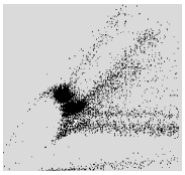
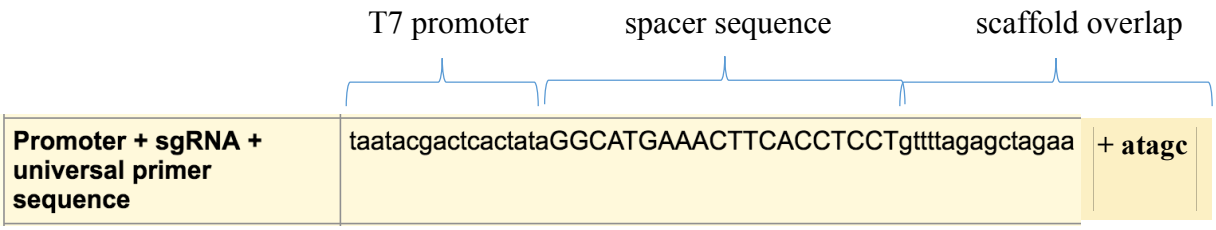


THE GOODELL LABORATORY

Authors	Michael Gundry and Lorenzo Brunetti	9/2016
Title	**CRISPR-based gene disruption in human hematopoietic cells**	
Introduction	This protocol describes a fast and efficient CRISPR-based protocol for editing the genome of human hematopoetic cells	
Citation	**Gundry M, Brunetti L, Lin A, et al. "Highly efficient genome editing of murine and human hematopoietic progenitor cells by CRISPR/Cas9. 2016, Cell Reports 17, 1453–1461 http://dx.doi.org/10.1016/j.celrep.2016.09.092**	
Key Materials	1. **sgRNA Forward primer**: The fwd primer sequence is unique for each sgRNA. Design this oligo on http://www.crisprscan.com/ with modifications (see sgRNA design) 2. **Reverse universal primer**: 5′-3′ → AGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC. This is the universal oligo that anneals to the scaffold portion of the forward primer used in the sgRNA synthesis PCR reaction (see sgRNA synthesis). 3. **KAPA HiFi HotStart ReadyMix PCR Kit (Kapa Biosystems)** 4. **PCR purification kit**: Qiagen QIAquick PCR Purification Kit 5. ***In vitro* transcription kit**: NEB HiScribe™ T7 High Yield RNA Synthesis Kit (E2040S) (see *in vitro* transcription step) 6. **RNA purification kit**: Zymo RNA Clean and Concentrate-25 (see in vitro transcription step) 7. **Cas9 protein** (IDT or PNA Bio) 8. **Neon Transfection System** (Invitrogen) 9. **RNaseZap RNase** (Thermo Fisher) or similar	

Protocol	A. sgRNA design	*Notes*
	The goal of this step is to design the forward primer for sgRNA synthesis. The forward primer consists of 3 parts: 1. T7 promoter 2. Target sequence, also known as spacer (indicated as "NNNN…N")	***Final Forward Primer Sequence*** = *TAATACGACTCACTATAGG + NNNNNNNNNNNNNNNNN + GTTTTAGAGCTAGAA**ATAGC*** ***For further info on this step please see: Shao et al. Nat Protocols 2014 (PMID: 25255092)***
1.	Use http://www.crisprscan.org/ to design your sgRNA. The CRISPRscan algorithm provides a list of potential target sequences. For each target sequence, CRISPRscan provides an "integrated" forward primer sequence that includes the T7 promoter, the target sequence, and the plasmid scaffold overlap sequence (see below in yellow). **Additional modifications to make to CRISPRscan's forward primer sequence: Add "ATAGC" at the end (highlighted in bold in the "final forward primer sequence" above)**	*-In CRISPRscan, enter the gene of interest and navigate to the region of the gene to be edited. The potential sgRNA target sequences are listed along with a corresponding CRISPRscan score.* *-Select a target sequence with a relatively high score and no (or the lowest number of) off-targets.* *-Click on the target sequence to see details*



Example of one **forward primer** generated with the CRISPRscan UCSC browser track. Note the "ATAGC" sequence added to the 3' end.

Protocol	B. sgRNA DNA template synthesis	*Notes*
	The purpose of this step is to join the sgRNA forward primer sequence with the scaffold sequence via overlapping PCR. The product of this PCR reaction will be a single dsDNA product with the guide target sequence linked to the scaffold sequence.	***Materials:*** 1) Rev primer 5′-3′ (**HPLC purified**): AGCACCGACTCGGTGCCACTTTTTCA AGTTGATAACGGACTAGCCTTATTTT AACTTGCTATTTCTAGCTCTAAAAC 2) Fwd primer (see "sgRNA design" section) 3) KAPA HiFi HotStart ReadyMix PCR Kit 5) Nuclease-free water 6) Thermocycler
1.	Dissolve and dilute the sgRNA fwd and universal rev primer to a final concentration of 10μM with nuclease-free water	
2.	To perform overlapping PCR, mix the following in PCR strip tubes: <u>20μl reaction</u> 2μl Fwd primer (10μM) 2μl Universal Rev primer (10μM) 10μl KAPA HiFi 2x 6μl nuclease-free H20	
3.	Run the PCR with the following program: 1. 95C for 3 min 2. 98C for 5 sec 3. 60C for 5 sec 4. 72C for 10 sec 5. Go to 2 for 6 cycles 6. 72C for 1 min 7. 4C forever	*The total time of the program is ~20mins. After the program is complete, the samples can be kept at 4C.*
4.	Purify the PCR product with the Qiagen MinElute PCR Purification Kit. Follow the kit protocol, and elute with <u>11.5μL</u> of EB buffer.	*Tip: prior to purification of the PCR product, run 1uL on a 2% agarose gel to confirm a single band of ~130bp and disappearance of the starting oligo components.*
5.	Measure the concentration of the PCR products on a spectrophotometer.	*Typical DNA concentrations obtained are in the 50-80ng/μL range.*

Protocol	C. In Vitro Transcription of sgRNA	*Notes*
	The purpose of this step transcribe the sgRNA from the DNA template (PCR product made in Part B)	***Materials:*** 1) NEB Hi-Scribe T7 High Yield RNA Synthesis kit 2) PCR products (from Part B) 3) Nuclease-free water 4) RNaseZap (or similar) 5) Any device that allows for 37C incubation
1.	Mix the following components in PCR strip tubes (reagents are from the NEB Hi-Scribe T7 High Yield RNA Synthesis kit): <u>10uL reaction</u> **Example**: Your DNA elution has a concentration of 60ng/uL. 4µL of eluted DNA (240 ng) 4µL of dNTPs 1µL of 10x Reaction Buffer 1µL of T7 RNA pol enzyme mix	*-We recommend applying RNaseZAP (or similar) to the working surface and gloved hands prior to starting this step.* *-Use half of the recommended reaction volume in the kit to save reagents (10uL instead of 20µL).* *-Use **200-500ng** of DNA for IVT (we always use 4ul of the elution from the DNA purification step)*
2.	Incubate the samples at 37C for at least 4 hours.	*We run IVT for 4hrs to overnight, with similar yields.*
3.	Bring each RNA sample up to a total volume of 50uL with nuclease-free H20. Run 1µL on a 1.5% agarose gel to assess RNA yield.	*The purpose of running 1uL on the gel is to confirm that the IVT worked, prior to purifying the RNA. We expect to see a strong visible band for each sample.*

Protocol	D. Purification of sgRNA	*Notes*
	The purpose of this step is to purify the sgRNA synthesized in Part C. Any extraneous contaminants may be detrimental to cell viability/health when the sgRNA is electroporated.	***Materials:*** 1) RNA purification kit: Zymo RNA Clean-up and concentrator 25 2) Nuclease-free H20 3) RNAseZAP
1.	Apply RNaseZAP (or similar) to gloved hands, and follow the protocol in the Zymo RNA Clean-up and Concentrator Zit.	*The starting volume of the sgRNA samples is 50uL, so add 100uL of the binding buffer, followed by 150μL of 100% EtOH.* *Elute in 25μL or 50μL of RNAase free water, depending on how concentrated you want your guide to be.*
2.	Measure the concentration of the eluted sgRNA on the Nanodrop	*The expected yield after purification is 50-80* ***ug*** *of total RNA (i.e. conc of 1 to 1.5 μg/uL, if the RNA was eluted in 50μL).*
3.	Aliquot the sgRNA into PCR strip tubes (~3-4μL per tube, depending on needs). Use immediately or store at -80C for the long-term.	

Protocol	**E. Electroporation of sgRNA-Cas9 RNP complexes**	*Notes*
	The purpose of this step is to bind the eluted sgRNA to the Cas9 protein to form sgRNA-Cas9 complexes and to transfect the complexes into the cells of interest through electroporation. **Note: the protocol is optimized for Neon 10μL tips. If you want to use 100μL tips, you need to scale the reagents up.**	***Materials:*** 1) Eluted sgRNA 2) Cas9 protein (IDT or PNA Bio) 3) Cells of interest 4) PBS 5) Cell counter 6) Culture medium
1.	Count cells and calculate the volume needed to get desired total # of cells (i.e. 150.000 – 250.000 per replicate)	*Note: keep in culture human CD34+ HSPCs for 36-48 hours in presence of cytokines prior to electroporation to increase knock-out efficiency. We recommend the following medium: Stem Span II (StemCell Tech) with hFLT3L, hTPO and hSCF (all at 100ng/mL)*
2.	Wash cells twice in PBS in 1.5ml microcentrifuge tubes and carefully remove the supernatant	*Remove the supernatant **completely** to avoid larger volumes in the electroporation step (see below)*
3.	In the meantime, prepare the sgRNA-Cas9 RNP complexes by incubating **15-30 minutes RT 1.5μg Cas9** with **1μg of <u>total</u> sgRNA** in PCR tubes for each replicate, except the Cas9 only control.	*-Concentration of Cas9 stock is 10 μg/μl for IDT (ready to use) and 2 μg/μL for PNA Bio (25μL of H20 added to 50μg of lyophilized Cas9). Since you will need 1.5 μg of Cas9 per replicate, it is recommended to further dilute aliquots of IDT Cas9 before the experiment to be more accurate.* *-If more than one guide is used, you will need to calculate the volume of each sgRNA to mix, so that the total amount of sgRNAs adds up to 1μg.*
4.	You are ready to electroporate.	

Detailed electroporation protocol and tips for success (NEON Transfection System)

Preparing the post-electroporation well-plates and media for cells

1. Prepare well-plates with the appropriate media for your cells, and label the wells.
   -For CD34+ cells: Stem Span II with cytokines (see Section E)
   -For AML cell lines: complete RPMI without antibiotics

Electroporation (performed under sterile conditions in a tissue culture hood)

1. Calculate the total volume of Invitrogen Resuspension Buffer. **Rule of thumb = 10μL of buffer per electroporation.** *Resuspend in a little extra volume (i.e. 110uL for 10 electroporations). Use Buffer R for cell lines and Buffer T for primary cells.*

2. Aliquot the desired amount of resuspended cells into each PCR tube containing pre-mixed sgRNA-Cas9 RNP (i.e. 10 μL for single replicates)

3. Set up the Neon Transfection system:
   -Add 3mL of electrolytic buffer E into a new plastic cuvette, and slide the cuvette into the Neon Transfection System (use buffer E2 for 100μL tips).
   -Select the desired protocol on device: **AML cell lines (1350V, 35ms, 1 pulse)**, **T-cells/CD34 HSC (1600V, 10ms, 3 pulses)**. The electroporation is performed with a special Neon pipette, which has a claw that latches onto the Neon electroporation pipette tip. *Extend the claw, grab the disc inside the pipette tip, and then firmly press the pipette down to secure the tip.*

4. Pipette the sample up and down 10 times to mix. Take out 10μL, **making sure there are no bubbles**, and insert the pipette tip directly into the electrolytic buffer in the plastic cuvette. Try not to touch the walls of the cuvette. Press start on the screen. After the "complete" message appears, remove the pipette, and dispense contents into a well with media. *Note: the pipette somehow always draws more than 10μL, so that's why we prepared a small excess (1-1.5μL) of each sample.*

5. Repeat for all the samples. Change pipette tips between samples, only if necessary.

6. Check cells under the microscope. Incubate at 37C and allow the cells to recover from the electroporation.

7. Test KO efficiency using PCR followed by NGS or surveyor assay; or using flow cytometry 3-4d after electroporation (if targeting cell surface marker)
   -We recommend using dual guides for easy assessment of CRISPR efficiency by identifying deletions in the amplicon PCR without the need for sequencing
   -If using single guide, we do not like the endonuclease based assays and often perform amplicon sequencing by spiking many samples onto a colleagues Nextseq or Miseq run (<1% of total run)

Additional notes on sgRNAs

The above protocol provides instructions for *in vitro* transcription (IVT) of sgRNA. However, we often will purchase synthetic sgRNA from Synthego (www.synthego.com) or TriLink for long-term projects for which we plan on performing many experiments with these sgRNA. Synthetic sgRNA clearly have a lower toxicity and result in much cleaner experiments allowing for analysis of RNA/protein at earlier timepoints.