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## Gene Tagging with the CRISPR-Cas9 System to Facilitate Macromolecular Complex Purification

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Abstract The need to generate modified cell lines that express tagged proteins of interest has become increasingly important. Here, we describe a detailed protocol for facile CRISPR/Cas9-mediated gene tagging and isolation of modified cells. In this protocol, we combine two previously published strategies that promote CRISPR/Cas9-mediated gene tagging: using chemically modified single-stranded oligonucleotides as donor templates and a co-selection strategy targeting the ATP1A1 gene at the same time as the gene of interest. Altogether, the protocol proposed here is both easier and saves time compared to other approaches for generating cells that express tagged proteins of interest, which is crucial to purify native complex from human cells.

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Keywords (separated by '-') CRISPR/Cas9 - Co-selection - Complex purification - Single-stranded oligonucleotide donor

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## Gene Tagging with the CRISPR-Cas9 System to Facilitate Macromolecular Complex Purification 2 3

Sylvain Geny, Simon Pichard, Arnaud Poterszman, and Jean-Paul Concordet 4 5

### Abstract 6

The need to generate modified cell lines that express tagged proteins of interest has become increasingly important. Here, we describe a detailed protocol for facile CRISPR/Cas9-mediated gene tagging and isolation of modified cells. In this protocol, we combine two previously published strategies that promote CRISPR/Cas9-mediated gene tagging: using chemically modified single-stranded oligonucleotides as donor templates and a co-selection strategy targeting the *ATPIA1* gene at the same time as the gene of interest. Altogether, the protocol proposed here is both easier and saves time compared to other approaches for generating cells that express tagged proteins of interest, which is crucial to purify native complex from human cells. 7 AU1 8 9 10 11 12 13 14

**Key words** CRISPR/Cas9, Co-selection, Complex purification, Single-stranded oligonucleotide donor 15 16

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### 1 Introduction 17

Using cells that express a genetically tagged subunit is a simple and efficient way to undertake affinity purification of a macromolecular complex of interest [1]. Antibodies to specific subunits could also be used but their isolation is often challenging, and they are seldom available. This obstacle can be overcome by expressing a fusion of the protein of interest with a peptide tag for which high affinity reagents are already available and well characterized. The method presented here consists in using optimized gene editing with the CRISPR-Cas9 system to introduce the coding sequence for a small peptide tag at the 5' or 3' end of the chosen subunit in order to facilitate affinity purification. Importantly, in contrast to transfection experiments, where the tagged protein is expressed from an exogenous promoter, the tagged protein is expressed from the endogenous locus and subject to the full extent of physiological control of gene expression. This approach therefore minimizes the 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32

risk of complex perturbation due to inappropriate expression levels associated with traditional transfection methods. 33 34

The CRISPR-Cas9 system has revolutionized many fields of life sciences by making it possible to modify the genome sequence with unprecedented efficiency in a great number of biological systems [2, 3]. Using a specific guide RNA, the Cas9 nuclease can be directed to generate a double-strand break (DSB) at virtually any chosen genomic site. To ensure their survival, cells will repair the damaged DNA. If DSB repair proceeds by end-joining repair pathways, small insertions or deletions are introduced at the site of the DSB while if template donor DNA is introduced together with the CRISPR-Cas9 nuclease, homology-directed repair (HDR) can take place and precise genome editing is achieved [4]. Donor DNA that can be used is either double-stranded plasmid or single-stranded oligonucleotides (ssODN). Precise genome editing with the CRISPR-Cas9 system is therefore a powerful method to introduce tag-coding sequences into genes of interest and greatly facilitates their analysis. However, HDR is generally less efficient than end-joining and HDR-based gene editing needs to be carefully optimized to achieve efficient insertion of tag-coding sequences. 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52

The precise gene editing method presented here is optimized at two levels, based on previously published studies. First, chemically modified ssODNs are used to increase the efficiency of precise gene editing [5] and second, a co-selection strategy is applied to increase the proportion of cells with the gene modification of interest. 53 54 55 56 57

The co-selection strategy was devised by the Doyon laboratory [6]. It consists of co-targeting the *ATP1A1* gene with a specific guide RNA and ssODN designed to introduce an *ATP1A1* mutation conferring resistance to ouabain, a toxic glycoside. After ouabain selection, the proportion of cells with gene editing at the locus of interest is significantly increased. The principle and efficiency of co-selection were initially reported in gene editing experiments with zinc finger nucleases [7]. The cellular mechanism for co-selection is not characterized, but it is thought that successful HDR at the selection gene locus likely corresponds to favorable cellular conditions for simultaneous HDR at the target locus, for example, during S/G2 cell cycle phases. 58 59 60 61 62 63 64 65 66 67 68 69

The co-selection of ouabain-resistant cells devised by the Doyon lab is more convenient than previous co-selection procedures [8] because it does not require the introduction of exogenous selection cassettes and can be very efficient, resulting in up to ten-fold enrichment. Importantly, co-selection generally makes it easier to isolate cells with modification of multiple alleles of the targeted gene. When tagging the subunit of a macromolecular complex, modification of multiple alleles increases the proportion of the complex that can be purified from edited cells. Furthermore, if all alleles are modified, it is easier to confirm that protein tagging does not perturb activity and regulation of the macromolecular 70 71 72 73 74 75 76 77 78 79 80

complex of interest. Finally, the protocol proposed here is very 81  
efficient which is critical to achieve gene tagging with ssODNs 82  
that are too small to include selection cassettes in addition to the 83  
tag-coding sequence. The lymphoblastoid cell line, K562, has been 84  
chosen for gene editing since the cells can be grown in to large-scale 85  
volumes in suspension, enabling the production of sufficient 86  
amounts of complex for purification. 87

The ATP1A1 co-selection strategy can be applied to the 88  
plasmid or ssODN donors [6]. ssODNs are particularly appealing 89  
to achieve efficient insertion of tag-coding sequences because they 90  
alleviate the need to construct donor plasmids. Although commer- 91  
cially available ssODNs have a length restriction under 200 nt, they 92  
can generally be used because a majority of purification tags require 93  
only a short-coding sequence. The ATP1A1 co-selection approach 94  
using ssODNs makes it possible to generate cells with several 95  
different purification tags in parallel to determine the optimal tag 96  
for purification of the macromolecular complex of interest. This 97  
cannot necessarily be predicted in advance [9, 10]. 98

Here, we demonstrate the feasibility of the method by tagging 99  
the XPB subunit of the TFIHH complex that plays a major role in 100  
DNA nucleotide excision repair and transcription [11]. We show 101  
that despite low DNA cleavage efficiency of the XPB gene with the 102  
CRISPR/Cas9 system in our experimental conditions, our 103  
approach is robust enough to isolate cells with insertion of 104  
tag-coding sequences by screening of only a small number of 105  
clones. We provide a detailed protocol applicable to any gene of 106  
interest to purify macromolecular complexes that cannot be 107  
isolated following previously described purification methods. 108

---

## 2 Materials 109

Prepare all solutions using ultrapure water and analytical grade 110  
reagents. Perform all cell culture work under sterile conditions. 111  
Diligently follow all waste disposal regulations when disposing 112  
waste materials. 113

Procedures described here need access to standard equipment 114  
for molecular biology, cell culture, and protein purification and 115  
require basic knowledge in these fields is required. Specific equip- 116  
ment and material is detailed below. 117

### 2.1 Oligonucleotides and Plasmids

1. Oligonucleotides for construction of sgRNA expression plas- 118  
mids are listed in Table 1. 119
2. ssODN donor oligonucleotide for ATP1A1 and XPB (Table 2). 120
3. Forward and reverse primers for genotyping (Table 3). 121
4. Expression plasmid for sgRNA: MLM3636 (Addgene cat. 122  
no. 43860). 123

t.1 **Table 1**

**Target sequences for sgRNAs. The 20 nt-long target sequence ( $N_{20}$ ) of each guide RNA and adjacent PAM are indicated. Oligonucleotides for cloning into the BsmB1 site MLM3636 should contain the appropriate 5' and 3' extensions: 5'ACACC (G)  $N_{20}$  G 3' for the sense oligonucleotide and 5'AAAA  $n_{20}$  C3' for the antisense oligonucleotide extension where  $n_{20}$  is the reverse complement of  $N_{20}$ . Online applications such as CRISPOR design the sequences to be ordered and examined if the target sequence starts with a G (required for efficient U6 transcription). If this is not the case, an extra G is added 5' to the guide sequence in order to ensure efficient transcription from the U6 promoter**

Guide RNA Plasmid	Target sequence	PAM
sgRNA-ATP1A1	GAGTTCTGTAATTCAGCATA <i>TGG</i>	<b>TGG</b>
sgRNA-XPB1	TAGGAAATGATGCTTAGGCA <i>GGG</i>	<b>GGG</b>
sgRNA-XPB2	TTAGGAAATGATGCTTAGGC <i>AGG</i>	<b>AGG</b>
sgRNA-XPB3	CGCTTTAGGAAATGATGCTT <i>AGG</i>	<b>AGG</b>

5. Expression plasmid for SpCas9: JDS246 (Addgene cat. no. 43861). 124

6. Plasmid sgG3-ATP1A1-Cas9 (Addgene cat. no. 86611). 126

**2.2 Cell Culture**

1. Tissue culture flasks (25, 75, and 175 cm<sup>2</sup>), reagent glass bottles of various sizes (250 mL ( $h = 105$  mm,  $d = 95$  mm, opening = 32 mm), 1000 mL ( $h = 222$  mm,  $d = 101$  mm, opening = 32 mm), and 5000 ml ( $h = 314$  mm,  $d = 182$  mm, opening = 68 mm), Duran® GLS 80® laboratory bottles) and gas permeable adhesive seal. Bottle caps are removed replaced with an aluminum foil and sterilized by autoclaving (134 °C, 15 psi, 20 min). 128AU4

2. RPMI 1640 with L-Glutamine. 136

3. Fetal bovine serum (heat inactivated) (FBS). 137

4. Penicillin-Streptomycin 10,000 U/mL. 138

5. Human chronic myelogenous leukemia K562 cells (ATCC, cat. no. CCL-243). 139

6. Phosphate-buffered saline. 141

7. Cell counter. 142

8. Cell counting chambers. 143

9. 0.4% Trypan blue dye solution in PBS. 144

10. Humidity, CO<sub>2</sub>, and temperature controlled orbital shaker fitted for 50 mL to 5 L glass bottles with 50 mm orbital and shaking speed of up to 150 rpm (InforsMultitron™). 145

11. Centrifuge with adaptors for 15 mL, 50 mL, 250 mL, and 1 L. For the 1 L tubes, use preferably a fixed angle rotor. 148

**Table 2**  
**Sequences of the single-stranded donor oligonucleotides. Homology arms are indicated in lower case. Epitope tag sequences 3Flag, His8, HiBiT, and cleavage site PreScission are in uppercase and underlined. Many different tags have been characterized and can be chosen for specific applications (Wood, 2014). For affinity purification, peptide tags are frequently used and small tags such as 3 × Flag and polyhistidine are generally preferred because they are considered less likely to affect protein function. Linker regions and stop codons are in uppercase. Phosphorothioate linkages are marked with an asterisk. The sequence of the ssODN mutATP1A1 donor is from [6]**

Donor ON	Sequence
mutATP1A1:	C * A * ATGTTACTGTGGATTGGAGCGATTCTTTTGTTCCTTGGCTTATAGCATCAGAGCTGC TACA GAAGAGGAAACCTCAAAAACGATGACGTGAGTTCCTGTAATTCAGCATAATCGAATTTGTAGTACAC ATCAGATAATC * T * T
XPB-HiBibit	c * g * cccagcaaacatgtacaccgcctcttcaagcgcttttaggaaa <u>GGTTCGGTGAGCGGGCTGGGGCTGTTCAA</u> <u>GAAGATTAGCTGA</u> cagggtactctgttcaagaccggcgcttggcacccttgttggga * a * a
ON1 XPB-Flag3X-His8	c * g * cccagcaaacatgtacaccgcctcttcaagcgcttttaggaaa <u>GGTTCGGACTACAAAAGACCCATGACGGTGATTA</u> <u>TAAAGATCATGACATCGAATTAACAAGGATGACGATGACAAGGGCAGGGCCCATCATCACCCAC</u> <u>CATCACCCACCAITTAG</u> cagggtactctgttcaagaccggcgcttggcacccttgttggga * a * a
ON2 XPB-PreScission-His10-CaptureSelect	c * g * cccagcaaacatgtacaccgcctcttcaagcgcttttaggaaa <u>AAGGTTCCCTGGAAAGTTCGTTCACAGGGGCC</u> <u>ctgggataccCATCATCACCCATCACCCATCACCCATCATCACCCATCACCCATCAAGCCCTGAAGCCCTAGCTAG</u> cagggta Ctttggttcaagaccggcgcttggcacccttgttggga * a * a
ON3 XPB-PreScission-Flag3X	c * g * cccagcaaacatgtacaccgcctcttcaagcgcttttaggaaa <u>GGTTCCTGGAAAGTTCGTTCACAGGGGCC</u> <u>CTGGGATCCGACTACAAAAGACCCATGACGGTGATATAAAGATCATGACATCGAATTA</u> <u>CGATGACGATGACAAAGTAG</u> cagggtactctgttcaagaccggcgcttggcacccttgttggga * a * a

t.1

AU2

AU3

t.2

t.3

t.4

t.5

t.6

t.7



t.1 **Table 3**  
**Sequences of the primers used for genotyping the clones**

t.2	XPBfw	AGACAGTAAGCGATCTGTAAACA
t.3	XPBrv	ACCCCACTCCCCAAAAAGTT

<b>2.3</b>	1. Tissue culture plate, 6 wells.	151
<b>Nucleofection AMAXA</b>	2. AMAXA cuvettes (Lonza).	152
	3. AMAXA nucleofector machine (Lonza).	153
	4. AMAXA pipettes (Lonza).	154
	5. Solution V AMAXA (Lonza).	155
		156
<b>2.4 Nano-Glo HiBiT</b>	1. ViewPlate 96-wells, white.	157
<b>Detection System</b>	2. Nano-Glo HiBiT Lytic detection system (Promega).	158
	3. Microplate Reader Wallac Victor 1420 (Perkin Elmer).	159
		160
<b>2.5 Ouabain</b>	1. Tissue culture plate, 6 wells.	161
<b>Selection, Pool and</b>	2. Tissue culture plate, 96 wells.	162
<b>Clone Analysis</b>	3. Ouabain octahydrate.	163
	4. DNA lysis buffer (Viagen).	164
	5. Phusion polymerase (New England Biolabs).	165
	6. dNTPs set.	166
	7. Agarose powder.	167
	8. Ethidium bromide.	168
	9. Tris-Borate EDTA UltraPure 10X.	169
	10. Bromophenol blue.	170
	11. 1 Kb Plus DNA Ladder (Invitrogen).	171
		172
<b>2.6 Biochemical</b>	1. Bioruptor (Diagenode) or any small-scale sonicator.	173
<b>Characterization and</b>	2. PBS containing 30% w/v glycerol.	174
<b>Purification</b>	3. RIPA buffer: 20 mM Tris-HCl or HEPES pH 7.5 120 mM KCl	175
	1% NP-40 0,1% SDS, 1 mM EDTA, 0,5% Na-Deoxycholate,	176
	supplemented with protein inhibitor cocktail (Roche™) and	177
	0.5 mM 1,4-dithreothiol (DTT) (Sigma Aldrich).	178
	4. Lysis buffer: 20 mM Tris or HEPES, 250 mM KCl, 20%	179
	glycerol, NP-40 0.05% supplemented with protein inhibitor	180
	cocktail (Roche™) and 0.5 mM DDT.	181
	5. DNase and RNase.	182
	6. M2 agarose beads.	183
	7. FLAG peptide (dykddddd) solution (10 mg/ml, pH adjusted	184
	to 7.5) and/or purified Precision protease (1 mg/mL).	185

8. Temperature controlled thermomixer. 186
9. TBST: 20 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20. 187  
20. 188
10. Blocking buffer: 3% w/v dry skimmed milk or BSA solution in TBST. 189  
190
11. Laemmli buffer 4X: 60 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.0005% Bromophenol Blue, 355 mM  $\beta$ -mercaptoethanol. 191  
192  
193  
194

### 3 Methods

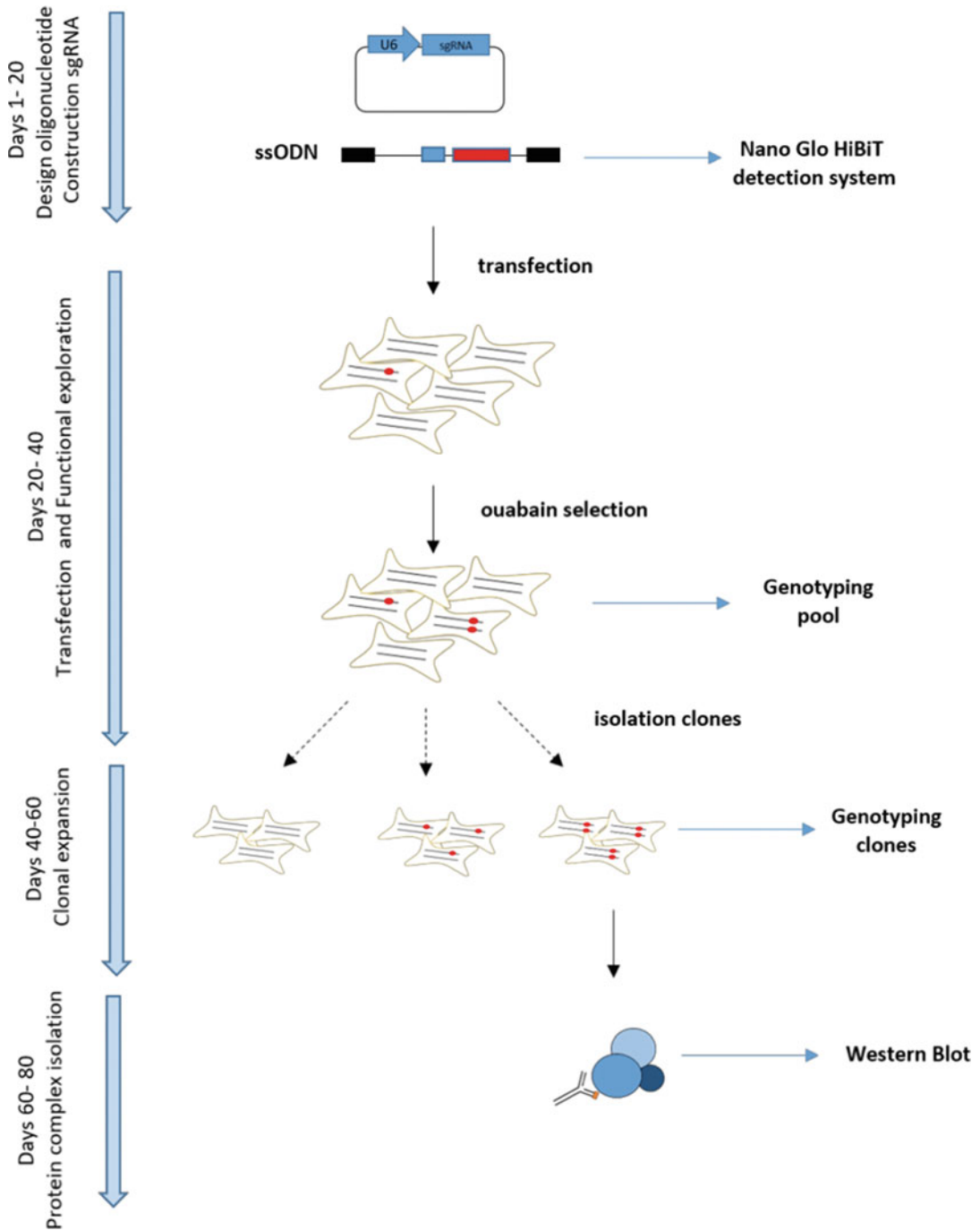
195

We detail the different steps of our gene editing protocol (Fig. 1.) 196  
The design of both sgRNAs targeting the gene of interest and the 197  
ssODN for the chosen peptide tag are detailed first (Subheadings 198  
3.1 and 3.2). Then, we describe how to take advantage of the newly 199  
developed Nano-Glo HiBiT Lytic Detection System to select the 200  
most efficient sgRNA for editing your target gene (Subheading 201  
3.3). Next, we provide the protocol for transfection of gene editing 202  
reagents into cells, ouabain selection (Subheading 3.4), and isolation 203  
of correctly modified cells (Subheading 3.5), carrying the 204  
tagged gene of interest. Finally, we report validation of the 205  
modified cell line by western blot analysis (Subheading 3.6) and 206  
large-scale suspension cultures to purify the target complex (Sub- 207  
heading 3.7). 208

#### 3.1 Design of sgRNAs for Targeting the Gene of Interest and Cloning into Guide RNA Expression Plasmid

Protein are usually tagged either at the N- or C-terminal end 209  
[12]. There is no easy method to predict how the tag will modify 210  
the protein structure and to conclude whether the protein and the 211  
tag will be accessible for their functions. Here, we show the step- 212  
by-step approach to introduce a purification tag at the C-terminal 213  
end of the protein XPB but a similar approach can be applied to tag 214  
the N-terminal end. To tag a protein at C-terminal end, the cut site 215  
introduced with the CRISPR/Cas9 system needs to be in the 216  
vicinity of the stop codon of the gene of interest. 217

1. Screen the genomic regions of interest using online CRISPR 218  
design tool CRISPOR (<http://crispor.tefor.net/>) to identify 219  
and rank the guide sequences. We recommend testing at least 220  
three sgRNAs with cut sites less than 20 bp away from the 221  
insertion site to find an optimal sgRNA. 222
2. Order the sense and antisense oligonucleotides for cloning of 223  
sgRNA sequences into plasmid MLM3636 from your preferred 224  
supplier. The oligonucleotide sequences needed are given by 225  
the CRISPOR website by clicking on the "Cloning/PCR primers" 226  
hyperlink for the selected guide RNA in the CRISPOR 227  
results web page. Resuspend the oligonucleotides for each 228

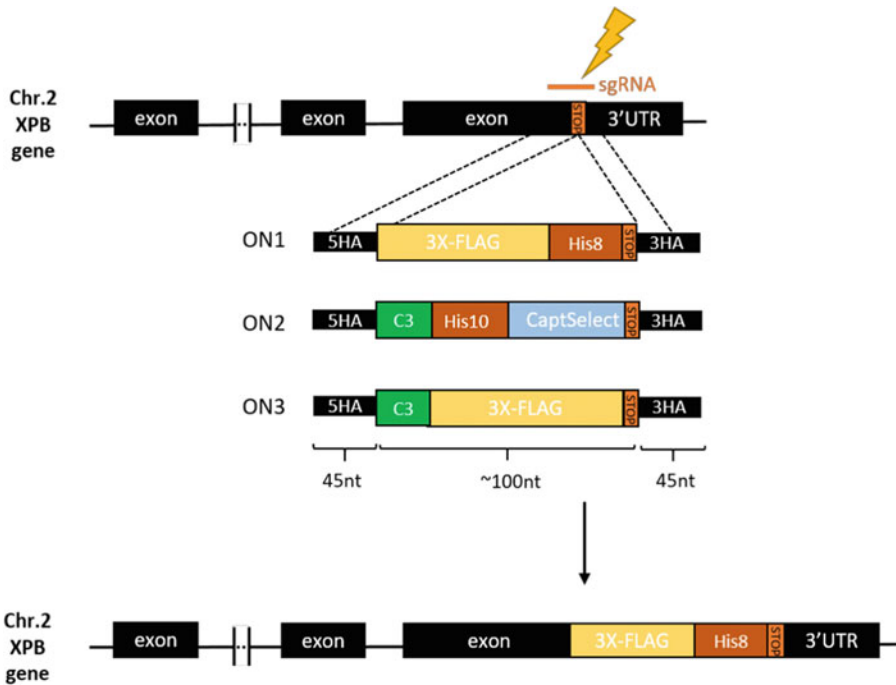


**Fig. 1** Timeline of a gene tagging experiment for protein complex purification. The main steps for a gene tagging experiment for protein complex purification are depicted. sgRNA guide sequences are cloned into an expression plasmid. Single-stranded donor oligonucleotides and plasmids expressing sgRNA and SpCas9 are transfected into cells. Treatment with ouabain is performed to enrich for gene-tagged cells among the total cell population. Finally, positive cells are clonally expanded to derive isogenic cell lines that express the tagged subunit protein to isolate the full macromolecular complex

- sgRNA at a concentration of 100  $\mu\text{M}$ . Prepare the following mixture for annealing the sgRNA oligos: 1  $\mu\text{L}$  of each oligonucleotides (100  $\mu\text{M}$ ), 2  $\mu\text{L}$  buffer NEB2x10, and 16  $\mu\text{L}$  water.
3. Anneal the oligonucleotides in a thermocycler by using the following parameters: 37  $^{\circ}\text{C}$  for 15 min; 95  $^{\circ}\text{C}$  for 5 min; ramp down to 25  $^{\circ}\text{C}$  at 5  $^{\circ}\text{C min}^{-1}$ .
  4. Digest 10  $\mu\text{g}$  plasmid MLM3636 with restriction enzyme BsmBI. Purify the linear plasmid using PCR cleanup kit. This step is sufficient to eliminate the short linker sequence between the two BsmBI sites of MLM3636.
  5. Set up a ligation reaction for each sgRNA: 1  $\mu\text{L}$  of pre-digested plasmid vector sgRNA (50 ng/ $\mu\text{L}$ ), 0.5  $\mu\text{L}$  of your annealing solution of sgRNA oligonucleotides, 1  $\mu\text{L}$  of T4 DNA ligase, 1.5  $\mu\text{L}$  of T4 DNA ligase buffer, 11  $\mu\text{L}$  of water.  
We recommend also setting up a no-insert, vector only negative control for ligation to observe the background amount of undesired ligation. Incubate the ligation reactions at room temperature for 1 h.
  6. Heat inactivate T4 DNA ligase with incubation at 65  $^{\circ}\text{C}$  for 10 min. Treat the ligation reaction for 30 min with BsmBI restriction enzyme to reduce background (arising from residual amounts of undigested plasmid or self-ligation of plasmid that was only digested once with BsmBI).
  7. Transform the ligation product into a competent *E. coli* strain, according to the protocol supplied with the cells. We recommend the DH5 $\alpha$  or XL10 strains for quick transformation. Briefly, add 3  $\mu\text{L}$  of the ligation product into 30  $\mu\text{L}$  of ice-cold chemically competent cells, incubate the mixture on ice for 10 min, heat-shock it at 42  $^{\circ}\text{C}$  for 30 s and return it immediately to ice for 2 min. Add 500  $\mu\text{L}$  of SOC medium and incubate 1 h under shaking at 37  $^{\circ}\text{C}$ . Plate the mixture onto an LB plate containing 100  $\mu\text{g/ml}$  ampicillin. Incubate it overnight at 37  $^{\circ}\text{C}$ . Pick up a colony to grow overnight at 37  $^{\circ}\text{C}$  in an appropriate volume of LB medium with antibiotics.
  8. Extract plasmid DNA using a Midi or Maxi Prep kit depending on the amount of plasmid required. Confirm successful sgRNA cloning by DNA sequencing.

### 3.2 Design of the Single-Stranded Oligonucleotide (ssODN) Donor

The oligonucleotide donors contain the coding sequence for one or several purification tags flanked by two homology regions as depicted in Fig. 2. Total length of the donor oligonucleotides should remain less than 200 bases to ensure efficient synthesis. For optimal HDR efficiency, we recommend designing homology regions of at least 45 bases on either side of the insert. If necessary, silent mutations in the guide RNA or PAM sequence should be added to prevent cleavage by Cas9 after oligonucleotide sequence



**Fig. 2** Schematic of the tagging strategy for the XPB locus. CRISPR/Cas9-induced DSB takes place in vicinity of the stop codon of the XPB gene with the selected guide RNA (sgRNA). The various single-stranded donor oligonucleotides tested (ON1,ON2,ON3) contain tag sequences (3X-FLAG/His8, His10/CaptSelect, and 3X-FLAG, respectively) flanked by two homology arms (5HA, 3HA) identical to the 5'- and 3'-genomic sequences adjacent to the cleavage site, respectively. As an example, the XPB locus is shown after integration of ON1 during repair of the DSB introduced by the complex of Cas9 and the sgRNA

integration. In that case, the homology arm should be chosen to be at least 45 bases long starting from the corresponding mutation. Two phosphorothioate linkages are added at both 5' and 3' ends between the three last nucleotides to protect donor oligonucleotides from degradation by cellular exonucleases and increase genome-editing efficiency [5]. No specific additional purification step, such as PAGE purification, is required after synthesis. If possible, we also recommend testing both sense and antisense oligonucleotides for optimal efficiency.

**3.3 Optimal sgRNA Selection with the Nano-Glo HiBiT Lytic Detection System**

We detail here a simple and highly sensitive protocol to find the optimal guide RNA for editing the gene of interest. A standard method for testing guide RNA efficiency is to measure the proportion of mutant sequences induced at the target site with the T7 endonuclease assay. However, this method is not very sensitive and does not reliably detect mutation rates below 5%. As we could not detect robust CRISPR/Cas9 induced-cleavage assay at the XPB locus using the T7 endonuclease assay, we used a more sensitive and quicker Nano-Glo HiBiT detection system to determine the

optimal sgRNA guide. The guide RNAs are used to stimulate integration of ssODN coding for a Hibit tag-coding sequence. The Nano-Glo HiBiT Lytic Detection System is highly convenient to detect HiBiT-tagged proteins in cell lysates through luciferase detection [13]. HiBiT is an 11-amino-acid peptide tag that can be fused to the N- or C-terminal end of a protein by genome editing with the CRISPR/Cas9 system. The Nano-Glo HiBiT detection system relies on a NanoLuc enzyme, which exhibits a 150-fold increase in luminescence compared to the traditional luciferases [14]. This approach increases the sensitivity of the method and allows robust detection of Hibit-tagged proteins, even when expressed at low levels such as transcription factors. In this protocol, we used distinct oligonucleotides containing either the purification tag or the HiBiT tag. If possible, it is very convenient to introduce HiBiT and purification tags in tandem. This allows integration efficiency to be tested easily and enables the isolation of modified cells from those exhibiting the highest integration efficiency.

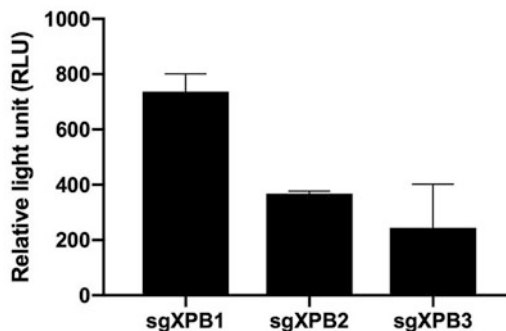
1. Thaw K562 cells using your favorite protocol. Cells are grown in RPMI 1640 with L-Glutamine supplemented with FBS (10%) and PenStrep (1%).
2. Split K562 cells 1/10 every 3–4 days by transferring 1 mL of suspension in a new tissue culture flask containing 9 mL of pre-warmed fresh media. We limit the number of passages to 30 to minimize genetic drift.
3. One day before nucleofection, determine the cell concentration, control that the viability is better than 95% and split cells in order to reach a cell count between 500,000–1,000,000 cells/mL.
4. Prepare DNA master mixes in Eppendorf tubes containing: 6  $\mu\text{g}$  of the donor oligonucleotide HiBiT, 2  $\mu\text{g}$  of guide sgRNA plasmid (sgXPB), and 2  $\mu\text{g}$  of plasmid expressing the SpCas9 protein (JDS246). The total volume should be lower than 10  $\mu\text{L}$  to avoid lower nucleofection efficiency.  
In a control well, we recommend transfection of 6  $\mu\text{g}$  of the donor oligonucleotide HiBiT on its own to measure the luminescent background signal in the experiment.
5. Count cells and centrifuge the required number of cells ( $1 \times 10^6$  cells per sample) at 90 g at room temperature for 10 min. Discard supernatant completely so that no residual medium covers the cell pellet. Resuspend cells in Nucleofector<sup>®</sup> solution V at  $10^6$  cells/100  $\mu\text{L}$ .
6. Mix the DNA master mixes with 100  $\mu\text{L}$  of cell suspension and transfer to a cuvette. Process the samples quickly to avoid storing the cells longer than 15 min in Nucleofector<sup>®</sup> Solution V.

7. Insert the cuvette into the Nucleofector<sup>®</sup>, select the cell type-specific program X-001, and press the start button. Using the provided pipette, immediately remove sample from the cuvette and transfer into the 6-well plate containing 2 mL of RPMI +1% PenStrep. 340-344
8. Incubate the 6-well plate at 37 °C, 5% CO<sub>2</sub> for 3 days then spin down and wash the cells with PBS. Resuspend the cells in 100 μL PBS and transfer to an opaque tissue culture plate to minimize absorption of the emitted light and cross-talk between wells. 345-349
9. Prepare a master mix of Nano-Glo HiBiT Lytic Detection System depending on the number of samples in your assay. Dilute the LgBiT Protein 1:100 and the Nano-Glo HiBiT Lytic Substrate 1:50 into an appropriate volume of Nano-Glo HiBiT Lytic Buffer at room temperature. Add 50 μL of Nano-Glo HiBiT Lytic Reagent in each well and mix. Wait at least 15 min for equilibration of LgBiT and HiBiT in the lysate. 350-356
10. Measure luminescence using settings specific to your instrument and consider the sgRNA with the highest luminescent signal as the optimal guide RNA plasmid for the following steps in the protocol. 357-360

The results of the Nano-Glo HiBiT Lytic Detection System is shown in Fig. 3 (See Note 1). 361-362

### 3.4 Transfection of Gene Editing Reagents into K562 Cells and Ouabain Selection 363

After selecting the optimal sgRNA guide, we proceed to genome editing with CRISPR/Cas9 to generate cell lines that express an endogenously tagged protein of interest. To ensure efficient integration in the cell genome, we combine chemically modified single-stranded oligonucleotides as homology donors and a co-selection strategy for enrichment in homology-driven repair during 364-369



**Fig. 3** Comparison of integration efficiency between the various sgRNAs targeting XPB. K652 cells were transfected with HiBiT ssODN donor, indicated guide RNA plasmid and JDS246 plasmid expressing the protein SpCas9. After 3 days, the cells were lysed using Nano-Glo HiBiT lytic detection system to measure luminescence. Higher luminescent signal correlates with higher integration of the donor HiBiT in cells



CRISPR/Cas9-mediated genome editing. This strategy generates a double integration at two genomic loci. The first integration corresponds to the tag insertion and is located downstream or upstream of the gene of interest. The second integration occurs in the ATP1A1 gene coding for the sodium-potassium pump. Introducing a specific known mutation via ssODN in the gene ATP1A1 confers to the cell resistance to ouabain, an ATP1A1 inhibitor of the enzyme responsible for sodium/potassium pump. The cells that have integrated ssODN at the ATP1A1 gene are more likely to integrate another ssODN at the locus of interest.

The selection with ouabain is performed according to the protocol described by Agudelo et al. [6] as detailed below.

1. Prepare stock solution of ouabain octahydrate (68.6 mM), by dissolving 50 mg of in 1 mL of water by heating the solution at 90 °C for 5–10 min and vortexing until complete dissolved.
2. Prepare by serial dilution 1/10, 1/100, and 1/1000 solutions. All dilution steps should be carried out in warm water to ensure the homogeneity of the solution. Add 7.3 µL of solution 1/1000 (68.6 µM) for each milliliter of media to reach a concentration of 0.5 µM.
3. In each transfection, add 6 µg of the donor oligonucleotide ON1/ON2/ON3, 2 µg of guide RNA plasmid (gRNA) vector, 4 µg of the donor oligonucleotide mutATP1A1 and 2 µg of plasmid sgG3-ATP1A1-Cas9.
4. After 72 h following nucleofection, split cells from each transfection into two 6-well plates. In both plates, add 1 mL of the cells for each condition and top up with RPMI 1640 with L-Glutamine supplemented with FBS (10%) and PenStrep (1%). In the first plate, add 15 µL of ouabain solution 1/1000 in each well. The second plate serves as a control to monitor the death of cells in the first plate (*see Note 2*).
5. Extract genomic DNA from the transfected cells using Quick-Extract DNA extraction solution according to the manufacturer's recommendations. Dilute genomic DNA at a final concentration around 50 ng/µL.
6. Amplify the locus of interest by PCR as described in Table 4. (*see Note 3*).
7. Run 2 µL of PCR products on 1% agarose gel TBE 0.5x at 100 volts for 90 min (*see Note 4*).
8. Visualize gel using a UV transilluminator.

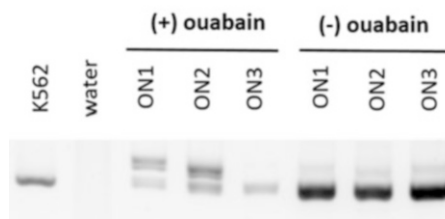
After selection, we assess the integration of our donor oligonucleotide in the pool of transfected cells by a PCR where primers bind in the genome regions flanking the cut site.

Tag sequence insertion increases the PCR fragment size (between 75 and 100 bp for the various combination of tags used



t.1 **Table 4**  
**Composition of the PCR mix used to amplify the genomic site XPB. Use the following PCR program: 98 °C/30s 98°C/10 s 65 °C/20 s 72 °C/20 s 30 cycles 72 °C/5 min 4 °C/Hold**

t.2	Genomic DNA(50 ng/μL)	1 μL
t.3	dNTPs (25 mM)	0.4 μL
t.4	Phusion DNA polymerase (2000 U/mL)	0.5 μL
t.5	Buffer PCR 5X	10 μL
t.6	Forward primer (10 μM)	2.5 μL
t.7	Reverse primer (10 μM)	2.5 μL
t.8	H <sub>2</sub> O	33.1 μL



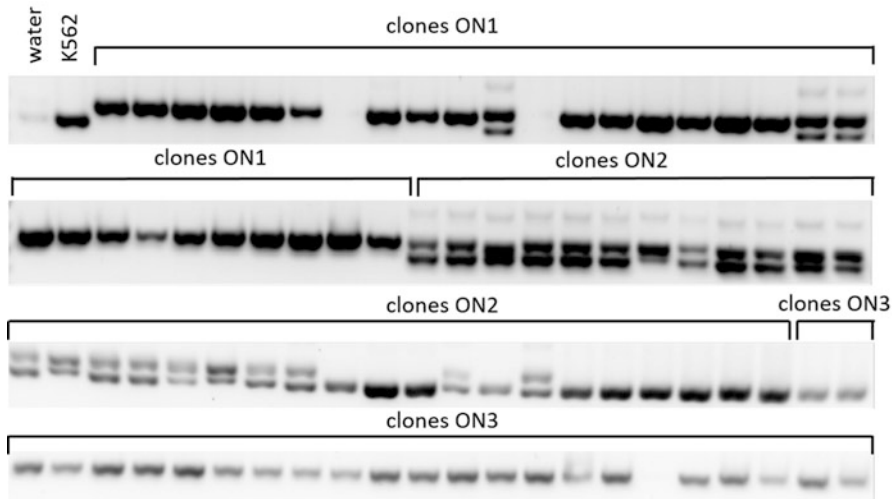
**Fig. 4** PCR Analysis of the mixed cell pools. Cell pools were collected after transfection with various ssODNs (ON1, -2, or -3 as indicated), with or without ouabian selection. Targeted genomic integration of the tag-coding sequence gives rise to a PCR fragment with bigger size (upper band) compared to the wild-type PCR fragment (lower band). Ouabain selection significantly increased the proportion of cells with integration of tag-coding sequences for ON1 and ON2

here) and can be readily compared between different experimental conditions (e.g., comparing sense and antisense donor oligonucleotides). An example of the analysis is shown in Fig. 4. If the proportion of PCR products with tag insertion is low (corresponding to less than 5% of the PCR products), the proportion of cells carrying the modified allele is probably too low to consider isolating a clone of modified cells by single-cell cloning. The protocol may best be repeated using another donor oligonucleotide design or after further optimization of transfection conditions.

**3.5 Isolation of Gene-Edited Cells by Single-Cell Cloning**

Different approaches can be used to isolate clonal populations of gene-edited cells. Cell types can vary greatly in their responses to FACS or serial dilution. For K562 cells, we used successfully both methods.

1. Sort single cells into 96-well plate with your favorite method (*see Note 5*).



**Fig. 5** Genotyping of the clones. Integration of ssODN donor at the XPB locus gives rise to a bigger PCR product (upper band) compared to wild-type PCR product (lower band). The majority of clones generated with ssODN ON1 are homozygous for integration whereas the majority are heterozygous with ssODN ON2. None of the clones generated with ON3 integrated the ssODN

2. Two weeks after cell sorting, visually screen plates using a microscope to identify the wells containing cells colonies. Split cells from each positive well into two 96-well plates (P1 and P2).
3. The day after, spin down and wash with PBS the cells from the plate P1. Extract genomic DNA directly in the wells using 25  $\mu$ L of commercially available DNA lysis buffer.
4. Proceed to PCR amplification for each clone following a similar protocol to the PCR analysis of the pool. Run 2  $\mu$ L of the PCR on 1% agarose TBE 1x gel at 100 volts for 1 h. Visualize gel using a UV transilluminator. In this genotyping, both knock-in and non-knock-in alleles could be amplified, showing whether both, only one or none of the alleles were modified to generate either homozygote or heterozygote clones. An example of gel is shown (Fig. 5).
5. Sequence the PCR fragment of positive clones to ensure correct sequence tag insertion. For homozygous clones, Sanger sequencing chromatograms display only one sequence. For heterozygous clones, Sanger sequencing chromatograms will contain mixtures of sequences that can be de-convoluted separately using online tool CRISP-ID (<http://crispid.gbiomed.kuleuven.be/>).
6. Expand positive clones from plate P2 and prepare frozen stocks, e.g., 10 batches of  $10 \times 10^6$  frozen cells.

<b>3.6 Validation of Gene Tagging by Western Blot</b>	Having isolated a clonal population of modified cells, expression of the tagged gene is validated at the protein level. We generally use approximately $2.5 \times 10^7$ cells and validate the presence of the tagged protein either directly in a whole cell extract or after immunopurification.	457 458 459 460 461 462
<b>3.6.1 Preparation of Cell Pellets</b>	<ol style="list-style-type: none"> <li>1. Seed several T175 flasks containing 25 mL RPMI 1640 supplemented with 10% FBS, L-Glutamine, and antibiotics.</li> <li>2. Harvest cells by centrifugation at 1200 g for 10 min at 4 °C, wash twice with ice-cold PBS containing 30% w/v glycerol, snap freeze in liquid nitrogen, and stored at -80 °C. We usually prepare batches of <math>1.5 \times 10^6</math> and <math>2.5 \times 10^6</math> cells.</li> </ol>	463 464 465 466 467 468 469
<b>3.6.2 Analysis of Tagged Proteins in the Whole Cell Extract</b>	<ol style="list-style-type: none"> <li>1. Resuspend <math>1.5 \times 10^6</math> cells in 150 µL of RIPA buffer and incubate for 10 min with periodic pipetting.</li> <li>2. Sonicate 2 times 30 s on ice. We use a Bioruptor™ sonication system (amplitude 30 and 0.5 s pulse on ice) (Optional).</li> <li>3. Centrifuge 15 min at 14000 g at 4 °C, collect the supernatant, and estimate the total protein concentration using a Bradford assay. A concentration of 3–4 mg/mL is expected.</li> <li>4. Heat 20 µg of total protein from the soluble extract prepared in Laemmli buffer at 95 °C for 5 min and centrifuge samples at 10,000 g for 30 s to bring down the condensate and remove insoluble debris.</li> <li>5. Load the centrifuged sample on a polyacrylamide gel, electrophorese, and transfer proteins from the gel matrix to a solid-support membrane using your favorite device (<i>see Note 6</i>).</li> <li>6. Block the membrane for 1 h at room temperature or overnight at 4 °C, incubate the membrane with an appropriate dilution of a primary antibody directed against the affinity-tag or the tagged subunit in the same buffer for 1 h at room temperature or overnight at 4 °C.</li> <li>7. Wash the membrane three times for 5 min in TBST, incubate with the recommended dilution of conjugated secondary antibody in TBST at room temperature for 1 h.</li> <li>8. Develop the western blot.</li> </ol>	470 471 472 473 474 475 476 477 478 479 480 481 482 483 484 485 486 487 488 489 490 491 492 493
<b>3.6.3 Analysis of the Target Complex after Immunopurification</b>	<ol style="list-style-type: none"> <li>1. Resuspend <math>2.5 \times 10^6</math> cells in 1 mL of lysis buffer (<i>see Note 7</i>) and sonicate 2 times 30 s on ice with a Bioruptor (amplitude 30 and 0.5 s pulse on ice).</li> <li>2. Centrifuge 15 min at 14,000 g, transfer the supernatant into an Eppendorf tube containing 20 µl of anti-FLAG affinity resin, and incubate for 3–4 h with gentle agitation. All manipulations should be performed on ice or at 4 °C.</li> </ol>	494 495 496 497 498 499 500

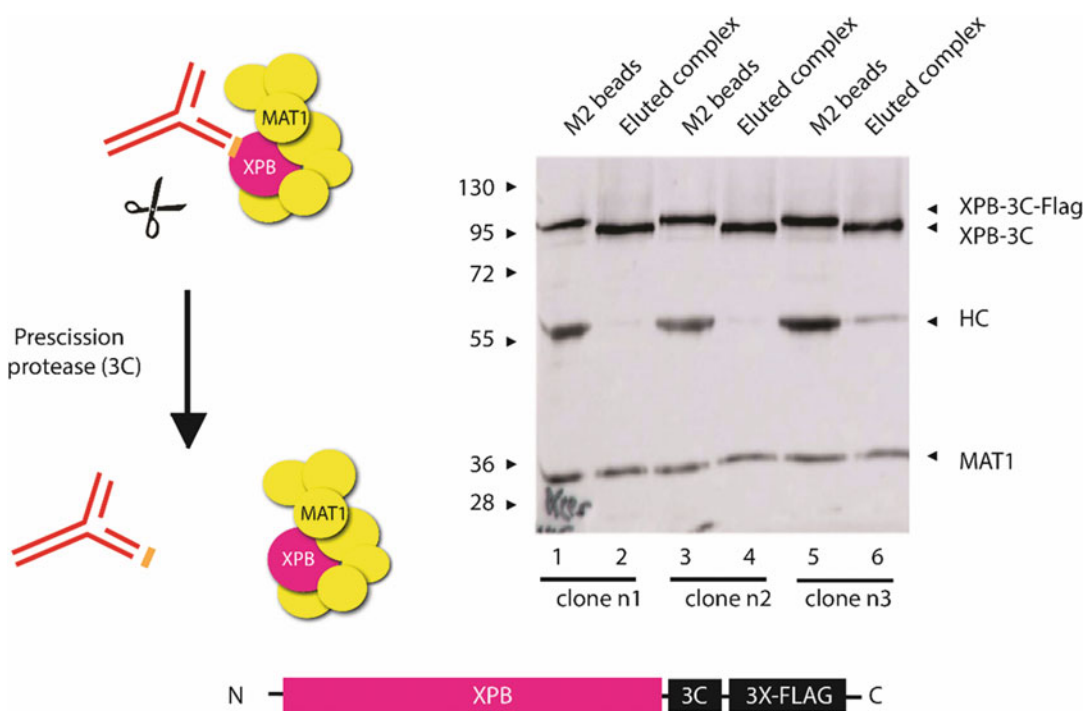
3. Centrifuge at 1000 g for 2 min to sediment the resin and discard the supernatant. Resuspend the resin in 1 mL of lysis buffer and pellet the beads again. Repeat this washing step twice and carefully remove the supernatant using a pipette with a narrow-end pipette tip.
4. For analysis under denaturing conditions, elute bound proteins by addition of: 60  $\mu$ L of Laemmli buffer. For analysis in native condition, add 60  $\mu$ L of lysis buffer containing 0.5 mg/mL FLAG peptide or 5  $\mu$ g/mL of PreScission protease. After incubation for 12 h at 4 °C under gentle agitation, sediment the resin by centrifugation, and mix 45  $\mu$ L of the eluate with 15  $\mu$ L of 4X Laemmli buffer.
5. Heat the Laemmli-containing eluate at 95 °C for 5 min, and after centrifugation at 10000 g for 30 s load the sample on a polyacrylamide gel and perform a western blot analysis as detailed in Subheading 3.7.2 (*see Note 8*).
6. The result of an experiment in which the gene encoding the XPB subunit of the TFIIH transcription/DNA repair was edited to add a C-terminal PreScission protease cleavage site (3C) followed by a 3X-FLAG peptide is shown (Fig. 6).

### 3.7 Suspension Cultures and Purification

After validation of the modified cell line, one clone is selected for scale-up and purification. We generally use batches of  $10^9$  cells for the first purification trials and adapt subsequently. In this section, we detail large-scale suspension cultures and immunopurification.

#### 3.7.1 Large-Scale Suspension Cultures

1. Thaw a vial of a positive clone using your favorite protocol. As above, cells are grown in RPMI 1640 with L-Glutamine supplemented with 10% fetal bovine serum (FBS) and 1% PenStrep.
2. Expand cells in a tissue culture flask until a total of  $15 \times 10^6$  cells with a minimum viability of 95% is reached.
3. Transfer cells in a sterile 250 ml glass bottle and dilute with fresh medium to a cell density  $\sim 0.2\text{--}0.4 \times 10^6$  cells/mL in a total volume of 40–60 mL, cover the opening with gas permeable adhesive seal and incubate at 37 °C, 5% CO<sub>2</sub>, 75% humidity under agitation (90 rpm).
4. After 24–48 h, when a cell density of  $\sim 0.7\text{--}1.0 \times 10^6$  cells/mL is reached transfer cells into a 500 ml glass bottle and dilute to a cell density  $\sim 0.2\text{--}0.4 \times 10^6$  cells/mL in a total volume of 80–120 mL and incubate as above.
5. Repeat **step 4** to seed successively a 1000 ml bottle with a working volume of 150–250 ml and four 5000 ml bottles with working volumes of 750–1250 mL.



**Fig. 6** Validation of engineered cell lines by western blot analysis. The gene encoding the XPB subunit of the TFIIH transcription/DNA repair was edited to fuse its C-terminus to a PreScission protease cleavage site (3C) followed by a 3X-FLAG peptide. For validation of selected clones, the corresponding soluble extracts were incubated with anti-FLAG affinity resin, and after extensive washing, bound proteins were eluted in denaturing conditions with Laemmli buffer (lanes 1, 3, and 5). Alternatively, immobilized complexes can be eluted in native condition by addition of FLAG competitor peptide (not shown) or PreScission protease to cleave the tag (lanes 2, 4, and 6). Immuno-purified proteins were resolved using a 12% SDS-polyacrylamide gel and subjected to western blot analysis. Antibodies directed against XPB and MAT1, another TFIIH subunit were used for protein detection

6. When the cell density of  $\sim 0.7\text{--}1.0 \times 10^6$  cells/mL is reached in the 5000 mL bottles add fresh medium and incubate further. 545  
546
7. Harvest cells by centrifugation at 1000 g for 10 min at 4 °C, wash twice with ice-cold PBS containing 30% w/v glycerol, snap freeze in liquid nitrogen and store at  $-80$  °C. We usually prepare batches of  $10^9$  cells. 547  
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A typical amplification workflow is shown in Fig. 7a. As modified cell lines can be unstable and prone genetic drift, we carefully monitor the number of passages and always start with a freshly thawed vial. 551  
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### 3.7.2 Immunopurification

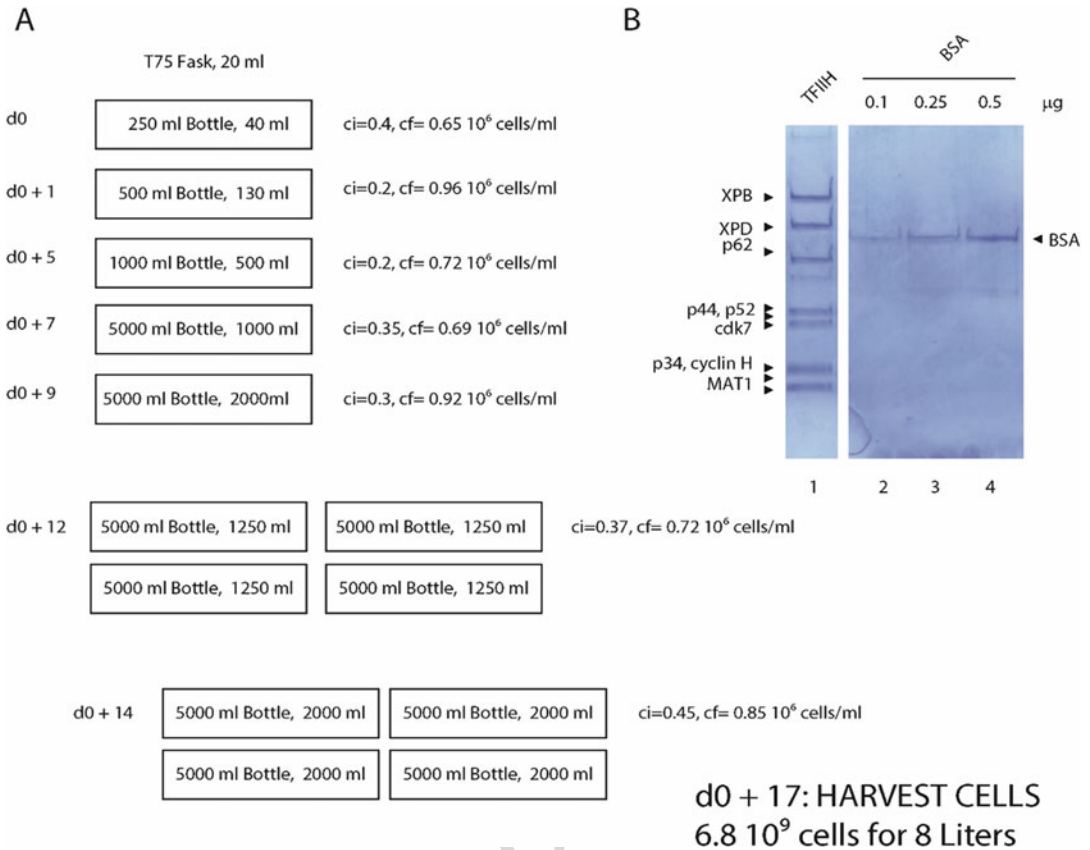
1. Resuspend one batch of  $10^9$  cells in 20 mL of lysis buffer (*see Note 7*) and sonicate 4 times 30 s on ice with a Bioruptor (amplitude 30 and 0.5 s pulse on ice). 556  
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2. Add DNase and RNase to obtain a final concentration of 12.5  $\mu\text{g}/\text{mL}$  for both nucleases and incubate for 20 min at room temperature under gentle agitation.
3. Centrifuge 60 min at 50,000 g.
4. Load the extract on a 5 ml heparin column and collect the fractions containing the complex of interest.
5. Incubate the extract or the fractions containing the complex with 50  $\mu\text{l}$  of anti-FLAG affinity resin during 3–4 h under gentle agitation in a 15 ml Falcon tube. All manipulations should be performed on ice or at 4  $^{\circ}\text{C}$ .
6. Centrifuge at 1000 g for 2 min to sediment the resin and discard the supernatant. Resuspend the resin in 1 mL of lysis buffer, transfer into a 1.5 mL Eppendorf tube and pellet the beads again. Repeat this washing step twice and carefully remove the supernatant using a pipette with a narrow-end pipette tip.
7. Add 50  $\mu\text{L}$  of lysis buffer containing 0.5 mg/mL FLAG peptide and incubate for 12 h at 4  $^{\circ}\text{C}$  under periodic agitation (30s every 5 min).
8. Collect the supernatant and repeat **step 7** with an incubation of 2 h and pool the two eluates.
9. Mix 20  $\mu\text{L}$  of the eluate with 5  $\mu\text{L}$  of 4X Laemmli buffer and analyze on an SDS-PAGE (12.5% acrylamide). The corresponding gel is shown in Fig. 7b.
10. Aliquot and snap freeze the purified sample.

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#### 4 Notes

1. The relative luciferase values detected here with Nano-Glo HiBiT Lytic Detection System were lower than in the other experiments performed at various genomic loci and in other cell lines. This is consistent with the results obtained in T7 endonuclease assay that indicate a very low activity of sgRNAs targeting the stop codon of the XPB gene.
2. Within a few days, only the cells that have gained ouabain resistance will proliferate. Nonetheless, subsequent splitting of the cells is essential to avoid interference in molecular analyses from the genomic DNA of dead cells. After 2 weeks of ouabain treatment, the cell population is considered stable.
3. Suitable PCR primers to the target region can be designed with CRISPOR by clicking on the “Cloning/PCR primers” hyperlink for the selected guide RNA on the CRISPOR results web page or using the online tool Primer3 (<http://www.bioinfo.ut>).



**Fig. 7** Large-scale cultures and immunopurification. (a) Freshly thawed cells of a representative modified K562 cell line were expanded in a T75 tissue culture flask and then successively transferred into 250, 500, 1000, and 5000 mL glass bottles. Initial and final cell densities (ci and cf., respectively) are indicated. (b) Purified TFIH resolved on a Coomassie-stained 12.5% SDS-polyacrylamide gel

ec/primer3/). PCR primers should be designed that flank the DSB site within a total length of between 300 and 800 bp. 601 602

4. Make sure to run the electrophoresis for a sufficient length of time because the separation between the PCR fragments is challenging due to the short length of the purification tag sequence relative to the amplified DNA region. 603 604 605 606
5. Some adherent cell lines do not tolerate single-cell dilution in 96-well plates and may need using conditioned medium or can alternatively be dilution plated in 10 cm cell culture dishes. 607 608 609
6. For most proteins, we run a 12.5% a 1 mm thick polyacrylamide gel for 1 h 30 (35 mA) with a TGS running buffer (25 mM Tris-HCl pH 8.3, 190 mM glycine, 0.1% SDS). Do not omit to include a sample prepared from a non-modified cell line as negative control. 610 611 612 613 614



7. The composition of the RIPA and lysis buffers can be adjusted 615  
by using a few detergents (ionic and nonionic detergent) and 616  
different salt concentrations (low, medium, and high salt). 617  
High ionic strength enhances solubility of many proteins but 618  
can lead to complex dissociation. For intracellular proteins, 619  
care should be taken to maintain a reducing environment. 620
8. As analyses were performed in native conditions, antibodies 621  
directed against the affinity-tag or the tagged protein as well 622  
as against another subunit of the complex can be used. 623

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