Fast and simple DNA preparation for the screening of CRISPR/Cas9-generated knockout cell lines

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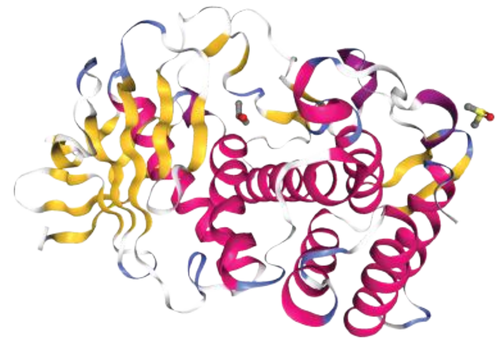

MicroGEM's *prep*GEM structure model

Introduction

CRISPR/Cas9 is an immune response mechanism utilized by prokaryotes, which allows for the recognition and destruction of foreign pathogenic DNA. Molecular biology has adopted the CRISPR/Cas9 mechanism to perform genome-editing in eukaryotes with the aim of supporting basic biological research, developing biotechnology products, and treating diseases. Although this technique is very efficient, some steps can be time consuming such as the time between the editing and the genotyping analysis itself. In fact, after the transfection of the cells with the CRISPR/Cas9 system, in order to evaluate the editing efficiency and select the clones carrying the desired genetic alteration, cells are often initially seeded individually in a 96-well plate and then have to be expanded in order to perform genotyping which can take up to 3-4 weeks.

The *prep*GEM Universal DNA extraction kit (#PUN1000) provides a flexible extraction method. It is based on a single tube and temperature-driven approach without the need for washing and centrifugation steps, thus allowing DNA extraction from 1 to up to 96 samples in up to 7 min.

In this application note, *prep*GEM Universal was used for the extraction of DNA from single cell colonies after CRISPR-mediated editing to perform screening of the clones.

The extraction was performed 10 days after the transfection using a 96-well plate. The data here reported shows that *prep*GEM Universal is well suited for CRISPR screening and provides a rapid and simple method to extract DNA suitable for PCR and Sanger sequencing analysis.

Methods and Results

In vitro testing of the protospacers by Cas9 nuclease assay

Three to five different candidate protospacers, specifically targeting exon sequences of the gene of interest, were selected from published CRISPR Knockout libraries such as "Brie" (Doench et al., 2016), ''Gecko v2" (Sanjana et al., 2014) or "Yusa" (Tzelepis et al., 2016). The selected protospacers were manufactured in the format of 2nml of crRNA by Integrated DNA Technologies (IDT) according to the Alt-R®CRISPR-Cas9 product line.

In order to evaluate the *in vitro* performance of the different crRNAs, a nuclease assay was performed.

In the first step, genomic DNA was extracted by QIAamp DNA Mini Kit (Qiagen) to amplify a region containing the predicted cut sites between 200-400 bp by PCR using Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific) following the manufacturer's guidelines. The correct product size was evaluated by running 10% of the PCR product on 1%-agarose gel stained with SYBR-Safe (Thermo Fisher Scientific).

Subsequently, the specific sgRNAs were constituted by mixing the 2µl of crRNA (20µM) with 2µl of the corresponding tracrRNA (20µM) and 5.6µl of nuclease-free IDTE buffer and then incubated at 95°C for 5 min and cooled down to room temperature (Alt-R®CRISPR, IDT). Afterwards, 1µl of sgRNA was incubated with 1µg of purified NLS-Cas9 enzyme (homemade) for 5 min to form the RNP complex, which was then incubated with 150ng of the PCR product, 5µL of 10x NEB 3.1 buffer (New England Biolabs) and filled with Ultra-Pure Nuclease-Free water (Thermo Fisher Scientific) up to 15µl. The reaction was incubated for 1h at 37°C, 1µL of RNAse A (4µg/µL) was added for an additional 15 min incubation to degrade the RNA. The reaction was finished by incubating the mix for 15 min with 1µL of Stop-solution (25% glycerol, 1.2% SDS, 250 mM EDTA) and the final product was loaded on a 1%-agarose gel to select the best working sgRNAs (Fig. 1).

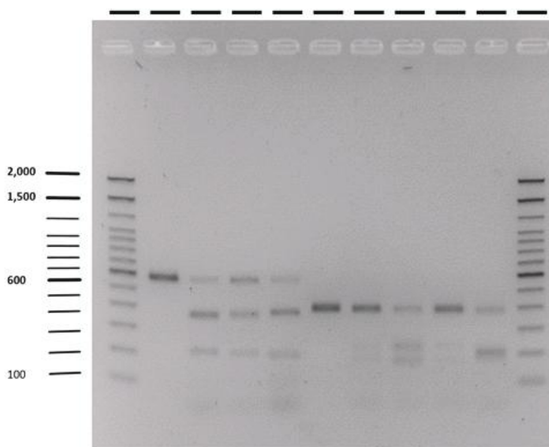

Figure 1: In vitro Cas9 nuclease assay using different sgRNA. Lane 1 - size marker, Lane 2 - uncut control, Lanes 3 to 5 - digested PCR products with individual guides, Lane 6 - uncut control, Lanes 7 - 10: digested PCR products with individual guides, Lane 10 – size marker

Cell line electroporation and single cell sorting

After selection of the best protospacer sequence, $4x10^5$ target cells were electroporated either with a non-targeting RNP control complex or an RNP complex containing individual crRNAs and tracrRNA conjugated with ATTO550 fluorescent dye, using a 10µL tip of the Neon™ transfection system (Thermo Fisher Scientific), following the user guide for genome editing protocol from IDT. Three days after the transfection, between 192 to 288 viable $ATTO550^{hi}$-labelled cells (Fig. 2) were individually sorted into a well of a U-bottom 96-well plate containing 150µL of proper media using a BD FACSAria Fusion (Beckton Dickinson). One week later, the proliferative single colonies (determined by media colour change) were reorganized into a single 96-well plate and fresh media was added.

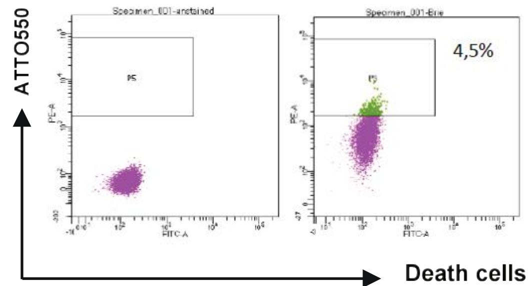

Figure 2: Cell sorting of cell positive for sgRNA:Cas9 complex. The electroporated cells with the target RNP were sorted three days after using as marker the tracrRNA conjugated with ATTO550.

DNA extraction in 96-well plate format and clonal analysis via PCR and sequencing

Ten days after transfection, half of the volume containing approximatively between 5000 and 20000 cells was taken and centrifuged for 2 min at 2200 rpm. Then, the DNA was extracted using the *prep*GEM Universal (#PUN1000, MicroGEM) in a volume of 50µl/well (Fig. 3). The *prep*GEM Universal components were mixed as follows:

Reagents	50µl
Blue Buffer	5µl
***prep*GEM**	1µl
Water	44µl

Table 1: Extraction mix volume of 50µl for 1 sample.

The extraction mix of 50µl was added to each well of the 96-well plate containing the cell pellet. The 96-well plate was incubated in a standard laboratory thermocycler as follows:

Degrees	Thermocycler
75°C	5 min
95°C	2 min

Table 2: Incubation steps for thermocycler.

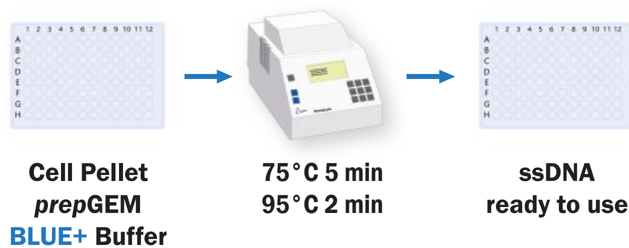

Figure 3: Workflow of the extraction with prepGEM Universal (#PUN1000) in a thermocycler. Sample and reagents are mixed and placed in a 96-well plate and the extraction is run on a standard thermocycler. The extraction occurs at 75°C and at the end of the extraction, the 95°C step irreversibly inactivates the proteinase and denatures the DNA to become single-stranded, making it ready for PCR-based applications.

In order to analyse the clones, 1µL of extracted DNA was used as template to run an end-point PCR using Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific) following the manufacturer's guidelines. Fig. 4 shows that the DNA from low cell number was efficiently extracted with *prep*GEM Universal in high throughput format and the DNA was suitable to be amplified and allow clone selection. The PCR products from the promising candidates were cleaned up using either QIAquick PCR purification Kit (Qiagen) or PureLink™ Pro 96 PCR Purification Kit (Invitrogen) and then sent for sanger sequencing (Fig. 5). Finally, the knock-out probability for every clone was determined using the ICE CRISPR Analysis Tool (Synthego).

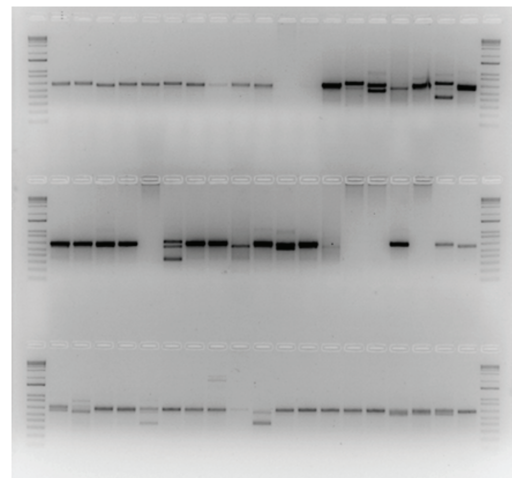

Figure 4: Single clone colonies analysis after DNA extraction in 96 wells with prepGEM Universal: End point PCR analysis.

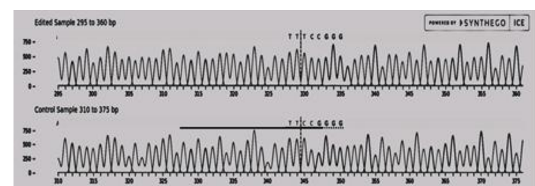

Figure 5: Single clone colonies analysis after DNA extraction in 96 wells with prepGEM Universal. Sanger Sequencing analysis.

Conclusions

The data shown in this application note demonstrates that the *prep*GEM Universal kit is suitable to rapidly extract gDNA from low cell numbers and is adaptable to a 96-well format. Therefore, this product is well suited for genome editing screening such as CRISPR/CAS9 clonal selection. This data is supported by Yang et al., 2014 who showed that *prep*GEM Universal allowed DNA extraction from single clone colonies after 12 days from the editing, greatly reducing the time for the analysis that on average lasts 3-4 weeks. In conclusion, *prep*GEM Universal offers several advantages for genome editing screening:

- Short waiting time for single cells to grow in a colony with sufficient cells for the extraction (10-12 days or less)

- DNA extraction can be conducted in a 96-well format, increasing throughput

- Rapid extraction procedure for up to 96 samples in 7 minutes

- The reaction mix can be scaled up/down based on cell numbers from a minimum of one cell to a large number of cells

- Extracted DNA is suitable for PCR-based analysis as well as Sanger Sequencing

In conclusion, *prep*GEM Universal (#PUN1000) offers a more efficient extraction method for CRISPR genotyping.

References

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