TEG-seq: an ion torrent-adapted NGS workflow for *in cellulo* mapping of CRISPR specificity

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GUIDE-seq was developed to detect CRISPR/Cas9 off-target. However, as originally reported, it was associated with a high level of nonspecific amplification. In an attempt to improve it, we developed target-enriched GUIDE-seq (TEG-seq). The sensitivity level reached 0.1–10 reads-per-million depending on the NGS platform used, which was equivalent to 0.0002–1% measured by Targeted Amplicon-seq. Application of TEG-seq was demonstrated for the evaluation of various Cas9/gRNA configurations, which suggests delivery of Cas9/gRNA ribonucleoprotein results in significantly fewer off-targets than Cas9/gRNA plasmid. TEG-seq was also applied to 22 gRNAs with relatively high *in silico* ranking score that targeted the biological relevant SNPs. The result indicated the initial selection of gRNAs with high score is important, although it cannot exclude the possibility of off-target.

The evolution of genome editing technologies holds promise for the concept of directly correcting mutations or disrupting abnormal genes in order to treat diseases, particularly inherited genetic disorders. Current databases indicate 135,588 variant–disease associations (VDAs), between 83,002 SNPs and 9169 diseases and phenotypes [1,2] in over 3874 genes [3]. These SNPs/mutations are potentially correctable or disruptable using genome editing tools such as CRISPR–Cas9 [4–7], TALEN [8–11] and ZFN [12–14]. As they are known to induce off-target mutations at sites with homology to the target sites, gene and cell therapeutic applications of these nucleases will require a comprehensive knowledge of their off-target effects to minimize the risk of deleterious outcomes.

Many strategies have been explored to improve the specificity of targeted nucleases. Modifications to the FokI dimerization domain increased the specificity of ZFNs and TALENs by requiring formation of obligate heterodimers to cleave the target DNA in a prescribed orientation and spacing [15,16]. The inactivation of Cas9 nuclease domains to create Cas9

nickases or dCas9–FokI fusions has also increased specificity by requiring two gRNA/Cas9 complexes [17–20]. Likewise, reducing the gRNA length from 20 to 17 nucleotides has been shown to increase specificity [21]. Recently, structure-guided protein engineering [22–24] and phage-assisted continuous evolution [25] have been used to develop novel Cas9 variants that show reduced off-target cleavage. These improvements have reduced initial concerns over the specificity of CRISPR/Cas9 nucleases. However, regardless of the nuclease technology, it is difficult to determine the full spectrum of off-target cleavage events in a complex genome under various experimental conditions. That said, an efficient, unbiased and reliable genome-wide off-target detection method is crucial for the application of genome editing-based gene and cell therapy as well as for benchmarking the fidelity evaluation of different gene editing tools.

Various methods have been developed to identify off-target, nuclease-cleaved sites. These methods rely on the double-strand breaks (DSBs) caused by nucleases that can be marked by a DNA tag either through

in vitro ligation or *in cellulo* tag integration. The marked DSB site is then amplified and sequenced using next-generation sequencing (NGS). Several *in vitro* methods including BLESS-seq [26], HTGTS [27] and CIRCLE-seq [28] have been developed for off-target detection. These methods, by nature of the fact that the genomic DNA substrate has been removed from a cellular context and stripped of all protein, tend to identify all possible on- and off-target cleavage sites for a particular gRNA. The data analysis can also be challenging due to the potentially high nonspecific sequence reads as a result of the PCR amplification used to enrich the marked DSB. Digenome-seq [29], another *in vitro* method that relies on whole-genome sequencing, may reduce the noise and false positive calls, but likely sacrifices the sensitivity necessary to faithfully identify off-target sites that are cleaved at low frequencies. *In cellulo* approaches, in which off-target cleavage events occur and are tagged in living cells, more closely simulate the cellular nuclease-based gene editing environment. Two methods, IDLV-seq [30] and GUIDE-seq [31] were developed with this goal. IDLV-seq relies

METHOD SUMMARY

TEG-seq is a target-enriched GUIDE-seq using an ion-torrent next-generation sequencing platform for unbiased genome-wide CRISPR off-target detection. It reduces nonspecific amplification, and therefore increases its sensitivity for off-target detection.

on the integration of a linear viral DNA to the DSB site. However, the viral DNA is not chemically protected, which could lead to partial degradation and low efficiency of tag integration into DSBs and low sensitivity of detection. GUIDE-seq uses phosphorothioate-modified double strand DNA oligos (dsOND) as tags to reduce degradation and improve tag integration efficiency at DSBs in living cells. GUIDE-seq also requires an exponential PCR amplification step to enrich the marked DSB sites that may cause higher nonspecific noise and potential false-positive calls.

We report here a modification of the GUIDE-seq method, termed target-enriched GUIDE-seq (TEG-seq), in which 5' phosphate primers are used for PCR amplification and differentially mark the amplicon containing the DSB site from nonspecifically amplified products. This is followed by preferential magnetic bead enrichment of marked DSB amplicons. These improvements significantly reduce nonspecific amplification and improve sensitivity of DSB detection. We also developed a 96-well format workflow that enables the study of multiple samples in parallel. The sensitivity of this method reached the level of Targeted Amplicon-seq, which has been widely used to validate the known and predicted gRNA off-target sites through direct amplification and NGS [28,31].

Materials & methods

Oligonucleotides, double strand DNA Tag & adaptors

The oligonucleotide sequences for TEG-seq and Targeted Amplicon-seq are listed in Supplementary Table 5. The double strand DNA (dsTag) and adaptors were annealed in the reaction containing 1 x TE buffer, 100 mM NaCl and 50 μ M of top and bottom oligos, at 75°C for 5 min and gradually cooled down to room temperature in 20 to 30 min.

gRNA preparation

The gRNAs were synthesized using *in vitro* transcription as described in a previous publication [32]. Briefly, the 80 bp cr/tracrRNA constant region was PCR amplified from the GeneArt® CRISPR Nuclease Vector (1 ng) using the Constant Forward and Universal Reverse oligos (10 μ M). The 80 bp cr/tracrRNA PCR products (0.15 μ M) were equally mixed with universal forward and reverse oligos (10 μ M) as well as target-

specific forward and reverse oligos (0.3 μ M). The *in vitro* transcription of gRNA was carried out using the TranscriptAid T7 High Yield Transcription Kit (ThermoFisher Scientific, MA, USA) following the manufacturer's protocol.

SpCas9 expression

For the plasmid format, the SpCas9 and gRNA were cloned in the GeneArt CRISPR Nuclease Vector (ThermoFisher Scientific) following the manufacturer's protocol. The recombinant wild type SpCas9 (Platinum™ Cas9) were purchased from ThermoFisher Scientific).

TEG-seq using the ion torrent platform

See the protocol provided in the Supplementary data.

Ion-torrent NGS & data analysis for TEG-seq & Targeted Amplicon-seq

Ion-torrent platform was used for all sequencing. PGM™ with 318-chip or Proton with PI-chip was used for TEG-seq to obtain at least one million reads per sample. Proton with PI-chip or S5 with 540-chip were used for Target Amplicon-seq to obtain at least 10,000 reads per sample. Reads were first aligned to the human genome reference, hg19. Mapped reads were further processed through in-house developed plug-in software, named Motif-Search, which performs the following: sorts barcodes for different samples, stacks and counts reads for all amplicons, and scans the genome and identifies the sequence and genome position for potential off-targets based on the guide and PAM sequence. The candidates for potential CRISPR/Cas9-induced DSB sites were compared with the control sample lacking CRISPR/Cas9 treatment (dsTag only) to determine if the candidates were related to CRISPR/Cas9-induced DSB sites. To compare different samples from various experiments and different NGS runs, reads from all samples were normalized using reads per million (RPM of mapped read).

NGS reads from the Targeted Amplicon-seq experiments were aligned to the corresponding reference PCR sequence. The mapped reads were further processed using the in-house developed plugin, named 'CELFT' (cut efficiency for low frequency target) to visualize the cleavage that contains 'large

indel' and/or dsTag integration and calculated percentage of cleavage events. To minimize the false positive for the Targeted Amplicon-seq validation, caused by NGS error, especially the area with homopolymer in cleavage loci, only the large indel for having at least three or more bases was counted as a positive.

Results & discussion

The TEG-seq workflow

GUIDE-seq [31] and other genome-wide off-target detection methods [26–28,30] have been developed to provide relatively unbiased mapping of on- and off-target cleavage events. These technologies depend on the exponential amplification of tagged segments of the genome followed by sequencing and identification of those amplicons. While this technology has proven to be an advancement to the field, it can be associated with high background reads and a technically challenging workflow. Using a similar dsTag as previously reported [31], we tested the GUIDE-seq workflow using ion-torrent NGS (Ion-GUIDE-seq; Supplementary Figure 1 and 'Materials & methods'). The sequencing data showed that only a small portion of amplicons contained the desired dsTag sequence with the majority of the high-read hits not related to CRISPR/Cas9 editing (data not shown). It was hypothesized that the majority of PCR products amplified using the dsTag-specific primers (F or R) paired with the adaptor primer (P1) were actually the P1/P1 product (Supplementary Figure 1). This was confirmed by using the P1 primer alone under various conditions for multiple samples (Supplementary Figure 2). This over-representation of the P1/P1 product likely impaired the sensitivity of Ion-GUIDE-seq for genome-wide off-target detection.

To reduce the undesired P1/P1 product amplified in Ion-GUIDE-seq and enrich for the targeted DSB amplicons for NGS, we developed the TEG-seq sample preparation procedure. As shown in Figure 1, we used 5'-phosphorylated primers paired with the P1 primers for the second-round PCR amplification. This resulted in a 5'-phosphate on the products (5P-F2/P1 and 5P-R2/P1) but not on the P1/P1 product. In the next step, a nonphosphorylated barcoded adaptor (BC-A), containing the ion sequencing primer A was specifically ligated to the phosphorylated product

but not the P1/P1 product, which lacks the 5'phosphate required to ligate the nonphosphorylated barcode adaptor. A third round of PCR was then performed using P1 and 'A-tail' primers to further enrich the P1-A product. The BC-A-tail primer contains an internal spacer that stops the polymerase-mediated nucleotide extension and leaves a single-strand tail at the end of the PCR product. Finally, the dsTag-specific A-P1 product was further enriched from the remaining P1/P1 nonspecific product using a biotinylated oligo complementary to the tail sequence and captured by streptavidin (SA) magnetic bead selection. The barcoded A-P1 product was then sequenced. By selectively modifying target PCR amplicons using 5'phosphorylated primers followed by bead enrichment for the targeted fragments, TEG-seq reduces nonspecific amplification and therefore likely increases sensitivity. We also used barcoding to multiplex the workflow to improve robustness and throughput.

Validation of TEG-seq accuracy using Targeted Amplicon-seq

To evaluate the sensitivity level of TEG-seq, we compared it with Targeted Amplicon re-sequencing using NGS (Targeted Amplicon-seq), which we believe is currently the most sensitive method to detect nuclease-induced editing at predicted chromosomal locations. The two gRNAs using targeted loci HEK4 and VEG1 (previously identified to have a high and medium number of off-target sites [31] with poor *in silico* ranking score (Supplementary Table 1) were chosen. We detected over 300 and 30 off-target candidates for HEK4 and VEG1 respectively from the TEG-seq experiments and confirmed 253 positive for HEK4 and 28 positive for VEG1 by Targeted Amplicon-seq. The rarest off-target event detected by TEG-seq was approximately 1 RPM for HEK4 from 5 million and 4 RPM for VEG1 from 2.4 million total reads (Figure 2A and 2B, Supplementary Table 2). The rarest off-target detected by Targeted Amplicon-seq was 0.0004% for HEK4 and 0.0002% for VEG1. There was a reasonable correlation between TEG-seq and Targeted Amplicon-seq for both HEK4 and VEG1 with the correlation factor R value at 0.9070 and 0.9329 respectively.

Taken together, these data suggest that using NGS platforms such as PGM that produce several million total reads

in a single run, TEG-seq is capable of detecting off-target events at levels as low as 1–10 RPM, which corresponds to the cleavage rate as low as 0.001–1% as detected by Targeted Amplicon-seq (Figure 2). The combination of TEG-seq for profiling off-targets followed by validation using Targeted Amplicon-seq could be a good approach to identify appropriately edited cell lines and to validate cells destined for use in cell and gene therapy.

Off-target detection sensitivity under various sequencing depths

The sensitivity of TEG-seq and GUIDE-seq detection is directly proportional to the sequencing depth. The data from our lab and others [28,31] were generated using either Ion-Torrent PGM or Illumina MiSeq, which yield approximately 1–5 million reads per run. The lower limit of detection using the PGM was approximately 1–10 RPM. It is likely that rarer off-target events could be detected by increasing the depth of

Figure 1. Diagram of ion-TEG-seq workflow. A dsTag was co-transfected with Cas9/gRNA ribonucleoprotein or plasmid that expresses Cas9 and gRNA. The genomic DNA was extracted and fragmented to 200–600 bp using enzyme-based ion-shear. The P1 adaptor was ligated to the fragmented DNA sample. The first PCR was performed in separated tubes using P1/F1 for the forward and P1/R1 for the reverse PCR. In the second (nested) PCR, 5' phosphorylated primers (5P-F2 or 5P-R2) were used that generated PCR products containing a 5' phosphate only in the F2/P1 or R2/P1 products, but not P1/P1 product. A nonphosphorylated barcode adaptor (BC-A) was specifically ligated to the F2/P1 or R2/P1 products, but not to the P1/P1 product. A third PCR was performed using P1/A-tail primer followed by a bead enrichment via a biotinylated capture oligo that was complementary to the A-tail primer. The enriched amplicons were then applied to next-generation sequencing. dsTag: Double strand DNA tag; SA: Streptavidin.

Figure 2. Comparison of off-target detection level between TEG-seq and Targeted Amplicon-seq. (A) HEK4 off-targets detected by TEG-seq is plotted in RPM against the percentage of cleavage detected by Targeted Amplicon-seq. The correlation factor R value is indicated on the upper right side. The on-target activity is indicated by red color. **(B)** VEG1 off-targets detected by TEG-seq in RPM is plotted to the percentage of cleavage detected by Targeted Amplicon-seq. The correlation factor R value is indicated on the upper right side. The on-target activity is indicated by red color. RPM: Reads-per-million.

sequencing. To verify this, we used the Ion Proton and re-sequenced the HEK4 sample that was delivered with wild-type

SpCas9 RNP. The Proton generated 42 million mapped reads, which is approximately 23-fold higher than the number

of reads (1.8 million) from PGM. With this increased depth, we detected 32 additional off-target events. The read number per

Figure 3. Off-target detection of HEK4 gRNA-directed cleavage at different sequencing depths. (A) Read count of HEK4 on- and off-targets. **(B)** RPM of HEK4 on- and off-targets. Asterisks indicate on-target events. RPM: Reads-per-million.

Figure 4. Off-target mapping using Cas9/gRNA plasmid and RNP delivery formats. (A) On- and off-target events detected using plasmid or RNP Cas9 with HEK4 site gRNA. (B) On- and off-target events detected using plasmid or RNP Cas9 with VEG1 site gRNAs. (C) Pairwise comparison of the on-/off-target ratio for those targets that were positive from both plasmid and RNP delivering formats. RNP: Ribonucleoprotein; RPM: Reads-per-million.

target from the Proton is approximately a one order of magnitude increase over PGM (Figure 3A). However, their off-target profiles were similar after normalizing the read number to RPM with the additional, more rare 32 off-targets (Figure 3B). All 32 additional off-target events detected by Proton were below 8 RPM and ranged

down to 0.04 RPM (Supplementary Table 3). 26 of the 32 additional off-target events detected by the Proton were also detected by PGM when the plasmid format was used. However, six appeared novel and not previously detected in this or other studies published [28,31]. These data indicate that with the throughput of 1–5 million reads

generated by the PGM, off-target loci can be detected at the level of 1–10 RPM, which is equivalent to 0.001–1% from Targeted Amplicon-seq. Deeper sequencing platform such as Ion Proton or ion S5 would be recommended to detect lower than 1–10 RPM off-targets.

Figure 5. On- and off-targets detected from 22 gRNAs targeting biological relevant SNPs. 22 gRNAs with *in silico* ranking score from MIT CRISPR design tool targeting eight disease-associated SNPs (Supplementary Table 4). Their on- and off-targets were detected by TEG-seq and measured by PRM.

Table 1. The detailed result of five gRNAs detected with a single off-target.

Target no	Gene	gRNA	PAM	MIT <i>in silico</i> score	(RPM) TEG-seq	Ratio (On/off)	(%) Amplicon-Seq	MIT <i>in silico</i> predicted
4-on	<i>SMCHD</i>	GTTTCTCTATCATATAAGAA	TGG	49	2303			
4-off		: : : : : _ : C : : : : : : : :	TGG		39	59	0.29%	No
7-on	<i>HBB-2</i>	GTAACGGCAGACTTCTCCTC	AGG	80	18599			
7-off		: G : : : C : : : : : _ : : : : :	AGG		95	196	0.20%	No
13-on	<i>INS</i>	CTAGGTAGAGAGCTTCCACC	AGG	74	14342			
13-off		GC : : : : : : : C : : : : : : :	AGG		1171	12	0.08%	Yes
21-on	<i>PAH</i>	TAGCGAACTGAGAAGGCCG	AGG	75	801			
21-off		CTTG : : : : _ : T : : : : :	TGG		228	3.5	30.98%	No
22-on	<i>PAH</i>	TTCATCAGGTGCACCCAGAG	AGG	63	2700			
22-off		CG : : : : : CA : : : : : : : :	GGG		8	338	0.01%	Yes

'_' in gRNA sequence is the missing base on genome that forms a 'bulge' mismatch.
RPM: Reads-per-million.

Comparison of genome-wide off-target cleavage using plasmid & RNP CRISPR/Cas9 formats

In comparison with Cas9 delivered to the cell via plasmid DNA, direct transfection of Cas9 protein/gRNA ribonucleoprotein complexes (RNPs) provides a transient burst of activity with no opportunity for direct integration and persistent cleavage activity [32,33]. The Cas9/gRNA RNP is quickly turned over by the cell, which likely lowers the cellular concentration and thus the opportunity for off-target cleavage. CRISPR/Cas9 RNP delivery has grown in popularity but validation of genome-wide off-target effects among wild-type and various 'high fidelity' Cas9 as RNPs has only been reported recently [28]. This type of specificity evaluation benchmarking of various RNPs will be important particularly for therapeutic applications where the Cas9 plasmid format is not optimal. In an effort to more clearly define the differences in specificity between Cas9 plasmid and RNP delivery, we applied TEG-seq to HEK4 and VEG1 gRNAs and found that RNP delivery yielded approximately nine- and six-fold fewer detectable off-target events (25 vs 252 and 4 vs 27) respectively as compared with Cas9 plasmid delivery (Figure 4A & B). In general we found significantly lower off-target RPM with the RNP format than the plasmid format while their RPM of on-targets were similar. More importantly, the ratio of on-/off-target was considerably higher with RNPs than with the plasmid format in a pairwise comparison to those positive off-targets from both formats (Figure 4C). In contrast to plasmid delivery, we did not detect any off-target events from the RNP delivery format that had higher cleavage

activity than the on-target (the ratio of on-/off-target <1). Although the results showed marked improvement with RNP delivery, a subset of significantly active off-target sites remain.

These results support the idea that using Cas9 as RNP reduces the number of detectable off-target events and improves overall specificity over plasmid DNA as measured by the ratio of on-target to off-target events. This is important when considering cell line development where a tenfold difference in on-target activity to the next highest off-target event represents an approximate one in ten chance of isolating a cell line with both events occurring in the same genome. Conversely, for therapeutic applications of Cas9 RNPs in nondividing primary cells, complete elimination of off-target events would be desirable as clonal isolation is generally not achievable prior to application of cells to the patient.

Genome wide off-targets on 22 gRNAs with relative high ranking score designed from *in silico* tool

Significant effort has been made to create a rule set to enable better *in silico* prediction of CRISPR specificity [34,35]. However, there remains a relatively large discrepancy between off-targets detected by nonbiased approaches with those predicted using the current *in silico* design

The logo for Bertin Instruments, featuring the word "bertin" in a bold, lowercase sans-serif font, with "INSTRUMENTS" in a smaller, uppercase sans-serif font below it. The text is white and set against a dark blue background with a subtle pattern of glowing blue lines.

AIR SAMPLING

ASSAY KITS

A horizontal flowchart consisting of four circular icons connected by arrows. The first icon is blue with a white swirl symbol. The second icon is blue with a white test tube symbol. The third icon is blue with a white grid symbol. The fourth icon is red with a white cell symbol. The flow starts from left to right, with the final cell icon having a line extending downwards towards the "CELL IMAGING SYSTEM" label.

SAMPLE PREPARATION

CELL IMAGING SYSTEM

A photograph of the INCELLIS Smart Cell Imager, a white and black laboratory instrument. It features a large, square, black screen on top displaying a colorful, abstract image. Below the screen is a black base with a white "bertin" logo. The device is shown from a slightly elevated angle.

INCELLIS® SMART CELL IMAGER

- **High quality images:** due to its unique CMOS sensor
- **Embedded cell culture applications:** to save time
- **User-friendly:** smart interface

tools [28,31]. It's worth noting that the gRNAs (HEK4 and VEG1) evaluated in this study and those in previous work [28,31] had poor *in silico* ranking score (Supplementary Table 1). They were chosen specifically due to their propensity for high off-target cleavage. We felt it pertinent to apply the nonbiased approach to evaluate the gRNAs with relative high *in silico* ranking score to simulate the application scenario of gene correction. We chose 22 gRNAs targeting the SNPs that cause eight genetic disorders (Supplementary Table 4) using the wild-type SpCas9 RNP delivery format. Surprisingly, only five (23%) of 22 resulted in detectable single off-target cleavage events by TEG-seq and confirmed by Targeted Amplicon-seq (Figure 5 and Table 1). The off-target RPMs from TEG-seq were at least one order of magnitude lower compared with their corresponding on-target except the #21. The percentages of off-target cleavage from Amplicon-seq were all <0.29% except the #21. The *in silico* ranking score seem not well-correlated to the off-target result. For example, three targets (#7, #13 and #21) with the detectable off-targets have a relatively high ranking score. Of the five targets with detected off-target, only two were predicted by the design tool; three were not predicted, in which a 'bulge' mismatch was contained. The result implies that the initial selection of gRNA with high *in silico* ranking score is important although it cannot exclude the off-target. The probability of having the off-target is low if using Cas9/gRNA RNP delivery format with a high *in silico* score gRNA. To minimize detectable off-target, it is wise to design and test two to three gRNAs for each target.

This study reports an adaptation of the originally reported GUIDE-seq method to the Ion Torrent family of NGS instruments. The new protocol reduces nonspecific sequencing reads by enriching for relevant amplicons prior to sequencing while also enabling multiplexing of experiments through barcoding. TEG-seq represents an improved tool for validating CRISPR nuclease specificity in a cellular context. The nonbiased detection methods such as TEG-seq could be used for initial specificity screening of gRNAs followed by validation of the final edited cell clones using deep Targeted Amplicon-seq to identify appropriately edited cell lines and

to validate cells destined for use in cell and gene therapy.

Author contributions

Pei-Zhong Tang developed the method, designed and performed most of bench work, analyzed and generated data and drafted the manuscript. Lansha Peng performed some bench work. Bo Ding and Vadim Mozhayskiy developed bioinformatic tools. Jason Potter and Jonathan D Chesnut edited the manuscript.

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Supplementary data

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