Application Note

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Use of an Engineered Restriction Site to Estimate CRISPR Homology-Directed Repair Efficiency

Authors

Kyle Luttgeharm,
Chava Pocernich, and
Kit-Sum Wong
Agilent Technologies, Inc.

Abstract

The Agilent 5200 Fragment Analyzer system coupled with the Agilent CRISPR Discovery Gel kit provides high-throughput analysis for detection of CRISPR-induced homology-directed repair (HDR) knock-in of novel restriction enzyme sites. The presented method enables determination of the approximate frequency of HDR repairs in both pooled and individual cell line systems when using CRISPR gene editing.

Introduction

CRISPR gene editing is rapidly becoming the preferred method for gene knockout studies. CRISPR gene editing uses the Cas9 endonuclease combined with a guide RNA to cleave a targeted genomic sequence and induce a double-stranded break[1,2]. After cleavage, the cut DNA is repaired either by the nonhomologous end joining (NHEJ) pathway or the homology-directed repair (HDR) pathway. More commonly used, but error prone, the NHEJ pathway randomly inserts or deletes DNA bases (InDels) to repair the double-stranded break[3]. These modifications can result in a loss of gene function by introducing a frameshift mutation or a premature stop codon[4]. The most common InDels are ±1 or 2 base pairs (bp), but larger InDels can occur[5,6]. In contrast to the error prone NEHJ pathway, the HDR pathway uses a donor sequence containing flanking homologous ends separated by a novel DNA sequence. During repair of the double-stranded break, the cell recognizes the homologous sequences and inserts the donor template, containing the novel sequence, at the site of the double-stranded break.

To expedite the creation of knockout lines, many are using HDR repair to knock-in novel restriction enzyme sites allowing for simultaneous disruption of the open reading frame and rapid identification/quantification of edited lines. Quantification of HDR events can be accomplished by amplification of the edited locus followed by restriction enzyme treatment. Since only amplicons containing the HDR event will be cleaved, the percent cleaved should equal the percent HDR. Restriction digest samples were analyzed to determine the actual percent cleaved with the Agilent CRISPR Discovery Gel kit on the Agilent 5200 Fragment Analyzer system.

Experimental

Amplification and restriction digests

Synthetic genes representing a wildtype (WT) gene and a variety of NHEJ events (±1, ±2, ±10, and all possible SNP) were synthesized with a gene containing a BamHI restriction enzyme site (pHDR), representing an HDR event. The plasmids were mixed at various percentages to model various percent HDR modification with and without NHEJ events. A 410 bp fragment was amplified for 30 PCR cycles or 15 PCR cycles using DreamTaq (Thermo Fisher Scientific, #EP0701) according to the manufacturer's instructions. After 30 PCR cycles, 200 ng of unpurified PCR product was digested with 1 µL Fast Digest BamHI (Thermo Fisher Scientific, #FD0054) in a 20 µL reaction and incubated at 37 °C for 15 min. After a limited amplification with 15 PCR cycles, the PCR product was concentrated using the Monarch PCR and DNA Cleanup Kit (New England Biolabs, #T1030) and eluted in 10 µL NEB elution buffer. Following purification, BamHI digests were completed as described previously.

Restriction digest analysis

All digested samples were analyzed using the CRISPR Discovery Gel kit (p/n DNF-910-K1000CP) on the 5200 Fragment Analyzer system by diluting 2 µL of sample in 100 µL 0.1X TE. The percent cleaved was calculated using the CRISPR main plugin for the Agilent ProSize data analysis software. The CRISPR main plugin calculates the percent cleaved using Equation 1.

Agarose gel electrophoresis was performed using a 2 % agarose gel loaded with about 200 ng DNA. The agarose gel was post stained with 0.5 µg/mL ethidium bromide for 30 minutes followed by visualization with a 300 nm UV transilluminator.

$$\% \text{ Cleaved} = \frac{\frac{\text{nmol Digested Fragment 1} + \text{nmol Digested Fragment 2}}{2}}{\frac{\text{nmol Digested Fragment 1} + \text{nmol Digested Fragment 2}}{2} + \text{nmol Undigested Fragment}}$$

Equation 1.

Results and Discussion

While techniques have been developed to increase the frequency of HDR integration, NHEJ repair remains a more efficient repair pathway, resulting in a mixture of HDR integrations and NHEJ repair events. To model this, the WT and NHEJ genes were mixed 1:1 (m/m). These gene mixtures were then mixed with pHDR so that the final solution contained 0, 6, 11, 25, and 40 % (m/m) pHDR, followed by amplified for 30 PCR cycles. Digestion of the amplicons with BamHI resulted in the following percent cleaved: 0, 0.7, 1.8, 7.0, and 24.9 % respectively, well under the theoretical values (Figure 1). Further examination of the electropherogram revealed the presence of heteroduplexed DNA produced by mixed template PCR. Thus, resulting in the inaccessibility of the BamHI restriction site and indigestible HDR events (Figure 2). These data showed that the percent cleaved produced by restriction digest was less than the actual number of HDR events present in a cell pool due to the presence of indigestible heteroduplexed DNA.

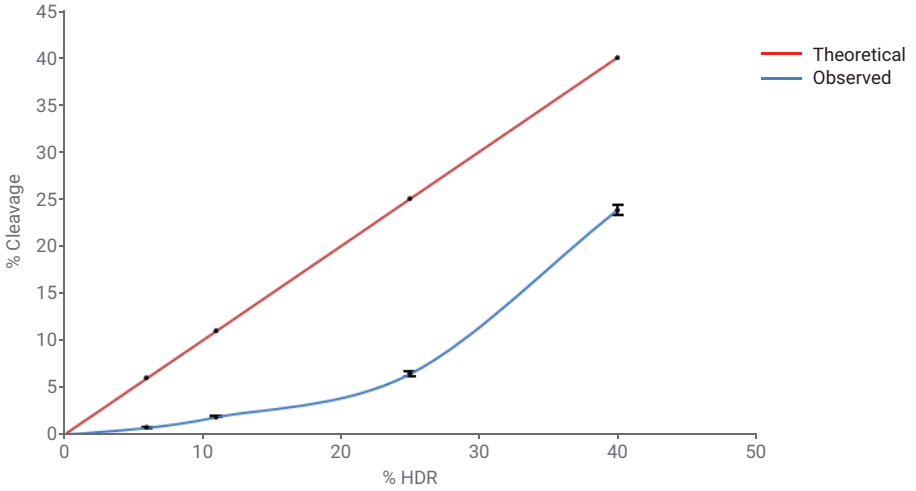

Figure 1. Observed levels of percent HDR compared to the theoretical value from BamHI digested mixed template PCR of various %pHDR. The observed percent HDR was significantly less than the theoretical level of HDR presumably due to large amounts of indigestible heteroduplexes formed during mixed template PCR. All PCR reactions were completed using a mix of NHEJ events containing different amounts of an HDR model. Amplifications were done using 30 cycles. Data shown as the mean ± s.e. (n = 8).

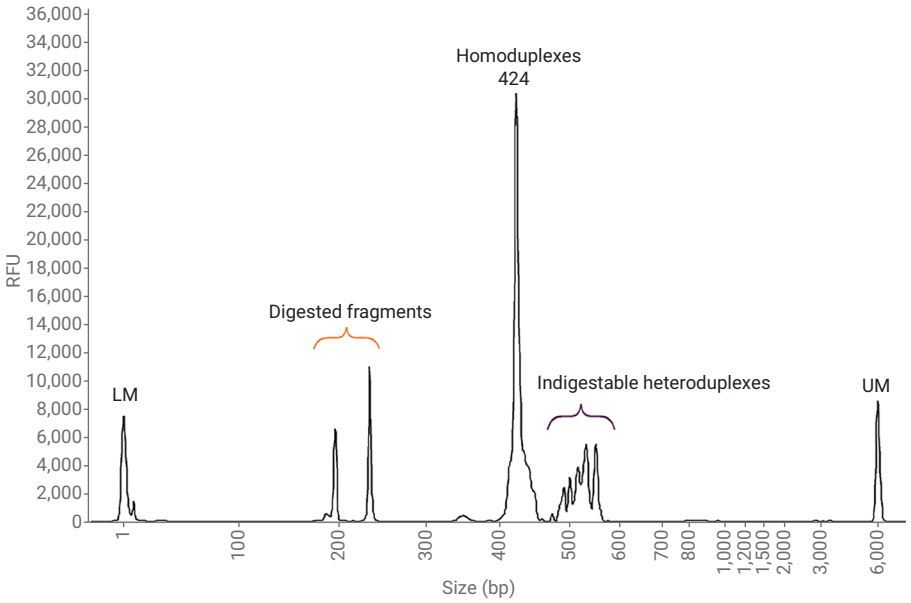

Figure 2. Analysis of BamHI digested from 40 % pHDR mixed template PCR using the Agilent 5200 Fragment Analyzer system with the Agilent CRISPR Discovery Gel kit. Heteroduplex formation was easily seen on the 5200 Fragment Analyzer system, providing for rapid assessment of cleavage accuracy. LM = lower marker, UM = upper marker.

Separation of the same 6, 11, and 25 % pHDR digestion mixes were compared between agarose gel electrophoresis and the 5200 Fragment Analyzer system. Agarose gel electrophoresis was not sensitive enough to detect cleavage products from the 6, 11, and 25 % pHDR digestion mixes, in contrast to the 5200 Fragment Analyzer system (Figure 3). Only the 40 % pHDR digestion mix produced enough cleavage products for detection by agarose gel electrophoresis, with the homoduplexed and heteroduplexed bands indistinguishable from each other. The enhanced sensitivity of the 5200 Fragment Analyzer system with the CRISPR Discovery Gel kit provided for detection of low levels of cleavage products and provided excellent resolution between homoduplexed and heteroduplexed formations, which were not possible with agarose gel electrophoresis.

To determine how the spontaneous heteroduplex formation would impact the percent cleaved when no NHEJ events were present, mixes between the WT and the HDR models were made with the percent pHDR varying from 0 to 100 % (Figure 4). The only point at which the observed percent cleaved matched the theoretical percent HDR was 50 % pHDR. Interestingly, all points below 50 % pHDR were underestimated and all points above 50 % were overestimated, while the 50 % pHDR/50 % WT mix resulted in approximately 50 % cleavage.

At any point above or below a 50 % mixture of pHDR, the heteroduplex formation has a large impact on number of cleavable HDR homoduplexes. This can be explained through the following theoretical mathematical examples (Table 1). For instance, if a 100 nM sample with 20 % pHDR resulted in half heteroduplexed formations, only 10 nmol cleavable homoduplexes would remain, representing a 50 % decrease in cleavable fragments. In this scenario,

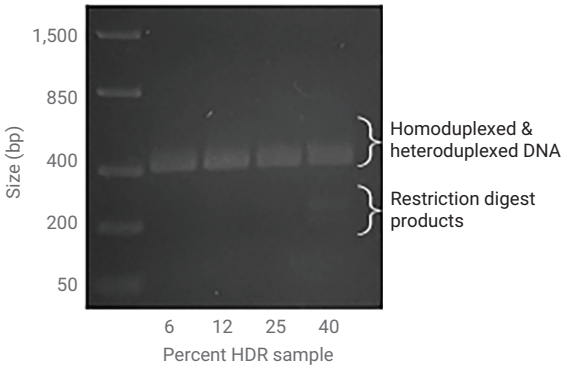

Figure 3. Agarose gel electrophoresis analysis of BamHI digested from mixed template PCR of 6, 12, 25, and 40 % pHDR. Restriction digest product was only detected with the 40 % pHDR sample. There was no visible separation between the heteroduplex and homoduplex fragments.

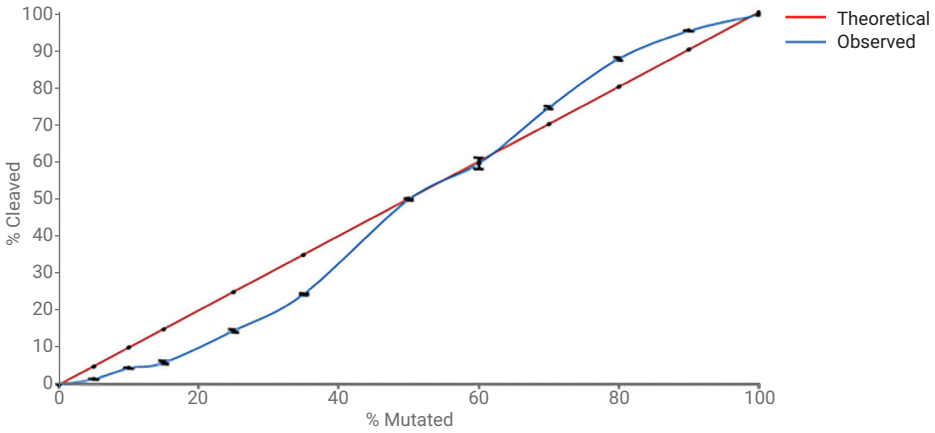

Figure 4. Observed levels of percent HDR compared to the theoretical value when only WT and varying levels of HDR events are present. The observed percent HDR was significantly less than the level expected below 50 % pHDR and greater than that expected above 50 % pHDR. Only the 50 % pHDR model returned values consistent with those expected. All PCR reactions were completed using a mix of NHEJ events containing different amounts of an HDR model. Amplifications were done using 30 PCR cycles. Data shown as the mean ± s.e. (n = 5).

Table 1. A theoretical mathematical HDR model demonstrating how spontaneous heteroduplex formation resulting from mixed template PCR impacts the observed level HDR integration.

% pHDR	nMols HDR	nMols WT	nMol Spontaneously Heteroduplexed	nMol Homoduplex HDR	nMol Homoduplex WT	Measured % HDR (% Cleaved)
20	20	80	20	10	70	13%
50	50	50	20	40	40	50%
80	80	20	20	70	10	88%

there were originally 80 nmols of WT fragments, with 20 nmols forming heteroduplexes with the HDR event, leaving 70 nmols WT homoduplexes. The percent cleaved is based on the amount of homoduplexed DNA and equals 13 % instead of the expected 20 % (Table 1). Conversely, when the percent HDR is greater than 50 % the above scenario is reversed, increasing the observed percent cleaved above the actual HDR event (Table 1). At the 50 % pHDR/50 % WT ratio, no matter the percent of heteroduplex formation, there will always be 50 % cleavage occurrence (Table 1).

Due to the random nature of mixed template PCR heteroduplex formation, it is impossible to determine the exact percent mutated DNA from the percent cleaved values. Steps can be taken to reduce the possibility of heterduplex formation and ensure percent cleaved values more closely reflect the theoretical HDR event. The simplest solution to minimize the possibility of heteroduplex formation is to significantly decrease the number of PCR cycles. This can result in low levels of PCR amplicon, thus requiring a concentration step. To test this, the number of PCR cycles was reduced from 30 to 15 followed by a concentration step. After digestion of these samples, the 5200 Fragment Analyzer system revealed fewer heteroduplex formations (Figure 5B). The percent cleaved from the 15 cycle PCR samples were closer to the theoretical values and were statistically different from the 30 cycle PCR data (Figure 5A). Additionally, the percent error decreased by as much as 40 % when compared to the 30 cycle PCR data (Table 2).

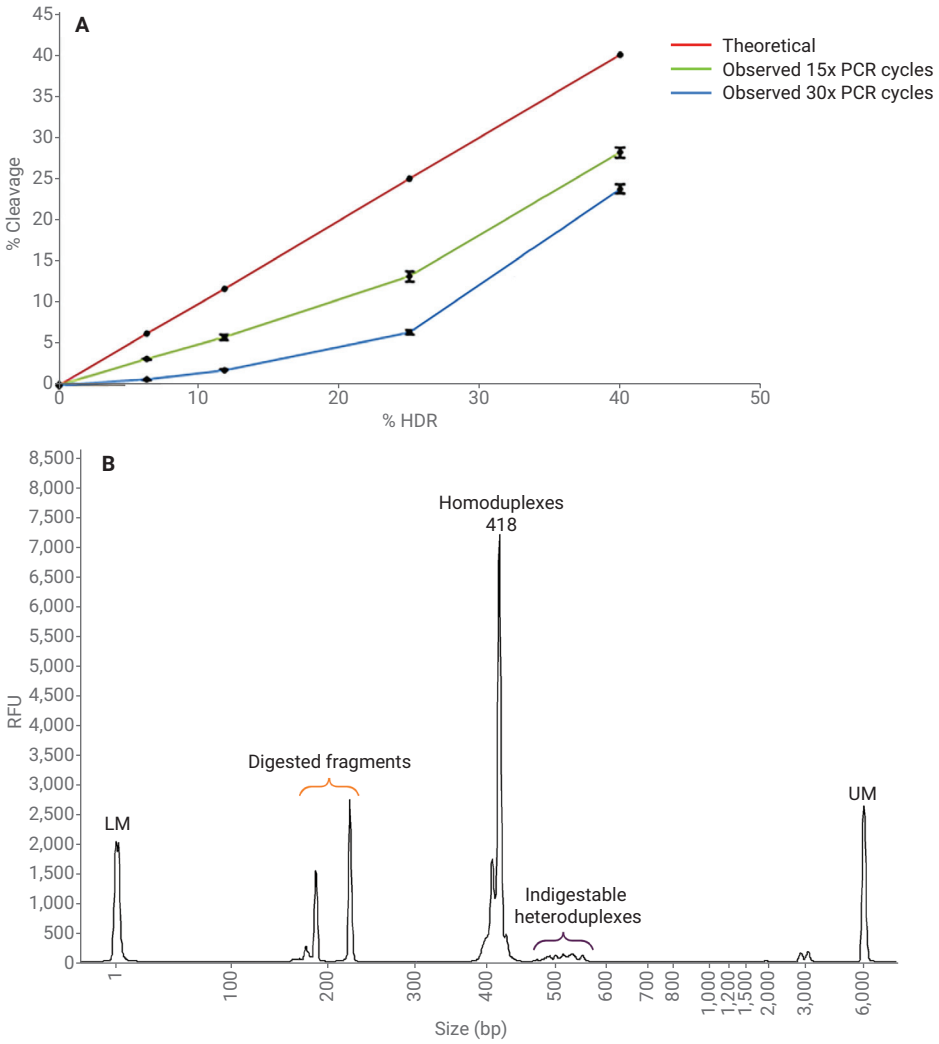

Figure 5. (A) Observed levels of percent HDR compared to the theoretical value. The observed percent HDR was significantly higher when fewer PCR cycles were used ($P < 0.01$). All PCR reactions were completed using a mix of NHEJ events containing different amounts of an HDR model. Amplifications were done using either 15 or 30 cycles. Data shown as the mean ± s.e. (n = 8). (B) Lower amounts of heteroduplex formation were observed when 15 PCR cycles were used compared to 30 cycles (See Figure 2). LM = Lower Marker, UM = Upper Marker.

Table 2. A summary of observed percent cleaved and percent error[1] resulting from 15 and 30 PCR cycles.

	15 PCR Cycles		30 PCR Cycles	
% HDR	Average % Cleavage	% error	Average % Cleavage	% error
6.25%	3.22% ± 0.06	48%	0.74% ± 0.02	88%
11.76%	5.82% ± 0.34	51%	1.87% ± 0.07	84%
25%	13.05% ± 0.68	47%	6.42% ± 0.26	74%
40%	28.2% ± 0.64	30%	23.79% ±0.53	41%

1% error to expected % cleaved value

In addition to estimating the amount of HDR events in pooled cell lines, the presence of a novel restriction enzyme site also provides for rapid genotyping of individual diploid cell lines and organisms. When both alleles in a diploid organism have been modified to contain the novel restriction site, digestion with the restriction enzyme will cleave all fragments producing 100 % cleavage. At any point above or below a 50 % mixture of pHDR, heteroduplex formation has a large impact on the number of cleavable HDR homoduplexes (Figure 4, 100 % HDR point). A monoallelic mutation will demonstrate approximately 50 % cleavage, while cleavage will not be observed when HDR events are not present.

Conclusions

The data presented here demonstrates the ability of the Agilent 5200 Fragment Analyzer system with the Agilent CRISPR Discovery Gel kit to quickly genotype individual cell lines and approximate the percent HDR in pooled cell samples, while demonstrating the effect heteroduplex formation from mixed template PCR can have on the estimation of percent HDR modification. The high resolution of the 5200 Fragment Analyzer system enables separation of the homoduplex and heteroduplex formations, thus aiding in optimization of PCR conditions to limit the effect of spontaneous heteroduplex formation from mixed template PCR. Additionally, the increased sensitivity of the 5200 Fragment Analyzer systemprovides detection of low levels of HDR events compared to agarose gel electrophoresis analysis, reducing time spent on screening samples.

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