer (see reagent set up)

Live cell imaging and cell cycle analysis

- SiR-DNA (SPIROCHROME, cat. no. SC007)
- CO_2-independent imaging medium without phenol red (Gibco, cat. no. ME080051L1)
- Silicon grease

REAGENTS SET UP

Agarose gel Resuspend the correct amount (%w/v) of agarose in 1X TBE. Boil until agarose is dissolved and cool down to ~50-60ºC. Dilute SYBR® Safe DNA Gel Stain 100,000 fold in the gel prior to polymerization.

Cell lysis buffer Prepare a solution containing 10% glycerol, 1 mM DTT, 0.15m EDTA, 0.5% TritonX100 in 1x PBS. Dissolve 1 tablet of cOmplete EDTA-free protease inhibitor and 1 tablet of PhosStop in 10ml cell lysis buffer. Prepare buffer freshly prior use.

Denaturation Solution Dissolve NaOH pellets to a final concentration of 0.5M and NaCl to 1.5M in ddH_2O. Prepare freshly.

DIG1, 10X Dissolve Maleic Acid to a final concentration of 1M and NaCl to 1.5M in ddH_2O. Adjust pH to 7.5 with NaOH pellets. Careful Maleic acid only dissolves at a pH above ~6.5-7 and has hardly any buffering capacity.

DIG1, 1X Dilute 10X DIG1 buffer to 1X with ddH_2O.

DIG1 wash buffer Dilute 10X DIG1 buffer to 1X with ddH_2O and add 0.3% TWEEN® 20.

DIG3 Dilute stock solutions to final concentrations of 0.1M NaCl and 0.1M Tris (pH9.5).

DIG block, 10X Weigh out 10% w/v Roche blocking agent and dissolve in 1X DIG1. Heat the solution to ~65ºC bringing the blocking reagent into solution. Make aliquots and store at -20ºC. Use a fresh aliquot every time due to the risk of contamination.

DNA lysis buffer Dilute and dissolve the following reagents in ddH_2O: 0.45% TWEEN® 20, 0.45% Triton X100, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH8.3. Store at room temperature and add 100ug/ml proteinase K prior to use.

DTT, 1M Prepare the solution in ddH_2O and divide it into aliquots. Store them at -20 °C. Use a new aliquot for each reaction, as DTT is easily oxidized.

FACS buffer Dilute 2% FBS in PBS, filter through a 0.22µm membrane.

HeLa growth medium High Glucose DMEM supplemented with 10 % FBS, 100 U/ml Penicillin, 100 µg/ml Streptomycin and 2 mM L-Glutamine. Supplemented DMEM medium is abbreviated as complete DMEM.

Imaging medium CO_2 independent medium without phenol red supplemented with 20% FBS, 2 mM L-Glutamine and 1 mM sodium pyruvate. Filter through a 0.22µM membrane to clear the medium of precipitants.

LB medium Dissolve 1% NaCl, 1% peptone and 0.5% yeast extract in ddH_2O. Sterilize by autoclaving.

LB agar medium Dissolve 15 g Agar in 1l LB medium. Sterilize by autoclaving.

NaCl, 5 M Dissolve the appropriate amount of NaCl in ddH_2O. Filter sterilize or autoclave.

Neutralisation solution Dissolve NaCl to a final concentration of 1.5 M and Tris to 0.5 M in ddH_2O. Adjust pH to 7.0 using HCl.

PBS, 1X Prepare 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 in ddH_2O. Adjust the pH to 7.4 with HCl and autoclave.

PBS-T Add 0.1% TWEEN® 20 into 1X PBS.

SDS PAGE running buffer Dilute 20X NuPAGE MOPS SDS Running Buffer to 1X with ddH_2O.

SDS sample buffer 4X NuPAGE® LDS Sample Buffer was supplemented with 100 µM DTT and diluted with whole protein extracts to 1X.

20%SDS Weigh out 20% w/v SDS pellets and dissolve in the appropriate amount of ddH_2O by heating to 65-68ºC and sterile filter the solution.

SSC, 20X Dissolve NaCl to a final concentration of 3 M and sodium citrate to 0.3 M in ddH_2O. Adjust pH to 7.0 with 1 M HCl due to weak buffering capacity.

Stripping solution Dissolve NaOH pellets to a final concentration of 0.2 M NaOH in ddH_2O and add 0.1% SDS. Prepare freshly.

TBE, 1X Dilute a 10X stock solution with ddH_2O. The final concentration of 1X TBE is 89 mM Tris pH 7.6, 89 mM boric acid, 2 mM EDTA.

1M Tris pH9.5 Dissolve Trizma base to a final concentration of 1M in ddH_2O and adjust the pH to 9.5 with concentrated HCl. Filter sterilize or autoclave.

1M Tris pH8.3 Dissolve Trizma base to the final concentration of 1M in ddH_2O and adjust pH to 8.3 with concentrated HCl. Filter sterilize or autoclave.

Western transfer buffer Dilute NuPAGE® (20X) Transfer Buffer in ddH_2O to 1X and add 10% pure Ethanol.

Western blot blocking solution Resuspend 5% w/v non-fat dry milk powder in PBS-T. Store at 4ºC for up to a few days.

EQUIPMENT

- NuncDish 24 well (Thermo Scientific, cat. no. 142475)
- NuncDish 6 well (Thermo Scientific, cat. no. 140675)

- NuncDish 96well (Thermo Scientific, cat. no. 167008)
- Cryotube™ vials Thermo nunc (Thermo Scientific, cat. no. 366656)
- Falcon® 12 x 75 mm Tube with Cell Strainer Cap (Falcon, cat.no. 352235)
- Falcon® 12 x 75 mm Tube Snap Cap (Falcon, cat.no. 352058)
- Falcon 14ml polypropylene tubes (Thermo Scientific, cat.no. 352059)
- CellStar tubes 15ml, PP, conical bottom (Greiner, cat. no. 188271)
- Falcon™ Disposable Polystyrene Serological 5ml Pipets (Falcon, cat. no. 357543)
- Falcon™ Disposable Polystyrene Serological 10ml Pipets (Falcon, cat. no. 357551)
- Falcon™ Disposable Polystyrene Serological 25ml Pipets (Falcon, cat. no. 357525)
- Filter tips: 1000 µl, 200 µl, 20 µl, 10 µl
- 3MM Whatman paper
- Genescreen Plus Hybridization Transfer Membrane (Perkin Elmer, cat. no. NEF976001PK)
- Immobilon-P Transfer Membrane (PVDF membrane, Merck, cat. no. IPVH00010)
- 96 Well SCREENSTAR Microplate (Greiner, cat. no. 655866)
- Imaging plates 96CG, glass bottom (zell-kontakt, cat. no. 5241-20)
- Glass hybridization flasks 40x300mm
- 0.22µm Filters
- Disposable cuvettes made of PS, semi-micro, 1.5-3.0 ml (neoLab, cat. no. E-1642)
- RED™ Imaging System (Biozym Scientific GmbH)
- Mini Trans-Blot®Electrophoretic Transfer Cell (BioRad, cat no. 1703930)
- ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices)
- PowerPac™ Basic Power Supply (BioRad, cat. no.164-5050)
- Rocker or shaker
- Hybaid Oven Mini10
- EI9001-XCELL II Mini Cell (NOVEX) gel chamber
- Transferpette-8 electronic 1-20 µl (Brand, cat. no. 705400)
- Transferpette-8 electronic 20-200 µl (Brand, cat. no. 705404)
- Submerged Horizontal agarose gel electrophoresis system

PROCEDURE

gRNA and donor design TIMING 1 d

1| *gRNA design.* The gRNA should bind over or as close as possible to the target site, e.g. ATG or Stop codon. Homologous recombination will occur most efficiently within 100bp of the target site and drops dramatically beyond this region. Design two different gRNA pairs if possible. gRNAs were designed using either http://crispr.mit.edu/ or https://benchling.com/crispr [5,35,36]. Chose gRNAs with predicted off-targets that contain at least 2nt mismatches located within the seeding region 8-12nt 5'of the PAM sequence.

2| *Design of oligos for gRNA cloning.* The vector can be digested using BbsI, and a pair of annealed oligos are to be cloned scarless into the vector before the sgRNA scaffold. The oligos are designed based on the target site sequence (20bp) and need to be followed on the 3' end by a 3bp NGG PAM sequence, but do not contain the PAM site. BbsI sites were added to the gRNAs to enable cloning the oligos into pX335 vectors as described on the webpage https://www.addgene.org/crispr/zhang/ (see Table 1).

3| Order the oligos as described in Figure 11 and Table 1.

Donor design and synthesis TIMING 14-21 d

4| *Donor design.* The donor plasmid contains the fluorescent marker gene (e.g. mEGFP or mCherry) which is flanked by 500-800bp homology arms of the gene of interest. The fluorescent marker replaces either the start or the stop codon and is in-frame with the gene of interest. If the ends of the homology arms are GC rich or contain any repetitive regions, they should be shortened, otherwise 800bp works well. A linker should be placed between the tag and the gene to maintain functionality of the tagged protein. 5xGlycine or 5x Glycine/Serine repeats can be used as linker [37]. However, if there is a known linker that has already been used for the protein of interest, it is best to use that one. The inclusion of additional cloning sites between the homology arms and the insert enables the exchange of the tag or linker if required (Figure 12).

CRITICAL No selection marker for mammalian cells is contained in the donor plasmid. If antibiotic selection in mammalian cells is used, random integration of the donor plasmid into the cell genome will increase. Also, no CMV, RSV or any other exogenous promoter is present within the donor plasmid, otherwise the expression levels of the tagged protein will not be physiological and artefacts will occur. Clone the tag in frame with the endogenous promoter. If two proteins of interest have to be tagged**,** the least expressed protein should be tagged with mEGFP since it is brighter and more stable than mCherry. Use monomeric fluorescent tags such as mEGFP, mCherry or mCer3. The donor design usually depends on the gRNAs as it should differ from the endogenous gene otherwise the gRNA will also cut the donor, causing a decrease in HDR efficiency. Therefore it is best to use gRNAs across the start or stop codon (depending on N- or C-terminal tagging, respectively), so that the donor differs to the endogenous locus due to the insertion of the tag.

5| *Gene synthesis of donor* The donor plasmids were synthesized by either Geneart (Thermo Scientific) or Genewiz (Sigma Aldrich).
CRITICAL The synthesis of the donors can take up to 21 days depending on the G/C % and repetitive sequences in the homology arms. During the production period, proceed with the gRNA cloning and the T7E1 assay.

Cloning gRNAs into the pX335-U6-Chimeric-BB-CBh-hSpCas9n(D10A) vector
TIMING 4 d

6| Resuspend the forward and reverse strand for each gRNA oligo to a final concentration of 100µM in ddH_2O.

7| Use 4 µl of each 100µM stock solution of forward and reverse oligo and mix well by pipetting.

8| Anneal forward and reverse oligo (Figure 11, Table 1) in a thermocycler by using the following parameters: 95ºC for 5min. and subsequently cool to 25ºC with a ramp of 0.1ºC/sec (~ 5ºC/min.)

9| Digest pX335-U6-Chimeric-BB-CBh-hSpCa9n(D10A) vector with BbsI (BpiI):

Component	Amount	Final concentration
pX335-U6-Chimeric-BB-CBh-hSpCa9n(D10A) vector	2 µg	
FastDigest BbsI (BpiI)	2 µl	
10X Fast Digest Buffer	4 µl	1X
ddH_2O	up to 40 µl	

Incubate digestion mix at 37ºC for 30min.

10| After the incubation period, run the digestion product on a gel and purify the digested vector: cast a 1% (w/v) agarose gel in TBE buffer with SYBR Safe dye.

11| Purify the digestion product of 8.4kb using the QIAquick gel purification kit according to the supplier's manual. Elute the DNA in 30 µl of EB buffer (part of the kit).

12| *Ligation of annealed oligos into BbsI-digested pX335 using the Roche Rapid Ligation Kit.* Prepare for each gRNA and a no-insert negative control the following ligation reaction:

Component	Amount
BbsI-digested pX335 vector	50-100 ng
Annealed gRNA oligos	1 µl (replace with water for no-insert control)

5X DNA dilution buffer	Up to 10 µl
T4 DNA ligation buffer	10 µl
T4 DNA ligase	1 µl

Incubate the ligation mix at room temperature for more than 5min.

CRITICAL In former protocols[5], phosphorylation of the annealed oligos and dephosphorylation of the vector were performed, thereby causing an elevated number of false positive colonies or no colonies at all. The above protocol is reliable, although at times there are only a few colonies, but almost all of them are positive for the correct insertion of gRNA oligos.

13| *Transformation of ligation reactions into a competent E.coli strain.*

(i) Add 10 µl of each ligation reaction into 50 µl ice-cold chemically competent NEB 5alpha competent bacteria, incubate on ice for 30min., perform a heat-shock at 42ºC for 45sec and place on ice immediately for 2-3min.

(ii) Add 1ml room temperature SOC medium to the mixture and incubate at 37ºC with agitation for 60 min. Subsequently spin for 1min. at 3000g, remove supernatant and resuspend bacteria pellet in 100 µl LB medium.

(iii) Plate the suspension on a LB agar plate containing 100 µg/ml ampicillin and incubate at 37ºC overnight.

14| Day 2: Examine the LB plates and pick a few colonies, inoculate these into 5ml LB medium containing 100 µg/ml ampicillin. Incubate these cultures at 37ºC overnight.

15| Day 3: Isolate the plasmid DNA from the LB cultures by using QIAprep spin miniprep kit according to the supplier's manual.

16| Validate the insertion of the gRNA into pX335 plasmids by Sanger sequencing using the following oligo: 5' TGCATATACGATACAAGGCTGTTAG 3' (Table 1)

TROUBLESHOOTING (Table 3)

Functionality test of gRNAs using the T7E1 assay TIMING 5 d

17| *Maintenance of HeLa Kyoto cells.*

(i) HeLa Kyoto cells are cultured in High Glucose DMEM medium supplemented with 10% FBS, 1000 unit/ml PenStrep, 2 mM glutamine, 1 mM sodium pyruvate at 37 °C and 5% CO_2.

(ii) To split the cells, remove the medium and rinse the cells once with 1X PBS. Add an adequate amount of trypsin and incubate at RT until cells are almost detached, remove trypsin and resuspend cells in an adequate amount of growth medium.

(iii) Transfer 1:5-1:10 of the cell suspension into a new TC dish and put them back into an incubator with 37ºC, 5% CO_2.

18| *Transfection of HeLa Kyoto cells with gRNA plasmids.*

(i) Seed $1.5x10^5$ cells per well on a 6 well TC dish in 2ml of normal growth medium

one day prior to the transfection. The cells should be at least 50% confluency during the transfection.

(ii) Dilute the gRNA plasmids in 200 µl of jetPrime buffer as indicated in the table below.

(iii) Mix well and add 4 µl of jetPrime solution, incubate at room temperature for 15min.

(iv) Add transfection complex onto the cells.

(v) After 4h incubation at 37ºC and 5% CO_2 replace transfection medium with normal growth medium.

Sample	**CRISPR/Cas9 nickase sense**	**CRISPR/Cas9 nickase antisense**	**Buffer**	**JetPrime**
CRISPR Oligo 1 (sense)–negative control	2 µg		200 µl	4 µl
CRISPR Oligo 2 (antisense)–negative control		2 µg	200 µl	4 µl
Paired CRISPRs	1 µg	1 µg	200 µl	4 µl
Untransfected cells-negative control	-	-	-	-

19| Day 2: Passage cells if they are more than 80% confluent.

20| Day 3: *Harvest cells for genomic DNA extraction 72h post transfection*. Wash cells with 1X PBS, add 1ml trypsin and wait until cells almost detach. Remove trypsin and resuspend cells in 1ml growth medium. Transfer cell suspension into an Eppendorf tube, spin for 1 min. at 200g, wash cell pellet in 1X PBS and spin again for 1min. at 200g.

21| Prepare genomic DNA from cell pellets using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma) or a similar genomic DNA preparation kit.

22| *Touchdown PCR.* The PCR product should be 700 to 1000bp. The primers should be designed that the CRISPR/Cas9 target site results in two fragments which differ 100-200bp in length and are at least 300bp in total. The region of interest is amplified using HotStar HighFidelity PCR kit and each reaction was set up as described below:

Component	**Volume**
5x HotStar PCR buffer	4 µl
T7 Primer_fw (10 µM)	2 µl
T7 Primer_rev (10 µM)	2 µl
HotStar HighFidelity polymerase	1 µl

Q Solution	4 µl
Template DNA (200 ng)	
ddH_2O	Up to 20 µl

Amplify the region of interest using the following cycling conditions:

Cycles	Denature	Anneal	Extend
1	95 °C, 5 min		
2-21	95 °C, 30s	Start temperature is 5ºC higher than T_m of oligos, - 0.5ºC/cycle, 45s	72ºC, 0.5kb/1min.
22-43	95 °C, 30s	End temperature of last cycle = 5ºC lower than T_m, 45s	72ºC, 0.5kb/1min.

23| Run 2 µl of PCR product on a 2% agarose gel. One single amplified fragment should be present. Keep 3 µl of PCR product for later analysis.

24| Use 15 µl of PCR product and perform the following denaturing/reannealing cycle to generate heteroduplexed DNA:

Temperature	Time
95 °C	5 min
95 °C – 85 °C	-2 °C/s
85 °C – 25 °C	-0.1 °C/s
25 °C	10 min

25| Digest 15 µl of heteroduplexed DNA by adding 1.8 µl of 10X NEB buffer 2 and 1.2 µl of T7 Endonuclease I. Incubate at 37ºC for 30min.

26| Analyze 18 µl of T7E1 digested product on a 2% agarose gel. Run 3 µl of undigested PCR product to compare the cleavage efficiency. The negative controls should only have one band as the PCR product band whereas the paired nickase sample digested with T7E1 should have several cleavage bands as predicted. The gRNA pair with the highest cleavage efficiency will be used for introducing the fluorescent marker gene at the locus of interest.

CRITICAL An alternative to the T7E1 assay is TIDE. TIDE consists of the amplification of the target region, Sanger sequencing and analysis of the sequence traces by a specially developed decomposition algorithm that identifies the major induced mutations in the projected editing site and accurately determines their frequency in a cell population [39].

TROUBLESHOOTING (Table 3)

Delivery of paired CRISPR/Cas9 approach using plasmids

CRITICAL The paired CRISPR/Cas9 approach has been used in a number of mammalian cell lines. The transfection conditions for each cell line must be established separately. The conditions described below are best for HeLa Kyoto cells.

27| *Transfection of HeLa Kyoto cells with gRNA and donor plasmids.* Seed $1.5x10^5$ cells per well on a 6 well TC dish in 2ml OptimemI one day prior to transfection.

CRITICAL Homozygosity can be increased up to 2% when cells are seeded into medium without serum such as OptimemI causing starvation and G1 arrest. Consequently, the cells enter S-phase after the transfection. Homologous recombination occurs more frequently during this stage of the cell cycle.

28| Day 2: The cells should be at least 60% confluence during transfection. Dilute the gRNA plasmids and donor plasmid in 200 µl of jetPrime buffer as indicated in the table below. Mix well and add 4 µl of jetPrime solution, incubate at room temperature for 15min and add transfection complex onto the cells. After 4h at 37ºC and 5% CO_2 incubation replace transfection medium with normal growth medium.

CRISPR/Cas9 nickase sense	CRISPR/Cas9 nickase antisense	Donor plasmid	Buffer	JetPrime
0.5 µg	0.5 µg	1 µg	200 µl	4 µl

29| Day 3-10: Passage cells when they are ~80% confluent until single cell sorting which is performed 7-10 days post transfection.

Selection of single cell clones expressing fluorescent tag using FACS TIMING 10-12d

CRITICAL Single cell sorting can cause growth problems for some cell lines. Establish the best growth condition for the used cell lines such as conditioned medium. HeLa Kyoto cells survive single cell sorting quite well and the survival rate is 30-50%. Donor Plasmids have a cryptic promoter in the BacORI and therefore there is always some background expression of mEGFP occuring, which can result in false positive EGFP expression [29] during the FACS sort. Therefore, FACS sorting is done 7-10 days post transfection when dilution of the donor plasmid has occurred.

30| Transfected cells are grown and expanded until FACS. 7-10 days after transfection single cell FACS is performed. FACS is performed with at least one 10cm cell dish (if possible with 1-2 15cm dishes of cells) and the parental cell line is used as the negative control.

31| Prepare five 96well plates for single cell sorting: add 100 µl growth medium per well.

32| Prepare cells for FACS single cell sorting. Remove growth medium from 10cm dish of HeLa Kyoto cells. Wash cells with 5-10ml of PBS per 10cm dish. Add 1-2ml Trypsin-

EDTA 0.05% and incubate at room temperature until cells are almost detached from the dish (1-5min). Remove Trypsin-EDTA 0.05% and add 2ml FACS buffer. Resuspend cells thoroughly and filter cells through a tube with cell strainer. Use HeLa Kyoto cells as negative control for FACS.

33| Perform single cell sorting for cells expressing the fluorescent marker (usually mEGFP or mCherry) into 96 well plates containing 100 µl growth medium whereby at least five 96well plates containing single cells are generated. The fluorescent signal of endogenously tagged proteins are usually relative dim. The efficiency of positively tagged cells is in the range of 0.05-10% positive cells and is typically 0.5%.

34| Day 2-12: Let the cell clones grow up to a decent size and check for single cell clones (discard multi cell clones).

35| Day 10-12: Pick survivors and transfer them onto a fresh 96well plate, so that all clones are on one or two 96well masterplates.

36| Day 13-14: Split masterplates and prepare duplicates for DNA preparation.

37| Keep clones growing on 96well plates until the clones expressing the correctly tagged protein of interest are identified.

Validation of fluorescent marker integration at correct locus and test of homozygosity by junction PCR TIMING 1 d

38| *Design of oligos for junction PCR.* To test if the fluorescent marker is integrated into the expected locus, design a forward primer binding at the 5' end outside of the left homology arm and a reverse primer binding to the fluorescent marker gene (Table 1). This primer set should result in one PCR product of the expected size. To test if all alleles are tagged with the fluorescent marker at the correct locus (= homozygosity), design a forward primer located 5' outside of the left homology arm and a reverse primer 3'outside of the right homology arm. Two PCR products using this primer set will indicate heterozygote clones and one PCR product which runs at the expected size of the tagged gene will indicate homozygosity (Figure 13).

CRITICAL The junction PCR is not a quantitative PCR; consequently, the tagged-gene in heterozygous clones is sometimes not detected as there is a competition between the two PCR products (Figure 4). If problems occur in detecting the tagged gene, it is advisable to repeat the junction PCR with primers used for the T7E1 assay, which result in shorter PCR products, thereby improving amplification of both PCR products. However, using the T7E1 primers has the disadvantage that the primers are localized within the homology arms, which will also amplify randomly integrated donor plasmids. Homozygously tagged genes will be always resulting in one PCR product.

39| *DNA preparation for junction PCR.*

(i) Prepare DNA directly from 96well plates by removing the medium
(ii) Add 30 µl DNA lysis buffer.
(iii) Resuspend cells and transfer the DNA solution into PCR tubes.

(iv) Incubate DNA solution at 56ºC for 1h followed by 95ºC for 10min.
(v) Store the DNA at 4-8ºC until further use.

40| *Junction PCR.* The region of interest is amplified using the HotStar HighFidelity PCR kit, each reaction was set up as described below:

Component	Volume
5x HotStar PCR buffer	3 µl
Primer_fw (10 µM)	1.4 µl
Primer_rev (10 µM)	1.4 µl
HotStar HighFidelity polymerase	0.7 µl
Q Solution	3 µl
DNA	3-5 µl
ddH_2O	Up to 15 µl

Amplify the region of interest using the following cycling conditions:

Cycles	Denature	Anneal	Extend
1	95 °C, 5 min		
2-21	95 °C, 30s	Start temperature is 5ºC higher than T_m of oligos, - 0.5ºC/cycle, 45s	68ºC, 0.5kb/1min.
22-43	95 °C, 30s	End temperature of last cycle = 5ºC lower than T_m, 45s	68ºC, 0.5kb/1min.

41| *Agarose gel electrophoresis*.
(i) Prepare a 0.8% agarose gel using 1X TBE.
(ii) Submerge gel into electrophoresis tanks containing 1X TBE.
(iii) Apply 5µl of Gene Ruler as marker.
(iv) Add 3µl of 6x Loading dye to the PCR product and apply to a 0.8% agarose gel using 8-channel pipette.
(v) Run samples at 100-150Volt for ~1-1.5h. Expected result is shown in Figure 4 and 13.

Sanger sequencing TIMING 1-2 d

42| Use genomic DNA prepared with the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma) or a similar genomic DNA preparation kit according to the supplier's protocol.

43| Perform a PCR reaction using the conditions as for the T7E1 assay or junction PCR to check for integration of the tag (Table 1) and homozygosity.

CRITICAL Use primers resulting in short fragments, as there are many repetitive sequences and polyA regions within the homology arm that result in poor or disrupted

amplification. Furthermore, use a high fidelity proof reading Taq polymerase such as HotStar HighFidelity PCR kit from QIAGEN.

44| Gelpurify the PCR products for the tagged and untagged gene using the MinElute Gel Extraction Kit from QIAGEN according to the supplier's protocol.

45| Perform Sanger sequencing with primers used for the PCR, to detect possible mutations at the integration site of the tag and additionally, to detect mutations in the untagged alleles (Figure 14).

46| If only heterozygously tagged genes were generated, the untagged alleles are sequenced to find wildtype alleles to perform a second round of transfection with the aim to generate homozygously tagged genes.

TROUBLESHOOTING (Table 3)

Southern blot analysis TIMING 5 d

47| *Southern blot probe design and labelling.* The southern blot probe consists of 100-400bp where the GC% content should be less than 40%. The probe for the gene of interest should bind outside of the homology arms. In addition, a probe against the fluorescent marker is designed.

(i) Design oligos to amplify the probe from genomic DNA using the HotStar HighFidelity PCR kit as described above.

(ii) The amplified probe is inserted into the Zero Blunt TOPO PCR Cloning Kit accordingly to the manufacturer's protocol.

(iii) Use the probe-TOPO plasmid to label the southern blot probe using the PCR DIG probe Synthesis kit from Roche according to the supplier's protocol.

(iv) Run 2 µl of product on a gel next to known quantities of marker to estimate the amount of synthesized probe.

(v) Labelled probe migrates less than the unlabeled one. Labelled probe can be stored at -20ºC or can be used directly.

(vi) Use 10ng labelled probe/ml of hybridization buffer (normally 20-35ul in 25ml hybridization buffer). Hybridization temperature is dependent on the probe and is calculated as follows:

i. $T_m = 49.82 + 0.41\ (\%\ G + C) - (600/l)$ [l = length of probe in base pairs]

ii. $T_{hyp} = T_m\ (-20 \text{ to } -25°C)$

48| *DNA sample preparation.* Prepare genomic DNA using the Epicentre MasterPure DNA purification kit (or a similar genomic DNA purification kit to prepare high concentrated genomic DNA) following manufacturer's instructions. Two 10cm cell dishes result in enough DNA for 2-3 Southern blots. Examine 1 µl of genomic DNA on a 0.8-1% agarose gel to detect if the DNA is fragmented and to estimate the amount of DNA.

CRITICAL Nano Drop cannot measure genomic DNA precisely.

49| *Digestion of genomic DNA.* Select enzyme(s) such that you will have a detectable difference in size between integrated and non-integrated samples with your probe. Use

restriction nucleases that are not sensitive to methylation of CpG dinucleotides and that produce a relatively small fragment.

(i) Digest ~10-20ug of DNA overnight at the appropriate temperature in 40 µl with 1/10v enzyme(s).
(ii) Load samples on a 0.6-0.8% TBE gel and electrophorese overnight to get a good separation.

50| *Transfer of DNA onto nylon membrane.*

(i) Take a picture of the gel next to a fluorescent ruler and trim off excess gel. Cut the bottom of the right corner for later orientation.
(ii) Wash gel 2 x 20min in denaturation solution (~300-400ml for a 20cm x 20cm gel) on a shaking platform.
(iii) Rinse once with ddH_2O and wash 2-3 x 20min with neutralization solution. Check the pH of the gel which should not be higher than 7.5. If the pH is higher, replace the neutralization solution and continue with washes until the pH of the gel is 7.5.
(iv) Build the transfer stack as described in Figure 15 and below.
(v) Fill a tank with 2-3L 10 X SSC.
(vi) Place a glass plate on top of the tank whereby the glass plate does not cover the whole tank and there is some space in the front and back of the tank.
(vii) On top of the glass plate place 2 wicks of 3MM Whatman filter paper which are 2-3cm wider than the gel and soaked together in 10X SSC. The ends of the wicks rest in 10X SSC.
(viii) Roll out air bubbles with a plastic pipette.
(ix) Wet 1 piece of filter paper the same size as the gel and place on top of the wicks.
(x) Roll out air bubbles with a plastic pipette.
(xi) Slide the gel onto a glass plate and place it onto the wet filter paper.
(xii) Put saran wrap or parafilm around the edges of the gel on all sides to stop evaporation and short circuits.
(xiii) Cut the Genescreen PlusR nylon membrane to the size of the gel. First pre-wet Genescreen nylon membrane in ddH_2O and subsequently soak it in 10X SSC. Put the membrane on top of the gel (try not to move it too much!).
(xiv) Soak 2 pieces of filter paper in 10X SSC and place on top of the membrane, roll with a pipette gently to remove air bubbles.
(xv) Place 2 dry pieces of filter paper on top and then stack paper towels on top of the filter paper.
(xvi) Place a glass plate on top of the paper towels and two 100ml bottle half full of water on top as a weight. (the weight should not be too heavy as it will crush the gel). Leave to transfer overnight (>16h).

51| The following day, remove the paper towels and paper. Mark the wells of the gel on the membrane using a pencil and turn the damp membrane upright (DNA side is on top.) Keep either some wet filter paper underneath of the membrane or put the membrane onto filter paper soaked in 2X SSC. Crosslink the membrane using a Stratalinker® UV Crosslinker and autocrosslink twice. Afterwards rinse the membrane with ddH_2O and either continue with hybridization or dry the membrane on filter paper. The dried membrane can be stored at 4ºC.

52| *Hybridization.* The hybridization temperature is dependent on the probe (see above).

(i) Preheat the hybridization oven.

(ii) If the membrane is dry, rinse it briefly with ddH_2O or 2X SSC. Do not allow the membrane to dry after this point.
(iii) Place the membrane in the hybridization bottle, by rolling the membrane around a pipette, put the pipette into the bottle and slowly unroll the membrane onto the walls of the bottle. Air bubbles between membrane and glass should be avoided. If the membrane is big and overlaps itself in the hybridization bottle, use a nylon mesh cut 0.5cm bigger than the membrane, pre-wet it in 2XSSC and roll up with the wet membrane and insert them together into the hybridization bottle.
(iv) Add 25-30ml pre-warmed DIG Easy Hyb buffer and incubate rotating in hybridization oven for at least 30 minutes.
(v) Denaturation of the probe: add 15-35ul probe (depending on the concentration of the probe) into 100 µl ddH_2O and incubate at 95ºC for 5-10min. Subsequently place the denatured probe directly onto ice for 2-3min. Spin briefly down to collect evaporated solution.
(vi) Add the denatured probe to freshly pre-warmed 25 ml DIG Easy Hyb buffer.
(vii) Exchange the DIG Easy Hyb buffer with the hybridization buffer containing the denatured probe.
(viii) Incubate at the specific hybridization temperature in the oven rotating overnight.

53| *Detection of probe-target-hybrid.* After the hybridization, the membrane will be washed as described below. All wash steps are done on a shaking platform or with rotation in the hybridization oven.
(i) Wash membrane 2x5min in 2X SSCcontaining 0.1% SDS at room temperature shaking.
(ii) Wash 2x15min at 65ºC in high stringency wash buffer containing 0.1X SSC and 0.1% SDS (pre-heat wash buffer) with agitation.
(iii) Wash 1x10min with high stringency wash buffer at room temperature followed by a 1x5min wash in DIG1 wash buffer.
(iv) Dilute 10X DIG blocking buffer 1:10with 1X DIG1 buffer.
(v) Block the membrane at room temperature with rapid agitation for at least 30min.
(vi) Prepare a 1:20000 dilution of anti-digoxigenin-Alkaline Phosphatase Fab fragments in 1X DIG blocking buffer (= anti-DIG solution).
(vii) Remove the 1X DIG blocking solution from the membrane and replace with anti-DIG solution.
(viii) Incubate with agitation at room temperature for 30min. Do not increase this incubation time as background will increase if the incubation time exceeds 30-40min.
(ix) Wash membrane 3x20min with DIG1 wash buffer and 2x2min with DIG3 buffer with agitation.
(x) Drain membrane slightly and place on a plastic sleeve protector (DNA site upwards).
(xi) Add 4-8ml CDPstar chemiluminescent substrate onto the membrane and place cautiously on top the sheet of the sleeve protector avoiding air bubbles.
(xii) Incubate at room temperature 5min.
(xiii) Remove excess liquid and put the membrane sealed in the sleeve protector into a film cassette.
(xiv) Expose to films for 1-2h. If required, perform a second exposure overnight. Remove membrane and store in 2X SSC until further use.

54| *Stripping and re-probing.*
(i) Rinse the membrane a few times in ddH_2O.
(ii) Incubate 2x15min in stripping buffer at 37ºC with gentle agitation.

(iii) Wash twice with 2X SSC at room temperature for 5min.
(iv) Store membrane in 2X SSC at 4ºC or use with another probe and repeat the hybridization and detection steps.

TROUBLESHOOTING (Table 3)

Western blot analysis TIMING 3 d

55| *Preparation of protein lysates.* Harvest cells from a 6well cell culture dish by trypsinization and transfer the cell suspension into 2ml Eppendorf tubes.
(i) Pellet cells at 300 x g for 1min.
(ii) Wash cell pellet with 1X PBS.
(iii) Spin the cell suspension a second time and discard the supernatant.
(iv) Resuspend the cell pellet well with an equal amount of protein lysis buffer.
(v) Incubate cell lysate on ice for 30min and mix every few minutes by vortexing or pipetting.
(vi) Centrifuge the cell suspension at maximum speed (16000 x g) at 4ºC for 10min.
(vii) The total protein concentration of the lysate was determined using the Bio-Rad Protein Assay following the supplier's protocol.

56| *SDS PAGE.*
(i) 40 µg of total protein were mixed with 4X NuPAGE® LDS Sample Buffer supplemented with 100 µM DTT.
(ii) Incubate samples at 65ºC for 5-10min.
(iii) Apply sample to 4-12% NuPAGE® Bis-Tris Gels in a EI9001-XCELL II Mini Cell (NOVEX) gel chamber and run at 125-150V for approximately 90min. in NuPAGE MOPS SDS Running Buffer (1X, diluted in ddH_2O). PageRuler Plus Prestained Protein Ladder 10-250 kDa was used as a marker.

57| *Protein blotting.* The protein gel was transferred onto a PVDF membrane using the Mini Trans-Blot® Electrophoretic Transfer Cell.
(i) Prepare the transfer buffer.
(ii) Cut the PVDF membrane and 3MM Whatmann filter paper to the size of the gel.
(iii) Wet the PVDF membrane with methanol, rinse with ddH_2O and soak in transfer buffer.
(iv) Assemble the gel sandwich for the transfer as follows: Place the transfer cassette with the black side down, put pre-wetted fiber pads and 2 pre-wetted filter papers on the cassette. Place PVDF membrane on top of filter papers and put gel on top of the membrane. Place again two pre-wetted filter papers on top of the gel and roll with a pipette gently to remove air bubbles. Place pre-wetted fiber pad on top and close the transfer cassette.
(v) The transfer cassette was placed in the transfer cell such that the membrane site (black side) is placed towards the Anode (+).
(vi) Run transfer in 1 liter of transfer buffer at 4ºC with 400mA for 2h or at 60mA overnight.

58| *Immunodetection.* Disassemble the transfer cassette. All incubation and washing steps are performed on a rocker.

(i) Block the blots with 5% milk (w/v) in PBS+ 0.1% TWEEN® 20 (= PBS-T) at RT for 1h or at 4ºC overnight shaking.
(ii) Dilute the primary antibodies in 5% milk/PBS-T accordingly to Table 2 below.
(iii) Incubate the blot in diluted primary antibody at 4ºC overnight.
(iv) Day 2: Wash the blots 3x15 min with PBS-T at room temperature.
(v) Incubate with secondary antibodies (either rabbit or mouse as appropriate) diluted 1:5000 in 5% milk/PBS-T at room temperature for 1h.
(vi) Wash 4x20min. with PBS-T.
(vii) Incubate with Western LIGHTNING™ *plus*- or with Amersham™ ECL™ Prime Western Blotting Detection Reagents accordingly to the manufacturer's instructions.
(viii) Kodak BioMax MR or Amersham Hyperfilm MP films were placed on top of blots (protein site up) and developed in a Kodak RPX-OMAT processor.

59| *Stripping of blots.* All incubation and washing steps are done on a rocker at room temperature. Rinse blots with ddH_2O and wash for another 5 min. in ddH_2O. Incubate blots for 5-10min. in 0.2 N NaOH. Subsequently rinse the blots with ddH_2O and wash them for 5min in ddH_2O. Then perform the immunodetection protocol with the next primary antibody.

CRITICAL Use also antibodies against the protein of interest if available to detect homozygous expression of cell clones. Immunoprecipitations of the most promising clones are performed to test for the integration of the tagged protein in the expected complex.

Microscopy of promising tagged cell clones TIMING 2 d

60| To detect correct localization of the tagged protein, live cell imaging or immuno-staining with fixed cells can be performed.

(A) Live cell imaging

CRITICAL Live cell imaging has the advantage to follow a cell throughout the cell cycle and investigate if the tagged protein is performing as expected during cell division. Prior to live imaging set up the correct cell density and growth conditions on imaging plates. If cells do not grow on glass bottom plates, use plastic imaging plates.

61| Seed all cell clones, which have been positively tested for the integration of the fluorescent marker at the correct locus, onto a glass bottom 96well plate for imaging. The cell density should be 50-70% during imaging, this is usually 5000-7000 HeLa Kyoto cells per 96well.

62| Grow cells in normal growth medium at 37ºC, 5% CO_2 overnight.

63| Next day change medium to CO_2-independent medium without Phenol red and seal the edges of the plate with silicon.

64| Use a confocal microscope such as the Zeiss 780 laser scanning confocal microscope for live cell imaging. An incubation chamber should be installed to maintain cells growing at 37ºC. Images are taken with a 63× PlanApochromat numerical aperture (NA) 1.4 oil objective.

65| Image the cells during the cell cycle to observe if the fluorescently tagged protein behaves as expected.

(B) Imaging of fixed cells

CRITICAL Imaging of fixed cells has the advantage of allowing counter staining with antibodies if necessary.

66| Grow cells as described above on glass bottom plates or any other imaging plates which can be used on confocal microscopes.

67| Grow the cells in normal growth medium at 37ºC, 5% CO_2 overnight.

68| Fix them with 4%PFA in 1X PBS at RT for 15min.

69| Wash with 1X PBS twice.

70| If necessary counter stain nuclei with 0.1 µg/ml HOECHST in PBS.

71| Keep cells in 1X PBS for imaging.

72| Perform imaging as described in (A) as soon as possible. Fixed samples can be stored at 4-8ºC for 1-2 weeks.

Cell cycle analysis TIMING 4 d

CRITICAL Establish the best growth conditions and seeding density for the cell line to be analyzed. Cells should grow evenly, with a confluency of ~50% at the beginning of imaging. Adjust growth conditions for each cell line. If necessary use CO_2 and normal growth medium w/o phenol red for imaging. Any wide-field microscope with incubator can be used as long as it is equipped for autofocus and automated imaging (e.g. ImageXpress Micro XLS Widefield High-Content Analysis System from Molecular Devices or ScanR from Olympus). The conditions below are best for HeLa Kyoto cells.

73| *Seeding of cells.* Plate 7000 HeLa cells per well on a 96Well SCREENSTAR Microplate. Grow cells at 37ºC and 5% CO_2 overnight.

74| Day 2: Stain the nucleus with 50nM SiR-DNA in CO_2-independent imaging medium (w/o Phenolred) and seal the edges of the plate with silicon. Incubate at 37ºC (no CO_2) for 3h-4h.

75| Day 2-3: *Imaging.* Take images in the Cy5 Channel every 5 minutes for 24h using ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices). Perform an autofocus for all imaged wells and set up the imaging conditions so that the signal to noise ratio is 3:1.

Day 4: *Cell cycle analysis.* The CellCognition software [38] is used to automatically track single cells through the cell cycle and classify them into interphase and mitotic stages. The CeCogAnalyzer 1.5.2 is used.

The general documentation and the code for the cell cycle analysis are available on GitHub via https://git.embl.de/nwalther/Cecog_HMM_PostProcessing and the related wiki https://git.embl.de/nwalther/Cecog_HMM_PostProcessing/wikis/home.
The settings and documentation for CellCognition can be found on GitHub under the following link: https://git.embl.de/nwalther/Cecog_HMM_PostProcessing/wikis/Cellcognition. The CellCognition output is further processed with the custom-written MatLab code *cellfitness.m* to determine the mitotic timing including statistical analysis. The documentation for Cellfitness can be found on GitHub under the following link: https://git.embl.de/nwalther/Cecog_HMM_PostProcessing/wikis/Cellfitness.

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Table 1: Oligos

Step	oligo	Sequence (5 ->3)	Purpose
8	gRNA sense	caccgNNNNNNNNNNNNNNNNNNNN	Cloning into pX335
8	gRNA antisense	aaacNNNNNNNNNNNNNNNNNNNNc	Cloning into pX335
16	U6 promoter	TGCATATACGATACAAGGCTGTTAG	Sanger sequencing of modified pX335 containing gRNAs
38, 43	GFP_rev	GATGTTGCCGTCCTCCTTGA	Integration of GFP and sanger sequencing
38, 43	mCherry_rev	TCAGCTTCAGCCTCTGCTTGATCTCG	Integration of mCherry and sanger sequencing

Table 2: Primary antibodies

Name	Dilution	Supplier/ Reference
Anti-GFP (mouse)	1:1000	Roche
RFP antibody [3F5] (mouse)	1:500	ChromoTek
Anti-GAPDH (GC5) sc-32233	1:2000	Santa Cruz Biotechnology
Cas9-HRP antibody ab202580	1:2000	Abcam

Table 3: Troubleshooting

Step	Problem	Possible reason	Possible solution
14	No colonies	Ligation failure, incomplete digestion of cloning plasmid, too concentrated annealed gRNA oligos	reanneal sgRNA oligos; titrate sgRNA oligo concentration during ligation; redigest pSpCas9(BB) or pUC19
26	Inefficient or increased T7E1 cleavage	T7E1 activity is sensitive to the DNA:enzyme ratio, as well as incubation temperature and time	Use less DNA or more T7E1; decrease incubation time
46	No clear Sanger sequences	Pool of different tagged alleles, noisy sequence	Subcloning into vector (e.g. TOPO) and Sanger sequencing of several single clones
47	Low yield of DIG-labeled PCR product	Too much DIG-dUTP in reaction	Reduce the concentration of DIG-dUTP in the reaction
47, 53	Many non-specific bands	G/C content of probe too high, unspecific binding of probe	Design new probe

FIGURE LEGENDS

Figure 1: Paired CRISPR/Cas9 nickase approach

The RuvC nuclease domain is mutated in the Cas9D10A nickase resulting in single strand DNA breaks whose subsequent repair occurs preferentially through HDR [12, 15-17]. Two gRNAs are required to cleave the antisense and sense strand simultaneously when using Cas9D10A nickase which results in more specificity and less off-target cleavages. In the presence of the DNA repair template, a donor plasmid containing the fluorescent marker flanked by homology arms (500-800 bp), HDR takes place and the gene of interest is inserted into a specific locus. The efficiency of HDR varies between cell lines and it is advisable to test the efficiency of homologous recombination in the cells of interest[18].

Figure 2: Validation pipeline of genome-edited cell lines

After the cells have been transfected and selected for fluorescently expressing clones, the validation pipeline consists of junction PCR, Southern blot analysis, Sanger sequencing, microscopy, Western blot and cell cycle analysis.

Figure 3: FACS of genome-edited cell lines tagging NUP358 at the N-terminus

HeLa cells are used as negative control and mEGFP-NUP358 cells were selected for mEGFP expression. GFP-expressing single cells were sorted into 96well plates. SSC = Sideward Scatter, FSC = Forward Scatter.

Figure 4: Junction PCR of HeLa mEGFP-NUP358 cells.

(a) PCR to test the integration of mEGFP into the correct locus. (b) Test of homozygosity at the correct locus with primers outside of the homology arms and (c) inside the homology arms.

Figure 5: **Southern blot analysis of HeLa mEGFP-NUP358 cell clones.**

Southern blot was performed with different HeLa cell clones expressing mEGFP-NUP358 using GFP or NUP358 probes. Green asterisks in the blot with GFP probe indicate extra integration of mEGFP. Red asterisks in the blot with NUP358 probe reveal rearrangements of the chromosome within this region. (*) = additional or shifted DNA fragments

Figure 6: Sanger sequencing at the target site (Start codon).

PCR was performed using primers around the target site, start codon of NUP358, followed by sanger sequencing. The sequences of tagged and untagged NUP358 was aligned to the donor

plasmid used as DNA repair template during transfection. The green box indicates mEGFP which is missing in HeLa wt and untagged NUP358 as expected.

Figure 7: Western blot analysis of HeLa mEGFP-NUP358.
Antibodies against GFP (upper panel) and GAPDH (loading control, lower panel) were used.

Figure 8: Live cell imaging of HeLa mEGFP-NUP358 cell clones.

Figure 9: Analysis of mitosis.
(a) Automated live cell imaging of SiR-DNA stained cells. (b) Automated classification and (c) analysis of mitotic cell tracks using CellCognition software. (d) Statistical evaluation of the duration from pro- to anaphase onset.

Figure 10: Mitotic duration of HeLa mEGFP-NUP358 cell clones.

Figure 11: gRNA design – oligos for cloning.
The PAM (NGG) is always at the 3' end of the gRNA, but is not included in the oligos for cloning. At the end are the cloning sites indicated in red.

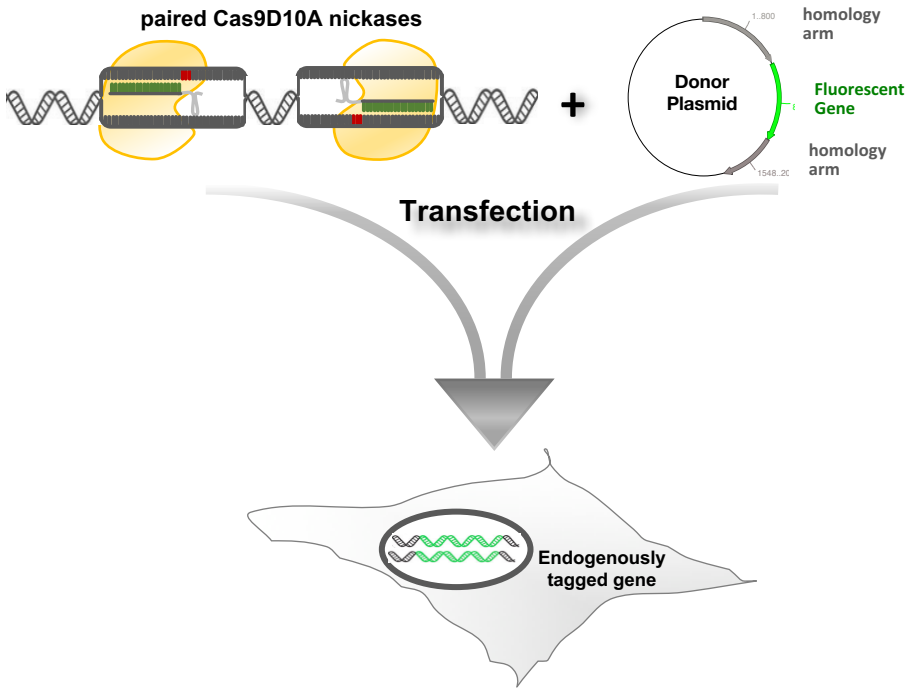
Figure 12: Donor plasmid.

Figure 13: Scheme of expected junction PCR result.

Figure 14: Example of sequences containing point mutation.

Figure 15: Southern blot transfer of DNA.

Figure 1


paired Cas9D10A nickases
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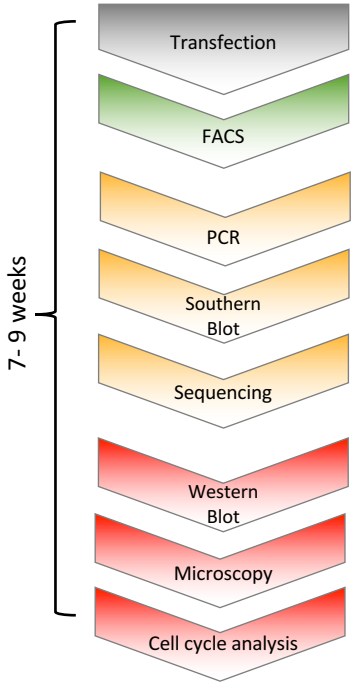
Donor
Plasmid
homology
arm
Fluorescent
Gene
homology
arm
Transfection
Endogenously
tagged gene

Figure 2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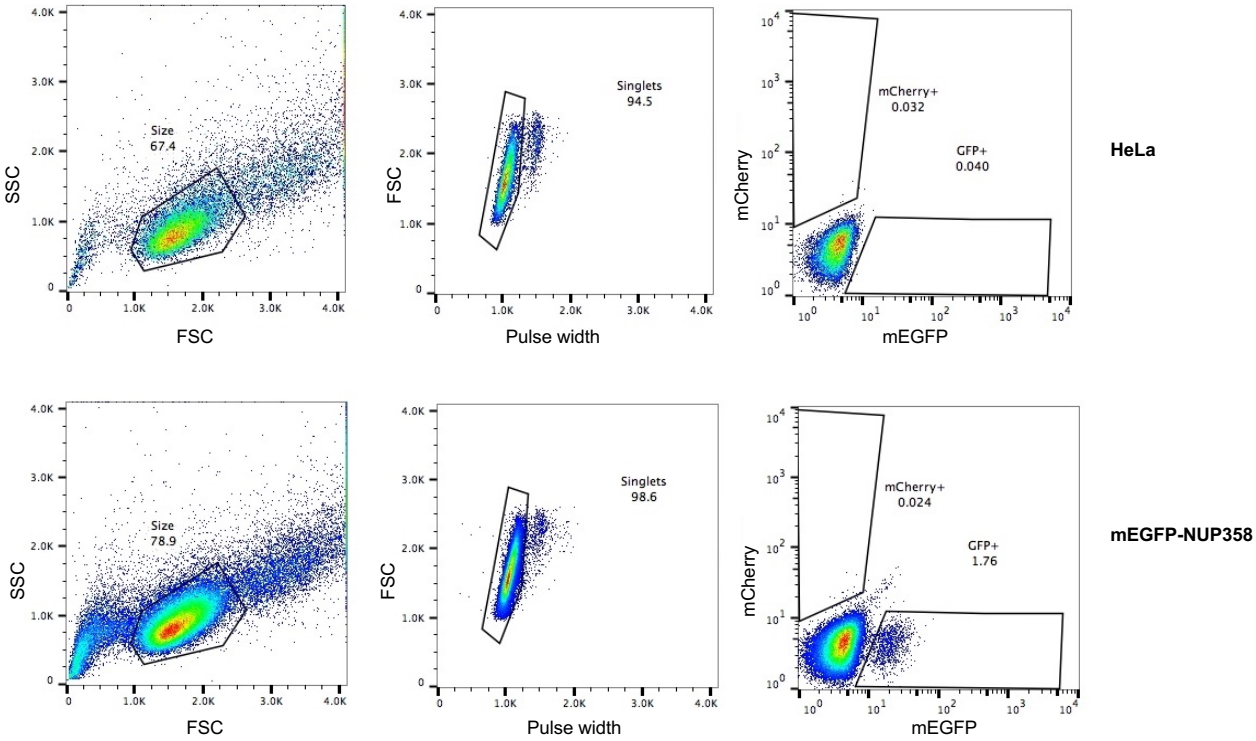
Transfection
FACS
PCR
Southern Blot
Sequencing
Western Blot
Microscopy
Cell cycle analysis

Figure 3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Pulse width
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SSC
FSC
Size
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GFP+
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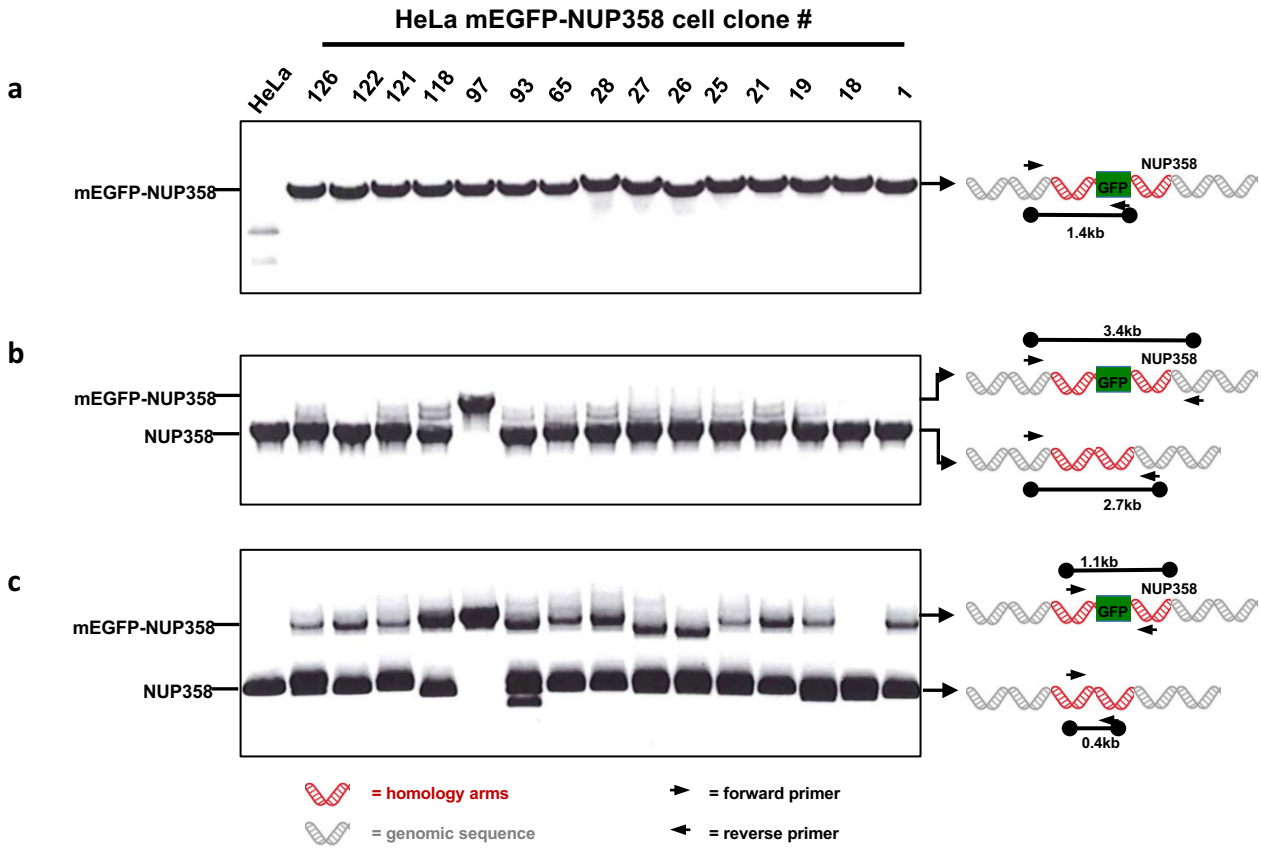
mEGFP-NUP358

Figure 4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18
1
mEGFP-NUP358
NUP358
GFP
1.4kb
b
mEGFP-NUP358
NUP358
3.4kb
NUP358
GFP
2.7kb
c
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NUP358
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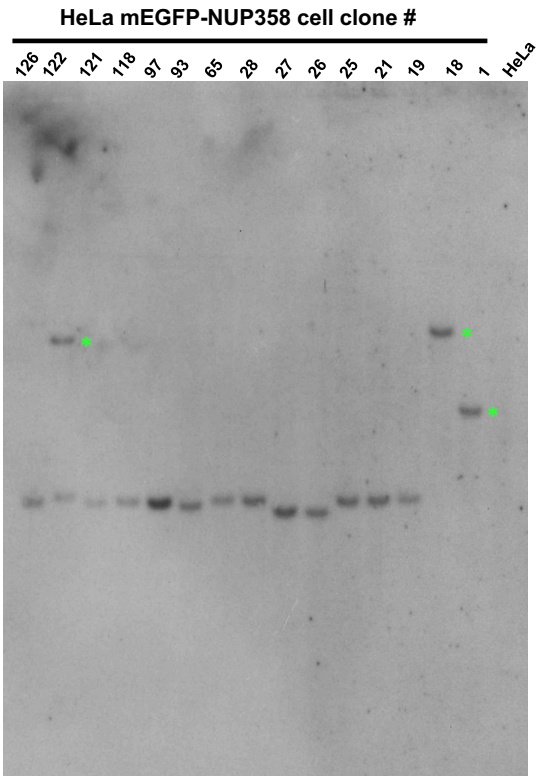
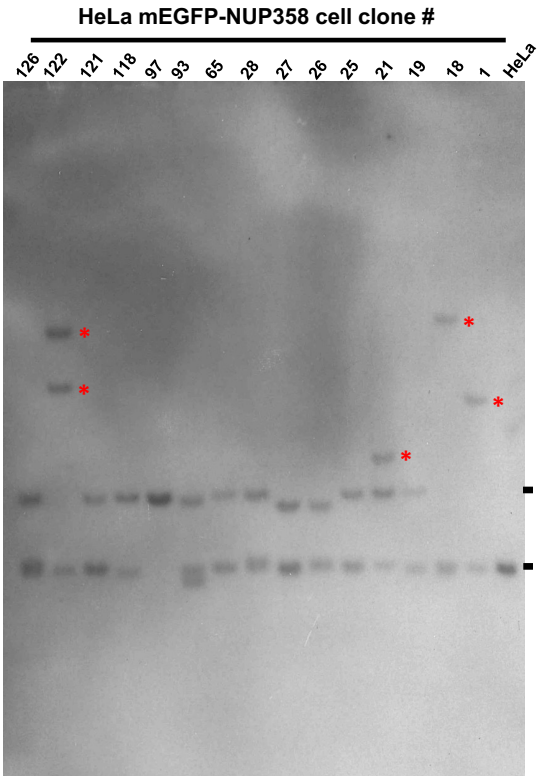
= homology arms
= genomic sequence
= forward primer
= reverse primer

Figure 5



GFP probe



NUP358 probe

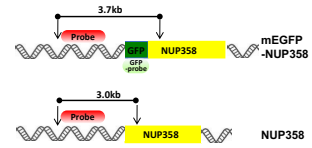


Figure 6

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                 #118 untagged 103>TCGCCAACGTGGCGCACACTGCTGACGCAGTACGCAAGTTCGTCACAGTGGTCCTCCGCCGGCTGCGCCGCAAGTTCGTCACAGTGGTCCTCCGCCGGCT>202
            #118 mEGFP-tagged  106>TCGCCAACGTGGCGCACACTGCTGACGCAGTACGCAAGTTCGTCACAGTGGTCCTCCGCCGGCTGCGCCGCAAGTTCGTCACAGTGGTCCTCCGCCGGCT>205
             #97 mEGFP-tagged  104>TCGCCAACGTGGCGCACACTGCTGACGCAGTACGCAAGTTCGTCACAGTGGTCCTCCGCCGGCTGCGCCGCAAGTTCGTCACAGTGGTCCTCCGCCGGCT>203

                                   *         *         *         *         *         *         *         *         *         *
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                     HeLa wt  204>ACGGCGCTGCGTCACTGGTTTGCAGGCGCTTTCCTCTTGGAAGTGGCGACTGCTGCGGGCCTGAGCGCTGGTCTCACGCGCCTCGGGAGCCAGGTTGGCG>303
                 #118 untagged 203>ACGGCGCTGCGTCACTGGTTTGCAGGCGCTTTCCTCTTGGAAGTGGCGACTGCTGCGG----------------------------------------->260
            #118 mEGFP-tagged  206>ACGGCGCTGCGTCACTGGTTTGCAGGCGCTTTCCTCTTGGAAGTGGCGACTGCTGCGGGCCTGAGCGCTGGTCTCACGCGCCTCGGGAGCCAGGTTGGCG>305
             #97 mEGFP-tagged  204>ACGGCGCTGCGTCACTGGTTTGCAGGCGCTTTCCTCTTGGAAGTGGCGACTGCTGCGGGCCTGAGCGCTGGTCTCACGCGCCTCGGGAGCCAGGTTGGCG>303

                                   *         *         *         *         *         *         *         *         *         *
mEGFP-NUP358 donor plasmid 1996>GCGCGACTAGTatggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccacaagttcagcgt>2095
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                 #118 untagged 260>---------------------------------------------------------------------------------------------->260
            #118 mEGFP-tagged  306>GCGCGACTAGTATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGT>405
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                                                                   mEGFP
                                   *         *         *         *         *         *         *         *         *         *
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                     HeLa wt  311>---------------------------------------------------------------------------------------------->311
                 #118 untagged 260>---------------------------------------------------------------------------------------------->260
            #118 mEGFP-tagged  406>GTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACC>505
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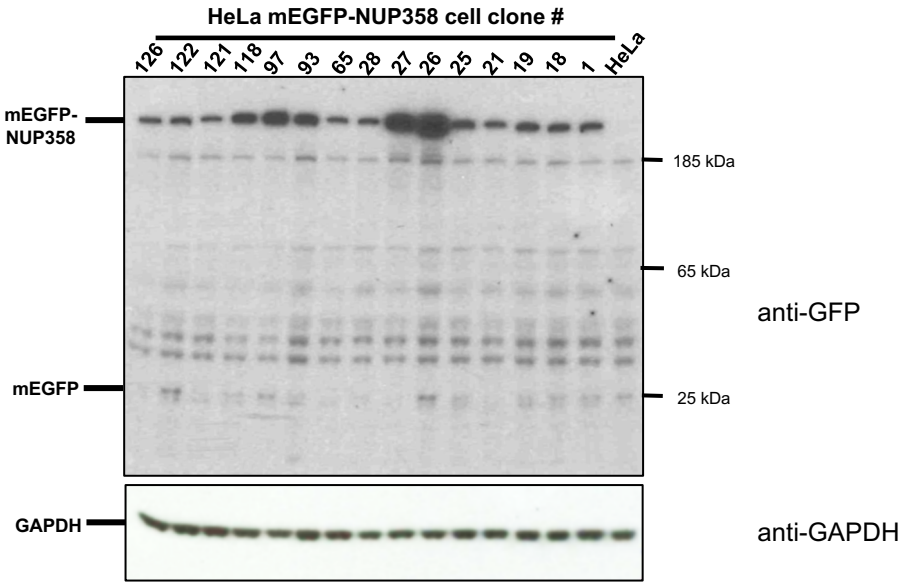
Figure 7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anti-GFP
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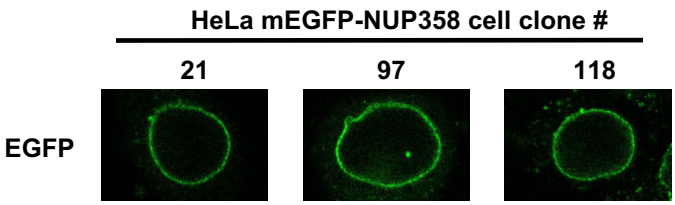
GAPDH
anti-GAPDH

Figure 8



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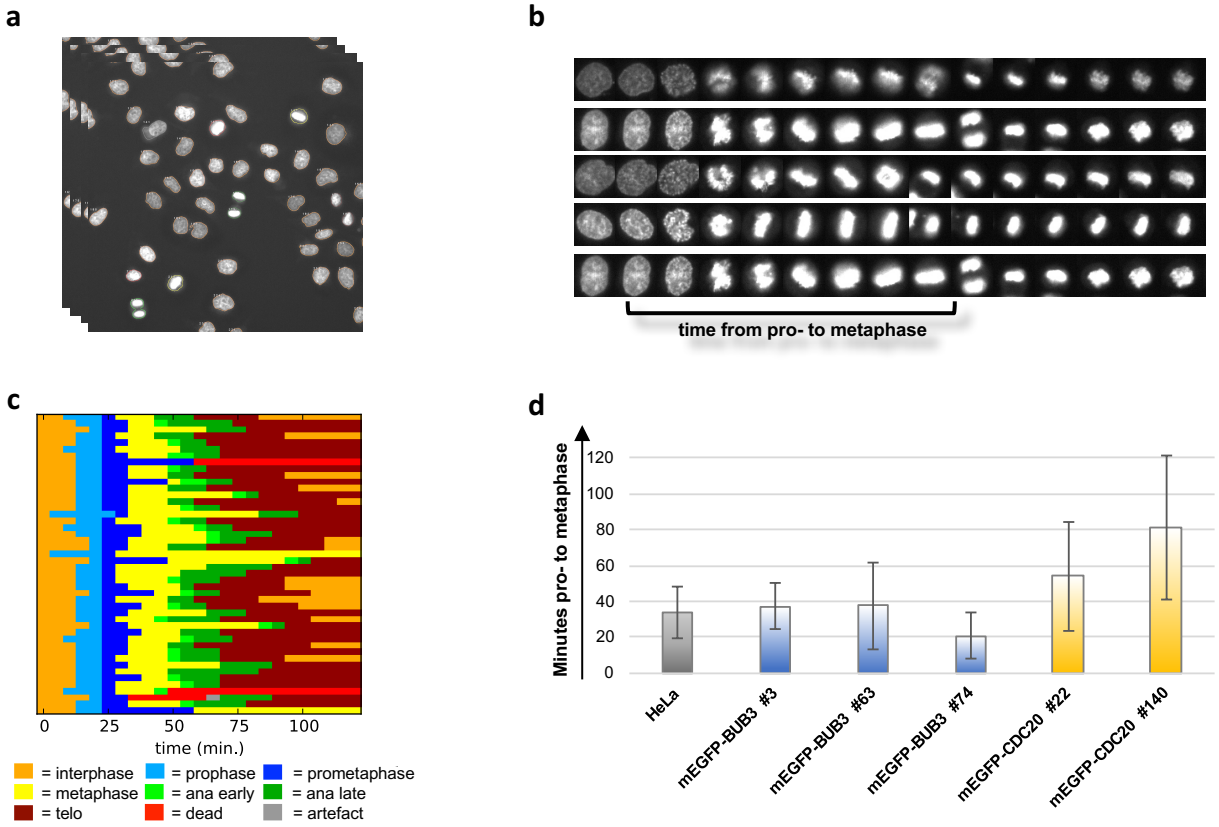
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c
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50
75
100
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= prophase
= prometaphase
= metaphase
= ana early
= ana late
= telo
= dead
= artefact
d
Minutes pro- to metaphase
120
100
80
60
40
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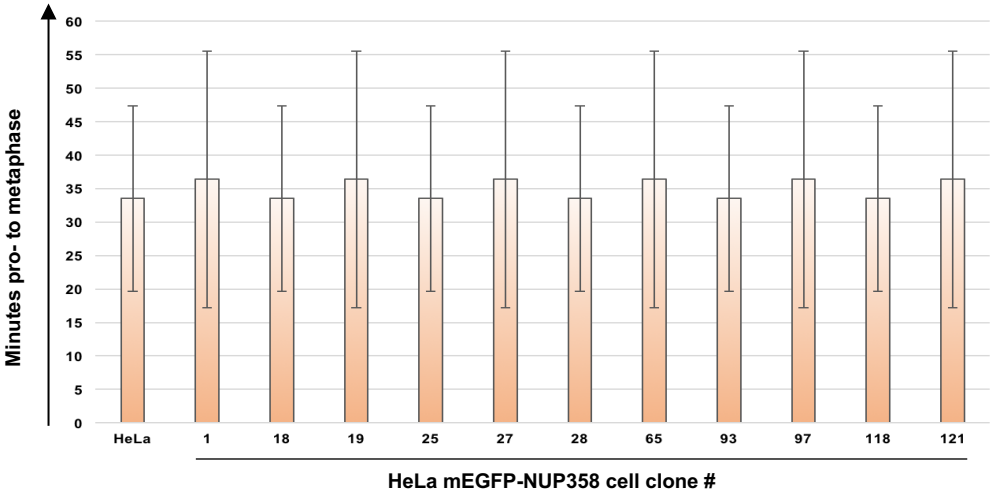
Figure 10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19
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28
65
93
97
118
121
HeLa mEGFP-NUP358 cell clone #

Figure 11

For 5’ caccgNNNNNNNNNNNNNNNNNNNN 3’
Rev 3’ cNNNNNNNNNNNNNNNNNNNNcaaa 5’

Figure 12

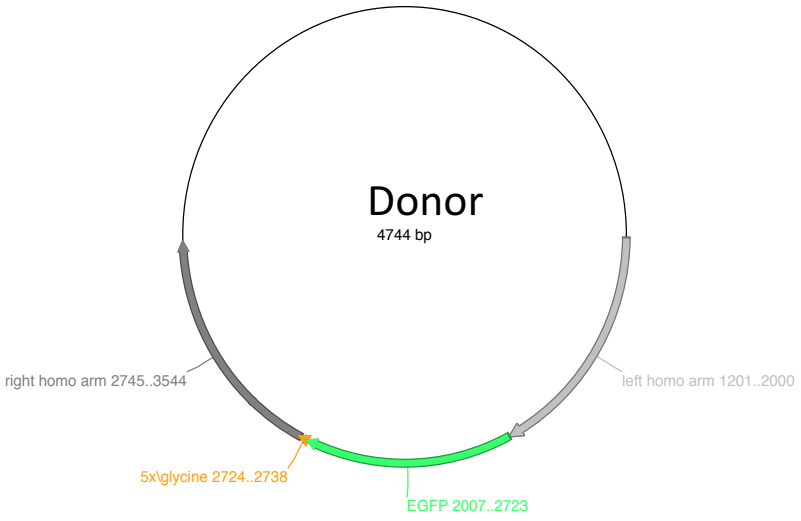
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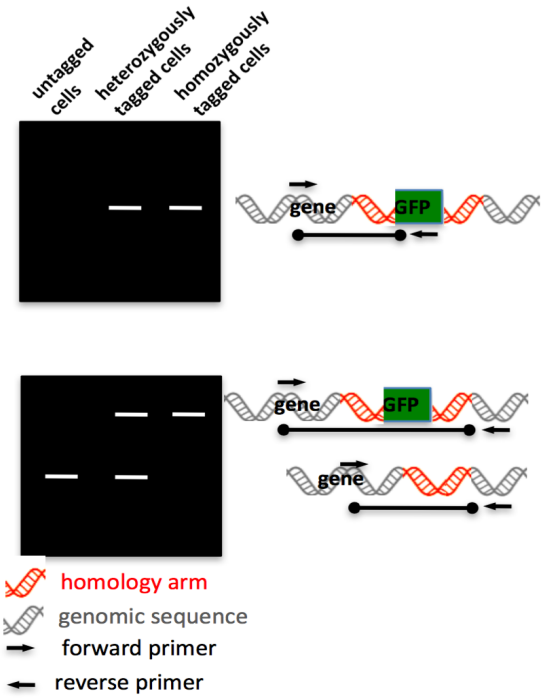
Figure 13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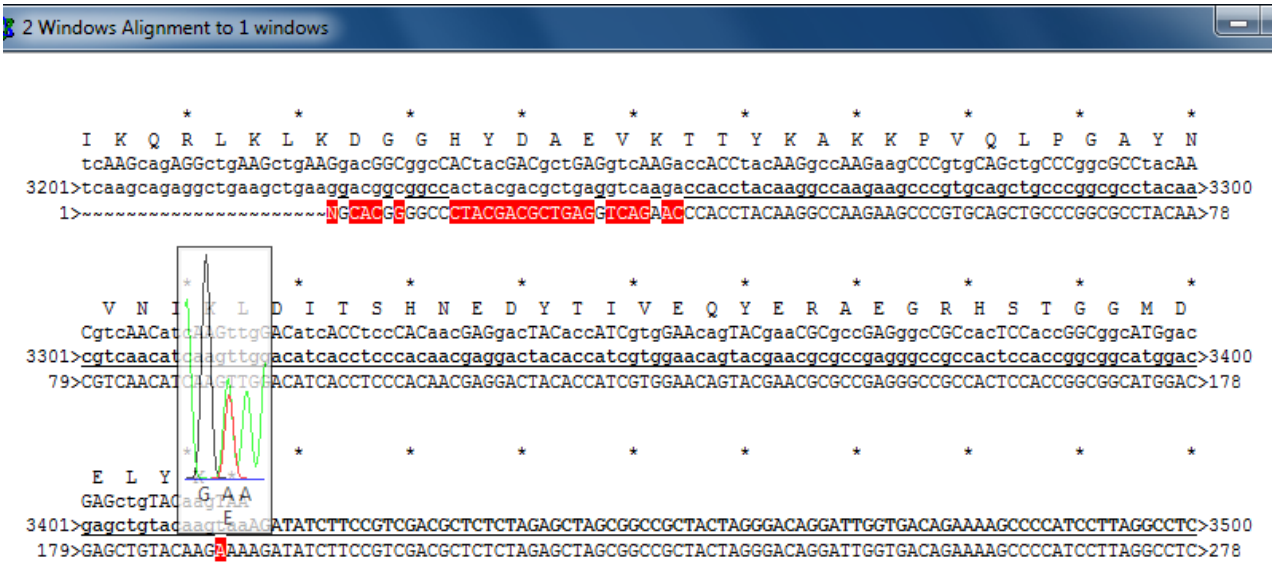
GFP
gene
homology arm
genomic sequence
forward primer
reverse primer

Figure 14


2 Windows Alignment to 1 windows
I K Q R L K L K D G G H Y D A E V K T T Y K A K K P V Q L P G A Y N
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1>~~~~~~~~~~~~~~~~~~~~~~NGCACGGGGCCCTACGACGCTGAGGTCAGAACCCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACAA>78
V N I K L D I T S H N E D Y T I V E Q Y E R A E G R H S T G G M D
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E L Y
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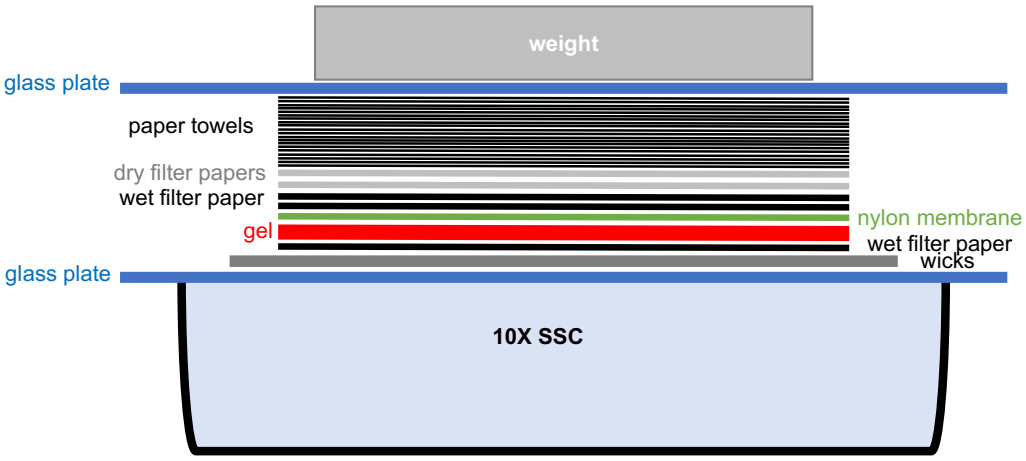
Figure 15


weight
glass plate
paper towels
dry filter papers
wet filter paper
gel
nylon membrane
wet filter paper
wicks
glass plate
10X SSC