



Nov 12, 2024

ChrX Variant Installation Protocol

DOI

<https://dx.doi.org/10.17504/protocols.io.5jyl8dmndg2w/v1>

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Protocol Citation: Eric Che, Ting Wu, Basheer Becerra, Jayoung Ryu, Marlena Starrs, Estelle Lecluze, Benjamin P. Kleinstiver, Richard Sherwood, Guillaume Lettre, Luca Pinello, Daniel E. Bauer 2024. ChrX Variant Installation Protocol. **protocols.io**
<https://dx.doi.org/10.17504/protocols.io.5jyl8dmndg2w/v1>

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Protocol status: Working

We use this protocol and it's working

Created: November 07, 2024

Last Modified: November 12, 2024

Protocol Integer ID: 111776

Keywords: set of gwas variant, gwas variant, genetic variants influence erythroid differentiation cellular phenotype, chrX variant installation protocol genome, associated variant, genetic variants influence erythroid differentiation, functional genomic, genome, functional genomics of trait, identified numerous single nucleotide polymorphism, red blood cell trait, adenine base editor, pamless adenine base editor, deletion variant, numerous single nucleotide polymorphism, cellular phenotype, grna editing outcome, enrichment variant, erythroid precursor differentiation, handling grna mapping, less crispr base, gwa, crispr, variant, sentinel variant, enrichment grna, functional consequences of these variant, base editing screen, genetic variant, guide rna

Funders Acknowledgements:

Pinello

Grant ID: HG012010



Abstract

Genome-wide association studies (GWAS) have identified numerous single nucleotide polymorphisms (SNPs) associated with various traits and diseases, yet understanding the functional consequences of these variants remains challenging. In this study, we focused on Chromosome X, selecting 1,852 variants (577 sentinel variants and the rest as proxies) associated with red blood cell traits, primarily located in non-coding regions, along with 55 clinically relevant variants catalogued in ClinVar, all associated with haematological disorders. We employed a near-PAMless adenine base editor (ABE8e-SpRY), and D10A-SpRY without deaminase as a control, in a proliferation and differentiation screen within erythroid precursor HUDEP-2 cells. By including ~5 guide RNAs (gRNAs) installing or perturbing each variant positioned along the gRNA editing window, we designed a total of 11,036 gRNAs. We are also developing a comprehensive computational pipeline tailored for PAM-less CRISPR base editing screens to analyze the large datasets generated from these screens. For instance, our current pipeline is capable of handling gRNA mapping despite self-editing of the gRNA cassette along with characterization of per-gRNA editing outcomes by processing the gRNA sensor construct containing a target-mimicking “surrogate” sequence. Our screen successfully identified a set of GWAS variants on Chromosome X that significantly impacted erythroid precursor differentiation. By comparing ABE8e and D10A using MAGeCK and BEAN, we identified 90 deletion variants and 69 enrichment variants, as well as 300 depletion gRNAs and 282 enrichment gRNAs with FDR ≤ 0.01 . Ongoing work seeks to experimentally validate causal variants and determine their molecular mechanisms, including based on single cell perturbation analysis. This study aims to enhance our understanding of how genetic variants influence erythroid differentiation cellular phenotypes. Additionally, the development of a robust computational pipeline for base editing screens will provide a valuable resource for future studies aimed at exploring the functional genomics of trait-associated variants.

Guide library preparation

1 Library structure:

5'flank-[19-20-nt gRNA]-FEscaffold-terminator-[32-nt target][4-nt barcode]-revcomp-r2seq-[14-nt BE sublibrary primer]

tggaaggacgaaacaccg[19-20-nt
gRNA]GTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTTTAAATAAGGCT
AGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT[32-nt target (6-nt
upstream, 20-nt gRNA, 6-nt PAM)][4-nt barcode]
AGATCGGAAGAGCACACGNNNNNNNNNNNNNNNN

2 Reconstitute ssDNA oligo pool:

2.1 Spin down lyophilized ssDNA oligo pool

2.2 Prepare 2 ng/μL stock by resuspending in TE buffer with low EDTA (10mM Tris-Cl pH 8.0, 0.1 mM EDTA)

2.3 Aliquot into 3 tubes to limit chances of contamination and to reduce freeze/thaw cycles.

2.4 Store at -80 °C

3 Restriction Digest of pHKO9-BsmBI library backbone:

3.1 Cut 10 μg pHKO9-BsmBI and isolate 8 kB band from 1% SYBR gel

3.2 Add components in the following order:

	A	B
	H2O	85 - x μL
	NEB 3.1	10 μL



	A	B
	pHKO9-BsmBI	x μ L
	BsmBI	5 μ L

3.3 Incubate at 55 °C for 4 hours

3.4 Run gel-PCR

Note: Use this vector for plasmid library cloning ONLY, not for single guides (scaffold will be cut off during digestion)

4 **PCR1:**

Forward Primer for PCR1:

010415_sgRNA_60bp_fw,

TAACCTTGAAAGTATTTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG

Reverse primers are library specific, for example library A primer:

042921_BEsubA_rv,

TCACCTTGCTACGTCGTGT

4.1 PCR1 Reaction – 20 μ L:

	A	B
	Oligo pool @2ng/ μ L	1 μ L
	2x Q5 mix	10 μ L
	DMSO	0.5 μ L
	F primer @10 μ M	1 μ L
	R primer @10 μ M	1 μ L
	dd H2O	6.7 μ L

Cycling:

	A	B	C	D	E	F
	98 °C	98 °C	63 °C	72 °C	72 °C	4 °C
	30 sec	10 sec	30 sec	30 sec	5 min	remaining

The cycle number depends on the ratio between the number of guides in your library and the total oligos in the pool. Smaller libraries, <10% of the total, use 10 cycles for PCR1. For larger libraries, 7 cycles will be sufficient. For very small libraries (<1%) use 15 cycles, and for single guides, use 20 cycles.

5 qPCR2:

Primers for PCR2 Forward:

010415_sgRNA_60bp_fw,

TAACTTGAAAGTATTTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG

Reverse:

052522_r2seq_6ntbc_pHK09_BsmBlx3,

ATTTGTCTCAAGATCTAGTTACGCCAAGCTT

CAGAAGACGGCATAACGAGAT CTNNNNNN GTGACTGGAGTTCAGA

CGTGTGCTCTTCCGATCT

5.1 qPCR2 Reaction – 15 uL:

Using 0.25 uL of unpurified PCR1 product, perform 15 uL qPCR with PCR2 primers to determine optimal PCR2 cycles.

	A	B
	PCR1 product	0.25uL
	2xQ5 mix	7.5ul
	Fw	1.5ul
	Rev	1.5ul
	evaGreen	0.25ul
	H2O	15ul

5.2 Run qPCR, get Ct cycle and Tm value



5.3 Run product on 2% EtBR gel to ensure clean 299 bp product

6 PCR2:

6.1 PCR2 Reaction – 100 uL:

Use 10 uL unpurified PCR1 in 100 uL reaction [Note-- scale up to 20 uL in 200 uL reaction for larger libraries] for cycles determined by qPCR with:

	A	B
	PCR1 product	10 uL
	2x Q5 mix	50 uL
	DMSO	2.5 uL
	F primer @10uM	5 uL
	R primer @10uM	5 uL
	dd H2O	6.7 uL

Cycling:

	A	B	C	D	E	F
	98 °C	98 °C	65 °C	72 °C	72 °C	4 °C
	30 sec	10 sec	30 sec	60 sec	5 min	remaining

6.2 Product should be 299 bp. Isolate band from gel and clean with zymo kit:

- pHKO9 vector, MW 5073100 g/mol, 0.05pmol = 254ng.
- PCR2 product, use 3X molar amount of vector, 0.15 pmol = 28 ng gel-purified library PCR2 product

7 Gibson assembly:

7.1 For small libraries, perform 20 uL 2-way NEBuilder. This can be scaled up proportionally as needed to 100-200 uL total reaction for larger libraries. Here we perform 100ul 2-way NEBuilder. The amount of vector and PCR2 products can be adjusted based on the number of guides.

- pHKO9 vector, MW 5073100g/mol, 0.05pmol would be 254ng.
- 28 ng gel-purified library PCR2 product (0.15 pmol = 3X molar amount of vector)

	A	B
	Total	20 uL
	2xHiFi assembly mix	10 uL
	Vector	0.05pmol (254ng)
	Purified PCR2 products	0.15pmol (28ng)
	50 degree, 1h	

Total: 20 uL

	A	B
	Total	100 uL
	2xHiFi assembly mix	50 ul
	Vector	0.25 pmol (1270 ng)
	Purified PCR2 products	0.75 pmol (140 ng)
	50 degree, 1h	

Total: 100 uL



8 Cleanup/concentration:

- 8.1 Add 1 ul GlycoBlue, 2 ul 50mM NaCl, 100 ul Isopropanol to the Gibson reaction system
- 8.2 Vortex, incubate at room temperature for 15 min
- 8.3 SPIN > 15.000 g for 15 min
- 8.4 Carefully remove liquid without disturbing pellet
- 8.5 Wash with 300 ul 80% EtOH and SPIN >15.000 g for 5 min
- 8.6 Remove most liquid with P1000 and spin at >15,000 g for 1 min
- 8.7 Carefully remove all liquid with p200, making sure tube has no liquid left, and air-dry pellet 3-5 min by keeping cap open and leaving at room temp
- 8.8 Add 8.25 ul EB, warming at 55C 10min to fully resuspend

9 Electroporation:

- 9.1 Add 2uL of NEBuilder product to 25uL of Lucigen Endura electrocompetent cells, repeat 4x
- 9.2 Electroporate using the following parameters:
 - 1mm cuvette
 - 10 μ F
 - 600 Ohms
 - 1800 Volts
- 9.3 Immediately add 1mL recovery media from Lucigen



- 9.4 After electroporation, pool all samples from the same library in 14mL round bottom tube, add another 1mL media to each cuvette to wash, then add to 14 mL tube, total 8mL
- 9.5 Shake at 37 °C for 1 hr
- 9.6 Plate 1/10,000, 1/40,000 and 1/100,000 dilutions. Dilute 2uL bacteria in 200uL media, then further dilute:
- 1/10,000- 80uL dilute in 100uL
 - 1/40,000- 20uL dilute in 100uL
 - 1/100,000- 8uL dilute in 100uL
- 9.7 Transfer the remaining into 400 mL LB + Amp. Shake overnight, spin down for maxiprep as 2 × 200 mL aliquots, perform maxiprep on 1 × 200 mL aliquot, keeping the second as backup
- 10 **Validation:**
- 10.1 Aim for for 50-1000X as many colonies as there are unique library members
Ex: On 100,000- fold plate, count 20 colonies, library contains 500 guides
 $(100,000 \times 20) / 500 = 4,000$ colonies per guide
- 10.2 Pick 16 colonies to sequence, either perform colony PCR using the primers:
F- pX330_seq_fw: GAGGGCCTATTTCCCATGAT
R- 111219_postPT_rv: CTAGGCACCGGATCAATTGC
- OR
- Miniprep 16 colonies and send for sequencing with the primer:
F- pX330_seq_fw: GAGGGCCTATTTCCCATGAT
- 10.3 Ensure the guide sequences match the correct library and that the spacer and target sequences are the same for each guide

Lentivirus Production

11 Lentivirus production:

- 11.1 1 Day before: Seed 6 plates 293T cells per library, media DMEM +10% FBS +1%PS

- 11.2 Day of infection: Ensure plates are around ~70-80% confluence
For each plate, use 13.3ug PsPAX2, 6.7ug VSVG and 20ug plasmid library
- 11.3 In one tube, combine all packaging plasmids (Pax2 and VSVG) and library plasmids (for a single library) with OptiMEM up to 500uL per plate.
- 11.4 In a second tube, combine 180uL PEI and 320uL OptiMEM per plate

Example for 6 plates, assuming all plasmid concentrations are at 1 ug/uL:
Tube 1 (DNA): 79.8uL Pax2 +40.2uL VSVG +120 uL Library plasmids, add 2.76mL OptiMEM.

Tube 2 (PEI): 1.08mL PEI + 1.92mL OptiMEM
- 11.5 Add the DNA mixture (3 mL) to the PEI mixture, incubate 20 mins at room temperature
- 11.6 Add 1 mL mixture to 1 plate 293T cells, dropwise, repeat for each plate
- 11.7 8-12 hours after transfection, remove media and add 20mL fresh pre-warmed media
- 11.8 48 hours after transfection, add 6mL fresh media
- 11.9 72 hours after transfection, centrifuge virus:
 - Transfer supernatant to 50mL falcon tubes, spin down at 4500xg for 15 minutes to spin down cell fragments
 - Filter supernatant using Stericup-HV Sterile Vacuum Filtration System (Millipore sigma S2HVV01RE) 150 mL process volume, 0.45 µm pore size.
 - Transfer supernatant to polystyrene tubes set inside ultracentrifuge rotor tubes, fill with PBS to the top, ensure proper balance and centrifuge at 24000xg at 4°C for 2 hours
- 12 **Harvest virus:**
- 12.1 Aspirate media from polystyrene, invert tubes onto kimwipe and dry for 1-2 minutes

- 12.2 Add 50 uL DMEM, cover tubes with parafilm and resuspend on tilt table at 4°C for 2 hours
- 12.3 Transfer resuspended virus to 1 mL microcentrifuge tubes and spin down at 4 °C max speed for 1 min
- 12.4 Aliquot 10 uL virus into 8 strip tubes and store at -80 °C

13 **Titration:**

- 13.1 Note: If you want to begin titration the day of concentration, freeze for a minimum of 1 hour at -80 (ensure liquid is frozen) since you want your titration to reflect the activity of frozen lenti

Take the small aliquot out for titrating the lenti on the relevant cell lines with appropriate polybrene concentration in 24-wells using defined cell number, using drug resistance as metric of efficiency. Do NOT re-freeze virus aliquot.

- 13.2 Use this titration value to calculate dosage for a complete experiment:

Sample titration protocol (scale as needed for specific application)

- 1M cells per group were used for titration
- Virus volume: 0ul, 0.3ul, 1ul, 2ul, 5ul, 10ul
- Day1, virus transduction
- Day2, evenly divide the cells of each group into two wells, add puro into one well
- Day4, count cells of each group. 30% infection efficiency (MOI = 0.3) usually have VCN of 1.
- Day 7, collect cells for ddPCR to check the VCN of all groups. You can choose to use VCN=1 or >1 for experiment.

14 **Lenti library infection:**

HUDEP2 cells with (1) No-deaminase, (2) ABE8e-SpRY stable expression cell lines 11 million cells will be needed for ~12,000 guides (x1000 coverage). Assume 30% of cells (assume MOI=0.3) will be selected by puro, 38.4 million for 16 wells (36.7 million) cells will be needed to start with

- 14.1 Transduction to be performed under the following conditions:
 - 16 wells for library infection (6 well plate)



- 2e6 cells/ml
- 1.2ml volume (2.4e6 cells)
- Add virus as planned from titer

- 14.2 6 hrs post-transduction, add 1 mL of media (final volume 2.2 mL).
- 14.3 Next morning, add 1 more mL of media, ~18 hrs post-transduction
- 14.4 At 24 hrs pool your experiment wells into one volume. Upon adding puro, set up a 24-well plate as below for assessment of transduction efficiency and survival. 50 uL cells should be enough for this purpose. Check cell count 48 hrs after adding puro.
- 14.5 Spin down all the other cells and resuspend in EDMII plus puro and count.
- 14.6 Count cells at 48hrs after puro. Mock should not be more than 7e5/mL per well.
- 14.7 Count cells every other day to monitor the cell growth if needed.
- 14.8 Media changes should be performed every other day. Split to 2e5/mL each time. Don't spin down, just add fresh media. In two days, this will grow to 8e5/mL. Growth will slow around D8 of erythroid differentiation so do not split harshly at later stages of experiment. Maintain cells in T175s in 60 mL (recommend 80 ml). At 2e5/mL in 60mL, you are maintaining 1000X coverage in a library of 12000 guides (because 1.2e7 cells)
- 14.9 Collect cells at D7 and D14, this is for proliferation screen. For sorting screens, check flow on day 7 to make sure the controls and library infected groups worked well, then do sorting on day 14
- 14.10 Proceed to sequencing the library

Library prep for sequencing

15 gDNA extraction:

- 15.1 Add 200 uL PBS for every 5 million cells (threshold for Qiagen Kit), divide into 200 uL per tube



15.2 Follow Qiagen DNEasy blood and tissue kit protocol

15.3 Elute with 100 uL H₂O per column

15.4 Pool like samples, record volume

15.5 qBit:

- Dilute 1 uL of gDNA in 10 uL H₂O
- mix = 198 uL buffer +1 uL dye per sample
- Add 1 uL diluted sample
- Standards: 188 uL buffer +1 uL dye + 10 uL standard

Also, we recommend checking gDNA concentration by using Nanodrop to make sure 260/280 were good, because the quality of gDNA will influence PCR.

15.6 Calculate % recovery:

1 cell contains ~ 0.0066 ng gDNA

Expected DNA yield (ug) = (recorded cell number * 0.0066)/1000

% recovery = actual yield (ug)/expected yield (ug) x100

16 **PCR1:**

- 16.1
- Make a table like this "example_spreadsheet_PCR1"
 - To calculate uL of gDNA for PCR1: recommended gDNA amount (ng)/concentration (ng/uL)
 - Reasonable range 10-50 uL
 - No more than 2.5 ug gDNA per 100 uL PCR1, ex 10 ug gDNA you will use 4 reactions, at LEAST 3 reactions even total gDNA was less than 7.5ug
 - H₂O (for a sample) = 45 * number of rxns - uL gDNA

16.2 Pre-PCR1 test (optional: to check whether the gDNA was good enough for PCR, if not, gDNA can be re-purified before start the PCR1. Also, suggest to have backup cell pellets):

Run a 20uL reaction using 1uL gDNA for each sample, 30 cycles, run all 20uL on 1.2% agarose gel



	A	B
	Q5	10 uL
	Fw (U6PE1)	0.5 uL
	Rv (P7 anchor)	0.5 uL
	H2O	8 uL

Cycling (30 cycles):

	A	B	C	D	E	F
	98 °C	98 °C	65 °C	72 °C	72 °C	4 °C
	10 min	10 sec	30 sec	60 sec	5 min	remaining

16.3 PCR1 reaction system for 100 uL reaction:

	A	B
	Q5	50 uL
	Fw (U6PE1)	2.5 uL
	Rv (P7 anchor)	2.5 uL
	H2O	45 uL

Number of reactions can be calculated by dividing ug gDNA by 2.5

Cycling:

	A	B	C	D	E	F
	98 °C	98 °C	65 °C	72 °C	72 °C	4 °C
	10 min	10 sec	30 sec	60 sec	5 min	remaining

16.4 Post PCR1 (for samples):



- Pool like samples, remove 15uL of pool and run an additional 20 cycles (total 33 cycles)
- Run all 15uL on gel
- To make sure all the PCR reactions worked well

17 **PCR1 reaction system for plasmid libraries:**

	A	B
	Q5	50 uL
	Fw (U6PE1)	2.5 uL
	Rv (P7 anchor)	2.5 uL
	H2O	44 uL
	Plasmid (diluted to 1/ng/uL)	1 uL

7 cycles, post-PCR1 for plasmid libraries: purify using Zymo kit, elute using 50 uL of warmed H2O

18 **PCR2:**

18.1 PCR 2 50 uL Reaction system:

	A	B
	Q5	25 uL
	Fw (i5 sequencing adapter)	1.25 uL
	Rv (P7 anchor)	1.25 uL
	H2O	16.5 uL

Aliquot 44 uL, add 6 uL unpurified PCR1 product

Cycling:

	A	B	C	D	E	F
	98 °C	98 °C	65 °C	72 °C	72 °C	4 °C
	3 min	10 sec	30 sec	60 sec	5 min	remaining

23 cycles

Run 5uL on gel, if over-amplified, repeat with reduced cycles, if under-amplified, purify pcr1 product (half of it) using bead purification method, first adding 0.5X beads to separate gDNA, then transfer supernatant to new tubes and add 1:1 beads and remove supernatant to separate primers, run PCR2 again. If bands clear but not too dark, proceed

18.2 Amplified Library sequence: ~295-300bp

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT[r1]
NNNNN[RS2]

ggaaaggacgaaacaccgNNNNNNNNNNNNNNNNNNNNNGTTTAAGAGCTATGCTGGAAACAG
CATAGCAAGTTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC
TTTTTTTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNAGATCGGAAGAGCAC
ACGTCTGAACTCCAGTCACNNNNNNNAGATCTCGTATGCCGTCTTCTGCTTG

022322_NEBNext_i501 AATGATACGGCGACCACCGAGATCTACAC

TATAGCCT AACTCTTTCCCTACACGACGCTCTTCCGATC*T

022322_NEBNext_i502 AATGATACGGCGACCACCGAGATCTACAC ATAGAGGC

AACTCTTTCCCTACACGACGCTCTTCCGATC*T

022322_NEBNext_i503 AATGATACGGCGACCACCGAGATCTACAC CCTATCCT

AACTCTTTCCCTACACGACGCTCTTCCGATC*T

18.3 PCR2 for plasmids:

	A	B
	Q5	25 uL
	Fw (i5 sequencing adapter)	1.25 uL
	Rv (P7 anchor)	1.25 uL
	PCR1 (purified)	22.5 uL

9 cycles, run on gel



19 **Bead purification of PCR2 (sample and plasmids) – SPRI selection by size:**

- 19.1 Let bead mixture stand at room temperature 30 mins
- 19.2 Shake well to mix
- 19.3 Add 45uL PCR2 and 45uL beads to the thicker 8 strip tubes (1:1)
- 19.4 Mix well – pipette and vortex
- 19.5 Let stand 2 mins
- 19.6 Place in magnetic rack for 2-3 mins until solution is clear
- 19.7 Discard supernatant
- 19.8 Add 180uL 85% Ethanol, wait 1 minute
- 19.9 Remove ethanol, repeat wash
- 19.10 Remove ethanol, spin down
- 19.11 Replace on magnet, wait 1 min, remove remaining EtOH with small pipette
- 19.12 Wait 2-3 minutes until beads have turned lighter brown (on magnet)



19.13 Add 30 uL H₂O, vortex, wait 2 minutes (off magnet)

19.14 Place on magnet rack, wait 2 minutes

19.15 Remove liquid to labeled tubes

19.16 Post-PCR2 prep for sequencing:

- Qbit all PCR2 samples (no need to dilute, usually around 20ng/ul to 80ng/ul)
- Pool
- Qbit final pool
- Run tapestation
- KAPA qPCR

19.17 Pooling:

Make a spreadsheet with all concentrations and recommended reads, calculate percentage of total reads for each sample, then scale by the factor your library is of the total. Example, your library is 80% of total- to make all the ng of samples you are pooling add to 100%, multiply by (100/80) or 1.25. Divide this by the concentration to get uL to pool. Then multiply those values by a factor that will give you reasonable volumes for pipetting.

Example:

https://docs.google.com/spreadsheets/d/1b2u_nt4Zsnw2EHLTCIUDHaDQAV1wgFIfV1ATXmFfbD4/edit#gid=1344171986

19.18 Tapestation:

- Aglient HS d1000
- Allow reagents to stand at room temperature 30 mins
- Vortex sample and reagents
- Ladder: 2uL sample
buffer + 2uL ladder in 1st tube of optical strip tube
- Sample: 2uL sample
buffer + 2uL sample
- Mix well, spin down
- Load into machine - remove caps, load into machine, make sure enough tips
- Load screentape, landscape orientation with QR code facing the green light
- Label samples on computer
- Run
- Peaks should be about 300 bp and no primer dimers

19.19 KAPA qPCR:

- Roche KAPA library quantification kit, illumina platforms
- Dilute library 10^4 x and 10^6 x (dilute 0.5ul in 50 H₂O, serially dilute 1uL into 100ul H₂O)
- Reaction system (20uL reaction)
- KAPA master mix 12.0 uL
- Rox Low 0.4 uL
- H₂O 3.6 uL
- Aliquot 16
- Add 4uL standard/sample/H₂O

	A	B	C	D	E	F
	Standard 1 20 pM	Standard 1 20 pM	Standard 1 20 pM	NTC	NTC	NTC
	Standard 2 2 pM	Standard 2 2pM	Standard 2 2pM			
	Standard 3 0.2 pM	Standard 3 0.2 pM	Standard 3 0.2 pM			
	Standard 4 0.02 pM	Standard 4 0.02 pM	Standard 4 0.02 pM			
	Standard 5 0.002 pM	Standard 5 0.002 pM	Standard 5 0.002 pM			
	Standard 6 0.0002 pM	Standard 6 0.0002 pM	Standard 6 0.0002 pM			
	10^4 dilution	10^4 dilution	10^4 dilution			
	10^6 dilution	10^6 dilution	10^6 dilution			

Step	Temp.	Duration	Cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	30 sec	35
Annealing/Extension/ Data acquisition	60°C	45 sec ¹	
Melt curve analysis ²	65 – 95°C		

Analyze using [KAPA analysis spreadsheet](#)

19.20 Pooling sampling based on nM from Tapestation into a single tube

Pooling Calculation:

$[(\text{desired read per sample})/(\text{desired total read across all samples})]/\text{Tapestation}$

nM*X000(random number to get a reasonably pipettable amount)

***Use no more than 1/3 of total volume 30 uL of purified PCR2

SPRI bead purification: to remove any undesirable bands (eg primer dimers) observed on gel

- Make a pool of sample, mix well, take ½ for SPRI
- Add dH2O or EB up to 50 uL
- Perform 0.9X bead purification (add 45 uL beads to pool) because product is ~300 bp.
- For gRNA library prep, don't worry about removing larger products (eg 500 bp) because we always see the second hump
- Second hump will be more intense if you overcycle PCR2

19.21 NGS:

Collect > 200 average reads per library member to be safe (so for 7,500-member library, aim for >1.5M reads)

- Read 1: >=50 nt
- Read 2: >=36 nt
- Index 1: 6 nt
- Index 2: 8 nt

19.22 NGS read analysis:

Read 1 (min 50 nt):



GACGTcgaTGGAAAGGACGAAACACCG NNNNNNNNNNNNNNNNNNNNNNNN

Note that SpRY has self-editing, so there may be A↯G/C↯T substitutions in the gRNA

Read 2 (min 36 nt):

[4-nt barcode in revcomp of orientation in library][32-nt target in revcomp of orientation in library]

Measure fraction of A↯G/C↯T editing to normalize phenotypic measurements of LDL uptake differences.

Index 1: 6-nt random barcode unique to each cloned gRNA

Index 2: i5 index