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-assy 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.1–5 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.1 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.6 In each allele the mutations can be different. Insertions or deletions can vary in size from one nucleotide up to several dozens of nucleotides.7 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) assay4 or the heteroduplex mobility assay (HMA)9 can be applied. They take advantage of the fact, that gDNA isolated from designer nuclease treated cells is a heterogenous mixture of mutated and unmutated alleles which hasper detection and quantification of in/del mutations.

This work was done in Witten, Germany.

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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. 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. 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 (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) 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. 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 or the human CCR5 gene as well as TALEN pairs specific for the human DMD gene and the human CCR5 gene. Furthermore, gDNA derived from patient derived CD34+ 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. One primer directly binds to the expected site of mutation. 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 pair21 were examined.

RESULTS
Evaluation of T7E1- and HMA for mutation detection
To detect in/del mutations T7E1 assays or the HMA21,23 based on HD formation of mutated and unmaturated 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 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 AAV51-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 nuclelease 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 8bp deletion at the expected CCR5-TALEN-cleavage site (Figure 3a). WT and mutated plasmids mixes were generated containing 0, 2.5, 5, 10, 20, 30, 40, 50, 60,
70, 80, 90, and 100% of the mutated target loci. Each mixture was adjusted to an equal DNA concentration of 1.5 ng/μl 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 mixtures. 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 pscAA-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. Plasmid 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/μl. 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) amplified containing 0, 5, 10, 50, and 100% of the modified target loci. For cFIXmut/cFIXmod the concentration of each mixture was 0.049 ng/μl. Here the mixture containing 0% (cFIXmod) represented 0% HR frequency and the mixture containing 100% (cFIXmod) simulated 100% HR frequency.

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 expression plasmid resulted in decreased mutation rates of 22.9% (Figure 5). As in the T7E1 assay 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

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**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.
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.

Mutation rates after transfection of nuclelease expression plasmids

- CCR5-TALEN
- CCR5-ZFN
- AAVS1-2xZFN
- DMD-TALEN

Untransfected
Transfected with 200 ng
Transfected with 400 ng

- 15.0%
- 20.9%
- 17.3%
- 14.3%
- 29.6%
- 22.9%
- 30.8%
- 27.3%

Figure 4

Figure 5

Quantitative PCR (q-PCR) to quantify different designer nucleases activity after transfection of nuclease expression plasmids. (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 ± 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+ human peripheral blood mononuclear cells (PBMCs). 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 factor 1a promoter in one construct (Figure 6a). Subsequently, we examined q-PCR performance relative to gDNA from untreated CD34+ 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 HMA224,25 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
subsequent readouts. Even the use of proof-reading polymerases is not always beneficial as HD DNA may form, eventually increasing the amount of recombined PCR products in which the potential mutation is corrected.26,27 Besides recombined PCR products allele drop-out during PCR amplification can affect sensitivity of the HD based assays limiting the estimation of mutation rates.26,27 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.28 Moreover, the ability of T7E1 to cleave all these structures differs. For example deletions are preferred over single base substitutions.26,28 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.29,30

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⁺ 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 effectiveness of different nucleases such as ZFN, TALEN, and clustered regularly interspaced short palindromic repeats/Cas that are directed against the same genomic locus.\textsuperscript{18,31,32} 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, in vivo studies using designer nucleases it may be necessary to compare different delivery approaches such as high pressure tail vain injection of nuclease expression plasmids\textsuperscript{33} or viral delivery.\textsuperscript{34,35} 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.\textsuperscript{36-38} 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. Another recently published method, the lacZ recovery/disruption assay,\textsuperscript{39} 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 pCT3-FokKKRR and pCDNA3-1-TnB-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-FlagAAVS1DEL-T2A-FlagAAVS1KR coexpressing a pair of ZFN specific for the human AAVS1 locus as well as pVax1-CCRS-ZFN-L and pVax1-CCRS-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 FuGENE6 transfection reagent (Promega, Madison, Michigan) applying a FuGENE6 to DNA ratio of 1:3. Fifty 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+ hPBMC, CCR5-ZFN expressing HDAdV5/35 vector production and infection of PBMCs with HDAdV5/35 was described elsewhere.\textsuperscript{37}

PCR amplifying designer nuclease binding sites

A 450 bp DNA fragment covering exon 51 of the human DMD gene, the human CCR5 locus (291 bp) as well as the human AAVS1 locus (468 bp) were amplified from 100 ng gDNA from cells transfected or cotransfected with the respective designer nuclease expression plasmids. The PCR was performed in a total volume of 25 µl using 12.5 µl 2x One Taq Mastermix (NEB, Ipswich, Massachusetts) and 0.3 µmol/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 were used to amplify the loci covering the respective nuclease binding sites as 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 µl 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 thermodrayer. For the gel retardation assay DNA was loaded directly onto a 6% sodium dodecyl sulfate- polyacrylamide gel electrophoresis gel using 0.5 x Tris-Borate-EDTA buffer, run at 200 V for 45 minutes and visualized using gel documentation (Bio-Rad Laboratories Hercules, California). For the T7E1 assay 8.5 µl of purified PCR products were supplemented with 1 µl NEB2 buffer, then heated to 95°C for 10 minutes and cooled down to 4°C with a cooling rate of 0.11°C/sec using a thermodrayer. Subsequently 0.5 µl T7E1 enzyme (NEB) was added and the restriction enzyme digestion 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 uncut PCR product using the formula % gene modification = 100 × (1 – (I – Ic) / Id).\textsuperscript{39}

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                  |
|---------------------|----------------------------|
| CCR5-TALEN          |                            |
| RM1 5’ TALEN binding site | 5’ TGTGGGCAACATGCCTGGTC    | 3’ |
| RM2 3’ TALEN binding site | 5’ AACTGCAAAGGCTGAAGA     | 3’ |
| Spacer              | 5’ ATCTCCATCCCAGTATA       | 3’ |
| CCR5 locus fwd (T7E1 and gel retardation) | 5’ AGATGGATTAATCAAGTGAAGTCC | 3’ |
| CCR5 locus rev (T7E1 and gel retardation) | 5’ CAAGGTCCCATGGGCGG       | 3’ |
| CCR5-TALEN fwd (q-PCR mutation detection) | 5’ TGTCATCCCTCATCTGGAT    | 3’ |
| CCR5-TALEN rev (q-PCR mutation detection) | 5’ AGATCCAGAGAAGAAGCTA    | 3’ |
| CCR5_ZFN            |                            |
| ZFN 5’ ZFN binding site | 5’ GTCATCCCATC              | 3’ |
| ZFN 3’ ZFN binding site | 5’ AAACGTCAAAAG             | 3’ |
| Spacer              | 5’ CTGAT                   | 3’ |
| CCR5 locus fwd (T7E1 and gel retardation) | 5’ AGATGGATTAATCAAGTGAAGTCC | 3’ |
| CCR5 locus rev (T7E1 and gel retardation) | 5’ CAAGGTCCCATGGGCGG       | 3’ |
| CCR5 ZFN fwd (q-PCR mutation detection) | 5’ GGTTGAAACAGATGGAT      | 3’ |
| CCR5 ZFN rev (q-PCR mutation detection) | 5’ CAGCTTTTCAGGGTTATCAG   | 3’ |
| DMD-TALEN           |                            |
| TN3 5’ TALEN binding site | 5’ AGGCTCTACTCAAGACT       | 3’ |
| TN8 3’ TALEN binding site | 5’ ACCCTGTGTACTAAGG        | 3’ |
| Spacer              | 5’ GTTACCTGGTGACACA        | 3’ |
| DMD locus fwd (T7E1 and gel retardation) | 5’ GAGTTTGGCTCAAAATGGTTACTCTT | 3’ |
| DMD locus rev (T7E1 and gel retardation) | 5’ AAATGGTCTAGGAGAATGAGT   | 3’ |
| Dyst fwd (q-PCR mutation detection) | 5’ AGACGTGAATGCAACACACC  | 3’ |
| DMD rev (q-PCR mutation detection) | 5’ TCAAGCAGAAGAAGCAGTCG   | 3’ |
| AAVS1-ZFN           |                            |
| ZFN 5’ ZFN binding site | 5’ ACCCCCAAGTGG            | 3’ |
| ZFN 3’ ZFN binding site | 5’ TAGGGACAGGAT            | 3’ |
| Spacer              | 5’ GCCCAC                   | 3’ |
| AAVS1 locus fwd (T7E1 and gel retardation) | 5’ TCCGAGTCACCTCTCACTCC   | 3’ |
| AAVS1 locus rev (T7E1 and gel retardation) | 5’ GGCTCCATCGTAAGCAA     | 3’ |
| AAVS1 fwd (q-PCR mutation detection) | 5’ ACAGTGCGGCGCCACTAGGG   | 3’ |
| AAVS1 rev (q-PCR mutation detection) | 5’ GATTGCATCCAGGAAATGGGG | 3’ |

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

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 PexA 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’-CGTCTCTGGCCTACGAATTCTAA-3’) and reverse (5’-CACCTGTGGTTACTAAGG-3’) and a hB2M specific HEX labeled probe (5’-AGATTCCAGAGAAGAAGCCTA-3’). 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 cycler (Bio-Rad Laboratories). For gDNA from nucleated 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: Δmutation 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 fragment containing intron 2 of cFIX and the nuclease binding site and a point mutation in exon II (mut), and pscAAV-cFIXmod where the nuclease binding site 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, 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 above. For HR detection in defined mut/mod mixtures a sequence of 204 bp was amplified using primer forward (5′-CATGGCTGCTATCCTCTA-3′) and reverse (5′-CAGGAAATAATCCCAGTCAAG-3′) primers and annealing at 60°C for 30 seconds. The PCR reactions were performed in triplicates in a 96-well format on a Biorad CFX one touch real time PCR cycler (Bio-Rad Laboratories). For gDNA from 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: Δmutation rate = 100 – (RFU of treated sample / RFU of untreated sample).

Statistical analyses

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

Ethics statement

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

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