

## ARTICLE

# Quantification of designer nuclease induced mutation rates: a direct comparison of different methods

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Designer nucleases are broadly applied to induce site-specific DNA double-strand breaks (DSB) in genomic DNA. These are repaired by nonhomologous end joining leading to insertions or deletions (in/dels) at the respective DNA-locus. To detect in/del mutations, the heteroduplex based T7-endonuclease I -assay is widely used. However, it only provides semi-quantitative evidence regarding the number of mutated alleles. Here we compared T7-endonuclease I- and heteroduplex mobility assays, with a quantitative polymerase chain reaction mutation detection method. A zinc finger nuclease pair specific for the human adeno-associated virus integration site 1 (AAVS1), a transcription activator-like effector nuclease pair specific for the human *DMD* gene, and a zinc finger nuclease- and a transcription activator-like effector nuclease pair specific for the human *CCR5* gene were explored. We found that the heteroduplex mobility assays and T7-endonuclease I -assays detected mutations but the relative number of mutated cells/alleles can only be estimated. In contrast, the quantitative polymerase chain reaction based method provided quantitative results which allow calculating mutation and homologous recombination rates in different eukaryotic cell types including human peripheral blood mononuclear cells. In conclusion, our quantitative polymerase chain reaction based mutation detection method expands the array of methods for in/del mutation detection and facilitates quantification of introduced in/del mutations for a genomic locus containing a mixture of mutated and unmutated DNA.

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## INTRODUCTION

For many different questions in biological research mutations or DNA double-strand breaks (DSB) have to be induced at discrete positions within genes under *in vivo* conditions. For this purpose designer nucleases such as the well-studied zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) or the clustered regularly interspaced short palindromic repeats (CRISPR)/associated Cas9 system are widely used for *in vivo* genome engineering.<sup>1–5</sup> Their molecular design and mode of action differ from each other, but they all have in common that they can be individually designed to specifically bind DNA sequences of interest and to introduce DNA DSB. For ZFN and first generation TALENs the DNA binding domain is fused to the FokI nuclease domain that is only active upon dimerization of two monomers. Therefore, ZFN and TALENs act as pairs to introduce a DSB at the desired cleavage site.<sup>1</sup> In contrast, for the recently introduced clustered regularly interspaced short palindromic repeats/Cas system dimerization is not required.

Designer nucleases are powerful tools for *in vivo* genome modification. In the absence of homologous DNA, eukaryotic cells repair DSB via nonhomologous end joining resulting in small insertion- or deletion mutations (in/dels) or complex combinations of deletions and insertions.<sup>6</sup> In each allele the

mutations can be different. Insertions or deletions can vary in size from one nucleotide up to several dozens of nucleotides.<sup>7</sup> Because of the heterogeneity of in/dels, commonly used mutation detection methods like single nucleotide polymorphism analysis are not suitable to detect all mutations introduced by designer nucleases. Moreover mutations are not induced in all cells and therefore, genomic DNA (gDNA) isolated from designer nuclease treated cells, tissue or organ represents usually a mixture of mutated and unmutated alleles which hampers detection and quantification of in/del mutations.

To detect in/del mutations, heteroduplex (HD) based assays such as the T7 endonuclease I (T7E1) assay<sup>8</sup> or the heteroduplex mobility assay (HMA)<sup>9</sup> can be applied. They take advantage of the fact, that gDNA isolated from designer nuclease treated cells is a heterogeneous mixture of mutated and unmutated alleles. Both assays are based on a polymerase chain reaction (PCR) product that was amplified from gDNA of designer nuclease treated cells which is denatured by heating and subsequently renatured by slow cooling. During renaturation single-stranded DNA fragments can anneal, also leading to mismatched nucleotides at the site of mutation. As a consequence these molecules have structural distortions at mismatched or unpaired bases whereas in/del mutations were introduced which can be recognized by the

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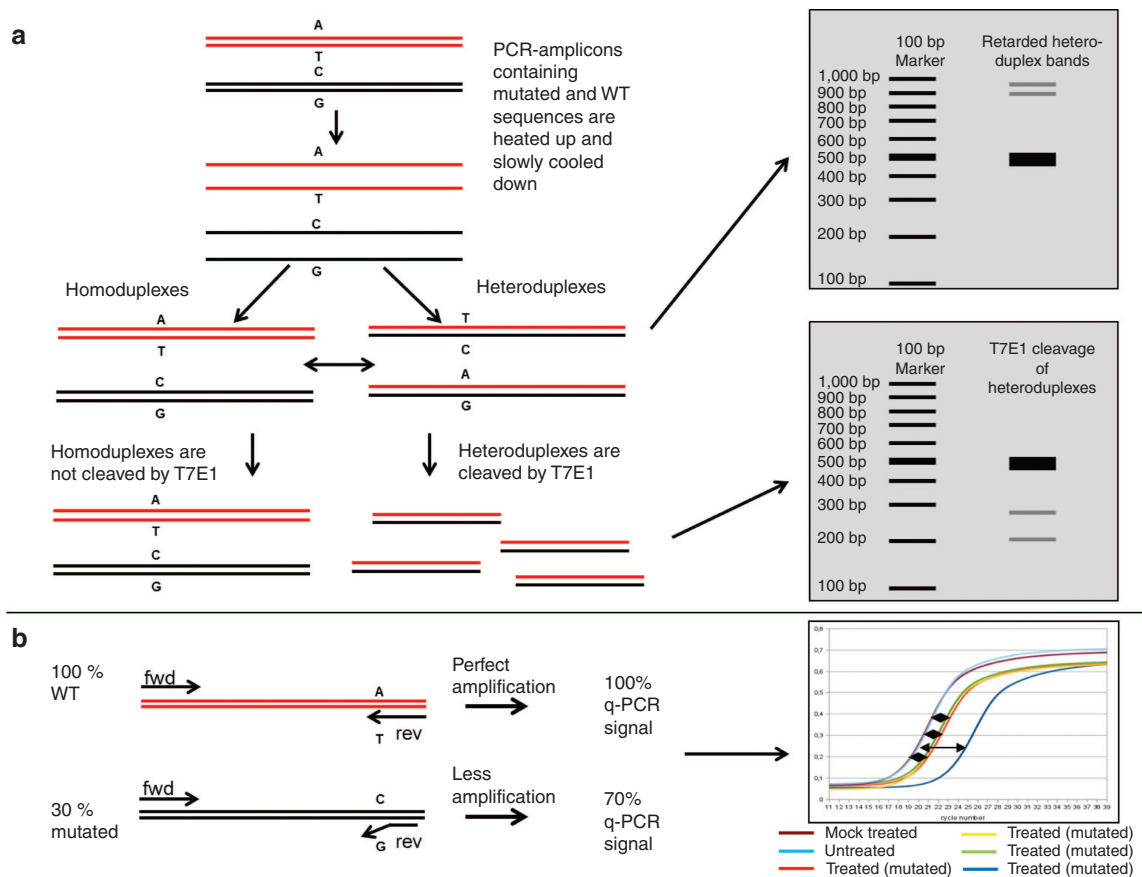
T7E1 enzyme that cleaves DNA close to the unpaired bases. Cleavage products can be visualized by gel electrophoresis (Figure 1a). Similar to the T7E1 assay, the HMA takes advantage of HD formation.<sup>9</sup> Here HDs indicative for in/del mutations are visualized in polyacrylamide gel electrophoresis. As HDs have conformational changes they migrate significantly slower during polyacrylamide gel electrophoresis than homoduplexes and the retardation is proportional to the differences between the two sequences.<sup>10,11</sup> In this way HDs of mutated and wildtype (WT) sequences can be distinguished from the homoduplexes of WT, and WT or mutated and mutated sequences<sup>12</sup> (Figure 1a). Despite the usefulness of these HD based assays to detect in/del mutations they are only semiquantitative as the number of HDs formed is not informative about the percentage of in/dels.

To overcome these disadvantages a quantitative PCR (q-PCR) mutation detection method based on the amplification refractory mutation detection system (ARMS)<sup>13</sup> can be used. This allows not only to detect, but also to quantify the percentage of in/del mutations caused by nonhomologous end joining after designer nuclease treatment.<sup>14</sup> In principle ARMS is optimized to amplify the respective wild type (WT) locus. The first primer binds near the predicted mutation site whereas the second primer is designed to bind to the wild type sequence of the predicted mutation site (Figure 1b). Since the primer pair is highly specific for the WT sequence, changes in the binding sites impairs amplification, thus enabling discrimination between mutated and WT sequences. In combination with q-PCR

this technique also allows to quantify the mutated sequences by relating the q-PCR signal of gDNA from designer nuclease treated cells to the q-PCR signal of gDNA from untreated cells. Thus the designer nuclease induced mutation rate can be measured as a decrease of the q-PCR signal relative to the control sample.

This principle can also be utilized for detection of homologous recombination (HR) events. In gene therapeutic approaches using designer nucleases in combination with a DNA cassette that is homologous to the regions flanking the nuclease cutting site HR can be used for specific sequence correction. The corrected sequence often differs only in a few bp, or even only in one single nucleotide. To detect these events via ARMS, the first primer binds near to the HR locus and enables amplification of unmodified and corrected sequences equally. It is combined with a detection primer that is highly specific for the corrected sequence resulting from HR. Thus, an increase in the q-PCR signal compared with a standard sample with no HR events correlates directly with percentage of HR events.

Here we present a comparison of this new mutation detection method to the commonly used T7E1-assay or HMA by challenging these assays with experimental samples of gDNA from HEK293 cells that were transduced with different designer nucleases, such as ZFN pairs specific for the human AAVS1 locus<sup>15</sup> or the human *CCR5*<sup>16,17</sup> gene as well as TALEN pairs specific for the human *DMD* gene<sup>18</sup> and the human *CCR5* gene.<sup>19,20</sup> Furthermore, gDNA derived from patient derived CD34<sup>+</sup> human hematopoietic progenitor cells that were ex



**Figure 1** Schematically shown is the principle of the different mutation detection assays. (a) Heteroduplex (HD) formation based assays. The genomic locus surrounding the expected mutation site is PCR amplified, denatured by heating and reannealed by slow cooling to form homoduplexes and HDs. Mutated and unmutated polymerase chain reaction (PCR) products can either be separated on SDS-polyacrylamide gel electrophoresis gels resulting in retarded bands or cleaved by T7-endonuclease I (T7E1) enzyme resulting in specific cleavage products. (b) Quantitative PCR (q-PCR) based mutation detection using a wild type (WT) DNA specific primer pair. WT sequences can be amplified with optimal PCR efficiency, when partially mutated DNA is used amplification is partially inhibited depending on the ratio of mutated to unmutated alleles.

*vivo* infected with a high-capacity adenoviral AdV5/35 chimeric vector expressing the CCR5-ZFN pair<sup>21</sup> were examined.

## RESULTS

### Evaluation of T7E1- and HMA for mutation detection

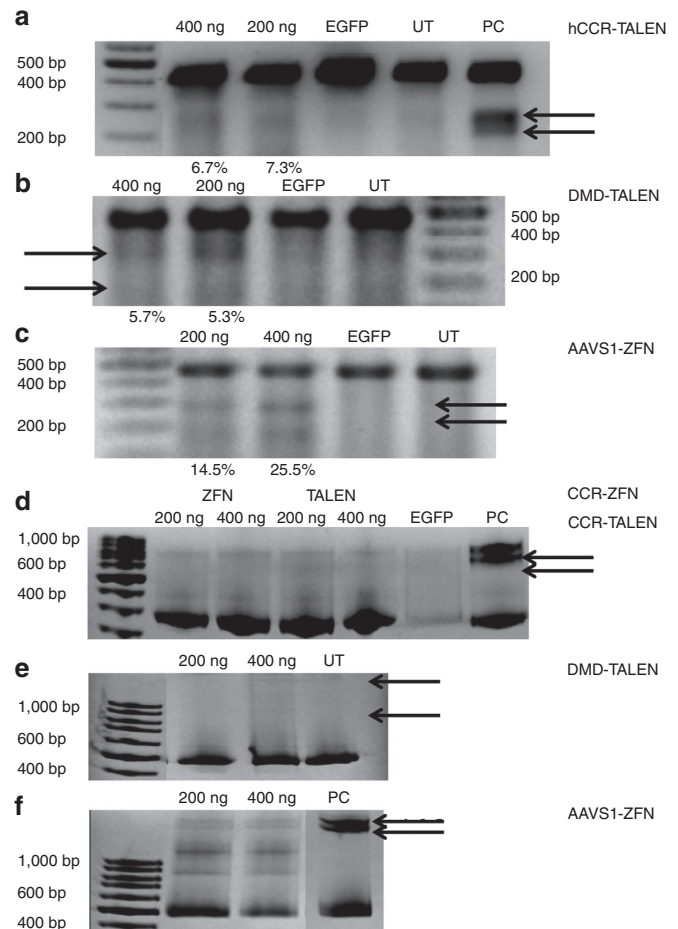
To detect in/del mutations T7E1 assays or the HMA<sup>22,23</sup> based on HD formation of mutated and unmutated DNA are widely used and the principles of these assays are schematically shown in Figure 1a. We challenged the T7E1 assay and HMA using gDNA isolated from HEK293 cells that were cotransfected with either 200 ng or 400 ng of expression plasmids for a *CCR5*-specific TALEN pair, a DMD-specific TALEN pair, and an *CCR5*-specific ZFN or an AAVS1-specific ZFN pair. All designer nuclease encoding constructs used in this study are schematically shown in Supplementary Figure S1. In the context of plasmid transfection all designer nucleases were expressed under the control of the cytomegalovirus (CMV) promoter.

After plasmid transfection of the *DMD*- and *CCR5*-specific TALEN pairs and the AAVS1-specific ZFN pair we performed a T7E1 assay which showed cleavage products indicative for successful introduction of in/del mutations at the expected position (Figure 2a,b,c). Cells that were cotransfected with 200 ng *CCR5*-TALEN expression plasmids showed 7.3% and cells that received 400 ng of respective plasmids showed a mutation rate of 6.7% (Figure 2a). Cells that were cotransfected with 200 ng *DMD*-TALEN expression plasmid showed 5.3% and cells that received 400 ng of respective plasmids showed a mutation rate of 5.7% (Figure 2b). Cells that were transfected with 200 ng of the AAVS1-ZFN expression plasmid showed 14.5% and cells that received 400 ng of respective plasmid showed a mutation rate of 25.5% (Figure 2c). Differences in mutation rates were observed when gDNA from cells treated with different nucleases were compared. When gDNA from AAVS1-ZFN treated cells was used, the mutation rate was higher compared with gDNA from cells treated with the same amounts of same *CCR5*- or *DMD*-TALENs. For gDNA from *DMD*-TALEN treated cells the lowest mutation rate were obtained (Figure 2a,b,c). Furthermore, the mutation rate increased with rising amount of transfected designer nuclease encoding plasmids in gDNA from cells treated with the *CCR5*-specific TALEN pair and the AAVS1-specific ZFN pair (Figure 2a,c). When gDNA from cells treated with the *DMD*-specific TALEN pair was used in this assay the mutation rate decreased with increasing amount of transfected expression plasmids (Figure 2b). In HMA, retarded bands corresponding to heteroduplexes were detectable indicating that in/del mutations were present among the respective PCR products (Figure 2d,e,f). The ratio of band strength of retarded PCR products to unretarded PCR products was highest when gDNA from AAVS1-ZFN treated cells was used (Figure 2f) whereas it was lowest when gDNA from *CCR5*-TALEN or *CCR5*-ZFN treated cells were used (Figure 2d). Differences in strength of specifically shifted bands was not observed after treatment with different amounts of transfected nuclease expression plasmids for gDNA from cells treated with AAVS1-ZFN and *CCR5*-TALEN or *CCR5*-ZFN. Only when gDNA from *DMD*-TALEN transfected cells was used, the band strength of retarded heteroduplexes increased with rising amounts of transfected plasmid (Figure 2d,e,f).

### Establishment of q-PCR based mutation detection and challenge with experimental conditions

As HD based assays are insufficient for mutation quantification, we developed a q-PCR mutation detection method based on an ARMS to specifically detect and quantify in/del mutations induced by designer nucleases (Figure 1b).

To show that we can detect and quantify given percentages of mutated sequences in the background of WT DNA, the q-PCR mutation detection assay was established using plasmids containing a 450 bp fragment of the *CCR5* locus surrounding the respective *CCR5*-TALEN binding site. Either these plasmids contained the WT *CCR5* sequence or contained an 8 bp deletion at the expected *CCR5*-TALEN-cleavage site (Figure 3a). WT and mutated plasmids mixtures were generated containing 0, 2.5, 5, 10, 20, 30, 40, 50, 60,

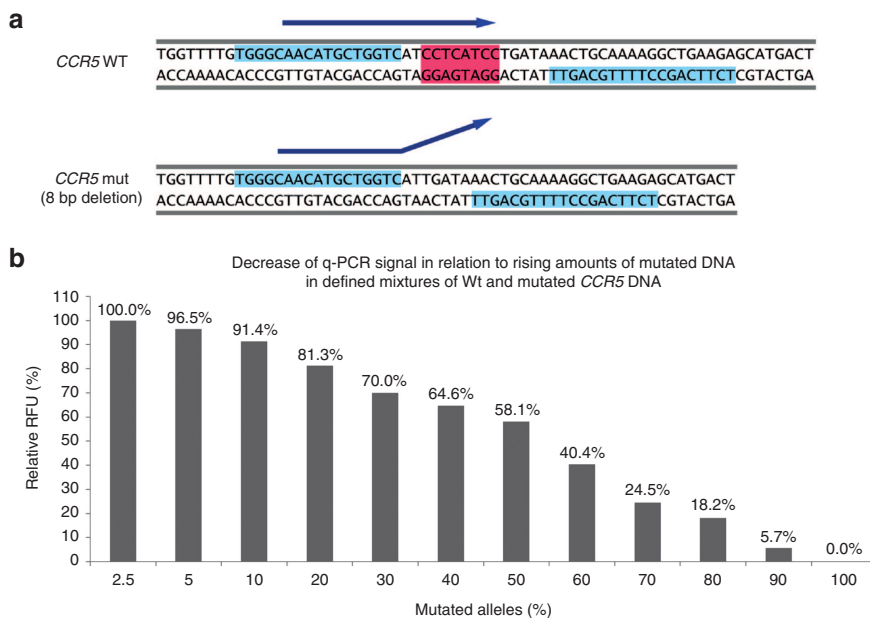


**Figure 2** Representative results of T7E1 assays and gel shift assays performed on gDNA of HEK293 cells transfected or cotransfected with varying amounts of nuclease expression plasmids. (a) T7E1 assay performed after cotransfection of plasmids pAC-CMV-TALE-RM1 and pAC-CMV-TALE-RM2 encoding TALENs binding to the *CCR5* locus. (b) T7E1 assay performed after cotransfection of plasmids pTn3 and pTn8 cotransfection cutting at the *DMD* locus. (c) T7E1 assay performed after cotransfection of plasmids pCMV-FlagAAVS1ELD-T2A-FlagAAVS1KKR transfection specifically binding to the AAVS1 locus. (d) Heteroduplex mobility assay (HMA) after cotransfection of p-*CCR5*-ZFN-L and p-*CCR5*-ZFN-R; pAC-CMV-TALE-RM1 and pAC-CMV-TALE-RM2 encoding a ZFN pair and a TALEN pair against the *CCR5* locus, respectively. (e) HMA assay after cotransfection of plasmids TN3 and TN8 binding to the *DMD* locus. (f) Transfection of the plasmid pCMV-FlagAAVS1ELD-T2A-FlagAAVS1KKR expressing a complete ZFN pair from one plasmid. Cleavage products of T7E1 assay a–c and HMA (d–f) indicating heteroduplexes of mutated DNA and wild type DNA are marked by arrows. a–c mutation rates measured for samples treated with designer nucleases are depicted below the respective lanes of the gel pictures. CMV, Cytomegalovirus; EGFP, cells transfected with EGFP only (negative control); UT, untreated cells (negative control); PC, positive control (PCR products from untransfected cells were mixed with equal amounts of PCR products from plasmid with defined deletions at the nuclease binding site).

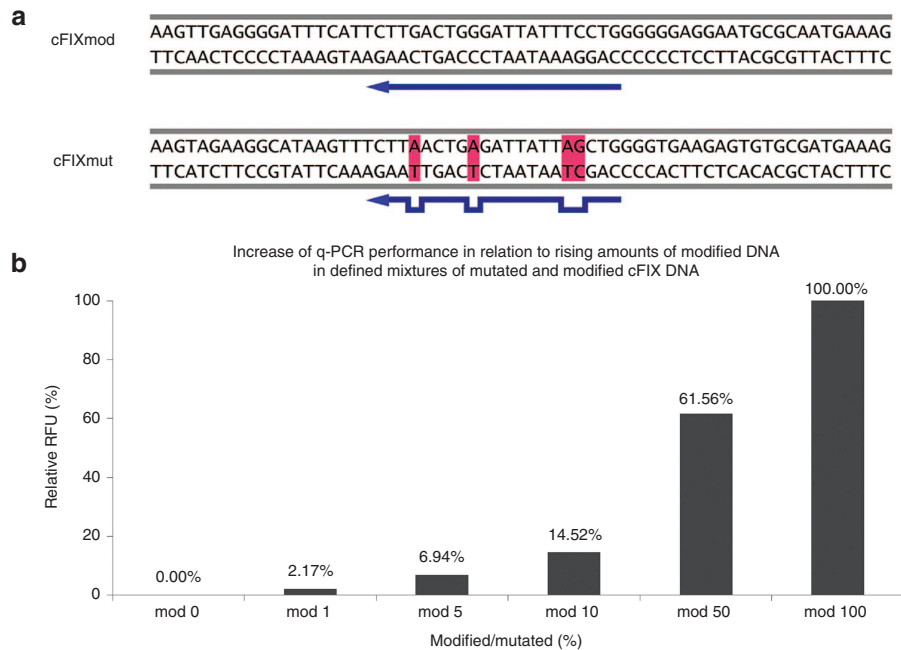
70, 80, 90, and 100% of the mutated target loci. Each mixture was adjusted to an equal DNA concentration of 1.5 ng/ $\mu$ l and subjected to q-PCR. The resulting q-PCR signal for each mixture was depicted relative to the maximum q-PCR signal obtained using the mixture that only contained WT plasmid. As shown in Figure 3b with increasing amounts of mutated DNA within the mixtures the relative fluorescent signal decreased in correlation to the incremental increasing percentage of mutated alleles within the respective mixes. q-PCR mutation detection limit was 5%. This shows that this assay is truly able to quantify the amount of mutated alleles within a mixture of WT and mutated sequences as it is the case for gDNA isolated from designer nuclease treated cells or tissues.

The q-PCR HR detection assay was established using plasmids containing 1,922 bp of the genomic canine coagulation factor IX (cFIX) locus. One plasmid pscAA-FIXmut, carried a single base pair exchange in the catalytic domain of the cFIX gene leading to the bleeding diathesis in dogs (Figure 4a). The second plasmid pscAAV-FIXmod contained a codon modified cFIX sequence (Figure 4a). This sequence was codon optimized at the nuclease binding sites and therefore, it can also function as donor for HR to correct the point mutation in the canine genome. Plasmids mixtures cFIXmut and cFIXmod were generated containing 0, 5, 10, 50, and 100% of the modified target loci. For cFIXmut/cFIXmod the concentration of each mixture was 0.049 ng/ $\mu$ l. Here the mixture containing 0% (cFIXmod) represented 0% HR frequency and the mixture containing 100% (cFIXmod) simulated 100% HR frequency. To normalize samples a second PCR (outPCR) amplifying a 283 region at the 5'-end of the 1,922 cFIX sequence identical in all used plasmids was performed for all samples. HR detection was performed with specific primers for cFIXmod (Figure 4a). PCR conditions were first established as shown in Supplementary Figure S2. This q-PCR based HR detection approach yielded accurate and sensitive calibration curves (Figure 4b). It detected nearly the same ratios of cFIXmod as used in the defined samples. This shows that the ARMS q-PCR can also be used to detect and quantify HR events.

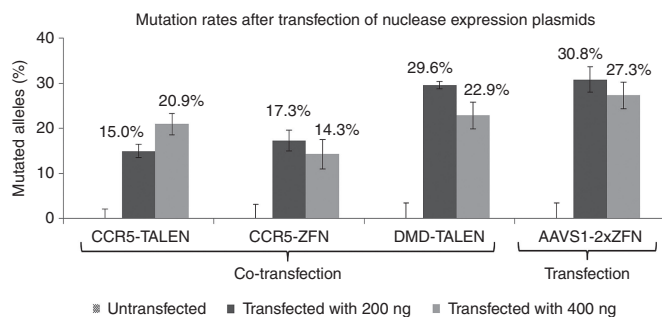
To challenge this q-PCR mutation detection assay with genomic targets derived from tissue culture experiments, the q-PCR analyses was performed using equal amounts of gDNA from HEK293 cells that were untreated or transfected with either 200 ng or 400 ng of different nuclease expression plasmids. Here we used the same gDNA that also used for T7E1 assays shown in Figure 2. Overall mutation rates obtained with the q-PCR assay are higher than demonstrating that q-PCR based mutation detection is very sensitive. For gDNA of cells cotransfected with 200 ng of CCR5-TALEN expression plasmids mutation rates of 15.0% were observed (Figure 5). When cells were cotransfected with 400 ng of CCR5-TALEN expression plasmids, the mutation rate increased to 20.9% (Figure 5). Mutation rates induced by cotransfection of 200 ng of the CCR5-ZFN expression plasmids induced mutations rates of 17.3% whereas after cotransfection of 400 ng of the CCR5-ZFN expression plasmids the mutation rate decreased to 14.3% (Figure 5). In contrast to the results obtained using the T7E1 assay cotransfection with DMD specific TALEN expression plasmids resulted in higher mutation rates compared with the CCR5 specific ZFN- or TALEN pairs. Treatment with 200 ng of each DMD specific TALEN expression plasmid resulted in 29.6% mutation rate. However, cotransfection of 400 ng of the TALEN expressing plasmid resulted in decreased mutation rates of 22.9% (Figure 5). As in the T7E1 after transfection of the plasmid coexpressing the AAVS1-ZFN pair, the highest mutation rates were observed. Transfection of 200 ng of plasmid resulted in a mutation rate of 30.8% and after transfection of 400 ng a mutation rate of 27.3% was measured (Figure 5). When comparing the effectiveness of the different designer nucleases used in this study, the q-PCR results differed from the results of the HD based assays, as CCR5-TALENs and ZFN showed lowest mutation rates. But again AAVS1-ZFN showed the highest mutation rates. Comparing the results obtained using gDNA from cells transfected with different amounts of nuclease expression plasmids, a dose effect can also be observed that is different from the results of the T7E1 assay in Figure 2. For CCR5-TALEN, DMD-TALEN, and AAVS1-ZFN treatment with 400 ng



**Figure 3** Establishment and testing of a quantitative PCR (q-PCR) approach to quantify designer nuclease activity. (a) Sequence overview of the wildtype (CCR5WT) and mutated (CCR5mut) human CCR5 locus used to establish q-PCR based mutation detection. Transcription activator-like effector nuclease (TALEN) binding sites are depicted as blue boxes. The red box indicates nucleotides that were deleted in CCR5mut. The binding site of the wildtype (WT) specific mutation detection primer is depicted as blue arrow. (b) Detection limit of q-PCR-based quantification of mutated DNA in defined samples. Decrease of q-PCR performance in relation to increasing ratios of mutated to WT DNA in defined mixtures. Relative fluorescence units (RFU) are displayed. Shown is the mean of a technical triplicate.



**Figure 4** Establishment and testing of a quantitative PCR (q-PCR) approach to detect homologous recombination (HR). (a) Sequence overview of the modified (cFIXmod) and mutated (cFIXmut) canine factor IX (cFIX) locus used to establish the q-PCR based HR detection. Red boxes indicate differences in the nucleotide sequence compared. Primer binding sites and mismatches of the detection primer are indicated by blue arrows. (b) Increase of q-PCR performance in relation to increasing ratios of modified (mod) to mutated (mut) DNA in defined mixtures. Relative fluorescence units (RFU) are displayed. As shown in the mean of a technical triplicate.



**Figure 5** Quantitative PCR (q-PCR) to quantify different designer nuclease activity after transfection of nuclease expression plasmid. (a) Schematic overview designer nucleases encoded on the plasmids used in this experiment. Transcription activator-like effector nucleases (TALENs) targeting the human *DMD*- and the human *CCR5* locus with respective repeat-variable diresidues (RVD) fused to the Fok I cleavage domain were expressed under the control of the cytomegalovirus (CMV) promoter (CMV-P). Zinc finger nucleases (ZFN) against the *CCR5* locus were also expressed under the control of the CMV-P. ZFNs against the AAVS1 site were expressed from a single plasmid under the control of the CMV-P. ZFN domains were separated by a 2A peptide cleavage site. (b) q-PCR mutation detection using gDNA from HEK293 cells transfected with varying amounts (200 ng and 400 ng) of different nuclease expression plasmids. As shown in the mean  $\pm$  SEM ( $n = 3$ ).

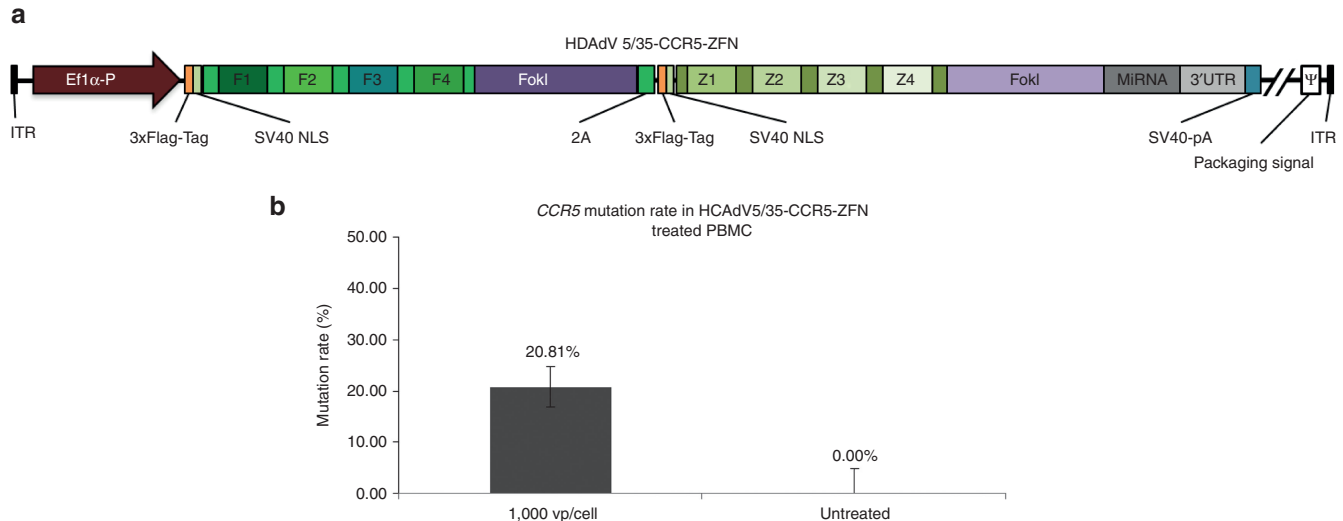
nuclease expression plasmids resulted in lower mutation rates compared with treatment with 200 ng nuclease expression plasmids. Only for the CCR5-ZFN treatment using 400 ng of the expression plasmids we measured increased mutation rates compared with treatment with 200 ng nuclease expression plasmids, respectively (Figure 5).

Designer nucleases are currently being tested in preclinical and clinical studies. To explore potential future applications for the q-PCR based mutation detection in the context of gene therapeutic

approaches, we investigated gDNA derived from CD34<sup>+</sup> human peripheral blood mononuclear cells (hPBMC). PBMCs were infected at 1,000 viral particles per cell with a chimeric high-capacity adenoviral vector HDAd5/35-CCR5-ZFN expressing the CCR5-ZFN pair under the control of the elongation factor1 alpha promoter in one construct (Figure 6a). Subsequently, we examined q-PCR performance relative to gDNA from untreated CD34<sup>+</sup> PBMCs. The q-PCR based mutation detection revealed a mutation rate of 20.8% (Figure 6b) whereas the T7E1 assay only showed an estimated mutation rate of up to 13% as previously published. First of all, this demonstrates that q-PCR based mutation detection is probably more sensitive than the T7E1 assay. Beyond that, even if not tested in the same cells these results indicate that efficient viral delivery of the nuclease expression construct increased the mutation rate of the of CCR5-ZFN when compared with mutation rates observed after plasmid cotransfection (Figures 2 and 5b).

## DISCUSSION

To detect designer nuclease induced in/del mutations, T7E1 assays or HMA<sup>12,24,25</sup> based on HD formation of mutated and unmutated DNA are widely used. HD based assays work independent of the size of in/dels and there is no need for special equipment. However, the complete procedure is work and time intensive and especially for the T7E1 assay several protocols exist. These differ in one or several parameters such as the type and amount of polymerase used for PCR, the PCR cleanup method, the cooling rate, presence and composition of hybridization buffers during HD formation, and the incubation time with the T7E1 enzyme. Hence, results of this assay are inconsistent and it may be challenging to compare results between different laboratories. It also needs to be emphasized that these critical steps can even negatively influence mutation detection as PCR amplification of the target locus may introduce further mutations that will result in unpredictable migration or cleavage in



**Figure 6** Quantitative PCR (q-PCR) to quantify *CCR5* specific zinc finger nuclease (ZFN) activity after transduction of human peripheral blood mononuclear cells (hPBMC) with high capacity adenoviral vector HDAd5/35-CCR5-ZFN. (a) Schematic overview of the genome organization of the high capacity adenoviral vector HDAd5/35-CCR5-ZFN. HDAd5/35-CCR5-ZFN encodes both *CCR5* specific ZFN domains fused to the FokI cleavage domain under the control of the elongation factor-1 alpha promoter (EF1 $\alpha$ -P). SV40, Simian virus 40; NLS, nuclear localization signal; F1-F4 and Z1-Z4, single fingers in the ZFN protein binding to the target DNA, SV40-pA, SV40 polyadenylation signal; BGH-pA, bovine growth hormone polyadenylation signal; miRNA, microRNA target site suppressing ZFN expression during viral vector production; ITR, adenoviral inverted terminal repeat. (b) q-PCR mutation quantification using gDNA from human peripheral blood mononuclear cells (hPBMC) transduced with high capacity adenoviral vector HDAd5/35-CCR5-ZFN at 1,000 viral particles per cell. As shown in the mean  $\pm$  SEM ( $n = 3$ ).

subsequent readouts. Even the use of proof-reading polymerases is not always beneficial as HD DNA may form, eventually increasing the amount of recombinant PCR products in which the potential mutation is corrected.<sup>26,27</sup> Besides recombinant PCR products allele drop-out during PCR amplification can affect sensitivity of the HD based assays limiting the estimation of mutation rates.<sup>26,27</sup> Another bias for HD based mutation detection is the random reannealing during renaturation. In addition to HD formation, homoduplexes of mutated strands can form which are then not recognized by the T7E1 enzyme. Moreover, the behavior of the T7E1 enzyme toward different tertiary DNA structures is challenging. The enzyme recognizes and cleaves HD DNA but also cruciform DNA structures, Holliday structures or junctions as well as nicked double-stranded DNA.<sup>28</sup> Moreover, the ability of T7E1 to cleave all these structures differs. For example deletions are preferred over single base substitutions.<sup>8,26</sup> Moreover we observed that homoduplex DNA can be cleaved to a certain extent if too much of the T7E1 enzyme was present, or if the incubation time and the temperature are suboptimal. As a result the background signal is relatively high resulting in the appearance of a smear which has been reported before.<sup>29,30</sup>

For the HMA assay we observed several retarded bands in analyzed samples from treated cells that are not always consistent with the band observed using the respective positive control. Therefore, it may be difficult to interpret which band may represent specific HDs (Figure 2d,e,f). Thus in concordance to the T7E1 assay, HD based assays are insufficient to quantify the percentage of mutated alleles. However, both assays will still retain their eligibility to detect in/del mutations as proof of principle method, but it remains to be emphasized that these methods are not useful for quantification of nuclease activity.

As shown in Figures 3b and 4b, the new q-PCR based mutation detection assay specifically detects designer nuclease induced in/del mutations and also HR events with high sensitivity and accuracy. In contrast to other commonly used assays it allows quantitative measurements avoiding biases such as mutation induction

through PCR or mutation correction by formation of recombinant PCR products, as well as misleading results resulting from unspecific tertiary structure formation and unspecific cleavage. Compared with conventional assays, the PCR approach is time saving and many samples can be analyzed in parallel. Nevertheless it has to be mentioned that primers binding to the expected mutation site have to be designed with respect to the predicted cutting site of the respective nuclease used to induce the DNA-DSB. It may be necessary to design several different primers and to test them carefully for their specificity/discrimination properties. Noteworthy for each primer pair the PCR setup including annealing temperature and the effect of additives such as Mg<sup>+</sup> or dimethyl sulfoxide (DMSO) needs to be optimized rendering the primer sequence specific to WT sequences. As it is possible that a small part of mutations that can be induced by nonhomologous end joining of designer nuclease induced DSB may be located outside of the primer binding site leaving the primer binding site used to discriminate mutated and WT sequences unchanged, these changes will remain undetected by our assay. But as the majority of mutations are induced at the position of the DSB that will affect the primer binding site, the small percentage of mutations that may be induced outside of the primer binding site may only lead to a minor underestimation of the true mutation rate that can be quantified by this assay. Moreover, for establishing a q-PCR approach it is crucial to use equal amounts of gDNA from treated and untreated cells. gDNA should be adjusted to the same concentration before conducting a q-PCR and final values should be normalized with the true amount of gDNA in each sample measured by a q-PCR amplifying a housekeeping gene such as human beta 2-microglobulin (hB2M) or other normalization PCRs with identical efficiencies in every sample.

For a direct comparison of the T7E1 assay and the q-PCR approach we quantified bands obtained after performing a T7E1 assay (Figure 2) and directly compared them with respective q-PCR results (Figure 5). We found that for different target loci cleavage efficiencies were lower when measured by T7E1 assay if directly compared

with the q-PCR suggesting that the q-PCR approach may be more sensitive.

The q-PCR based mutation detection represents a precise tool to compare the effectivity of different nucleases such as ZFN, TALEN, and clustered regularly interspaced short palindromic repeats/Cas that are directed against the same genomic locus.<sup>18,31,32</sup> As shown in Figures 2 and 5 the mutation rates induced by the respective nucleases differed dependent on which kind of nuclease was used. This may be especially interesting when effectiveness of different kinds of designer nuclease targeting the same genomic locus need to be compared, as shown here for ZFN-and TALEN pairs targeting the human *CCR5* gene. Furthermore, the amount and the way of delivering a nuclease expression construct can influence nuclease performance, as transfection of a single plasmid coexpressing the complete hAAVS1 ZFN pair induced higher mutation rates than cotransfection of two plasmids each carrying one half of a ZFN pair specific for *CCR5*. As displayed in Figures 2 and 5b for some nucleases tested here we observed an inverse dose-response effect for the q-PCR assays. Except for the *CCR5*-TALEN pair nuclease activity was less robust in samples which received the higher amount of nuclease encoding plasmid. Potentially this could be interpreted as some kind of saturation effect and that nuclease efficiency is decreased if accumulation of the respective nuclease occurs in transduced cells. This shows how important it is to optimize experimental setups for each nuclease used for a respective locus. Therefore, a sensitive quantification tool such as q-PCR based detection of nuclease induced in/del mutations is very useful to compare different approaches or to find the optimal conditions to achieve the best results for each approach.

In this study, we evaluated mutation rates of a ZFN pair targeting the *CCR5* locus which was delivered by an adenoviral vector into PBMCs. Also, for *in vivo* studies using designer nucleases it may be necessary to compare different delivery approaches such as high pressure tail vein injection of nuclease expression plasmids<sup>33</sup> or viral delivery.<sup>34,35</sup> The new quantitative q-PCR assay may also help to define the optimal therapeutic window to achieve high effectivity and low off target effect. Moreover, also for detection of HR events the q-PCR based detection method is feasible. It provides a valuable tool to detect and quantify the results of HR experiments using HR cassettes containing only the therapeutic sequence. Note that we are also using the q-PCR system to quantify clustered regularly interspaced short palindromic repeats/Cas9 induced mutations (not shown). This suggests that the q-PCR-based technique is independent of the nuclease used to induce the respective DSB. It measures the decrease of specific primer binding in relation to an untreated sample. The applied designer nuclease for induction of the respective mutation has no influence on the assay as it is not present in the purified gDNA isolated from nuclease treated cells or organs.

Besides the assays explored in this study, subcloning of PCR products that cover the respective nuclease binding site and subsequent sequencing of single clones is widely used. Although this technology is rather time and work intensive as many clones have to be analyzed, this method provides insights into the molecular basis of introduced in/dels. Next generation sequencing can also be used to quantify in/del mutations.<sup>36-38</sup> However, not every laboratory has access to this technology and expertise to analyze these data is mandatory. Apart from that the costs are still relatively high. Furthermore, it can be problematic to detect single nucleotide exchanges or long insertions using next generation sequencing.<sup>39</sup> Another recently published method, the lacZ recovery/disruption assay,<sup>40</sup> also provides a precise way to determine mutation rates

induced by designer nucleases. However, also this assay needs to be carefully designed and it is rather work intensive.

In conclusion, compared with conventional HD based mutation detection assays like HMA and T7E1 assays, the new q-PCR mutation detection method provides quantitative data on the mutation rates or HR events induced by designer nuclease treatment at the respective target sequence.

## MATERIALS AND METHODS

### Plasmids and designer nucleases used in this study

TALEN expression plasmids pcTn3-FokKKRS and pcDNA3\_1-Tn8-FokELDS expressing TALENs specific for human *DMD* gene were kindly provided by Charles Gersbach (Duke University, Durham, NC, USA) and TALEN expression plasmids pAC-CMV-TALE-RM1 and pAC-CMV-TALE-RM2 expressing TALENs specific for human *CCR5* were kindly provided by Toni Cathomen (University Freiburg, Germany). Plasmid pCMV-FlagAAVSIED-T2A-FlagAAVSIKKR coexpressing a pair of ZFN specific for the human AAVS1 locus as well as pVax1-*CCR5*-ZFN-L and pVax1-*CCR5*-ZFN-R expressing ZFN specific for the human *CCR5* gene were kindly provided by Jacob Giehm Mikkelsen (University Aarhus, Denmark). Sequences of respective nuclease binding sites and the spacer sequence between nuclease binding sites are listed in Table 1.

### Tissue culture, transfection of cells, and gDNA isolation

HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% Fetal Bovine Serum (Pan Biotech, Aidenbach, Germany). One day before transfection HEK293 cells were seeded into 24-well plates. Nuclease encoding plasmids and an EGFP expressing plasmid to normalize for transfection efficiency were cotransfected using FuGENE 6 transfection reagent (Promega, Madison, Michigan) applying a FuGENE6 to DNA ratio of 1:3. Forty eight hours post-transfection cells were harvested and gDNA isolated using the blood and tissue kit (Qiagen, Hilden, Germany). gDNA was used as starting material for PCR reactions followed by HMA- or T7E1 assay. Cultivation of CD34<sup>+</sup> hPBMC, *CCR5*-ZFN expressing HDAdV5/35 vector production and infection of PBMCs with HDAdV5/35 was described elsewhere.<sup>21</sup>

### PCR amplifying designer nuclease binding sites

A 450bp DNA fragment covering exon 51 of the human *DMD* gene, the human *CCR5* locus (291 bp) as well as the human AAVS1 locus (468bp) were amplified from 100ng gDNA from cells transfected or cotransfected with the respective designer nuclease expression plasmids. The PCR was performed in a total volume of 25  $\mu$ l using 12.5  $\mu$ l 2 $\times$  One Taq Mastermix (NEB, Ipswich, Massachusetts) and 0.3  $\mu$ mol/l of each primer. The PCR reaction was carried out with an initial denaturation step of 95°C for 4 minutes followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55 or 60°C respectively for 30 seconds, and elongation at 72°C for 30 seconds. A final elongation step was conducted at 72°C for 3 minutes. Primers used to amplify the loci covering the respective nuclease binding sites are listed in Table 1.

### Mutation detection using T7E1-assay and gel retardation assay

After electrophoresis using a 2% agarose gel, PCR products were gel purified using the Wizard SV Gel- and PCR Clean-Up System (Promega). For HD formation followed by gel retardation assay 8.5  $\mu$ l of purified PCR products were heated to 95°C for 10 minutes and cooled down to 4°C with a cooling rate of 0.1°C/sec using a thermocycler. For the gel retardation assay DNA was loaded directly onto a 6% sodium dodecyl sulfate- polyacrylamide gel electrophoresis gel using 0.5  $\times$  Tris-Borate-EDTA buffer, run at 200V for 45 minutes and visualized using gel documentation (Bio-Rad Laboratories Hercules, California). For the T7E1 assay 8.5  $\mu$ l of purified PCR products were supplemented with 1  $\mu$ l NEB2 buffer, then heated to 95°C for 10 minutes and cooled down to 4°C with a cooling rate of 0.1°C/ seconds using a thermocycler. Subsequently 0.5  $\mu$ l T7E1 enzyme (NEB) was added and the restriction enzyme digest was incubated at 37°C for 30 minutes and subsequently separated on a 2% agarose gel. Mutation rates were measured by relating the band strength of the specific cleavage products relative to the band of uncleaved PCR product using the formula % gene modification =  $100 \times (1 - (1 - \text{fraction cleaved})^{1/2})$ .<sup>41</sup>

As positive controls for HD assays PCR products amplified from gDNA of untransfected cells were mixed with equal amounts of PCR products amplified from plasmids containing the respective locus with defined deletions

**Table 1** Binding sites and respective spacers of designer nucleases used in this study, primer sequences used to amplify the target loci, and primer sequences used for q-PCR based mutation detection

| Binding sites                              | Sequences |                           |    |
|--|-----------|---------------------------|----|
| <b>CCR5-TALEN</b>                          |           |                           |    |
| RM1 5' TALEN binding site                  | 5'        | TGTGGGCAACATGCTGGTC       | 3' |
| RM2 3' TALEN binding site                  | 5'        | AACTGCAAAAGGCTGAAGA       | 3' |
| Spacer                                     | 5'        | ATCCTCATCTGATA            | 3' |
| CCR5 locus fwd (T7E1 and gel retardation)  | 5'        | AGATGGATTATCAAGTGCAAGTCC  | 3' |
| CCR5 locus rev (T7E1 and gel retardation)  | 5'        | CAAAGTCCCCTGGGCG          | 3' |
| CCR5-TALEN fwd (q-PCR mutation detection)  | 5'        | TGGTCATCTCATCTGAT         | 3' |
| CCR5-TALEN rev (q-PCR mutation detection)  | 5'        | AGATCCAGAGAAGAAGCCTA      | 3' |
| <b>CCR5_ZFN</b>                            |           |                           |    |
| ZFN 5' ZFN binding site                    | 5'        | GTCATCTCATC               | 3' |
| ZFN 3' ZFN binding site                    | 5'        | AAACTGCAAAAG              | 3' |
| Spacer                                     | 5'        | CTGAT                     | 3' |
| CCR5 locus fwd (T7E1 and gel retardation)  | 5'        | AGATGGATTATCAAGTGCAAGTCC  | 3' |
| CCR5 locus rev (T7E1 and gel retardation)  | 5'        | CAAAGTCCCCTGGGCG          | 3' |
| CCR5 ZFN fwd (q-PCR mutation detection)    | 5'        | GGGTGGAACAAGATGGAT        | 3' |
| CCR5 ZFN rev (q-PCR mutation detection)    | 5'        | CAGCCTTTGCAGTTATCAG       | 3' |
| <b>DMD-TALEN</b>                           |           |                           |    |
| TN3-5' TALEN binding site                  | 5'        | AGCTCCTACTCAGACT          | 3' |
| TN8-3' TALEN binding site                  | 5'        | ACCTGTGGTTACTAAGG         | 3' |
| Spacer                                     | 5'        | GTTACTCTGGTGACACA         | 3' |
| DMD locus fwd (T7E1 and gel retardation)   | 5'        | GAGTTTGGCTCAAATGTTACTCTT  | 3' |
| DMD locus rev (T7E1 and gel retardation)   | 5'        | AAATGGTCTAGGAGAGTAAAGT    | 3' |
| Dyst fwd (q-PCR mutation detection)        | 5'        | AGACTGTTACTCTGGTGACACAACC | 3' |
| DMD rev (q-PCR mutation detection)         | 5'        | TCAAGCAGAGAAAGCCAGTCG     | 3' |
| <b>AAVS1-ZFN</b>                           |           |                           |    |
| ZFN 5' ZFN binding site                    | 5'        | ACCCACAGTGG               | 3' |
| ZFN 3' ZFN binding site                    | 5'        | TAGGGACAGGAT              | 3' |
| Spacer                                     | 5'        | GGCCAC                    | 3' |
| AAVS1 locus fwd (T7E1 and gel retardation) | 5'        | TTCGGGTCACCTCTCACTCC      | 3' |
| AAVS1 locus rev (T7E1 and gel retardation) | 5'        | GGCTCCATCGTAAGCAA         | 3' |
| AAVS1 fwd (q-PCR mutation detection)       | 5'        | ACAGTGGGGCCACTAGGG        | 3' |
| AAVS1 rev (q-PCR mutation detection)       | 5'        | GATGGCTCCAGGAAATGGGG      | 3' |

fwd, forward; rev, reverse; ZFNs, zinc finger nucleases; q-PCR, quantitative PCR; TALEN, transcription activator-like effector nuclease; T7E1, T7-endonuclease I.

at the nuclease binding site. For the *CCR5* locus a 450 bp fragment having a 8 bp deletion at the spacer between binding sites of the *CCR5* specific TALEN was synthesized and cloned into the Pex-A plasmid (Eurofins, Ebersberg, Germany). For the hAAVS1 locus a PCR product amplified from gDNA of ZFN treated cells having a 2 bp deletion between ZFN binding sites was cloned into the pGEM-Teasy plasmid (Promega).

#### Quantification of gDNA and mutation detection by q-PCR

Mutation detection q-PCR was established and quantification potential as well as the detection limit was tested using the plasmid pGEM-teasy-CCR5WT containing a 450 bp fragment of the wild type *CCR5* locus surrounding the respective CCR5-TALEN binding site. A second plasmid pGEM-teasy-CCR5mut contained the same locus contained but with an 8 bp deletion at the expected CCR5-TALEN-cleavage site (Figure 3a). WT and mutated plasmids containing 0, 2.5, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% of the mutated target loci were generated. Each mixture was adjusted to an equal DNA concentration of 1.5 ng/μl and subjected to q-PCR as described below. The resulting q-PCR signal for each mixture was depicted relative to the

maximum q-PCR signal obtained using the mixture that only contained WT plasmid. To determine the amount of gDNA isolated from cells, a q-PCR amplifying a 79 bp fragment of the hB2M gene was amplified (see Supplementary Figure S3) using primers forward (5'-GGA ATTGATTTGGGAGAGCATC-3') and reverse (5'-CAGGTCCTGGCTCTACAATTTACTAA-3') and a hB2M specific HEX labeled probe (5'-AGTGTGACTGGGAGATCATCCACCTTC-3').<sup>42</sup> q-PCR was performed in a total volume of 10 μl using 5 μl SSo Advanced Probe Supermix (Bio-Rad Laboratories), 150 nmol/l of each primer, 200 nmol/l of probe and 1 μl gDNA. PCR was carried out with an initial denaturation step of 95°C for 3 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and elongation at 60°C for 30 seconds. To generate a standard curve a gDNA derived from Jurkat cells (NEB) was used. According to the standard curve the amount of gDNA from the different samples was interpolated. Subsequently gDNA of the samples to be analyzed for mutation detection including negative controls were diluted to the same concentration. Following dilution, the concentration was measured by hB2M probe q-PCR again to obtain correction values to normalize q-PCR results in the subsequent mutation detection q-PCR. For mutation detection a 150 bp DNA fragment covering the target locus was amplified from equal amounts



of gDNA in a total volume of 20 µl using 10 µl iQ SYBR Green Supermix (Bio-Rad Laboratories) and 500 nmol/l of primers. q-PCR was carried out with an initial denaturation step of 95°C for 4 minutes followed by 40 cycles of denaturation at 95°C for 45 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 15 seconds. Subsequently a final elongation step at 72°C for 3 minutes was performed. Primers used for q-PCR based mutation detection are listed in Table 1. q-PCR reactions for hB2M and mutation detection using gDNA from TALEN treated cells as well as untreated control cells were performed in triplicates in a 96-well format on a Biorad CFX one touch real time PCR cyclers (Bio-Rad Laboratories). For gDNA from nuclease treated cells the mutation rate is depicted as reduction relative fluorescent units (RFU) relative to the RFU obtained from untreated/mock treated samples. Following formula can be used to calculate the respective values: %mutation rate = 100 - (RFU of treated sample / RFU of untreated sample).

Starting DNA concentrations of TALEN treated and untreated samples obtained by mutation detection q-PCR were normalized according to hB2M q-PCR results and depicted as relative reduction of starting DNA concentration (Figure 1b).

HR detection was established using plasmids pscAAV-cFIXmut carrying a 1.922 kb sequence of cFIX gene containing the nuclease binding site and a point mutation in exon 8 (mut), and pscAAV-cFIXmod where the nuclease binding site where codon optimized and the point mutation changed to WT. The target locus, containing hemophilia B mutation as well as base pair exchanges within the cFIXmod cassette and the nuclease binding sites, is illustrated in Figure 4a. Plasmids pscAAV-cFIXmut and pscAAV-cFIXmod were generated containing 0, 1, 5, 10, 50, and 100% of pscAAV-cFIXmod and adjusted to the same concentration of 0.049 ng/µl. These mixtures were subjected to q-PCR as described below. For HR detection in defined mut:mod mixtures a sequence of 204 bp was amplified using primer forward (5'-CGATCGGCTCAATTCTTCA-3') and mod specific reverse (5'-CAGGAAATAATCCCAGTCAAG-3'). To normalize all samples, a normalization PCR (outPCR) was performed using primer forward (5'-CCCCTGTAGTCTGTGGTG-3') and reverse (5'-CATGCCGTGGCAATCTTAC-3'), amplifying identical downstream target sequences present in all samples. For analysis the Pfaffl method was used to determine the ratios/percentage of mut:wt or mut:mod: ratio (percentage HR events) =  $E_{\text{detPCR}}^{\Delta C_T, \text{detPCR}(\text{calibrator-test})} / E_{\text{outPCR}}^{\Delta C_T, \text{outPCR}(\text{calibrator-test})} (E_{\text{detPCR}}^{\text{standard sample}} / E_{\text{outPCR}}^{\text{standard sample}})^{\text{test} - \text{analyzed samples}}$ ,  $\Delta C_T$  = subtractions of q-PCR  $C_T$  values for detPCR or outPCR;  $C_T$  = Cycle number). Both detPCR and outPCR were performed separately in a total volume of 10 µl using 5 µl of DNA sample, 2 µl 5x EvaGreen-qPCR-Mix-II (Bio-Budget, Krefeld, Germany) 200 nmol/l primer and 5% DMSO. q-PCR was performed with an initial denaturation step of 95°C for 15 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 20 seconds, and elongation at 72°C for 20 seconds.

### Statistical analyses

All experiments were performed using triplicates. Data were reported as mean ± standard error of mean (SEM).

### Ethics statement

Work including gDNA derived from human PBMCs were obtained from and approved by the University of Seattle, USA.

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### REFERENCES

- Gaj, T, Gersbach, CA and Barbas, CF 3rd (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol* **31**: 397–405.
- Ramalingam, S, Annaluru, N and Chandrasegaran, S (2013). A CRISPR way to engineer the human genome. *Genome Biol* **14**: 107.

- Carroll, D (2011). Genome engineering with zinc-finger nucleases. *Genet* **188**: 773–782.
- Mali, P, Esvelt, KM and Church, GM (2013). Cas9 as a versatile tool for engineering biology. *Nat Methods* **10**: 957–963.
- Bogdanove, AJ and Voytas, DF (2011). TAL effectors: customizable proteins for DNA targeting. *Science* **333**: 1843–1846.
- Kim, Y, Kweon, J, Kim, A, Chon, JK, Yoo, JY, Kim, HJ et al. (2013). A library of TAL effector nucleases spanning the human genome. *Nat Biotechnol* **31**: 251–258.
- Mladenov, E and Iliakis, G (2011). Induction and repair of DNA double strand breaks: the increasing spectrum of non-homologous end joining pathways. *Mutat Res* **711**: 61–72.
- Mashal, RD, Koontz, J and Sklar, J (1995). Detection of mutations by cleavage of DNA heteroduplexes with bacteriophage resolvases. *Nat Genet* **9**: 177–183.
- Delwart, EL, Shpaer, EG, Louwagie, J, McCutchan, FE, Grez, M, Rübsamen-Waigmann, H et al. (1993). Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 env genes. *Science* **262**: 1257–1261.
- Delwart, EL, Herring, B, Rodrigo, AG and Mullins, JI (1995). Genetic subtyping of human immunodeficiency virus using a heteroduplex mobility assay. *PCR Methods Appl* **4**: S202–S216.
- Delwart, EL, Sheppard, HW, Walker, BD, Goudsmit, J and Mullins, JI (1994). Human immunodeficiency virus type 1 evolution *in vivo* tracked by DNA heteroduplex mobility assays. *J Virol* **68**: 6672–6683.
- Ota, S, Hisano, Y, Muraki, M, Hoshijima, K, Dahlem, TJ, Grunwald, DJ et al. (2013). Efficient identification of TALEN-mediated genome modifications using heteroduplex mobility assays. *Genes Cells* **18**: 450–458.
- Newton, CR, Graham, A, Heptinstall, LE, Powell, SJ, Summers, C, Kalsheker, N et al. (1989). Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res* **17**: 2503–2516.
- Yu, C, Zhang, Y, Yao, S and Wei, Y (2014). A PCR based protocol for detecting indel mutations induced by TALENs and CRISPR/Cas9 in zebrafish. *PLoS One* **9**: e98282.
- Hockemeyer, D, Soldner, F, Beard, C, Gao, Q, Mitalipova, M, DeKelver, RC et al. (2009). Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. *Nat Biotechnol* **27**: 851–857.
- Lombardo, A, Genovese, P, Beausejour, CM, Colleoni, S, Lee, YL, Kim, KA et al. (2007). Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nat Biotechnol* **25**: 1298–1306.
- Perez, EE, Wang, J, Miller, JC, Jouvenot, Y, Kim, KA, Liu, O et al. (2008). Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. *Nat Biotechnol* **26**: 808–816.
- Ousterout, DG, Perez-Pinera, P, Thakore, PI, Kabadi, AM, Brown, MT, Qin, X et al. (2013). Reading frame correction by targeted genome editing restores dystrophin expression in cells from Duchenne muscular dystrophy patients. *Mol Ther* **21**: 1718–1726.
- Mussolino, C, Alzubi, J, Fine, EJ, Morbitzer, R, Cradick, TJ, Lahaye, T et al. (2014). TALENs facilitate targeted genome editing in human cells with high specificity and low cytotoxicity. *Nucleic Acids Res* **42**: 6762–6773.
- Mussolino, C, Morbitzer, R, Lütge, F, Dannemann, N, Lahaye, T and Cathomen, T (2011). A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. *Nucleic Acids Res* **39**: 9283–9293.
- Saydaminova, K, Ye, X, Wang, H, Richter, M, Ho, M, Chen, H et al. (2015). Efficient genome editing in hematopoietic stem cells with helper-dependent Ad5/35 vectors expressing site-specific endonucleases under microRNA regulation. *Mol Ther Methods Clin Dev* **1**: 14057.
- Zou, S (1997). A practical approach to genetic screening for influenza virus variants. *J Clin Microbiol* **35**: 2623–2627.
- Zou, S, Stansfield, C and Bridge, J (1998). Identification of new influenza B virus variants by multiplex reverse transcription-PCR and the heteroduplex mobility assay. *J Clin Microbiol* **36**: 1544–1548.
- Nerys-Junior, A, Costa, LC, Braga-Dias, LP, Oliveira, M, Rossi, AD, da Cunha, RD et al. (2014). Use of the heteroduplex mobility assay and cell sorting to select genome sequences of the CCR5 gene in HEK 293T cells edited by transcription activator-like effector nucleases. *Genet Mol Biol* **37**: 120–126.
- Ota, S, Hisano, Y, Ikawa, Y and Kawahara, A (2014). Multiple genome modifications by the CRISPR/Cas9 system in zebrafish. *Genes Cells* **19**: 555–564.
- Judo, MS, Wedel, AB and Wilson, C (1998). Stimulation and suppression of PCR-mediated recombination. *Nucleic Acids Res* **26**: 1819–1825.
- Lahr, DJ and Katz, LA (2009). Reducing the impact of PCR-mediated recombination in molecular evolution and environmental studies using a new-generation high-fidelity DNA polymerase. *Biotechniques* **47**: 857–866.
- White, MF, Giraud-Panis, MJ, Pöhler, JR and Lilley, DM (1997). Recognition and manipulation of branched DNA structure by junction-resolving enzymes. *J Mol Biol* **269**: 647–664.
- Sakurai, T, Watanabe, S, Kamiyoshi, A, Sato, M and Shindo, T (2014). A single blastocyst assay optimized for detecting CRISPR/Cas9 system-induced indel mutations in mice. *BMC Biotechnol* **14**: 69.
- Vouillot, L, Thélie, A and Pollet, N (2015). Comparison of T7E1 and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. *G3 (Bethesda)* **5**: 407–415.

31. Ousterout, DG, Kabadi, AM, Thakore, PI, Majoros, WH, Reddy, TE and Gersbach, CA (2015). Multiplex CRISPR/Cas9-based genome editing for correction of dystrophin mutations that cause Duchenne muscular dystrophy. *Nat Commun* **6**: 6244.
32. Ousterout, DG, Kabadi, AM, Thakore, PI, Perez-Pinera, P, Brown, MT, Majoros, WH *et al.* (2015). Correction of dystrophin expression in cells from Duchenne muscular dystrophy patients through genomic excision of exon 51 by zinc finger nucleases. *Mol Ther* **23**: 523–532.
33. Bloom, K, Ely, A, Mussolino, C, Cathomen, T and Arbuthnot, P (2013). Inactivation of hepatitis B virus replication in cultured cells and *in vivo* with engineered transcription activator-like effector nucleases. *Mol Ther* **21**: 1889–1897.
34. Weber, ND, Stone, D, Sedlak, RH, De Silva Felixge, HS, Roychoudhury, P, Schiffer, JT *et al.* (2014). AAV-mediated delivery of zinc finger nucleases targeting hepatitis B virus inhibits active replication. *PLoS One* **9**: e97579.
35. Li, H, Haurigot, V, Doyon, Y, Li, T, Wong, SY, Bhagwat, AS *et al.* (2011). *In vivo* genome editing restores haemostasis in a mouse model of haemophilia. *Nat* **475**: 217–221.
36. Chiu, H, Schwartz, HT, Antoshechkin, I and Sternberg, PW (2013). Transgene-free genome editing in *Caenorhabditis elegans* using CRISPR-Cas. *Genet* **195**: 1167–1171.
37. Cho, SW, Kim, S, Kim, Y, Kweon, J, Kim, HS, Bae, S *et al.* (2014). Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res* **24**: 132–141.
38. Hsu, PD, Scott, DA, Weinstein, JA, Ran, FA, Konermann, S, Agarwala, V *et al.* (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol* **31**: 827–832.
39. Cradick, TJ, Fine, EJ, Antico, CJ and Bao, G (2013). CRISPR/Cas9 systems targeting  $\beta$ -globin and CCR5 genes have substantial off-target activity. *Nucleic Acids Res* **41**: 9584–9592.
40. Hisano, Y, Ota, S, Arakawa, K, Muraki, M, Kono, N, Oshita, K *et al.* (2013). Quantitative assay for TALEN activity at endogenous genomic loci. *Biol Open* **2**: 363–367.
41. Guschin, DY, Waite, AJ, Katibah, GE, Miller, JC, Holmes, MC and Rebar, EJ (2010). A rapid and general assay for monitoring endogenous gene modification. *Methods Mol Biol* **649**: 247–256.
42. Goff, LK, Neat, MJ, Crawley, CR, Jones, L, Jones, E, Lister, TA *et al.* (2000). The use of real-time quantitative polymerase chain reaction and comparative genomic hybridization to identify amplification of the REL gene in follicular lymphoma. *Br J Haematol* **111**: 618–625.



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