RESEARCH NOTE

Luminescent peptide tagging enables efficient screening for CRISPR-mediated knock-in in human induced pluripotent stem cells [version 1; peer review: 1 approved with reservations]

Ralitsa R. Madsen, Robert K. Semple

Centre for Cardiovascular Science, The Queen's Medical Research Institute, University of Edinburgh, Edinburgh, EH16 4TJ, UK

v1 **First published:** 20 Feb 2019, **4**:37 (https://doi.org/10.12688/wellcomeopenres.15119.1)

Second version: 15 Apr 2019, **4**:37 (https://doi.org/10.12688/wellcomeopenres.15119.2)

Latest published: 11 Jul 2019, **4**:37 (https://doi.org/10.12688/wellcomeopenres.15119.3)

Abstract

Human pluripotent stem cells are increasingly used for CRISPR-mediated gene targeting in efforts to generate models of human diseases. This is a challenging task because of the high sensitivity of these cells to suboptimal conditions, including CRISPR-associated DNA damage and subsequent rounds of single-cell cloning. We sought to develop a sensitive method that enables rapid screening of CRISPR targeted cells, while preserving cell viability and eliminating the need for expensive sequencing of a large number of clones. A protocol was designed in which the luminescent peptide tag, HiBiT, is appended to the extracellular portion of an inert surface membrane protein (CD46), using synthetic CRISPR reagents and a widely distributed human induced pluripotent stem cell (iPSC) line. We find that this approach substantially reduces labour-intensive screening of CRISPR-targeted iPSCs and minimises the number of subcloning steps. Successfully edited iPSCs could be identified within a week of targeting, based only on extracellular luminescence detection in live cells. The total screening time in each round was less than 30 minutes and no sequencing was required. This method can be developed further to serve as a highly sensitive co-selection strategy in CRISPR knock-in experiments, particularly in the context of challenging cell lines.

Keywords

CRISPR, knock-in, human pluripotent stem cells, iPSCs, screening, HiBiT, tagging

Open Peer Review

Reviewer Status ✔ ?

	Invited Reviewers	
	1	**2**
REVISED **version 3** published 11 Jul 2019		
REVISED **version 2** published 15 Apr 2019	✔ report	? report
version 1 published 20 Feb 2019	? report	

1 **Sara E. Howden**, Murdoch Children's Research Institute (MCRI), Parkville, Australia

2 **Aron M. Geurts**, Medical College of Wisconsin, Milwaukee, USA
Maribel Marquez, Medical College of Wisconsin, Milwaukee, USA

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Robert K. Semple (rsemple@ed.ac.uk)

Author roles: Madsen RR: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Semple RK**: Funding Acquisition, Supervision, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This work was funded by the Wellcome Trust [210752]
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

How to cite this article: Madsen RR and Semple RK. **Luminescent peptide tagging enables efficient screening for CRISPR-mediated knock-in in human induced pluripotent stem cells [version 1; peer review: 1 approved with reservations]** Wellcome Open Research 2019, **4**:37 (https://doi.org/10.12688/wellcomeopenres.15119.1)

First published: 20 Feb 2019, **4**:37 (https://doi.org/10.12688/wellcomeopenres.15119.1)

Introduction

The development of powerful gene editing technologies such as clustered regularly interspaced palindromic repeats (CRISPR)/Cas9, alongside continuously improving protocols for the derivation and maintenance of induced human induced pluripotent stem cells (iPSC), have made this cell type the model of choice in many settings, be it mechanistic disease studies or high-throughput screens aimed at identifying novel drug targets[1].

Gene editing in human iPSCs is particularly powerful when applied to generation of series of isogenic cell lines differing only with respect to a specific pathogenic mutation. The technology also enables tagging of endogenous proteins for studies that require differential cell labelling or the ability to pull down a target of interest. Nevertheless, such knock-in experiments remain challenging in human iPSCs, in part due to their sensitivity to CRISPR-induced DNA damage[2] and poor survival as single cells[3]. Without *bona fide* selection, homology-directed repair (HDR) rates lower than 1 % are considered normal for human iPSCs and require picking of a relatively large number of individual colonies, or several rounds of sib-selection with subcloning[4–6]. This makes gene editing in human iPSCs costly because of expensive cell culture materials and the need for extensive sequencing of individual clones.

Faced with this challenge and the need to tag an extracellular surface protein for differential cell labelling, we sought to develop a method that allows for faster and cheaper screening of successfully targeted iPSCs, while minimising exposure to suboptimal culture conditions. Given the low HDR efficiency in human iPSCs, such a method would have to be highly sensitive in order to distinguish the presence of rare gene-edited cells in a mixed population consisting mainly of wild-type counterparts. Thus, we focused on adapting the HiBiT luminescence technology for efficient screening and co-selection of human iPSCs undergoing dual targeting. The HiBiT technology comprises a split version of the exceptionally bright NanoLuc luciferase. A small 11 amino acid peptide, HiBiT, can be fused to a protein of interest, and subsequent supplementation with the large subunit (LgBiT) results in strong binding to HiBiT, thereby reconstituting NanoLuc activity. CRISPR-mediated HiBiT tagging and luminescence detection were recently used to track HIF1α dynamics in response to a range of stimuli, demonstrating the high sensitivity of this method in HEK293, HeLa and primary human umbilical vein endothelial cells[7]. Importantly, the high sensitivity of the HiBiT system enables detection of very low amounts of target protein, down to femtomoles or lower[7].

A major advantage of this technology emerges when the tag is appended to the extracellular portion of a surface membrane protein because this allows luminescence detection of gene-edited cells without the need for cell lysis and DNA extraction for downstream PCR-based applications. Here, we demonstrate that tagging of the extracellular portion of the cell surface protein CD46 allows for efficient and cost-effective screening of CRISPR-targeted human iPSCs, reducing processing time to less than 30 minutes per round, eliminating the need for expensive genetic assays and minimising stress-inducing cell manipulations.

Methods

Routine cell culture

We used the male iPSC line WTC11 (Coriell # GM25256) due to its amenability to genome editing and known diploid karyotype. WTC11 whole-exome and wholegenome sequencing data are available via the Conklin lab website.

Prior to targeting, the cells were cultured in 6-well plates coated with hESC-qualified Geltrex (Thermo Fisher Scientific # A1413302) diluted 1:100 in DMEM/F12 (Sigma # D6421). Cells were maintained in Essential 8 Flex (E8/F) medium (Thermo Fisher Scientific # A2858501) and passaged every four days when 80-90 % confluent, with split ratios ranging from 1:10 to 1:15. ReLeSR (Stem Cell Technologies # 5872) was used to dissociate the cells nonenzymatically, and 1X RevitaCell (Thermo Fisher Scientific # A2644501) was included during the first 24 hours to promote survival.

Targeting design

We chose the human protein CD46 (ENSEMBL gene id: ENSG00000117335) for tagging because it is mainly involved in autologous cell protection against the complement system as well as acting as a costimulatory factor for T-cells to promote CD4+ T cell differentiation (Uniprot P15529); it is thus unlikely that tagging of this protein's extracellular portion will interfere with important biological functions of human iPSCs and most of their differentiated derivatives. We used our previously published RNAseq dataset to confirm that the gene is well-expressed in WTC11[6].

CD46 is a single-pass type I membrane protein, with the N-terminus exposed on the extracellular side of the plasma membrane. The first 34 amino acids correspond to an endoplasmic reticulum (ER)-targeting signal sequence, which is cleaved from the mature protein. Consequently, the tag has to be knocked in after the signal peptide to avoid downstream removal. We used a published three-dimensional structure of CD46 (PDB ID: 1CKL) to confirm that this region is not buried within the protein upon folding. According to our strategy, successful targeting would result in tagging of 15 of the 16 CD46 isoforms. A flexible triple Glycine (GGG) linker was inserted between the tag and the start of CD46's extracellular portion. This linker cannot be cleaved by chymotrypsin, factor XA, thrombin or trypsin (checked against the SynLinker database).

For guide RNA design, the following 240 bp sequence was submitted to the CRISPRko module of the Broad Institute's GPP web tool[8] (accessed 1 October 2018):

5 ' - T G C T A T G A G C A C T C A G G T A A A A G C A TGGAACAGTCATTT AAAATCTTGCCAAGGGCCTTTCTGTT TTTTCTGTACTACCT GCTGCCAGACCACAGTCCATGGCTG ATGAAAGTGATATCAG TACTTCATCTTCATGTTCCTATTCT CTTATCCCTAGATGCCTG TGAGGAGCCACCAACATTTGAA GCTATGGAGCTCATTGGT AAACCAAAACCCTACTATGAGA TTGGTGAACGAGTA-3'

Two guide RNAs from the output list were selected based on their on- and off-target ranks as well as cut distance from the insertion site. One of these, annotated sgRNA_CD46_rank13 (Table 1), was chosen for sub-sequent tagging experiments as it has previously been validated by IDT (Design ID: Hs.Cas9.CD46.1.AF).

Two 200 bp long HDR templates were designed manually, either including a sequence for HiBiT or a V5 tag (Table 1), followed by the flexible GGG linker. All three templates were designed asymmetrically following established rules[9], using a shorter homology sequence to the PAM-distal region and complementarity to the nontargeted strand. Known WTC11-specific SNPs in the targeted CD46 region were taken into account following examination of the publicly available WTC11 whole-genome sequence. No silent mutations were introduced into the template because successful tagging destroys the PAM site, thus preventing repeated targeting. Using ExPASy's PeptideCutter tool, we confirmed that the translated fusion products wouldn't be targeted by intracellular proteases.

Preparation of CRISPR/Cas9 targeting reagents

Single-stranded oligo DNA (ssODN) templates, ALT-R XT CRISPR RNA (crRNA), ALT-R trans-activating crRNA (tracrRNA) and ALT-R Cas9 Nuclease V3 were acquired from Integrated DNA Technologies (IDT).

Prior to use, the crRNA and the tracRNA were resuspended in pH 7.5 TE buffer (IDT # 11-01-02-02) to a final concentration of 100 μM. To prepare crRNA:tracRNA duplexes at 50 μM, equal volumes of each RNA were mixed and heated for 5 minutes at 95 °C, followed by controlled cool-off to 25 °C at ramp rate 0.1 °C/second. The formed duplexes were placed on ice until ready to use.

Table 1. ALT-R XT CRISPR RNA (crRNA) and single-stranded oligo DNA (ssODN) sequences for CD46 targeting.

Description	Sequence
crRNA-CD46	5'-AAATGTTGGTGGCTCCTCAC-3'
ssODN-CD46-HiBiT	5'-GTACTACCTGCTGCCAGACC ACAGTCCATGGCTGATGAA AGTGATATCAGTACTTCATC TTCATGTTCCTATTCTCTTAT CCCTAGATGCCGTGAGCGGC TGGCGGCTGTTCAAGAAGAT TAGCggaggtggaTGTGAGGA GCCACCAACATTTGAAGCTA TGGAGCTCATTGGTAAACC AAAACCCTACTATGAGATTG-3'
ssODN-CD46-V5	5'-GTACTACCTGCTGCCAGACC ACAGTCCATGGCTGATGAA AGTGATATCAGTACTTCATC TTCATGTTCCTATTCTCTTAT CCCTAGATGCCGGTAAGCCTA TCCCTAACCCTCTCCTCGGTC TCGATTCTACGggaggtggaTG TGAGGAGCCACCAACATTTGAA GCTATGGAGCTCATTGGTAAAC CAAAACCCTACT-3'

Ribonucleoproteins were prepared by mixing 5 μl each of crRNA:tracRNA duplex (50 μM) and recombinant Cas9 enzyme (61 μM), followed by incubation at room temperature for 20 minutes. Next, 200 pmol of each HDR template was added to the RNPs prior to delivery into iPSCs.

Nucleofection and luminescence-based screening of CRISPR-targeted iPSCs

Targeting was performed using healthy, subconfluent iPSCs (P51) pretreated with 1X RevitaCell (in E8/F) for 3 hours. The cells were dissociated with StemPro Accutase (Thermo Fisher Scientific # A110501) and 1e6 cells taken forward for nucleofection. Following low-speed centrifugation (100G, 3 minutes), the cell pellet was resuspended in 100 μl nucleofection solution (P3 Nucleofection Kit, Lonza # V4XP-3024). Of this suspension, 85 μl were transferred to the assembled RNPs, resulting in c. 850,000 cells in the final reaction. The nucleofection was carried out with an Amaxa 4D nucleofector, using programme CA137. Immediatley following nucleofection, 500 μl E8/F with 1X RevitaCell were added to the cell suspension and transferred to a Falcon tube containing 4.5 ml E8/F with 1X RevitaCell. Of this suspension, 100 μl were seeded into each well of a Geltrex-coated opaque-white Nunc 96-well plate (Thermo Fisher Scientific # 165306). The following day, the cells were replenished with 200 μl fresh E8/F without RevitaCell and again on day 3 post-nucleofection.

Luminescence-based screening for successfully targeted cells was carried out 4 days post-nucleofection, using Promega's Nano-Glo HiBiT Extracellular Detection System. Briefly, the iPSCs were washed once with 200 μl DPBS per well, followed by addition of 50 μl DPBS. Next, 50 μl Nano-Glo HiBiT and LgBiT mixture were added to each well, followed by mixing and detection of the luminescent signal within 10 minutes, during which time the plate was kept away from light. Luminescence was detected on a BMG Clariostar, using top reading with lid to preserve sterility. The focal height was set to 11, and integration time was 1 second. Immediately following detection, the cells were transferred back to the biosafety cabinet and the solution removed from each well. Following a single wash in 200 μl DPBS, each well was replenished with 200 μl fresh E8/F. Although cell survival was not compromised with this procedure, we recommend the use of PBS with Ca^{2+}/Mg^{2+} in future repeats as we noted that prolonged exposure to DPBS caused transient dissolution of cell-cell contacts within individual colonies.

Following recovery for 1-2 days, cells with a positive luminescence signal were expanded non-enzymatically (using ReLeSR) into a Geltrex-coated 24-well plate, prior to a round of subcloning into Geltrex-coated 96-well plates, seeding 12.5 cells/well in E8/F supplemented with 1X RevitaCell. From previous work, we know that this seeding density typically results in survival of 2-3 cells in each well and allows for efficient enrichment while minimising cell loss[6]. RevitaCell was removed 6 days post-subcloning and the cells processed for a second round

of luminescence-based screening once most wells in a plate contained at least one colony with diameter > 1000 μm.

PCR-based detection of CRISPR-mediated CD46 tagging

Genomic DNA (gDNA) was extracted from luminescence-positive cells before subcloning and was used for PCR amplification of the CRISPR-targeted CD46 locus (F primer: 5'-AAGTCCCATTTCCTCCACTAC-3'; R primer: 5'-ACAAGAAGAAAATCATCATCACCG-3'). The PCR was carried out using 50-100 ng gDNA and GoTAQ G2 Colourless Master Mix (Promega) with the following thermocycling conditions (Techne Prime Thermal Cycler): 95 °C for 1 minute, 30 cycles of 95 °C for 30 seconds - 60 °C for 20 seconds - 72 °C for 45 seconds, 72 °C for 45 seconds, 4 °C until collection. A FAM-labelled forward primer was used to enable detection of the PCR products (diluted 1:20) by capillary electrophoresis. This was carried out on a LabChip GX24 Nucleic Acid Analyser (Perkin Elmer) using the DNA 1K Reagent Kit (Perkin Elmer #CLS760673) according to the manufacturer's instructions. Of note, prior to analysis, the PCR products were diluted 1:20 in nuclease-free water; this causes slower migration of the fragments due to low ionic strength. The correct size of the fragments was confirmed by conventional agarose gel electrophoresis (raw gel image included via OSF[10]). We suggest dilution in TE buffer in future repeats.

Results

We report preliminary work seeking to develop an efficient and highly sensitive high-throughput method for detection of successful CRISPR-mediated knock-in in human iPSCs. To do this, we co-targeted the cell surface protein CD46 with a HiBiT peptide and a V5 tag, followed by luminescence-based screening of live cells to identify cells with successful editing of at least one allele. The results are summarised in Figure 1.

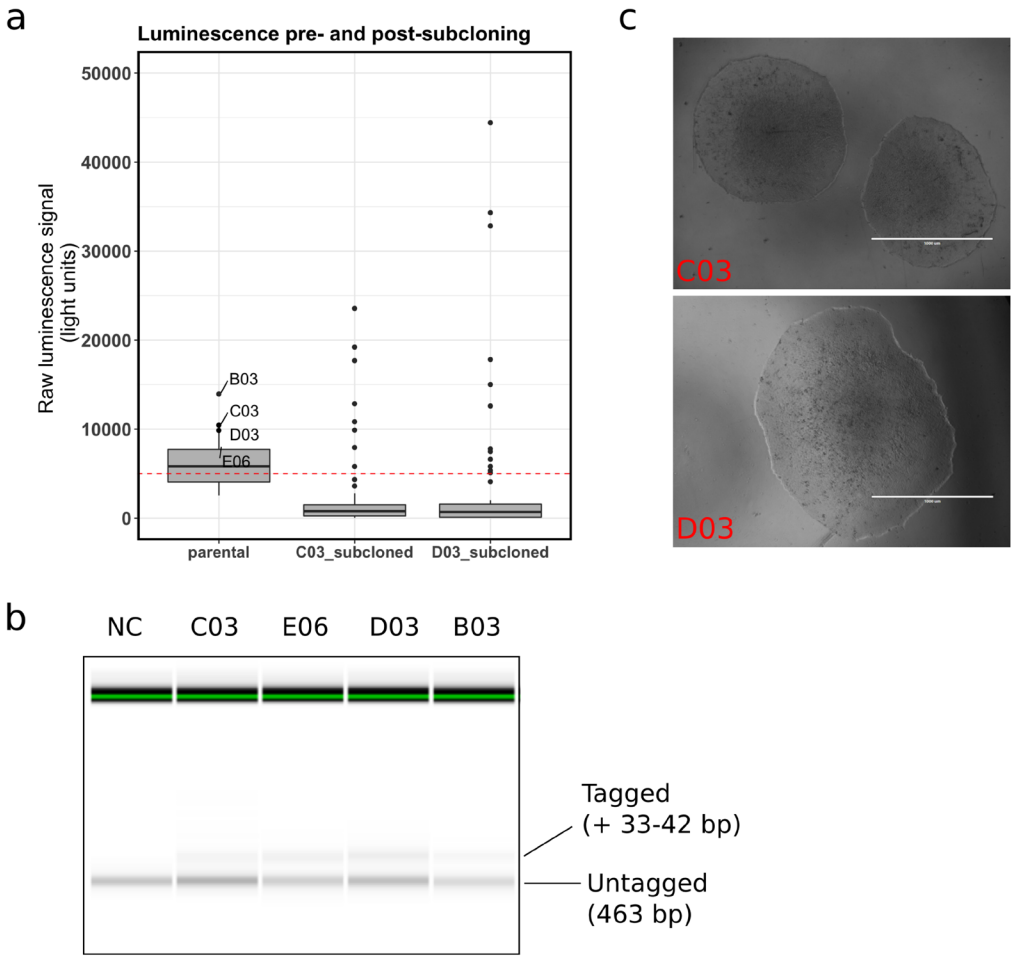

Figure 1. HiBiT-tagging of the membrane surface protein CD46 enables rapid screening for successful CRISPR-mediated knockin in human induced pluripotent stem cells (iPSCs). a. Boxplots depicting the HiBiT luminescence signal distribution before and after subcloning of CRISPR-targeted cells in the indicated wells. The dashed red line marks the background signal threshold, chosen based on initial standard curve measurements using recombinant HiBiT protein (not shown). **b**. Capillary electrophoresis following PCR amplification of the targeted CD46 region in non-targeted control (NC) iPSCs and four HiBiT/V5-targeted iPSC populations prior to subcloning. While a clear band-shift can be resolved in targeted cells, the resolution is insufficient to distinguish between a V5 (+42 bp) *vs* a HiBiT (+32 bp) knock-in. **c**. Example light micrographs of iPSC colonies following one round of subcloning, revealing healthy colony morphology with clearly defined edges. Scale bar = 1000 μm.

Prior to subcloning, the luminescence signal across wells with targeted cells exhibited a relatively broad distribution, with multiple wells reaching a signal just above background (set to 5000 light units) (Figure 1a). Only two wells had a signal between 10,000 and 15,000 light units. Cells from four wells with signal above 8000 light units were expanded, and two of these populations were subsequently used for subcloning into 96-well plates to enrich for HiBiT-positive cells. Within < 30 minutes, without the need for DNA extraction and Sanger sequencing of two 96-well plates, we were able to identify multiple wells with a substantial increase in luminescence signal relative to the overall population (Figure 1), indicative of successful enrichment. Importantly, we continued to use a stringent background threshold to limit the number of false positives.

PCR-based amplification of the targeted CD46 region in the original four wells chosen for expansion was used to visualise the presence of a longer product (Figure 1b). While neither method has the capacity to confirm whether or not the V5 tag has been incorporated into the second allele, the successful expression of HiBiT on the cell surface strongly suggests that at least one allele was targeted correctly in a subset of the examined cells.

The extracellular luminescence detection in live cells was compatible with pluripotent stem cell survival, with subcloned cells retaining a healthy colony appearance upon outgrowth (Figure 1c). Thus, the enriched cells can be used for subsequent replica-plating and immunofluorescent detection of V5 with high-content imaging systems, substantially reducing expenses by only limiting detection to a few wells of a 96-well plate.

Discussion

CRISPR-mediated knock-in in human iPSCs remains a challenge due to low efficiency, poor cell survival and the resulting need to screen a large number of clones. Combined witht the high cost of human iPSC maintenance, relevant disease models based on this cell system are prohibitively expensive to many academic labs. We report a novel targeting strategy that utilises the HiBiT luminescence system to enable efficient screening for successfully CRISPR-edited iPSCs. The protocol uses live cells, thus eliminating the need for replica-plating, and can be executed within 30 minutes without compromising cell health.

Prior to applying this system, a standard curve should be generated using recombinant HiBiT-tagged protein in conditions mimicking the final cell-based assay in order to estimate background levels. Although background levels may differ due to different kit lots and/or cell densities, keeping a conservative luminescence threshold across experiments is recommended to limit false-positive hits. It is notable that the luminescence signal distribution prior to subcloning was broad, but centred around the background threshold, which might reflect the high sensitivity of the HiBiT system whereby the presence of rare cells with successful CRISPR-mediated knock-in of the tag can be picked up in multiple wells. It is important that future studies seeking to use this approach determine its compatibility as "co-selection" marker when the second targeting locus is distinct from CD46. It also remains to be determined whether CD46 HiBiT-tagging will be equally efficient in other iPSC lines.

Finally, we note that this approach does not provide a quantitative measure of knock-in efficiency. The actual efficiency may be low overall, with detection in the current format only possible due to the high sensitivity of the HiBiT system. Although we were able to distinguish a CD46 band shift by PCR across the targeted region, detection of luminescence has the advantage of confirming that the tagged protein is processed and expressed normally. This is a substantial improvement compared to conventional approaches based on laborious sequencing of a large number of clones to rule out unwanted indels in a CRISPR-targeted cell population.

Conclusions

The HiBiT luminescence system can be used as a powerful tool to screen for successfully CRISPR-edited human iPSCs in knock-in experiments. The method is fast, cost-effective and minimises stress-inducing cell manipulations. Our preliminary data encourage additional development of the protocol by researchers using CRISPR to knock in point mutations or smaller tags into iPSCs or other challenging cell types. The system is versatile and tagged cells will be compatible with a wide range of downstream assays, including intravital imaging.

Data availability

Underlying data

Open Science Framework: Luminescent peptide tagging for efficient screening of gene-edited human iPSCs. https://doi.org/10.17605/OSF.IO/F82YD[10]

This project includes the following underlying data:

-2018-11-22_CRISPR_NP_CD46_tag_knockin_PSCs_before_subcloning_Hyperladder100bp.tif(Gelimage,validationgel)

– 2018 – 12 – 01 – H11C3 –p54 – 96well – 25cells.per.well – to – replica – plate – 40x – 2.tif(Rawmicrographimage, C03Figure1C)

– 2018 – 12 – 01 – H11D3 – p54 – 96well – 12.5cells.per.well – to – replica – plate – 40x – 1.tif(Rawmicrographimage, D03Figure1C)

2019-01-11_cap_electrophoresis_esultes.pptx (LabChipcapillaryelectrophoresisresults,withadditionaldescription)

LabChipGXRalitsa&Semple9DNA1ksamples11thJan2019.pdfLabChipcapillaryelectrophoresisrawoutputdata,Figure1B

–R_plots_for_publication.nb.html(RcodeunderlingFigure1a)

−Readme_LabChip_CapE_lectrophoresis_results.pptx(additionalinformationforinterpretationofFigure1B)

Grant information

This work was funded by the Wellcome Trust [210752].

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We would like to thank members of Prof Siddhartan Chandran's group for offering a space for iPSC culturing. In particular, we would like to thank James Cooper for advice on iPSC nucleofection. We thank Dr Pamela Brown (SURF Biomolecular Core) for help with capillary electrophoresis. We are also grateful to BMG Labtech and Promega specialists for their advice on assay set-up.

References

1. Hockemeyer D, Jaenisch R: **Induced Pluripotent Stem Cells Meet Genome Editing.** *Cell Stem Cell.* 2016; **18**(5): 573–586.
PubMed Abstract | Publisher Full Text | Free Full Text
2. Ihry RJ, Worringer KA, Salick MR, *et al.*: **p53 inhibits CRISPR-Cas9 engineering in human pluripotent stem cells.** *Nat Med.* 2018; **24**(7): 939–946.
PubMed Abstract | Publisher Full Text
3. Watanabe K, Ueno M, Kamiya D, *et al.*: **A ROCK inhibitor permits survival of dissociated human embryonic stem cells.** *Nat Biotechnol.* 2007; **25**(6): 681–6.
PubMed Abstract | Publisher Full Text
4. Miyaoka Y, Chan AH, Judge LM, *et al.*: **Isolation of single-base genome-edited human iPS cells without antibiotic selection.** *Nat Methods.* 2014; **11**(3): 291–3.
PubMed Abstract | Publisher Full Text | Free Full Text
5. Wang G, Yang L, Grishin D, *et al.*: **Efficient, footprint-free human iPSC genome editing by consolidation of Cas9/CRISPR and piggyBac technologies.** *Nat Protoc.* 2017; **12**(1): 88–103.
PubMed Abstract | Publisher Full Text | Free Full Text
6. Madsen RR, Knox RG, Pearce W, *et al.*: **Oncogenic PIK3CA promotes cellular stemness in an allele dose-dependent manner.** *bioarxiv.* 2018; 1–30.
Publisher Full Text
7. Schwinn MK, Machleidt T, Zimmerman K, *et al.*: **CRISPR-Mediated Tagging of Endogenous Proteins with a Luminescent Peptide.** *ACS Chem Biol.* 2018; **13**(2): 467–474.
PubMed Abstract | Publisher Full Text
8. Doench JG, Fusi N, Sullender M, *et al.*: **Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9.** *Nat Biotechnol.* 2016; **34**(2): 184–191.
PubMed Abstract | Publisher Full Text | Free Full Text
9. Richardson CD, Ray GJ, DeWitt MA, *et al.*: **Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA.** *Nat Biotechnol.* 2016; **34**(3): 339–344.
PubMed Abstract | Publisher Full Text
10. Madsen RR, Semple R: **Luminescent peptide tagging for efficient screening of gene-edited human ipscs**. 2019.
http://www.doi.org/10.17605/OSF.IO/F82YD

Open Peer Review

Current Peer Review Status: ?

Version 1

Reviewer Report 13 March 2019

https://doi.org/10.21956/wellcomeopenres.16495.r34940

?

Sara E. Howden
Murdoch Children's Research Institute (MCRI), Parkville, Victoria, Australia

In this study, Madsen and colleagues describe the use of HiBiT technology to tag the surface membrane protein CD46 in human pluripotent stem cells, in order to identify successfully gene-edited cells using luminescence detection in live cells. The authors claim that this makes gene editing in human iPSCs less costly because this approach substantially reduces labour-intensive screening of CRISPR-targeted iPSCs and minimises the number of subcloning steps.

- The authors should explain in more detail the purpose of the V5 tag. I am guessing that the purpose of this study is to use the HiBiT tag as a way to identify cells that have also incorporated the V5 tag on the second allele? If this is the case, this should be specifically stated. Perhaps it would be helpful if there was a diagram depicting the targeting strategy.

- How many wells of a 96-well plate are cells plated into following transfection? The authors state that “Only two wells had a signal between 10,000 and 15,000 light units”. Two out of how many? Please state as %.

- Is there a way to identify and specifically mark individual colonies within a well that have successfully incorporated the HiBiT tag? These could then theoretically be isolated by picking and expanding, thereby reducing subsequent rounds of subcloning.

- Lastly, although the authors claim this work is preliminary, it would be nice to see this particular study a little more complete. Of particular interest would be whether a pure population of cells harbouring both tags could in fact be obtained, how many rounds of subcloning are necessary to obtain this, and whether the cells maintain genomic integrity at the completion of this process.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
No source data required

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Stem cell biology, genome engineering

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 09 Apr 2019

Ralitsa Madsen, University of Edinburgh, Edinburgh, UK

We thank Dr Howden for her review of our Research Note on the use of a luminescence peptide for efficient identification of iPSCs undergoing successful CRISPR-mediated knock-in.

We have addressed Dr Howden's reservations as follows:

The authors should explain in more detail the purpose of the V5 tag. I am guessing that the purpose of this study is to use the HiBiT tag as a way to identify cells that have also incorporated the V5 tag on the second allele? If this is the case, this should be specifically stated. Perhaps it would be helpful if there was a diagram depicting the targeting strategy.

We agree with Dr Howden that this needs further clarification. The following paragraph has now been added to the Discussion section of the revised manuscript:

"Finally, we wish to highlight that our original aim was to test the use of HiBiT as an efficient and sensitive marker for successful editing of at least one CD46 allele. The V5 tag was co-introduced to allow additional immunofluorescence-based screening of HiBiT-positive wells by high-content imaging. We hypothesise that this will allow easier detection of cells with concomitant V5 knock-in into the second allele while reducing the need to stain entire plates. However, for logistical reasons, we have not been able to confirm this ourselves, but hope that this co-targeting strategy will be developed further by other researchers with access to our preliminary data."

How many wells of a 96-well plate are cells plated into following transfection? The authors state that "Only two wells had a signal between 10,000 and 15,000 light units". Two out of how many? Please state as %.

A total of 48 wells of a 96-well plate were seeded following nucleofection of the iPSC suspension.

This has now been added to the results section of the revised manuscript. However, we wish to refrain from "%" measures as this might wrongly be interpreted as a measure of the knock-in efficiency. As outlined in the Discussion section, this method does not provide a quantitative measure of knock-in efficiency which may be very low overall and the knock-in is only possible to detect due to the high sensitivity of HiBiT-based luminescence detection.

Is there a way to identify and specifically mark individual colonies within a well that have successfully incorporated the HiBiT tag? These could then theoretically be isolated by picking and expanding, thereby reducing subsequent rounds of subcloning.

This is not necessary with the sib-selection method that we are using. Its purpose is to avoid manual colony picking as described in the original paper on this method (Miyaoka et al. Nat Methods 2014). We have optimised the subcloning process for our iPSC line so that each well contains less than 5 colonies (the majority contain 2-3) and have previously used this method for rapid enrichment of a homogenous population of iPSCs with a knock-in modification of interest (Madsen et al. PNAS 2019). The extent of subcloning and the number of subcloning rounds will, however, depend on the starting knock-in efficiency and the sensitivity of the post-CRISPR screening method. This is why we chose HiBiT-based luminescence as a highly sensitive screening strategy, particularly in cases where the knock-in efficiency might be low.

Lastly, although the authors claim this work is preliminary, it would be nice to see this particular study a little more complete. Of particular interest would be whether a pure population of cells harbouring both tags could in fact be obtained, how many rounds of subcloning are necessary to obtain this, and whether the cells maintain genomic integrity at the completion of this process.

We agree with Dr Howden that further development of the method is needed. However, logistical hurdles have prevented us from doing this ourselves. Our hope is that this Research Note will be useful to others who might wish to improve the method and test its utility as co-targeting strategy. This has now been stated in the Discussion.

Competing Interests: No competing interests were disclosed.