Highly efficient genome editing in hematopoietic stem and progenitor cells

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Graphical Abstract:

Key points:
- IVTsgRNAs in K562 predict response to highly effective genome editing in HSPCs
- Low cost and efficient nucleofection protocols for RNP based editing in K562 and HSPCs
- Genome editing efficiencies in HSPCs up to 80% is independent of cell number, and CD34 subpopulations are equally sensitive for genome editing
- CRISPR-Cas9 gene editing does not impact cell proliferation and differentiation or long term P21 induced cellular senescence
Abstract

Achieving high knockout efficiencies in human hematopoietic stem progenitor cells (HSPCs) is of critical importance to study gene function and correlations.

Here we have evaluated the most critical parameters for achieving highly efficient genome editing in HSPCs and make valuable recommendations. We demonstrate a fast and efficient method for gRNA selection and to genome edit HSPCs.

We report knockout efficiencies up to 80% in human CD34+ HSPCs. Editing efficiency was similar between the different CD34+ progenitor and stem cell subpopulation including the immature CD34+CD38- subpopulation, which is enriched for hematopoietic stem cells. Ribonucleoprotein (RNP)-induced genome editing in human CD34+ HSPCs does not affect cell proliferation, differentiation or in vitro hematopoietic lineage commitment nor upregulate P21 expression.

Overall, this research demonstrates that RNP editing of CD34+ HSPCs can be used for fast and efficient genome editing in human HSPCs paving the road for therapeutic implementation.
**Introduction**

Genome editing is a powerful tool to study gene function and correlation, and the fast turnover time of generating knockouts makes this technique already indispensable for most research fields. Various genome editing technologies have been developed in the past decade, including zinc-finger nucleases (ZFNs) [1,2], transcription activator–like effector nucleases (TALENs) [3,4] and the RNA-guided Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) systems [5,6]. A variety of Cas proteins from multiple species have been identified [7], with *Streptococcus pyogenes* Cas9 (spCas9) and modification thereof being the most developed gene-editor to date with several updates that provide distinct features [8–12].

In mammalian cells, spCas9 and single guideRNA (sgRNA) were initially expressed using transfected plasmid DNA in immortalized cells [13] and modifications to the scaffold RNA were reported to further enhance activity [14]. Nonetheless, achieving high genome editing efficiencies in primary cells remained challenging. Several optimization steps were made to increase the efficiency in HSPCs. For instance, it was reported that direct Cas9 protein delivery, outperforms Cas9 delivered as mRNA or expressed through plasmid DNA [15]. Moreover, modifications that stabilize guide RNAs [15] prevent primary cells from an activated intracellular immune response [16,17], leading to higher genome editing efficiencies in HSPCs.

Despite the important progress that was made, genome editing in HSPCs can still be challenging and to date it is still unclear what parameters should be used for efficient genome editing in HSPCs. These include nucleofection-programs and buffers, Cas9 variants and sgRNA design. Moreover, there is only a limited number of studies that focused on the consequences of genome editing with respect to growth and differentiation. For this reason, we explored the most important parameters for genomically modifying HSPCs.

Various Cas9 modifications were reported to potentially enhance genome editing activity by the addition of nuclear localization signals (NLS), which might improve the delivery of RNPs to the nucleus [18,19]. RNP delivery can be performed using iTOP [20] or more commonly reported by nucleofection [21,22]. For nucleofection, various buffers have been reported that potentially might improve efficacy or reduce the costs of this relative expensive method [21,23].

Guide RNA design is of critical importance in experimental setup and multiple tools are available to accurately predict guide RNAs that have a low predicted off target effect. Importantly, numerous *in silico* based algorithms were developed by multiple groups [24–30],
reviewed by Guanqing and colleagues [31], and can aid in decision making for guide RNA selection. Furthermore, it was reported that the 4 nucleotides adjacent to the PAM site are critical for predicting efficacy and TT or GCC stretches should be avoided [32]. Despite all these models, it has been suggested that the sgRNA efficacy in HSPCs must be determined empirically by testing 5-8 guide RNAs for each target [21] and can therefore be costly in terms of CD34+ HSPCs donor cells as well as purchased sgRNAs.

Importantly, it was previously published that retinal epithelial cells as well human pluripotent stem cells show activation of the p53 pathway after genomic modification using CRISPR-Cas9 [33,34]. CRISPR Cas9 induces double strand breaks and thus might hamper proliferation and differentiation by induction of P21. This potentially generates a bias in using the technique for research purposes and results in the selection of cells with a defect in the p53 pathway for clinical applications. Similar results have been shown for genomically modified HSPCs, since multiple double strand breaks can activate the DNA damage response pathway [35]. DNA damage in hematopoietic stem cells coupled to failure of repair or faulty repair can lead to pathology, premature senescence, bone marrow failure and/or leukemias [36,37].

Here we have evaluated distinct important parameters that need to be considered for genomic editing of immortalized hematopoietic cells (or cell lines) as well as primary isolated CD34+ HSPCs. These parameters include optimal RNP concentration, cell numbers, nucleofection program and guide RNA design. Furthermore, we describe a time-and cost-efficient workflow, including buffers and nucleofection protocols, to genomically edit human CD34+ HSPCs. We show that the combination of the Doench and Wang score [24,38] is a good prediction tool and avoids exclusion of effective sgRNAs, however, screening of sgRNA is still needed and we show that RNP complexes that contain in vitro transcribed (IVT)-sgRNA in K562 predicts activity in HSPCs. Finally, our study showed no P21 induced senescence or cell growth defects.
Results

Highly efficient target validation using RNPs in K562

To setup efficient CRISPR Cas9 mediated gene editing in hematopoietic cells we first explored the optimal conditions to transfer spCas9 to leukemic K562 cells. After purification of recombinant spCas9 (Supplemental figure S1A-F), we compared the commercially available Lonza Amaza SF cell line kit, suited for K562 cells, to the K562 cell line electroporation solution that was previously reported [21]. K562 cells were nucleofected with an ATTO-labelled ribonucleoprotein (RNP) complex targeting CD33 and performance of both nucleofection solutions was assessed by measuring cell survival and knockout (KO) efficiency. Using the homemade nucleofection solution resulted in significant higher levels of transfected ATTO-labelled RNA molecules (Figure 1A). We observed a significant increase in knockout efficiency as measured by flow cytometry when using the home made nucleofection solution compared to the golden standard from Amaza and find no significant differences in cell survival (Figure 1B). Importantly, the homemade buffer can be stored for 3 months without loss of genome editing efficacy (Supplemental Figure 1G). Together these results indicate that the buffer reported by Bak and colleagues [21] should be preferably used for K562 cells.

Next we evaluated different recombinant spCas9 proteins with various nuclear localization signals (NLS), as it has been shown that the number and identity of these NLS sites can substantially influence genome editing efficiencies [18,19]. To test the activity of recombinant Cas9 with various NLS-sequences, we transduced K562 cells with a lentiviral bicistronic BFP-GFP-reporter, also expressing a gRNA against GFP and tested the distinct Cas9 variants in various concentrations (Figure 1C). We show that all transfected Cas9 proteins can generate knockout in a dose dependent manner, however, 3xNLS-spCas9 [19] is significantly more potent compared to the 1xNLS [39] and 4xNLS [18]. This indicates that fusion of specific nuclear localization motifs can significantly enhance genome editing efficiencies. Of note, KO efficiencies using the 3xNLS-Cas9 as an RNP in K562 reached a plateau between 0.6 and 1.0 RNP units in K562 (Figure 1D). We then sought to determine if this in-house generated low-cost RNP nucleofection protocol is suitable for high-throughput target validation in cell line lines. To test this, we developed sgRNAs for 10 genes that were identified in gene expression data set (data not shown) and used two guide RNAs on opposite strands (Figure 1E). Using two guide RNAs in the same exon can enhance the efficiency as shown previously [40], and is furthermore convenient for downstream quantification of successful genome editing as an additional sanger sequencing step is not required. Strikingly, we find that 9/10 genes show significant deleted parts within the exons, with efficiencies ranging between 50-100%, proving
that this system is highly suitable for fast and efficient target validation of multiple genes as well as gRNA selection (Figure 1F-G).

Highly efficient genome editing in human HSPCs is partly dependent on the nucleofection buffer

Encouraged by the findings of figure 1, we evaluated the current strategies to find the most optimal conditions for successful genome editing in CD34+ HSPCs. The transfer efficacy for nucleofection is largely dependent on the amplitude and frequency of electric pulses that temporarily opens the cytoplasmic membrane. The program EO100 is proposed by nucleofector developer Lonza. However, various programs are currently reported for HSPCs [19,21,41] and it is unclear what program works best. Here we confirm that the program EO100 works significantly better compared to the other programs, indicated by the nucleofection score that takes both survival and GFP positivity into consideration (Figure 2A). To maintain the cost-efficiency for genome editing HSPCs we investigated alternative buffers for Amaxa’s CD34 nucleofection buffer used for HSPCs. It was previously reported that a mannitol containing buffer (1M; supplemental table 1.2) can replace Amaxa’s CD34 nucleofection buffer (Amaxa P3) for HPSCs as well as for T-cells [21,23]. Knowing the composition of this nucleofection buffer, we decided to introduce variations in order to find the most optimal nucleofection buffer for CD34+ cells and compared this to Amaxa’s P3 buffer developed for HSPCs (Figure 2B). Nucleofection of three independent donors revealed that Amaxa P3 buffer performed best. The 1M buffer from Bak and colleagues [21] (Buffer 3), and similar buffers where the vendor of mannitol was changed (Buffer 1 and buffer 4), rank amongst the top performing buffers. We validated that mannitol is indeed more potent than sodium succinate (Supplementary Figure 2A), however, find that the vendor is not of critical importance for transfer efficacy (Supplemental figure 2B). We hypothesized that increasing the concentration of mannitol may further increase efficiency. Indeed, increased concentrations of mannitol resulted in significant increased survival while displaying comparable GFP positivity compared to Amaxa buffers (Figure 2C). Encouraged by these results, we performed similar experiments using RNPs and found that the cell survival for homemade buffers is comparable to the amaxa P3 buffer, there is however a ~10% reduction in genome editing efficiency observed (Figure 2D). Overall, we conclude that 150mM mannitol buffer (1M5) is the best buffer to transfec HSPCs with plasmid DNA, whereas Amaxa P3 shows the highest activity for RNPs. Nevertheless, the homemade buffers can provide high genome editing efficiencies and are therefore an excellent cost-effective alternative.
Genome editing efficiencies are equally represented within distinct human CD34+ HSPC subpopulations

Genome editing efficiencies might be highly dependent on the cell numbers that are used for nucleofecion as well as the concentration of RNPs. For this reason, we sought to determine whether lowering the cell numbers could potentially increase genome editing efficiency. Surprisingly, we find no differences in survival and knockout efficiency after nucleofection of various cell numbers using equal amounts of Cas9 RNPs (Figure 3A). Furthermore, we observe no significant differences in survival between Cas9 only and conditions treated with a RNP, suggesting that Cas9-induced double strand breaks are not toxic. Nonetheless, we observed that Cas9-only nucleofected cells have a significantly reduced survival compared to control cells, indicating that the nucleofection itself induces cell death. Next, we determined the optimal Cas9 RNP concentration to achieve efficient genome editing by using RNPs targeting CD45 (Figure 3B). We observe that 2xRNPs is the most cost-effective combination for generating KO in CD34+ HSPC, using a sgRNA targeting CD45 (Figure 3C). Increased concentrations of RNPs did not lead to increased cell death or shifts in the percentage of CD34+ subpopulations. CD34 positive cells can be divided in different subpopulations with distinct self-renewal and or differentiation properties, therefore we investigated the genome editing efficiency in the different CD34+ subpopulations. Our data shows that the knockout efficiency in CD34+CD38- immature hematopoietic stem cells, CD34+CD38+ hematopoietic stem and progenitor cells and CD34-CD38+ myeloid lineage committed cells was similar (Figure 3D-E). Further subdivision of the CD34+CD38- cells using CD45RA [42,43] that also in the most immature CD34+CD38-CD90+CD45RA- hematopoietic stem cells the knockout efficiency was similar to downstream progenitors, albeit that the number of events assayed in these experiments was rather low (Between 150 and 4200, Supplemental Figure 3). Overall, we show that genome editing of HSPCs is consistent between donors and that genome editing efficiencies are equal in CD34+ subpopulations.

IVTsgRNA screening in K562 predicts effectivity for HSPCs

Unlike K562, in which we successfully targeted most genes with 2 guide RNAs, sgRNA selection in CD34+ cells is more complicated. In silico modeling to predict sgRNA efficiency can be performed using numerous tools and algorithms that are available [31]. Nonetheless, it is unclear whether these algorithms also translate to RNPs, since most algorithms are based on lenti-virally expressed sgRNAs, which are in general more effective. Despite all these prediction tools that might help to enrich for finding effective sgRNAs, it is recommended to screen 5-8 guide RNAs per target as reported previously [21]. As sgRNA screening on a large scale is expensive and affects the stocks of precious CD34+ resources, we aimed to develop
a rapid screening method using K562 cells to test and select usefull sgRNAs for gene editing in HSPCs. To this end we used a combination of PCR and a cost-and time-efficient in vitro transcription protocol to generate in vitro transcribed sgRNAs (IVTsgRNAs) (Figure 4A, top). IVT templates to produce IVTsgRNA were generated and amplified using PCR. Notably no difference in IVT template formation was observed using different polymerases (Figure 4A, bottom). We compared IVTsgRNA-RNP to one of the commercially available kits and measured significant higher RNA yields (Supplementary Figure S4A) using the system that we have setup and show similar KO efficiencies (Figure 4B). Note that most genome editing is already observed 24h after nucleofection, which further increased at day 4. Next, we tested 6 genes and designed 5-10 guide RNAs per gene, based on high specificity scores calculated using CRISPOR. For most genes we find 2-3 guide RNAs that perform well (Figure 4C). We studied if KO efficiency in K562 cells is predictive for primary hematopoietic cells KO efficiency, by testing the top performing sgRNAs in CD34+ HSPC using synthetic sgRNAs. Tide analysis showed variable but substantial KO efficiencies (Figure 4D). Of note, we included a previously highly efficient CD45 sgRNA as a control and we observed 80% KO on protein level as well as TIDE indel score (Supplementary Figure 4B). The data shows that all the guides that we have identified in the K562 screen demonstrate activity in HSPCs, and we conclude that this screening system in k562 cells can be efficiently used to predict effective sgRNAs for genome editing in HSPCs.

As we now have validated six distinct sgRNAs from the IVTsgRNA prediction model, we sought to compare this to all the prediction tools available on CRISPOR website to see whether silico modeling can further refine guide RNA selection and screening. In agreement with Doench and colleagues [24] we show that their model has improved over time (Figure 4E) compared to the older version (Supplementary Figure 4C) and we find none of our validated sgRNAs in the lower Doench score region. sgRNAs with very low Doench score can thus be avoided, still screening of guide RNAs cannot be skipped. Comparison with the other models revealed that only the Wang score exclusively enriches for effective guide RNAs (Figure 4F), whereas Wu-, Chari- and Azimuth-scores potentially discriminate between potent and extremely potent sgRNAs (Supplementary Figure 4D). Doench and Wang scores are thus the only scores that select for potent sgRNAs without false negatives in the data set we have tested. Doench- and Wang-scores correlate according to our data set (Figure 4G) and we speculate that these scores might be combined to enhance in silico prediction. To test this, we multiplied the Wang and Doench-score and observed that this indeed enhanced the discrimination (Figure 4H). Note, that even with this cut-off only 6/35 guide RNAs were validated, indicating that in vitro screening is still of critical importance. Overall, we conclude that a combination of Wang-and Doench-score is the most efficient in silico first line prediction rule to enrich for potent guide
sgRNAs in a RNP system. However, as these prediction models are not sufficient to accurately predict effective guide RNAs in HSPCs, we encourage the use of the simple IVTsgRNA screening model that we have setup and test 8 guide RNAs in K562 to have the most cost-efficient method to generate KO in CD34+ HSPCs.

**P21 is not induced after highly efficient genome editing in human CD34+ HSPCs**

CRISPR-Cas9 induces double strand breaks, which has been reported to lead to p53 activation. An important downstream target of p53 in this DNA damage response pathway is the transcriptional activation of Cyclin dependent kinase inhibitor p21CIP1/Waf1 that regulates G1 cell cycle progression. Thus genome editing may potentially lead to selection of cells that are, to a variable degree, defective in their p53 response [33–35]. For this reason, we sought to determine the p53 response of Cas9-mediated genome editing in HSPCs as well as leukemic cell lines. Incubation with nutlin, an inhibitor of the interaction between MDM2 and p53 thus stabilizing p53, lead to a stark decrease in cell survival in p53 wild type AML cell lines but not in p53 mutant cell lines (Figure 5A). Nutlin induced P21 expression specifically in p53 WT cells but not in p53 mutant cells indicated by Western-blot (Figure 5B). Note that ML2 is weakly positive indicated by an increased intensity image (supplemental S5A). P21 induction was confirmed by flow cytometry and expression levels correlated with the western blot between the different cell lines (Figure 5C). Next, we assayed if CRISPR-induced DSBs in p53 WT cells lead to reduced cell survival as observed upon stabilizing p53 by nutlin. The bicistronic BFP-GFP-sgRNA CRISPR reporter gene was stably expressed in the 5 different cell lines (three p53wt and three p53 mutant lines; supplemental Figure S5B). Of note, nucleofection programs were optimized for all indicated cells (Supplemental Figure S5C-E). Next, we co-cultured the BFP-GFP-shRNA CRISPR reporter lines with their wildtype counterparts (1:1 ratio) in order to perform competition experiments. We hypothesized that BFP+ cells would be outcompeted by wild type cells if DSBs induce p53/p21-mediated cellular senescence (Figure 5D). Cas9 transfection led to a significant GFP reduction in all cell lines, however we did not observe a correlation with the loss of BFP+ cells (Figure 5E). This suggests that there is no P21 induced senescence in the p53wt cells as both cell lines with a P53wt status are not hampered in proliferation in this competition assay. These results encouraged us to assay the responses to RNP-induced DSBs in purified mobilized peripheral blood CD34+ HSPCs. As expected, CD34+ HSPCs upregulated p21 (Figure 5F) upon incubation with the p53 stabilizing agents nutlin, resulting in significant cell death after 16 hours (Supplemental Figure 5F). This indicates that CD34+ HSPC are responsive to p53 activation and react by upregulating p21. Next, we induced single DSB breaks using RNP complexes containing sgRNAs against CD33 or CD45 as well as multiple DSB using both CD33 and CD45
targeting RNPs at the same time. Knockout efficiency assayed after 4 days was 60% for CD33 and 50% for CD45 (Supplemental Figure 5G,H). The double knockouts resulted in a somewhat lower knockout efficiency of approximately 20%, which is mainly caused by sgCD45 that has a lower effect when multiplexing (Supplemental Figure 5I). In contrast to treatment with the p53 stabilizing agent nutlin, both the single and double RNP complex nucleofected CD34+ cells did not show significant upregulation of p21 (figure 5G). This suggests that p53 is either not activated due to RNP-induced DSB or that this activation remains too low to detect using p21 as a readout.

**CRISPR-Cas9 has no effect on the differentiation or proliferation of HSPCs**

To investigate whether CRISPR-Cas9 potentially hampers cell proliferation or differentiation of HSPCs we decided to generate a knockout in CD34+ cells and differentiated these in an erythroid *in vitro* culture model (Figure 6A). As CD45 and CD33 are not expressed on erythroid cells, the knockout dynamics were assayed using an RNP-complex targeting CD44. Using 1 RNP we observe 40% KO and limited cell death at day 5 indicating fast recovery (Figure 6B). Indeed, CD44 knockout was unchanged during erythroid proliferation and cells expanded similarly to control cells (Figure 6C). Furthermore, we observed no differences during differentiation indicated by erythroid immunophenotypic stainings of CD235 and CD71 (Figure 6D).

**Differentiation from HSPCs to lineage effector cells is maintained following CRISPR/Cas9 genome editing**

The nucleofection process and subsequent RNP-induced DSB leading to specific knockout of CD33, CD45 or both genes may affect the specific outgrowth of CD34+ HSPC to lineage effector cells. However, the frequency of CD13+ myeloid cells, CD235+ erythroid cells and CD41+ megakaryoid/HSPCs did not change between cells that were successfully genome edited or that were nucleofected with Cas9 only (Figure 6E). Importantly, the knockout efficiency within differentiated lineage cells was not altered compared to day 4. This showed that nucleofection as well as DSB induced by RNP complexes do not interfere with the specification to specific myeloid lineages. In addition, the data confirms that knockout of CD33, CD45 or the combination does not intrinsically interfere with differentiation to myeloid lineage.
Discussion

CRISPR Cas9 gene editing is a powerful tool to study gene functions in the hematopoietic system. The protocols that we have utilized here show that an optimized RNP system results in high KO efficiencies for K562 and is therefore an excellent method for target validation. The highly efficient KO in K562 is explained by 3 important parameters (i) two guide RNAs targeting one exon [Mandal 2014], (ii) the home made nucleofection buffer [21] and (iii) the improved Cas9-3xNLS [19]. Since we find that most other cell lines that we treated with Cas9-3xNLS showed similar or even higher levels of KO in the GFP reporter cells, we hypothesize that the RNP target validation method we describe applies for most hematological cell lines. The process is not only time saving but it might also reflect a better model as a heterozygous pool of KO is a more representative model to study gene function compared to clonal selected cells that have a distinct genetic or epigenetic cellular background due to cellular heterogeneity within cell lines [44].

Surprisingly, we do not find differences between 1xNLS [39] and 4xNLS [18], where it was previously demonstrated that 4xNLS significantly outperforms 1xNLS and 2xNLS-Cas9 proteins by local delivery in mouse brain [18]. Our experiments were performed in hematopoietic cells and thus this discrepancy may signify a cell type specific difference in effective nuclear localization. Both 1XNLS and 4xNLS proteins have their SV-40 sequence(s) (PKKKRKV) on the N-terminal of the protein. This in contrast to the plasmid expressing the 3xNLS Cas9 where the SV-40 NLS is on the C-terminal site of Cas9. As it was shown that the localization C-terminal or N-terminal does not affect Cas9 activity [45], we speculate that one of the other 2-NLS sites, that are derived from C-Myc and Nucleoplasmin [46], or the combination, substantially adds to localization of Cas9 to the nucleus in hematopoietic cells, and thereby explains the increased efficacy of the 3xNLS-Cas9 protein.

In general, we observe that genome editing in primary human HSPCs is less efficient compared to K562. We speculate that this might be caused by (i) the nucleofection buffer, which is different for K562 compared to primary cells, and (ii) the intrinsic nature of the cells that is clearly distinct for CD34+ cells (e.g. DNA repair machinery or nuclear transport). We have not performed nucleofection experiments of CD34+ cells with K562 nucleofection buffer. Based on a comparison between these two buffers, we speculate that ATP instead of Mannitol might provide the largest difference between the buffer that is used for K562 cells and the one that is used for CD34+ cells. Mannitol, used in the home made CD34+ buffer, was originally identified as a ROS quencher in plants [47] and electroporation efficacy balances with ROS production hence resulting in cell death [48]. We indeed observed that increased mannitol concentrations can improve CD34+ cell survival for plasmids, however, does not positively influence transfer efficacy. We furthermore speculate that the ion composition might be not
ideal in the 1M buffers that are used for CD34+ cells, and can possibly be improved by lowering the Mg2+ concentration and increase the K+ concentration, as Mg2+ and K+ have been linked to survival and transfer efficacy respectively [49]. Previously, ATP leaks are observed during electroporation as well as other cellular substances that are depleted from the cytosol [50,51]. Since the K562 buffer contains ATP we hypothesize that adding ATP or GTP to the nucleofection buffer might increase the efficacy as GTP is a critical substrate to activate nuclear import of cellular proteins with an NLS signal and thus RNPs.

CD34+ cells are p53wt in contrast to K562, and on mRNA levels P21 expression is observed in the first 24 hours after CRISPR Cas9 by making a single DSB in HSPCs on the y-chromosome of male cells [35]. Because the p53 pathway is involved in the NHEJ route we speculate that CD34+ cells are possibly more efficient in repairing the DSBs by their efficient p53 DNA repair machinery and thereby leading to decreased genome editing efficiencies. In fact, this suggests that transient p53 pathway inhibition using GSEA56 [35,52,53] can possibly enhance genome editing activities. It was previously shown that transient p53 inhibition using GSEA56 prevents a proliferation delay in genomically modified HSPCs using AAV6 and finally resulted in better engraftment, which was found initially delayed in AAV6 genomically edited cells [35]. We have not evaluated the p21 response in the first 48 hours after CRISPR, however, we do not observe any significant effect on p21 response at day 5 nor defects on long term proliferation and differentiation.

Overall, we find that sgRNA testing is the most challenging part for achieving high genome editing efficiencies in HSPCs. Routinely we follow the criteria to avoid sgRNAs with a low Doench-Wang score or a blocking motif adjacent to the pam-site [24,32,38]. However, we find that most guide RNAs do not work in HSPCs and we agree with previous findings [21], to routinely screen 5-8 guide RNAs to achieve high genome editing efficiencies in HSPCs. IVTsgRNAs trigger an immune response in primary cells by activating the RIG-1-IFN pathway leading to cell death and differentiation [54]. This can be partly reduced by phosphatase treatment of IVTsgRNAs, however, the IFN-1 pathway is not active in K562, in contrast to other cell lines tested, including Hela, HEK293, and HEPG2 [54]. For this reason, we speculate that additional phosphatase treatments are most likely not of additive value for the IVTsgRNA system we described and are thus not included in our protocols.

In conclusion, our work demonstrates the set-up of a time and cost-efficient system to select sgRNAs for genome editing in primary human HSPCs by screening in K562 cells. Furthermore, we provide guidelines and protocols for highly efficient genome editing in HSPCs. Importantly, our RNP nucleofection system for genome editing in HSPCs does not show any unwanted side effects, such as defects in proliferation, differentiation or lineage output. CRISPR-Cas9 RNP
nucleofection is a state-of-the-art technique to study the function of any particular gene in the hematopoietic system and paves the road toward implementation for many clinical purposes.

**Author contributions**

HJMPV, CK, AK, LR, SM, GM performed the experiments. CV provided the CD34+ HSPCs. Concepts were formulated by HJMPV, EA and CV. HJMPV, CV and EA wrote the initial manuscript and CK, AK, LR, SM, GM made improvements.

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Materials and methods

Cas9 Protein purification

pET-NLS-Cas9-6xHis was a gift from David Liu (Addgene plasmid # 62934), pET-21a 3xNLS SpCas9 protein expression was a gift from Scot Wolfe (Addgene plasmid # 114365), 4xNLS-pMJ915v2 was a gift from Jennifer Doudna (Addgene plasmid # 88917). Bacterial expression cultures were performed as previously described [20]. Bacteria were lysed in buffer A (20mM Tris, 0.5M NaCl, 50mM TCEP, 2.5mM MgCl2) + protease inhibitor cocktail (Roche) and 10 mM imidazole. His tag purification was performed using a AKTA purifier (GE, health care) in combination with 5 ml His-purification column (GE Healthcare, 17-5248). Washing and elution was done with buffer A+ 40 mM and 250 mM imidazole respectively. Pooled Cas9 containing fractions were concentrated using Amicon ultra centrifugal 100 kDa cut-off filters (UFC9100 Amicon) and subsequently loaded on HiLoad 16/60 Superdex 200 PG gel filtration column (GE28-9893-35, GE Healthcare) and purified fractions were concentrated with Amicon UFC9100 in buffer A + 0.5M NSDB-201 (Santa Cruz, sc-202237) and filtered using a Acrodisc Mustang E 0.22 uM Endotoxin removal filter (Pall, MSTG25KIT. Cas9 was snap-frozen and stored in an ultra-low freezer (-80oC). Protein concentration was determined using BCA protein assay including 0.1- 0.5M NSDB-201 to correct for background absorption.

Cell line culture and nucleofection

K562, EOL-1, NB4 and HL60 were cultured in Roswell Park Memorial Institute (RPMI) medium (130-046-703) containing 10% heat-inactivated FBS, while ML-2 and KG1A were cultured in RPMI medium containing 15% and 20% FBS respectively. All cell lines, concentration 1x10^6 -4x10^6 cells per condition, were nucleofected with Homemade nucleofection buffer [21] unless otherwise stated. Unless otherwise stated, NB4 and HL60 were nucleofected using program X-01, ML2 and EOL-1 at CA-137 and T-016 for K562. 5-10x volumes of room temperature medium was added to the cells after nucleofection and directly transferred to culture dishes without washing unless otherwise stated. Cells were grown in a 37oC incubator with 5% CO2 and all cell lines were tested negative for presence of mycoplasma.

IVTsgRNA production and purification

The forward oligo was developed including 1 or 2 extra guanines after the T7 promoter to enhance RNA expression [55] resulting in a 56-58 nt oligo containing the 20nt CRISPR sequence. A DNA template was formed by running a 35 cycle two step PCR (10 seconds
denaturation 98°C, 10 seconds elongation 68°C) using standard PCR mix as described below. 5 ul of PCR product was incubated with T7 transcription mix for 16-20 hours according to manufacturer's recommendations (Hi-SCribe T7, NEB E2040). DNAse I treatment using 1 ul of DNase I (NEB, M0303) was performed for 15 minutes at 37°C followed by 15 minutes of inactivation at 80°C. Next purification was performed by using monarch columns (NEB, T2040). RNA concentrations were estimated using NanoDrop and RNA was diluted to 0.5 ug/ul using Ultrapure water. RNA was transferred to 96-wells plate for easy downstream processing and stored at -80°C prior to use. The IVTsgRNA system as described here was validated and compared to Guide-it sgRNA In Vitro Transcription Kit (Takara Biosciences, 632635). Three different polymerases were validated to generate IVTsgRNA template: PrimeStar (Takara Bio Inc. R010A), Phusion High-Fidelity (Cat. #F553L, Thermo Scientific™) or Taq polymerase (Cat. #10342046, Invitrogen).

**High-throughput IVTsgRNA screening**

1 ug sgRNA, was incubated in 96 u-bottom well plates with 3ug-Cas9-3xNLS for 10 minutes at RT. K562 cells (0.2 x 10^6 cells/condition) were resuspended in 20 ul homemade nucleofection buffer and resuspended with a multi-channel. Hence the 20 ul of the RNP-K562 cell mixture was transferred to nucleofection strips and program T-16 (Amaxa I) or FF-120 (Amaxa 4-D) was used for nucleofection. 80 ul of culture media was added for cell recovery. The complete 100 ul nucleofection mix was transferred to 24 wells plates and cultured without washing. Nucleofection strips are washed 2 times using 100 ul Ultra-pure water and sterilized using 100 ul ethanol for 1 minute. The complete process of RNP formation, transfection and washing of the strips was performed using a multichannel and takes ~2-3 hours for 48 guide RNAs.

**High-throughput-DNA isolation**

200 ul of cell suspension was transferred to 96-wells V-well-plates. DNA was isolated using the NucleoVac vacuum system according to manufacturer's conditions NucleoSpin 8 Blood Core Kit (MN 740455.4) and isolated with Vacuum using the manifold (MN 740681). The whole process was performed using a multichannel pipet and isolation of 48 samples takes 1 hour. The HT-DNA isolation method was compared to the crude lysis method previously described [56] and Qiagen DNeasy Blood & Tissue Kit cat 69504. 2 ul of DNA isolates was amplified in a PCR.
Poly chain reaction (PCR)

For polymerase chain reactions (PCRs), 5-50 ng of DNA was amplified using Invitrogen Taq DNA Polymerase (Cat. #10342046), recombinant kit (Thermo Fisher #10342046) in 25 µl final reaction volume according to manufacturer’s instructions. Samples were then amplified in Veriti 96-Well Thermal Cycler, starting with 180 seconds of denaturation at 94°C followed by 40 cycles of denaturation (45 seconds, 94°C), annealing (30 seconds, 59-64°C) and extension (90 seconds per 1kb, 72°C) and one final extension of 10 minutes at 72°C.

PCR clean-up, sanger sequencing and indel analysis

PCR cleanup was performed according to NEBs manufacturer’s conditions by adding Exonuclease I (M0293) and Shrimp Alkaline Phosphatase (rSAP) M0371 to PCR products. Sanger sequencing was performed using the BigDye™ Terminator (BDT) Cycle Sequencing Kit (Thermo Fisher # 4337455) in a final reaction volume of 20 µl according to the manufacturer’s instructions and sequenced in Applied Biosystems™ 3730 DNA Analyzer. Indel score were analyzed under standard conditions using TIDE analysis open software (Brinkman et al 2014)

Guide RNA design and RNP formation

Guide RNAs were designed using CRISPOR by selecting guide RNA sequences with the highest specificity score. Guide RNA sequences targeting CD33 and CD45 were from [guide swap paper] and CD44 from [ref]. Synthetic Guide RNAs were dissolved in ultra-pure RNAse free water according to manufacturer’s recommendations for IDT and synthego. Guide RNA was mixed in ~ 1:1 molare ratio as suggested by [21,22] and incubated for 10 minutes at RT. 1xRNP units is 15 ug Cas9 + 5 ug Guide RNA.

CD34 cell isolation

Human material was obtained after informed consent, mobilized peripheral blood (MPB) was obtained from leukapheresis material, and cord blood (CB) was collected according to the guidelines of NetCord FACT (by the Sanquin Cord Blood bank, The Netherlands). Briefly, CD34+ cells derived from mobilized peripheral blood and cord blood were isolated using MACS beads according to manufacturer’s conditions (Miltenyi, 130-046-703) and cryo preserved in IMDM, 20% FCS including 5% DMSO. A more detailed protocol for isolation was previously described [Voermans et al 1999]. After isolation, the purity of CD34+ cells was determined by flow cytometry using CD41, CD34 and was usually >95% ± 4%.
CD34 nucleofection

Cryopreserved CD34+ cells were thawed and cultured in Cellquin media [57] supplemented with a cytokine maintenance cocktail containing SCF (100 ng/ml), FLT3 (100 ng/ml), and TPO (10ng/ml). All cells were pre-cultured for 16-30 hours prior to nucleofection. CD34 cells were collected and resuspended in nucleofection buffer 1M.3 and nucleofected using program EO100 unless otherwise stated. Directly after nucleofection, room temperature culture media was added for recovery and cells were spun to remove the residual washing buffer. All cells were then cultured with Cellquin media including IL3 (100 ng/ml), il6 (100 ng/ml), TPO (10ng/ml), SCF (100 ng/ml) at 37°C, 5% CO². Expansion and proliferation was performed as previously described [57].

GFP competition assays

Cell lines were transduced using retronectin (Takara biosciences, Cat T202) according manufacturers conditions. The GFP reporter was a kind gift from Kosuke Yusa (Addgene plasmid # 67980 and BFP+ cells were enriched using FACS, in case transduction efficiency was < 90%. Transduced cells were mixed 1:1 with their wild type counterparts prior to nucleofection and the BFP ratio was determined on cells that were not transfected with 5-10 µg Cas9-3xNLS.

Western Blot

Lysates for Western-blot were made in a 1% triton buffer including cOmplete protease inhibitor cocktail (Roche 11697498001) and Western-blot and flow cytometric staining was performed as described previously [58,59]. All antibodies for flow cytometry and Western-blot can be found in table 1.5.


Figure Legends

Figure 1. Highly efficient target validation using RNPs in K562

A) 1.0 RNP unit of Cas9-1xNLS-sgCD33-ATTO was nucleofected using three independent Amaxa kits and compared to the homemade K562 nucleofection buffer. Each point shows the relative amount of labeled RNA molecules detected at day 1 using flow cytometry and samples were compared with an unpaired student t-test. B) As Amaxa kits expire within 3 months after opening, we evaluated the performance over time as indicated by week numbers. Cell survival and knockout efficiency was measured after 1 and 4 days respectively post nucleofection using flow cytometry. Each point represents the average of a duplicate and samples where compared using a paired two tailed t-test (C) K562-CRISPR-GFP-reporter cells were nucleofected with spCas9 and analyzed for GFP and BFP expression after 4 days. Data points show the mean ±SD of a n=3 and comparison was made using a One-way ANOVA test followed by a post tukey test. (D) RNPs targeting CD45 were tested in various concentrations KO was measured at day 4 after nucleofection. Data points show the average ±SD of a n=2. (E) 20 Guide RNAs (2x gene) were designed for target validation in K562 cells using Broad institute sgRNA design. (F) K562 cells were nucleofected with 1xRNP per sgRNA and DNA was isolated 3 days post nucleofection. Images show PCR products where the KO band is indicated with an asterisk. (G) quantification of KO band/WT product by Image J software and comparison was made using a student t-test (*<p0.05 ,**p<0.01, ***p<0.001, ****p<0.0001). sgRNA sequences and primers are in tabel 1.0 and 1.1 respectively.

Figure 2. Highly efficient genome editing in human HSPCs is partly dependent on the nucleofection buffer

A) hCD34+ HSPCs were nucleofected with Amaxa P3 buffer and 1 ug of pMax-GFP plasmid on indicated programs. Cell survival and GFP-positivity were measured using flow cytometry 1 day post electroporation. Nucleofection score = % viable cells * % GFP+ cells normalized to the best performing program. B) hCD34+ HSPCs were nucleofected with 1 µg pMax-GFP plasmid, cell survival and GFP-positivity were measured 1 day post nucleofection. Nucleofection score = % viable cells * % GFP+ cells normalized to Amaxa P3. Buffers were ranked based on performance and the heatmap shows the relative composition of the buffers whereas the accurate composition can be found in supplemental table 1.2. C) Variations in the 1M2 buffer were made by changing the mannitol concentration, and efficacy was tested on hCD34+ HSPCs from 10 different donors. Survival and GFP-positivity were measured 1 day post nucleofection. Nucleofection score = % viable cells * % GFP+ cells normalized to
Amixa P3. D) CD34+ cells from 6 donors were nucleofected with indicated buffers and a 2xRNP targeting CD45, each dot represents a different donor and cell survival was measured at day 1 post nucleofection, and KO after 4 days. Statistics were calculated using a One-way ANOVA with Tukey’s post hoc test. (*) p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

**Figure 3. Genome editing efficiencies are equally represented within distinct human CD34+ HSPC subpopulations**

A) Indicated cell numbers were nucleofected with 1xRNP targeting CD45 or Cas9 only. Survival was measured 24 hours post nucleofection and KO after 4 days. B) hCD34+ HSPCs were targeted with indicated RNP concentrations and assessed for KO for CD45. C) 6 donors were analyzed for KO at day 5 (graph I) and cell survival after 1 day (Graph II) using flow cytometry. From the viable cells, the relative presence of distinct CD34 subpopulations was calculated. D,E) hCD34 HSPCs from 4 different donors were nucleofected with 1xRNP and at day 5 the KO was determined on indicated CD34 populations, P1 - P4 were gated on the viable cell fraction, P5 fraction was determined on the P1 fraction. The KO from the 4 different donors was calculated for CD33 and CD45. Statistics were calculated using a One-way ANOVA with Tukey’s post hoc test. (*) p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

**Figure 4 IVTsgRNA screening in K562 predicts effectiveness for HSPCs**

A) Illustration showing an overview of the PCR product that was generated to express guide RNAs. Gel images show the results from different primer concentrations and taq polymerases that were used. B) Comparison performance of IVTsgRNA products derived from Takara Bio kit and from our institute (left panel). 6 different IVTsgRNAs were nucleofected in K562 and analyzed for Indels at indicated times after nucleofection using TIDE analysis (right panel). C) 58 guide IVTsgRNAs were generated and tested in K562. Tide Indel scores were calculated on day 3 post nucleofection. sgRNA sequences can be found in table 1.4 and numbers indicate unique institute codes. (D) Six top candidate sgRNAs were ordered synthetically and were tested in CD34+HSPC cells for activity using 2xRNP. Tide indel Score were determined 3 days after nucleofection. Two examples chromatograms for sg23 and sg88, the guide is indicated in red and PAM site in orange. (E, F) sgRNA sequences that were tested using the IVT system were plotted for Doench and Wang scores using CRISPOR. Comparison was made between all the IVTsgRNAs tested relative to the 6 validated guides, and finally the 3 very potent guides previously used targeting CD33, CD44, CD45. G) plot shows the correlation between Wang- and Doench score from all the tested IVTsgRNAs. (H) combination of Wang and Doench for all
tested IVTsgRNA versus the validated sgRNAs. Statistics were calculated using a paired student t-test. (*p<0.05 , **p<0.01, ***p<0.001, ****p<0.0001).

Figure 5  P21 is not induced after highly efficient genome editing in human CD34+ HSPCs

A) AML cells were culture on 0.5x10^6 cells/ml and incubated with 10µM nutlin for 72 - 96 hours. Cell survival was analyzed using flow cytometry and graph presents the data from a n=3. Significance was calculated using a paired student t-test P-values. B) 0.5x10^6 cells/ml were incubated with 10µM nutlin for 2 hours and lysates were analyzed for P21 activation (note extra exposure of w-blot in supplementary figure 5A for increased contrast) C) simultaneously to the Western-blot from figure B, cells were analyzed using flow cytometry for P21 expression on the viable cell population. D) Model explaining competition assay designed for leukemic cell lines. 100% GFP+ cells were mixed to their WT-counterparts (1:1 ratio) and hence nucleofected with Cas9. In case P21 induces cellular senescence, BFP+ cells will lose the competition from WT cells over time. E) Representative graph from a single GFP competition experiment where cells were analyzed for BFP and GFP levels using flow at day 4 post nucleofection with Cas9 (left panel). The loss of genetically modified cells (right panel) was calculated by (BFP+ cell loss/ GFP knockout) (relative to non nucleofected cells). Graph shows the quantification of the loss of BFP+ cells at day 4 post nucleofection from a n=3. Data were analyzed using a One-way ANOVA. F) hCD34+ HSPCs were cultured in presence with 10uM Nutlin or PBS and P21 staining was measured using flow after indicated time points. Graphs show the expression of P21 for 4 different donors at indicated time points. Fold changes were calculated relative to control cells, and P values were calculated using a One-way ANOVA test and Tukey’s post-hoc test. G) CD34+ cells were nucleofected with 1xRNP for CD33, 1xRNP for CD45 and the combination represented as double KO. P21 staining was analyzed on day 5 after CRISPR. Graphs show the fold change relative to control cells and was analyzed using a One-way ANOVA with Tukey’s post hoc test. (*p<0.05 , **p<0.01, ***p<0.001, ****p<0.0001)

Figure 6  CRISPR-Cas9 has no effect on the differentiation or proliferation of HSPCs

A) Culture scheme that was used to study the growth dynamics of genomically modified cells. B) Flow cytometric measurement of genetically modified hCD34+ HSPCs analyzed for cell survival and KO efficiency on day 5. C) Expansion was determined using a casy-counter and fold change was calculated based on cell count at day 1 at the start of the experiment (left panel). KO stability was determined using flow cytometry on viable cells at indicated time points. D) Erythroid differentiation was analyzed at indicated time points (example left panel)
and quantified for two donors (right panel). E) CD34+ HSPCs with KO efficiencies of ~50% (Supplementary figure 5A), were analyzed for indicated cell lineage output as determined by flow cytometry, 9 days after CRISPR. On day 1-4 cells were cultured in media supplemented with a cytokine maintenance cocktail containing SCF (100 ng/ml), FLT3 (100 ng/ml), and TPO (10ng/ml), followed by 5 day’s cultured in media containing SCF 100 ng/ml, TPO 10ng/ml, EPO (2IU/ml), IL3 (100 ng/ml), IL6 (100 ng/ml) GM-CSF (100 ng/ml). Data was analyzed using a One-way ANOVA with Tukey’s post hoc test. (*<p0.05 , **p<0.01, ***p<0.001, ****p<0.0001).
Supplemental figure legends

Supplemental Figure S1 Cas9 purification and nucleofection buffer stability

**A**) General workflow Cas9 purification. 2-3 µg of Histrapp eluted fractions were ran on SDS-page gel (left panel), and Cas9 containing fractions were pooled and concentrated in 2-3 ml total volume. **B**) Size exclusion was performed on the pooled fractions to discriminate Cas9 monomers from oligomers to prevent protein aggregates in culture. Cas9 monomers typically elute around 66 ml (middle panel) and finally the purest fractions, indicated by SDS-page (right panel), were pooled and aliquoted snap frozen using liquid nitrogen and stored at -80ºC. **C**) For concentration determination, we recommend to dissolve the BSA standard in at least 5x diluted SEC buffer, since NSDB-201, an important zwitter-ionic compound added to the pooled Cas9 fractions, interferes in the BCA assay (undiluted curve). **D**) To check whether chaperone proteins are needed for active Cas9 expression, we compared parental BL21 cells (NEB) transformed with chaperone plasmids (Takara Biosciences Cat 3340), to Rosetta (DE3) cells. In both cases active Cas9 was purified, however, the yield of Rosetta cells is higher. **E**) Next we compared BL21-Star cells with Rosetta cells for protein production. Transformation efficiency of BL21-star cells was higher compared to Rosetta cells when 10 ng of DNA plasmid spCas9-3xNLS was used. **F**) Expression of spCas9 from cultures induced at different OD’s in LB media. In each lane 250 µl of cell culture was loaded, indicating that BL21-star cells provide higher yields compared to Rosetta cells and the optimal OD for protein induction is 0.6-0.7. **G**) Data points from figure 1A were plotted and correlated to the number of weeks past production to study stability of the buffer.

Supplemental Figure S2. Mannitol has a higher transfer capacity compared to sodium succinate and results are stable over distinct vendors.

**A**) CD34+ HSPCs were nucleofected with 1M buffer containing mannitol or replaced with Sodium succinate (both 75mM). Cell viability and KO efficiency of hCD45 was analyzed using flow cytometry at day 1 and day 5 respectively. **B**) CD34+ HSPCs were nucleofected with pMax-GFP and analyzed for cell viability and GFP expression. in total 6 donors were tested and data was analyzed using a One-way ANOVA with Tukey’s post hoc test. ns= not significant, (*<p0.05 , **p<0.01, ***p<0.001, ****p<0.0001) 

Supplementary Figure S3. Quantification of genomically modified CD34 cells

Column total events P5 shows the number of events that were measured and analyzed in Figure 3B and Figure 3C. CD34+CD38-CD45RA-CD90+ events, also referred to as the most potent CD34+ HSCs.
Supplementary Figure S4. Tide indel formation correlates to KO on protein level for hCD45.

A) Quantification of IVTsgRNAs that were generated using the commercial Takara kit versus the IVTsgRNA system that we developed. Hiscrbe T7 polymerase was used for the latter in a 20ul rxn volume and can be rescaled to 10 ul for an average of 50 ug sgRNA (Data not shown). B) Tide indel score analyzed on day 3 for CD34+ HSPCs targeted with 2xRNP for hCD45 and protein KO score analyzed on day 5 using flow cytometry. C) Comparison of validated sgRNA that were delivered using RNPs in HSPCs compared to indicated scores. Each data point represents a unique sgRNA and + indicates the moderate or ++ highly efficient. D) comparison of validated sgRNAs using CRISPOR. Each data point represents a unique sgRNA and + indicates the moderate or ++ highly efficient. Analysis was performed using an unpaired student T-test ****p<0.0001.

Supplementary Figure S5A-E. Cell line transductions and nucleofection optimization.

A) W-blot enhanced image from figure 5B showing that ML2 cells are also weakly positive for P21 induction. B) Cell lines after transduction and FACS sorting. Dark grey shows that all cells, except NB4 were <90% for the BFP-GFP reporter. C) Cell line was nucleofected on different programs with 1 ug pMax-GFP plasmid. Bars represent cell viability and GFP positivity measured 24 hours post nucleofection. D,E) GFP reporters cells of indicated cell lines were nucleofected with 4 µg Cas9-3xNLS. Cell survival was measured 24 hours post nucleofection, knockout after 96 hours. Overall these data suggest that X-01 can be used best HL60, whereas ML2 and EOL-1 perform better at CA-137.

Supplementary Figure S5F-I supportive data for P21 induction experiments

F) Cell survival CD34 cells measured using flow cytometry at indicated time points, data was analyzed using a student t-test. G) FACS plots belonging to figure 5G to show the KO analysis for each donor and each condition and quantification of single KOs and double KO’s is quantified in. H+I) showing the KO efficiencies for CD33 and CD45 in single and double KO. Note that CD45 sgRNA efficacy is significantly hampered in the double KO compared to single KO shown by student t-test analysis. (*<p0.05 , **p<0.01, ***p<0.001, ****p<0.0001)
Figure 1

A

K562

ATTO RNA MFI

FC relative to control cells

Amaxa Home made

B

K562

Knockout efficiency (%)

ns

**

Week

K562

Cell viability (%)

ns

ns

ns

Week

K562

nucleofection score

0

1

2

3

Week

K562

nucleofection score

0

0.5

1.0

1.5

2.0

D

K562

1 RNP = 15µg Cas9 + 5µg sgRNA

E

KO 10 genes in K562

Guide 1 (Watson)

Guide 2 (Crick)

1. Two exonic guides Watson / Crick
2. 20 bp crRNA
3. 40 bp - 700bp between sgRNAs

F

CDH6

LRP1

THRBI

TRSP1

NFI

KO WT

KO WT

KO WT

KO WT

KO WT

G

K562

% Knockout

CTRL KO

KO WT

KO WT

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Figure 3

A

CD34

Survival (%)

100

50

0

Cell number (10^6)

CTRL 1 2 3

RNP

Knockout efficiency (%)

100

50

0

Cell number (10^6)

CTRL 1 2 3

RNP

B

CD45

Normalized T cells

CTRL

1 RNP (15 µg Cas9 + 5 µg sgRNA)

2 RNP (30 µg Cas9 + 10 µg sgRNA)

3 RNP (45 µg Cas9 + 15 µg sgRNA)

C

Knockout efficiency (%)

100

80

60

40

20

CTRL 1 2 3

RNP

Survival (%)

100

50

0

CTRL 1 2 3

RNP

D

Bulk viable cells

CD34

CD38

CD45RA

CD90

CD34

CD38

sgCD33

CTRL

E

CD34+ 1xRNP CD33

Knockout efficiency (%)

100

80

60

40

20

P0 P1 P2 P3 P4 P5

ns

ns

ns

ns
Figure 4

A) IVTsgRNA PCR

B) TIDE indel score (%)

C) TIDE indel score (%)

D) CD34+ HSPCs

E) Doench’16 score

F) Wang-score

G) Doench - Wang correlation

H) Doench + Wang score

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Figure 5

A

Cell survival (ratio)

Nutlin (µM)

0.0

0.5

1.0

1.5

HL60

K562

NB4

EOL-1

ML2

p53 mutant

p53 wt

B

P53 mutant

P53 wt

2 hours Nutlin stimulation (10µM)

HL60

K562

NB4

EOL-1

ML2

P21

alpha-Vinculin

C

P53 mutant

P53 WT

Nutlin (10µM)

HL60

K562

NB4

EOL-1

ML2

E

Mean

Median

Fold change MFI P21

CTRL

6 nutlin

16 nutlin

G

Mean

Median

Fold change MFI P21

Cas9 only

sgCD33

sgCD45

Double KO

CTRL

6 nutlin

16 nutlin

GFP knockout (%)

Loss of BFP+ cells (%)

K562

HL60

NB4

ML2

EOL-1

P53 mutant

P53 WT

Loss of CRISPR cells (loss of BFP+ cells/ % GFP knockout)
Figure 6

A

Thaw
CD34+ cells

CRISPR

Erythroid blast expansion

Differentiation

t=0

TPO

IL3

Epo

Days post CRISPR

KO stability

Donor 1

Donor 2

50% of cells

B

Survival

FSC

SSC

CD44

Day 6

Day 13

Day 20

CD71

CD235

CTRL

sgCD44

KO

CD44 knockout (%)

C

Expansion

KO stability

Donor 1

Donor 2

Days post CRISPR

CD44 knockout (%)

D

Myeloid

Megakaryocytic

Erythroid lineage

Day 6

Day 13

Day 20

CD71

CD235

CTRL

sgCD44

ns

ns

ns

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Supplemental figure 1

A. 1ml fractions of HisTrap column
   - Pooled fractions
   - Cas9 160kD

B. SEC column
   - Pooled fractions
   - Cas9 160kD

C. BCA assay
   - OD (652 nm)
   - BSA (µg/ml)
   - Undiluted
   - 5x diluted SEC buffer
   - 20x diluted SEC buffer

D. 1ml fractions of HisTrap column
   - Yield 28 mg total protein
   - Yield 45 mg total protein

E. Colonies /10ng DNA plasmid
   - BE3-HF
   - Cas9-3xnl
   - Rosetta
   - BL21 star

F. Rosetta cells BL21 star cells
   - Cas9 160kD

G. Home made buffer
   - K562
   - Time in weeks
   - Nucleofection score

H. Comparison of nucleofection scores:
   - 0.4 - 0.9
   - Time in weeks
   - 0 - 1.5

---

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**Supplemental figure 2**

**A**

- **CD34+ HSPCs**
  - CTRL vs. Mannitol vs. Sodium succinate
  - ns

**B**

- **Normalized survival**
  - P3 A B C
  - Vendor Mannitol (50mM)
  - NS

- **Normalized GFP**
  - A B C
  - Vendor Mannitol (50mM)
  - NS

- **Nucleofection score**
  - P3 A B C
  - Batch 1 Batch 2
  - NS
## Supplemental Figure 3

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Supplementary figure S4

A

Yield IVTsgRNA (ug)

Takara  Sanquin

B

C

Doench'14

*p=0.0376

ns

Old Doench '16

*p=0.0293

D

Chari score

Xu-score

Wu-CRISPR-score

Moreno score

Azimuth score

CC-top score

Out of frame score

Lindel score
Supplemental figure 5

A

B

C

D

E

Wild type
GFP reporter
Supplemental Figure S5

**F**

F: Survival of cells treated with nutlin (10uM) compared to controls.

**G**

G: Flow cytometry analysis of KO efficiency for various conditions.

**H**

H: KO Efficiency (%CD33) for different conditions.

**I**

I: KO Efficiency (%CD45) for different conditions.

* p=0.0174