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TITLE:

HOX Loci Focused CRISPR/sgRNA Library Screening Identifying Critical CTCF Boundaries

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KEYWORDS:

CRISPR/Cas9, sgRNA library screening, CTCF boundary, *HOX* loci, one-step RT-qPCR, Indel mutation detection, Acute Myeloid Leukemia

SUMMARY:

A CRISPR/sgRNA library has been applied to interrogating protein-coding genes. However, the feasibility of a sgRNA library to uncover the function of a CTCF boundary in gene regulation remains unexplored. Here, we describe a *HOX* loci specific sgRNA library to elucidate the function of CTCF boundaries in *HOX* loci.

ABSTRACT:

CCCTC-binding factor (CTCF)-mediated stable topologically associating domains (TADs) play a critical role in constraining interactions of DNA elements that are located in neighboring TADs. CTCF plays an important role in regulating the spatial and temporal expression of *HOX* genes that control embryonic development, body patterning, hematopoiesis, and leukemogenesis. However, it remains largely unknown whether and how *HOX* loci associated CTCF boundaries regulate chromatin organization and *HOX* gene expression. In the current protocol, a specific sgRNA pooled library targeting all CTCF binding sites in the *HOXA/B/C/D* loci has been generated to examine the effects of disrupting CTCF-associated chromatin boundaries on TAD formation and *HOX* gene expression. Through CRISPR-Cas9 genetic screening, the CTCF binding site located between *HOXA7/HOXA9* genes (CBS7/9) has been identified as a critical regulator of oncogenic chromatin domain, as well as being important for maintaining ectopic *HOX* gene expression patterns in MLL-rearranged acute myeloid leukemia (AML). Thus, this sgRNA library screening

approach provides novel insights into CTCF mediated genome organization in specific gene loci and also provides a basis for the functional characterization of the annotated genetic regulatory elements, both coding and noncoding, during normal biological processes in the post-human genome project era.

INTRODUCTION:

Recent genome interaction studies revealed that the human nuclear genome forms stable topologically associating domains (TADs) that are conserved across cell types and species. The organization of the genome into separate domains facilitates and restricts interactions between regulatory elements (e.g., enhancers and promoters). The CCCTC-binding factor (CTCF) binds to TAD boundaries and plays a critical role in constraining interactions of DNA elements that are located in neighboring TADs[1]. However, genome wide CTCF binding data revealed that although CTCF mostly interacts with the same DNA-sites in different cell types, it often functions as a chromatin barrier at a specific site in one cell type but not in the other, suggesting that CTCF functions together with other activities in the formation of chromatin boundaries[2]. What remains unknown is whether the boundary elements (CTCF-binding sites) are directly linked to the biological function of CTCF, and how these links occur. Therefore, we hypothesize that specific CTCF binding sites in the genome directly regulate the formation of TADs and control promoter/enhancer interactions within these domains or between neighboring domains. The completion of the human and mouse genome sequencing projects and subsequent epigenetic analyses have uncovered new molecular and genetic signatures of the genome. However, the role of specific signatures/modifications in gene regulation and cellular function, as well as their molecular mechanism(s), have yet to be fully understood.

Multiple lines of evidence support that the CTCF-mediated TADs represent functional chromatin domains[3-5]. Although CTCF mostly interacts with the same DNA-sites in different cell types, genome wide CTCF ChIP-seq data revealed that CTCF often functions as a chromatin barrier in one cell type but not in the other[2]. CTCF plays an essential role during development by mediating genome organization[4,6,7]. Disruption of CTCF boundaries impaired enhancer/promoter interactions and gene expression, leading to developmental blockage. This suggests that CTCF mediated TADs are not only structural components, but also regulatory units required for proper enhancer action and gene transcription[5,8,9].

HOX genes play critical roles during embryonic development and they are temporally and spatially restricted in their expression patterns. The *HOXA* locus forms two stable TADs separating anterior and posterior genes by a CTCF-associated boundary element in both hESCs and IMR90 cells[1]. Recent reports demonstrated that *HoxBlinc*, a *HoxB* locus associated lncRNA, mediates the formation of CTCF directed TADs and enhancer/promoter interactions in the *HOXB* locus. This leads to anterior *HOXB* gene activation during ESC commitment and differentiation[10]. Furthermore, at specific gene loci including the *HOXA* locus, alteration of CTCF mediated TAD domains changed lineage specific gene expression profiles and was associated with the development of disease states[11,12]. The evidence supports a primary function for CTCF in coordinating gene transcription and determining cell identity by organizing the genome into functional domains.

Despite its role in the embryonic development, during hematopoiesis, *HOX* genes regulate hematopoietic stem and progenitor cell (HS/PC) function. This is done by controlling the balance between proliferation and differentiation[10,13-15]. The expression of *HOX* genes is tightly regulated throughout the specification and differentiation of hematopoietic cells, with highest expression in HS/PCs. *HOX* gene expression gradually decreases during maturation, with its lowest levels occurring in differentiated hematopoietic cells[16]. *HOX* gene dysregulation is a dominant mechanism of leukemic transformation by dysregulating self-renewal and differentiation properties of HS/PCs leading to leukemic transformation[17,18]. However, the mechanism of establishing and maintaining normal vs. oncogenic expression patterns of *HOX* genes as well as associated regulatory networks remains unclear.

CRISPR-Cas9 sgRNA library screening has been widely used to interrogate protein-coding genes[19] as well as non-coding genes, such as lncRNA[20] and miRNA[21] in different species. However, the cost to use the CRISPR-Cas9 sgRNA library to identify new genomic targets remains high, because high-throughput genome sequencing is often applied to verify the sgRNA library screening. Our sgRNA screening system is focused on the specific genome loci and evaluates the targeting sgRNAs through one-step RT-PCR according to the marker gene expression, such as *HOXA9*. Additionally, Sanger sequencing confirmed that the sgRNA was integrated into the genome, and Indel mutations can be detected to identify the sgRNA targeting site. Through the loci-specific CRISPR-Cas9 genetic screening, the CBS7/9 chromatin boundary has been identified as a critical regulator for establishing oncogenic chromatin domain and maintaining ectopic *HOX* gene expression patterns in AML pathogenesis[12]. The method can be widely applied to identify not only specific function of CTCF boundary in embryonic development, hematopoiesis, leukemogenesis, but also CTCF boundary as potential therapeutic targets for future epigenetic therapy.

PROTOCOL:

1. CTCF sgRNA library design using an online tool

1.1. Design the sgRNA targeting CTCF binding sites in the human *HOX* loci using the genetic perturbation platform (GPP) designer tool (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design).

1.2. Synthesize a total of 1,070 sgRNAs consisting of sgRNAs targeting 303 random targeting genes, 60 positive controls, 500 non-Human-targeting controls, and 207 CTCF elements or lncRNA targeting genes (**Figure 1, Table 1**). Each targeting DNA element is targeted by 5-10 different sgRNAs.

2. sgRNA library cloning

2.1. Clone the synthesized oligonucleotides into the CRISPR lentiviral backbone vector (lentiCRISPRv2).

2.1.1. Digest the LentiCRISPRv2 vector with BsmBI restriction enzyme at 37 °C for 2 h.

2.1.2. Look for the presence of the larger band (around 12,873 bp) on the gel after BsmBI digestion, and then purify it with the gel extraction kit.

NOTE: A 2kb small filler piece is also present on the gel after digestion, but this should be ignored.

2.1.3. Ligate the synthesized oligonucleotides and digested LentiCRISPR vector with 150 ng of digested LentiCRISPR DNA, 1 µL of 10 µM oligos, 2 µL of 10x T4 ligase buffer, 1 µL of T4 ligase, and then incubate them at 16 °C overnight.

2.2. Transform the lentiviral CRISPR/sgRNA library into electro-competent cells for amplification.

2.2.1. Prepare the electroporator at 1.8 kV, 200 ohms and 25 µF. Then pre-warm the recovery SOC media in a 37 °C water bath, and pre-warm LB ampicillin antibiotic plates at 37 °C.

2.2.2. Thaw the competent cells on ice for 10 min.

2.2.3. Prepare 1.5 mL micro-centrifuge tubes and 1 mm electroporation cuvettes on ice.

2.2.4. Mix 1 µL of a 10 ng/µL library plasmid DNA into 25 µL of competent cells in a 1.5 mL micro-centrifuge tube, and gently mix by flicking the bottom of the tube a few times manually.

2.2.5. Once the cuvette is cold enough, transfer the DNA/competent cell mixture to it. Tap twice on the countertop and wipe any water droplets from the exterior of cuvette with a tissue paper. Then place the cuvette in the electroporation module and press pulse.

2.2.6. Immediately add 975 µL of 37 °C pre-warmed SOC media. Mix by pipetting up and down and transfer to a 15 mL tube.

2.2.7. Rotate and incubate at 37 °C for 1 h.

2.2.8. Dilute 100 µL cells into 900 µL of SOC media and place 100 µL on a LB ampicillin antibiotic agar plate. Incubate overnight at 37 °C.

2.3. Extract the plasmid DNA from the combined colonies using a maxi-prep column as detailed in the manufacturer's protocol.

2.3.1. Scrape all the colonies from the LB agar plate and inoculate a starter culture of 2 mL of LB ampicillin antibiotic medium and incubate overnight at 37 °C with vigorous shaking (approx. 200 x g).

2.3.2. Dilute the starter culture 1:500 into 100 mL of LB ampicillin medium and incubate at 37 °C for 12-16 h with vigorous shaking (approx. 200 x g).

2.3.3. Harvest the bacterial cell pellet by centrifugation at 6,000 x *g* for 15 min at 4 °C.

2.3.4. Re-suspend the bacterial pellet in 10 mL of suspension buffer.

2.3.5. Lyse the suspended pellet with 10 mL of the lysis buffer, and vigorously invert 4-6 times. Incubate the lysate for 5 min at room temperature.

2.3.6. Neutralize the lysate with 10 mL of chilled Neutralization Buffer. Mix by gently inverting the tubes 4-6 times and incubate it for 20 min on ice.

2.3.7. Spin down at 13,500 x *g* for 30 min at 4 °C. Promptly transfer the supernatant containing the plasmid DNA to a new tube.

2.3.8. Repeat step 2.3.7, and promptly transfer the supernatant containing the plasmid DNA to a new tube.

2.3.9. Equilibrate the column by applying 10 mL of equilibration buffer and allow the column to empty by gravity flow.

2.3.10. Add the supernatant to the column and allow it to enter the resin by gravity flow.

2.3.11. Wash the column with 2 x 30 mL of washing buffer.

2.3.12. Elute the DNA with 15 mL of elution buffer.

2.3.13. Precipitate the DNA with 10.5 mL of room-temperature isopropanol to the eluted DNA. Mix and spin down immediately at 15,000 x *g* for 30 min at 4 °C, and gently decant the supernatant.

2.3.14. Wash the DNA pellet with 5 mL of 70% ethanol, centrifuge DNA pellet at 15,000 x *g* for 10 min and discard the clear supernatant.

2.3.15. Repeat step 2.5.14 twice more.

2.3.16. Centrifuge DNA pellet at 15,000 x *g* for 10 min, and gently decant the supernatant without disturbing the DNA pellet.

2.3.17. Air-dry the pellet for 5-10 min, and dissolve the DNA in a required volume of buffer (TE buffer, pH 8.0).

3. The high titer sgRNA library lentivirus generation

3.1. Cell preparation: Culture HEK293T cells in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1% (vol/vol) penicillin-streptomycin (PS) antibiotic in T-25 flasks. Place them in the incubator at 37 °C and 5% CO_2.

3.2. Package lentivirus: Co-transfect HEK293T cells with 20 µg of purified library vectors from step 2, 15 µg of the package plasmid (psPAX2) and 10 µg of the envelope plasmid (pMD2.G) for 48 h before harvesting the viruses.

3.3. Virus collection: After 48 h, collect the virus supernatant and filter the virus supernatant through a 0.45 µm low protein binding PVDF membrane.

3.4. Virus concentration: Concentrate the lentiviral supernatant by 50-fold using the concentrator and test the virus MOI in step 5.

3.5. Virus storage: Aliquot the concentrated viruses and store in a -80 °C freezer.

4. Optimized puromycin concentration

4.1. Leukemia cell culture: Culture MOLM13 AML cells in RPMI 1640 supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1x penicillin-streptomycin (PS) antibiotics in a T-125 flask. Place them in an incubator at 37 °C and 5% CO_2.

NOTE: Cells are typically passed every 4-5 d at a split ratio of 1:4 or 1:6, never allowing cells to reach more than 70% confluency.

4.2. Set up MOLM13 cells in a 12-well plate with a density of 1.0 x 10^4 cell/mL, at a total volume of 2 mL per well (2.0 x 10^4 cells).

4.3. Time-course assay: Treat MOLM13 cells with puromycin for 7 days in increasing concentrations (0.1 µg/mL, 0.2 µg/mL, 0.5 µg/mL, 1.0 µg/mL and 2.0 µg/mL)

4.3.1. Set up MOLM13 cells without puromycin treatment on day 0 and set up 3 replicate wells without puromycin treatment as a control from day 0 to day 7.

4.3.2. Treat MOLM13 cells with 0.1 µg/mL, 0.2 µg/mL, 0.5 µg/mL, 1.0 µg/mL and 2.0 µg/mL, separately, with each experimental condition containing 3 replicate wells.

4.3.3. Count the live cell ratio and make a survival curve from day 0 to day 7 containing all conditions.

4.4. Survival curve: Stain cells with Trypan blue and count viability daily to obtain the survival curves for each puromycin concentration.

4.5. Optimizing minimal puromycin concentration: Determine the minimal puromycin concentration through Trypan blue staining, in which all MOLM13 cells are killed between 5-7 days.

5. Titration of lentiviral library in MOLM13 leukemia cells

5.1. AML cells preparation: Collect MOLM13 AML cells with the transduction medium (RPMI 1640, 10% FBS, 1% PS, and 8.0 μg/mL coating medium) at a density of 1.5 x 10^6 cells /mL.

5.2. Place MOLM13 cells in the 12-well plate with 1.5 x 10^6 cells in each well.

5.3. Thaw the lentivirus: Remove the concentrated lentivirus from the -80 °C freezer and thaw it on ice.

5.4. Mix MOLM13 cells with a different dose of the concentrated lentivirus in separate wells, including 0, 1, 2.5, 5, 7.5 and 10 μL (total 6 groups).

5.5. Immediately centrifuge these mixtures at 1,000 x *g* for 2 h at 33 °C and transfer the 12-well plates back to the incubator at 37 °C and 5% CO_2 for 4 h.

5.6. After 4 h, spin down the infected cells at 400 x g for 5 min at room temperature.

5.7. Gently aspirate the supernatant without disturbing the cell pellet, and re-suspend the transduced cells with fresh media (RPMI 1640, 10% FBS and 1% PS), and then transfer them to T-25 flasks and incubate at 37 °C for 48 h without puromycin.

5.8. After 48 h, split these cells into 2 flasks (2 groups): an experimental group treated with 1 μg/mL puromycin for 5 days, and a control group without puromycin treatment for 5 days.

5.9. Carry out puromycin selection for 5 days with 1 μg/mL puromycin according to the step 4 until all the non-transduced control cells are dead. Exchange for fresh media every 2 days.

5.10. Measure the optimized MOI value for transduction by dividing the number of live cells treated with puromycin with the number of cells without puromycin treatment.

6. Transduction of the pooled CRISPR-Cas9 KO library

6.1. Transduction with lentivirus: Infect 1.5 x 10^6 MOLM13 cells with 0.3 MOI of sgRNA pooled lentivirus in medium (RPMI 1640, 10% FBS, 1% PS, and 8 μg/mL coating medium) in 6-well plate and use the cells without the lentivirus infection as a control.

6.2. Immediately centrifuge the 6-well plate at 1,000 x *g* for 2 h at 33 °C to spinfect the cells and transfer the plates back to the incubator at 37 °C and 5% CO_2 for 4 h.

6.3. Spin down the infected cells at 400 x g for 5 min at room temperature.

6.4. Gently aspirate supernatant without disturbing the cell pellet, and re-suspend the transduced cells with fresh media (RPMI 1640, 10% FBS and 1% PS), and then transfer them to T-25 flasks and incubate at 37 °C for 48 h without puromycin.

6.5. After 48 h, treat cells with 1 µg/mL puromycin for 5 days. Exchange for fresh media after 2 days and keep at an optimal cell density.

6.6. Seed the single clone in 96-well plates with limiting dilution methods and incubate these single clones at 37 °C and 5% CO_2. Culture them for 3-4 weeks.

6.7. After a single cell grows up into a population, transfer half of the cells into 24-well plates for further culture under puromycin selection and verify these clones in the next step. Keep the rest of the cells.

7. Screening of the pooled CRISPR-Cas9 KO library with one-step RT-qPCR

7.1. Determine the effectiveness of the sgRNA integrated clone screening by evaluating the expression of the marker gene *HOXA9* with one step reverse-transcriptase polymerase chain reaction (one-step RT-qPCR).

NOTE: *HOXA9* are highly expressed in MOLM13 AML cells in leukemogenesis[22,23].

7.2. Count the sgRNA integrated MOLM13 cell and transfer 1 x 10^4 cells per well to a 96-well PCR plate.

7.3. Centrifuge the tube at 1,000 x *g* for 5 min, and then thoroughly remove and discard the supernatant with a pipet without disturbing the cell pellet.

7.4. Wash cells with 125 µL of PBS buffer, and centrifuge the tube at 1,000 x *g* for 5 min. Then remove 120 µL of the supernatant using a pipette and retain approximately 5 µL of PBS in each well.

7.5. Add 50 µL of the cell lysis master mix containing 48 µL of cell lysis buffer, 1 µL of proteinase K solution (10 mg/mL) and 1 µL of DNase solution (1 mg/mL) to each well. Then pipet up and down 5 times to re-suspend the cell pellet.

7.6. Incubate the mix for 10 min at room temperature, followed by 5 min at 37 °C, and then 75 °C for 5 min.

7.7. Store the cell lysate at -80 °C freezer.

7.8. The preparation of one-step RT-qPCR reaction: Thaw the one-step reaction mix and other reaction components to 4 °C. Then spin down briefly to collect solutions at the bottom of tubes, and place on ice without light. Mix and spin gently.

7.9. Add 1 µL of cell lysate to the PCR wells with the RT-qPCR reaction mix, including 1 µL of the marker gene's forward primer (300 nM) and reverse primer (300 nM), 0.125 µL of reverse transcriptase (10 U/µL), and 5 µL of one-step reaction mix (2x).

7.10. Seal wells with optically transparent film, and gently vortex and mix the reaction components.

7.11. Place the 96-well PCR plate on a real-time PCR instrument.

7.12. Run the reverse transcription reaction for 10 min at 50 °C, followed by polymerase inactivation and DNA denaturation for 1 min at 95 °C.

7.13. Perform RT-PCR with 40 cycles of PCR reaction: denaturation for 15 s at 95 °C, annealing/extension and plate fluorescence reading for 20 s at 60 °C, and then melt curve analysis at 65-95 °C via 0.5 °C increments at 2-5 s/step.

7.14. Set up upregulated, downregulated and no change groups according to the expression levels of *HOXA9* gene by comparison to the control, separately. Use *β-actin* gene as a housekeeping gene control.

8. Verification of integrated sgRNAs positive clones through genotyping and Sanger sequence

8.1. Verify the *HOXA9* decreased expression clones through Sanger sequencing and perform PCR with 50-100 ng MOLM13 genome DNA, 5 µL polymerase reaction buffer (10x), 1 µL forward primer (10 µM) (AATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCG) and 1 µL reverse primer (10 µM) (TCTACTATTCTTTCCCCTGCACTGTTGTGGGCGATGTGCGCTCTG), 1 µL dNTP (10mM), 1 unit polymerase (5 U /µL). Perform the PCR reaction with the initial denaturation at 94 °C for 30 s, and then more denaturation at 94 °C for 20 s, annealing at 56 °C for 20 s, extension at 68 °C for 20 s (total 30 cycles), final extension at 68 °C for 10 min, and then holding at 4 °C.

8.2. Extract and purify the PCR products (size 285 bp) with a PCR Purification Kit.

8.3. Ligate the purified PCR products into the T vector with 2 µL T4 ligation buffer (10x), 50 ng T vector DNA (50 ng / µL), 25 ng purified PCR DNA (285 bp), 1 µL T4 ligase (3 units/µL), and place the ligation mix into an incubator at 16 °C overnight.

8.4. Transfer the ligation mix into DH5α competent cell, grow on a LB ampicillin antibiotic agar plate, and incubate overnight at 37 °C.

8.5. Pick the single clones from the LB plate and verify them by genotyping and Sanger sequencing.

9. Detection of sgRNAs induced Indel mutation by nuclease digestion assay

9.1. Detect the sgRNA integrated single clone induced Indel rates by a nuclease test assay.

9.2. Separately prepare PCR amplicons with 50-100 ng Indel mutant (test) and wild-type (WT, reference) DNA as PCR template, 5 µL polymerase reaction buffer (10x), 1 µL dNTP (10mM), 1 unit polymerase (5 units / µL), 1 µL forward primer (10 µM)(5'-GAGATGGCGGCGCGGAAG-3'), and 1 µL reverse primer (10 µM) (5'-AAATATAGGGCGGCTGTTCACT-3'). The PCR reaction was performed with initial denaturation at 98 °C for 30 s, and then denaturation at 98 °C for 20 s, annealing at 56 °C for 20 s, extension at 72 °C for 30 s (total 30 cycles), and final extension at 72 °C for 10 min, and holding at 4 °C.

9.3. Set up the heteroduplex mixture group with 200 ng of the "reference" (20 ng / µL) and 200 ng of "test" (20 ng / µL) PCR amplicons in 0.2 mL PCR tube, and the homoduplex mixture group with only 400 ng of "reference" PCR amplicons as a control.

9.4. Separately incubate the heteroduplex and homoduplex mixture at 95 °C for 5 min in a 1 L beaker filled with 800 mL of water and then cool down gradually to room temperature to anneal and form heteroduplex or homoduplexes.

9.5. Separately digest 400 ng of the annealed heteroduplex and homoduplex mixture with 1 µL indel mutation detection nuclease (2.5 units / µL) and 2 µL nuclease reaction buffer (10x) at 42°C for 60 min.

9.6. Analyze the digested samples with agarose gel electrophoresis, the heteroduplex mixture DNA should be cut into small fragments (70-250 bp), and the homoduplex DNA (320 bp) should not be cut.

REPRESENTATIVE RESULTS:

CRISPR-Cas9 technology is a powerful research tool for functional genomic studies. It is rapidly replacing conventional gene editing techniques and has high utility for both genome-wide and individual gene-focused applications. Here, the first individually cloned loci-specific CRISPR-Cas9-arrayed sgRNA library contains 1,070 sgRNAs consisting of sgRNAs targeting 303 random targeting genes, 60 positive controls, 500 non-Human-targeting controls, and 207 CTCF elements or lncRNA targeting genes in four *HOX* loci (**Figure 1, Table 1**). This library targets all CTCF core binding motifs, *HOX* gene associated lncRNAs, known regulatory elements, and several *HOX* genes as positive controls in the *HOX* loci. It also contains sgRNAs targeting random non-*HOX* genes, non-human genes and intergenic regions as negative controls. To enhance efficiency and specificity of CTCF site knock-out (KO) by lentiCRISPR transduction, each targeting site contains 5-10 sgRNAs (**Table 1**). In the protocol described here, sgRNA libraries are designed according to CTCF binding sites at the *HOXA/B/C/D* loci and lncRNAs in these loci, which is based on the Broad

Institute sgRNA tools (**Figures 1, 2**). After transduction at a low multiplicity of infection with a MOI of 0.3 in MOLM13 cells carrying the MLL-AF9 fusion, the infection rate is less than one sgRNA/cell followed by puromycin selection, and then the resistant clones grown from seeded single cell were screened for impairment of *HOXA9* gene expression.

The workflow for sgRNA library screening was briefly described (**Figure 3**). First, the virus containing sgRNA library were generated in HEK293T cells with the help of two vectors (psPAX2 and pMD2.G). sgRNA pooled library lentiviruses were concentrated and transduced into MOLM13 AML cells with polybrane (8.0 μg/mL). After a 48 h transduction, cells were treated with the optimal concentration of puromycin. After 5 days, the cells were seeded one cell/well into 96-well plates and the single clones were generated in the presence of puromycin. Finally, sgRNA single clones integrated into genome were identified by one-step RT-PCR, Sanger sequencing and Indel mutation detection (**Figure 3**). The puromycin resistant single clones are identified through one-step droplet digital RT-qPCR (RT-ddqPCR) according to altering expression of *HOXA9* oncogene (**Figure 4**). Genotyping and Sanger sequence were performed for sgRNA library construction and verification (**Figures 2, 4**).

sgRNA targeting MOLM13 positive clones in a 96-well PCR plate were further confirmed with the RT-qPCR method based on the expression levels of *HOXA9* genes through comparison with the control cells. Out of the 528 surviving clones screened, 10 clones exhibited more than 50% reduction in *HOXA9* levels (**Figure 4A**). sgRNAs integrated into the *HOXA9*-reduced, *HOXA9*-unchanged, and *HOXA9*-increased clones were further confirmed by PCR amplification of the sgRNA sequences using flanking vector primers. The purified PCR products were ligated into the T vector system through T4 ligase and sent out for identification by Sanger sequence (see step 8). The sequence data indicated that out of 30 clones sequenced, 21 clones included single sgRNA (**Table 2**). The categories of sgRNA were identified and analyzed according to the *HOXA9* expression levels. Six of ten clones showing a reduction in *HOXA9* levels contained sgRNAs targeting the CBS7/9 site, but not in the non-human genes, random human genes, and other CTCF site controls (**Figure 4 and Table 2**).

sgRNA integrated positive single clone-induced Indel mutations are determined by PCR-based genotyping and nuclease digestion based on the nuclease assay (**Figure 5**). The nuclease digestion assay has been performed to identify Indel mutations occurred in the CBS7/9 boundary in the representative *HOXA9*-reduced, *HOXA9*-unchanged, and *HOXA9*-increased clones. The results revealed that the CBS7/9 mutation has been found in 4 out of the 6 *HOXA9*-reduced clones: clones #5, 6, 28, and 121, but not in clones #15 and #31 (**Figure 5**). However, clone #15 contained the sgRNA targeting *HOTTIP* lncRNA site*,* while clone #31 contained several sgRNAs targeting *HOAIRM1* lncRNA, *HOTAIR* lncRNA, and *HOXD9/10* CTCF binding site (**Figures 4B, 5 and Table 2**).

FIGURE AND TABLE LEGENDS

Figure 1: Schematic diagram showing CTCF binding sites and lncRNAs in four *HOX* gene loci. Each targeting DNA element contains 5-10 different sgRNAs. CTCF ChIP-seq dataset was downloaded from GEO (GSM1335528) and visualized with Integrated Genomic Viewer (IGV). SgRNA targeting CTCF sites in *HOX* loci were labelled with orange scissors.

Figure 2: Schematic diagram representing the part of integrating sgRNA vector sequence and PCR amplification primers. The PCR amplification primers were designed according to the blank sequence of the sgRNA lentiviral vector. The forward primer (P1) was highlighted in yellow, the reverse primer (P2) was highlighted in red, and the sgRNA was highlighted in green in the sgRNA lentiviral vector.

Figure 3: Schematic diagram representing the workflow for sgRNAs library design, construction and verification. This workflow is as follows. First, the sgRNA library was designed and cloned into a lentiviral CRISPR vector, and then the lentivirus was packaged with the sgRNA library lentiviral vector, psPAX2 and pMD2.G vectors in the HEK293T cells. Next, MOLM13 cells were infected with a low MOI (0.3) virus and these cells underwent puromycin selection. Then, the single clone was seeded in a 96-well plate. Finally, the sgRNA single clones integrated into a genome were identified by one-step RT-qPCR, Sanger sequence and Indel mutation detection.

Figure 4: Pooled CRISPR-Cas9 KO library screening identified with one-step RT-qPCR and Sanger sequence. A. One step RT-droplet digital PCR screening of the *HOXA9* expression in single clones infected with lentivirus containing the sgRNA library. The screening of 528 sgRNA library infected clones for *HOXA9* expression levels is shown (528 dots). Ten of 528 clones exhibited more than 50% reduction in *HOXA9* levels (purple arrows). The red line signifies the boundary of a 2-fold decrease change by comparing with the control cells; the blue line signifies the boundary of a 2-fold increase change. **B**. The six clones #5, 6, 28, 121, 207 and 420 were targeted by the CBS7/9 specific sgRNA through Sanger sequence (green arrows). **C**. The RT-ddqPCR analysis of *HOXA9* levels in WT MOLM13 and the 21 clones containing single targeted sgRNA. The *HOXA9* expression data were grouped into five groups in accordance with the categories of sgRNA sequences: *HOXA7/9* CTCF site, non-human targets, other CTCF sites in the *HOX* loci, *HOX* associated lncRNAs, and other human targets (This figure has been modified from Luo et al.[12]). For statistics, this data was represented as the mean ± SD from three independent experiments with the Student's t-test.

Figure 5: Indel mutations of integrated sgRNAs positive clone confirmed with the PCR-based genotyping and nuclease assay. **A.** Genomic DNA was isolated from the representative CRISPR-Cas9 KO library screened clones that exhibited reduced, unchanged, or increased *HOXA9* expression levels. The heterozygous deletion of the CTCF site located between *HOXA7* and *HOXA9* genes (CBS7/9 boundary) was identified by PCR-based genotyping. The *HOXA9*-decreased clones #5, 6, 28, and 121 exhibited deletion in the CBS7/9 boundary location (black arrows). **B.** The Indel mutations in the CBS7/9 site were analyzed by the nuclease digestion assay from the representative clones that exhibited reduced (red line), unchanged (blue line), or increased (purple line) *HOXA9* expression levels. The *HOXA9*-decreased clones #5, 6, 28, and 121 exhibited mutations in the CBS7/9 boundary location (orange arrows). (This figure has been modified from Luo et al.[12])

Table 1. sgRNAs pool library targeting information (This data from Luo et al.[12]).

Table 2. Sanger sequencing results of sgRNAs presented in the selected *HOXA9*-decreased, *HOXA9*-unchanged, and *HOXA9*-increased clones. *HOXA9*-decreased, unchanged and increased clones are highlighted in red, blue and purple, separately. (This data from Luo et al.[12]).

DISCUSSION:

Protein-coding gene related sgRNA libraries have been applied in a functional screening system to identifying genes and networks regulating specific cellular functions through sgRNA enrichment[24-28]. Several non-coding region related sgRNA libraries were also shown in gene-specific functional screens for distal and proximal regulating elements, including *BCL11A*, *Tdgf1a* and drug-resistance regulating genes[28-30]. These sgRNA libraries were all generated by a detailed bioinformatics design, oligonucleotide synthesis, and sub-cloning the oligonucleotide pool(s) into vectors. The whole genome-wide screening approach is very powerful and useful but requires computational expertise for genome-wide sgRNA design and consistent funding for the expensive synthesis; thus, it is still challenging for most laboratories. However, our loci-specific sgRNA library screening approach is both convenient and efficient to identify the specific DNA element such as the CTCF binding site involved in chromatin organization and transcriptional regulation (**Figures 1 and 2**). By targeting the CTCF boundaries, we applied a one-step RT-PCR to evaluate sgRNA targeted clones according to the expression level of a specific marker gene, *HOXA9*. In addition, we performed Sanger sequencing to confirm these positive integrated sgRNAs clones (**Figures 2 and 4**). To functionally confirm these positive sgRNAs targeted clones, we carried out a PCR-based genotyping and mutation detection assay in order to determine whether the sgRNA induces the target site insert or deletion mutations (**Figure 5**). This gives us a promising method to target specific non-coding DNA elements and evaluate their biological function in mammalian cells.

In our protocol, we mentioned that a specific oligonucleotide design will ensure more efficient sub-cloning into lentiCRIPSRV2 vectors and more reliably generate an accurate sgRNA library (steps 1 - 2). In order to obtain the high titer sgRNA library lentivirus, the lentiviral supernatant should be concentrated 50-fold using the concentrator following the protocol (step 3), and stored in a -80 °C freezer in multiple aliquots (steps 3.1 - 3.5). An additional concern is finding the optimal MOI value for transduction. If the MOI is too low, the number of infected cells will decrease and lead to sgRNA screening failure. If the MOI is too high, it will integrate more than one sgRNA into a single cell, and it will interfere with the sgRNA library screening through the one-step RT-PCR and Indel mutation detection. Therefore, before screening, finding the optimal MOI for each group of cells through titration of the lentiviral library is an important step. Titration of the lentiviral library in MOLM13 leukemia cells and evaluation of the MOI will be carried out in the protocol (steps 5.1 - 5.10). Moreover, a thorough lysing of cells for reverse transcription can ensure successful one-step RT-PCR. This can be done by increasing the incubation time for lysis at all temperature stages in the protocol (steps 7.5 - 7.6). Therefore, in order to enhance the efficient for screening of the pooled CRISPR-Cas9 KO library, thorough cell lysis and reverse transcription play a critical role in determining the one-step RT-qPCR (steps 7.1 – 7.14). Additionally, increasing the quality of PCR products can ensure successful indel mutation detection, because low quality PCR products will affect the heteroduplex/homoduplex generation process (steps 9.1 – 9.6).

In addition, the method can be used to identify the role of CTCF in *HOX* gene regulation in early embryonic development and certain leukemia with aberrant *HOX* gene signature. For example, *HOX* genes play critical roles during embryonic development and all four clusters of *HOX* gens are temporally and spatially restricted in their expression patterns in embryonic development. Furthermore, NPM1 mutations are among the most common genetic abnormality in AML and account for 30% of AML patients with normal cytogenetic karyotype[31]. This subset of AML exhibits an aberrant *HOXA* and *HOXB* gene signature, which becomes a dominant mechanism of leukemic transformation[17]. It is critical to elucidate how *HOX* gene are regulated in normal development and dysregulated during leukemogenesis. We and others have shown that CTCF plays an essential role in chromatin organization and gene transcription in *HOX* loci[9,12]. Thus, the *HOX* loci focused sgRNA library screening provides a convenient means to entangle the specific function of the CTCF binding site in *HOX* gene regulation during development and hematopoietic malignancies. However, a limitation of the approach is the difficulty of finding a useful marker for the high-throughput next-generation sequencing. One of the future research goals will be to find a highly selective marker and carry out genome-wide next generation sequencing in order to see the marker's effects. Therefore, using a specific fluorescent marker-tagged gene as the tracking reporter will become a crucial tool in future research plans.

Enhancers play a multitude of critical roles in the regulation of promoter function and gene expression. However, it can also activate promoter activity from long distance in a position and orientation independent manner, and enhancers often regulate gene expression in a *trans* orientation. Thus, it is challenging to pinpoint the enhancer(s) for specific genes, especially in the post-genomic era. Traditional reporter assays and correlative functional analyses (e.g., chromatin immunoprecipitation and DNaseI hypersensitive assays) have been used to examine enhancer function[32,33]. Similarly, small scaled locus-focused screenings were also applied to explore the activities of distal and proximal regulatory elements for specific genes[34]. Recently, the pooled sgRNA-KO library strategy that targets non-coding regulatory elements in the *HOX* gene loci successfully identified a CTCF binding site located between *HOXA7* and *HOXA9* genes, as well as a *HOTTIP* lncRNA that is critical for controlling posterior *HOXA* chromatin domain organization, which drives ectopic *HOXA* gene expression in acute myeloid leukemia (AML)[12]. These studies demonstrated that the pooled sgRNA-KO library screening is also a powerful genetics approach to identify and evaluate biological function of non-coding elements in our genome in situ.

CTCF, as a chromatin insulator protein, plays an important role in genome organization by defining chromatin neighborhoods for specific gene expression patterns in specific cell type[11,35]. Alteration of topologically associated domain (TAD) structure changes the enhancer/promoter interactions, resulting in a diseased state[5,11]. CTCF is highly conserved in metazoan and is enriched at the TAD boundaries. However, it remains unclear whether and how CTCF contributes to maintain chromatin boundary structure and TAD formation. Although the pooled CTCF sgRNA-knockout library screening was focused on the four *HOX* loci, it proved to be a powerful method to identify and dissect CTCF boundaries, and define TAD domain as well as enhancer/promoter interaction and transcription within the TAD domain[12]. Additionally, this method can be

efficiently applied to identify the lncRNA elements and transcription factors that mediate chromatin conformation and accessibility activity in *HOX* loci. We are also trying to explore the CRISPR/sgRNA library containing the genome-wide CTCF sites through next generation sequencing identification according to the CTCF ChIP-seq and ChIA-PET data in future research. Thus, this strategy can be extended to a whole chromosome or even the whole genome.

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DISCLOSURES:

We have no conflicts of interest related to this report.

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Figure 1.Schematic diagram shows CTCF binding sites and lncRNAs in four HOX gene loci

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Figure 1

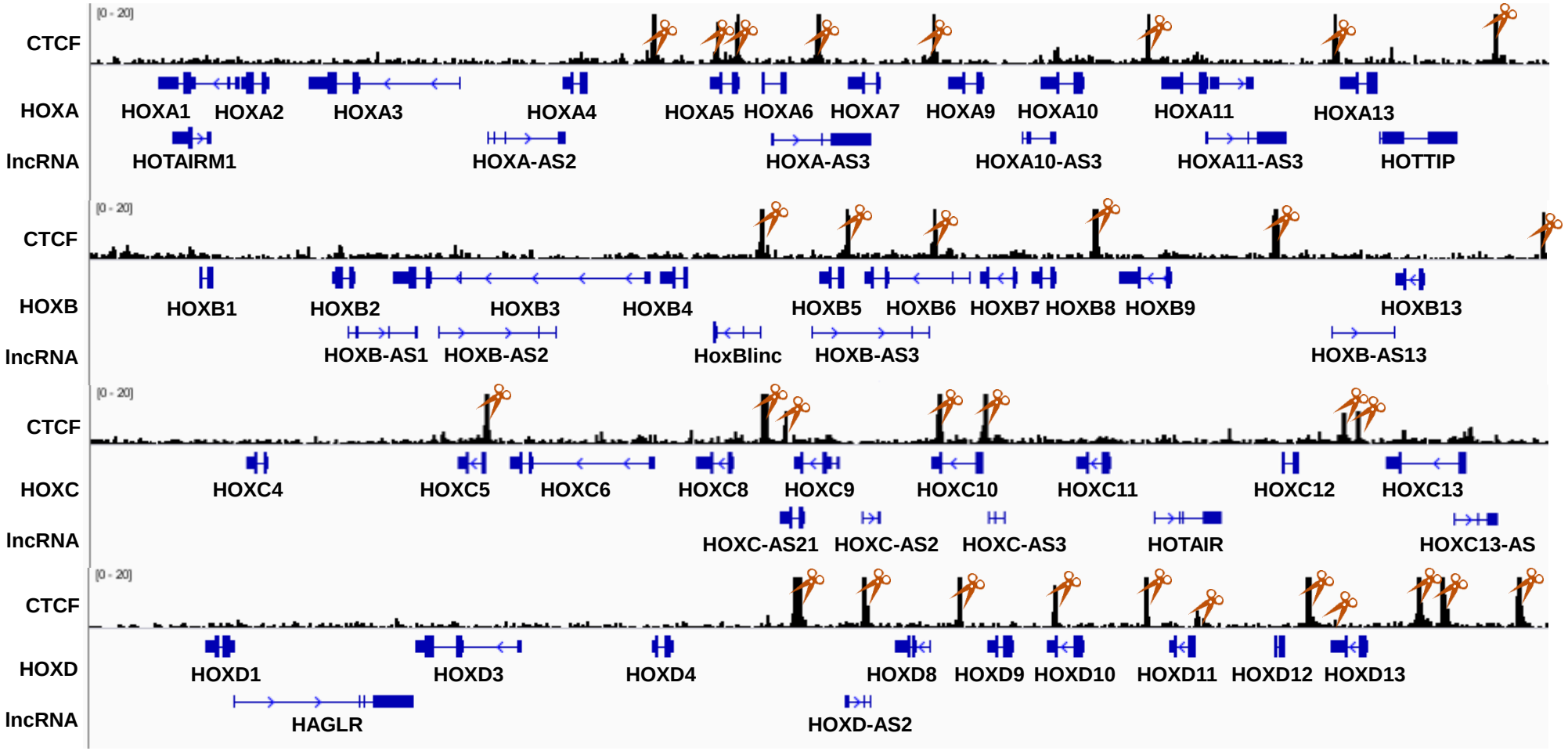
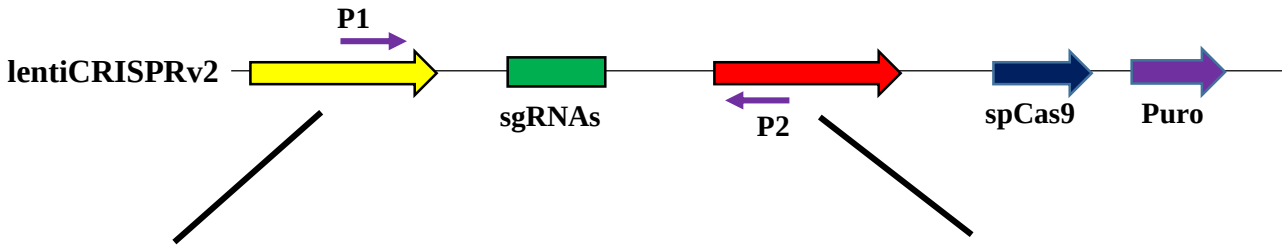


Figure 2.Schematic diagram represents the part of integrating sgRNA vector sequence and PCR amplification primers.

Figure 2



PCR product (~ 285 bp) is amplified from genomic DNA

AATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGG

ACGAAACACCGNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAG

TCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTGAATTCGCTAGCTAGGTCTTGAAAGGA

GTGGGAATTGGCTCCGGTGCCCGTCAGTGGGCAGAGCGCACATCGCCCACAACAGTGCAGGGGAAAGAA

TAGTAGA

PCR#1 Forward primer (P1): AATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCG

PCR#2 Reverse primer (P2): TCTACTATTCTTTCCCCTGCACTGTTGTGGGCGATGTGCGCTCTG

Figure 3. Schematic diagram represents workflow for sgRNAs library design, construction and verification.

Click here to access/download;Figure;Figure 3.pdf

Figure 3

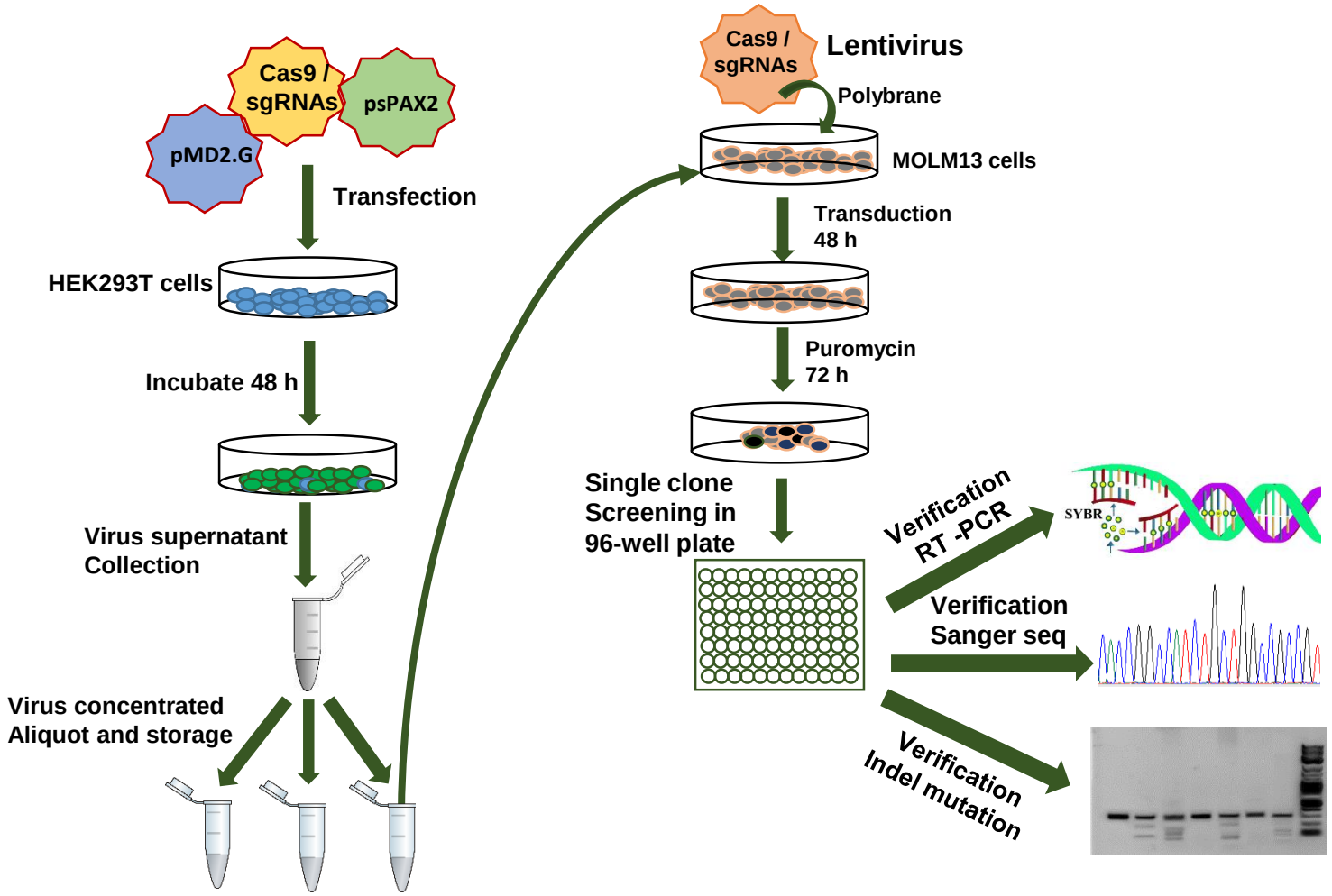
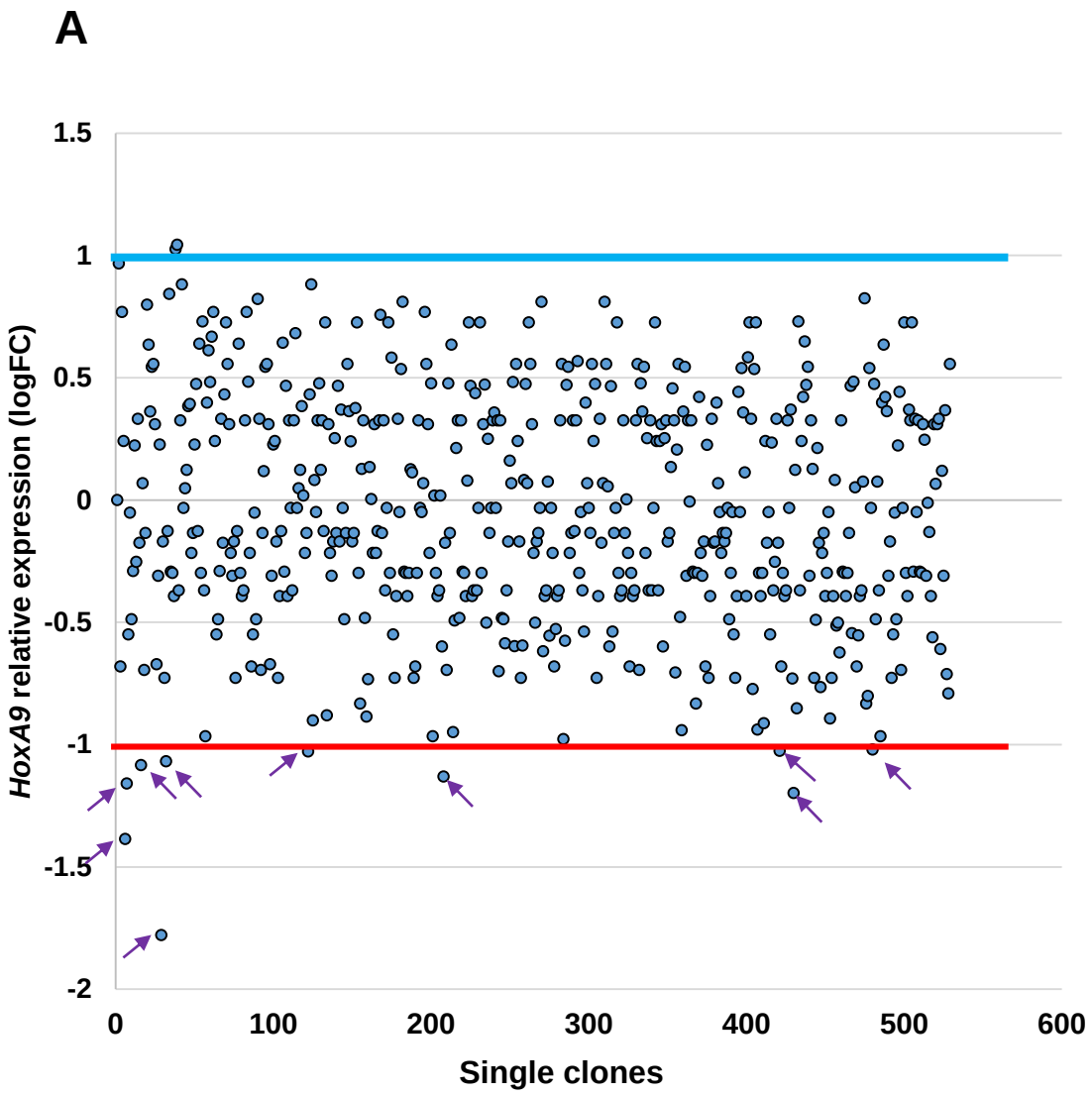
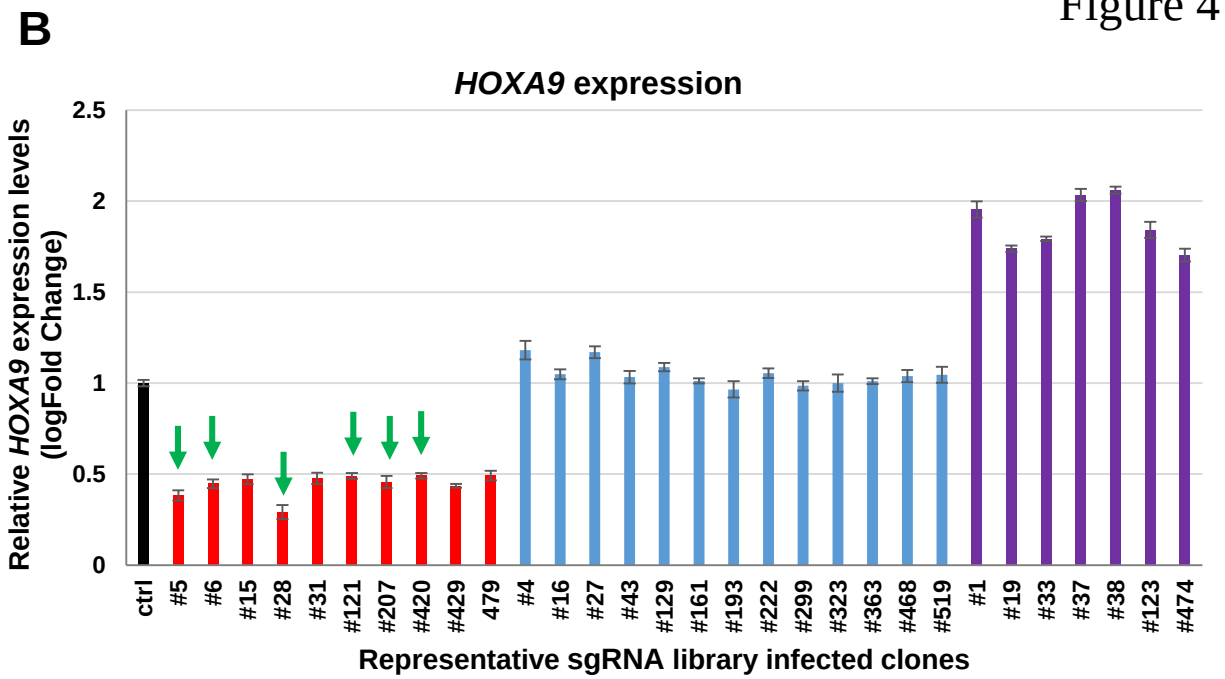


Figure 4. Pooled CRISPR-Cas9 KO library screening identified with one-step RT-qPCR and Sanger sequence.

Figure 4





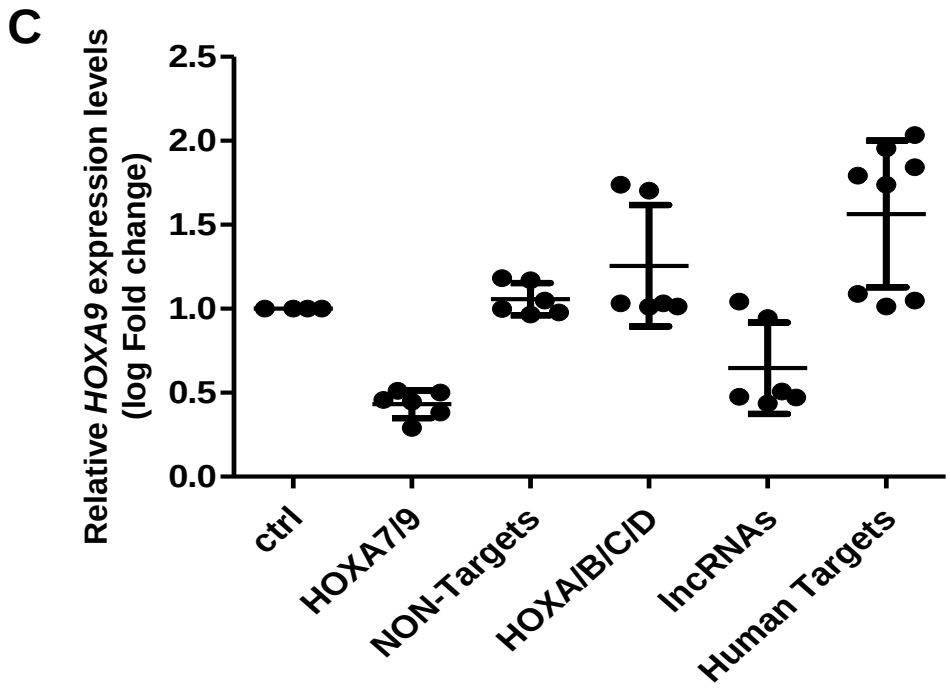
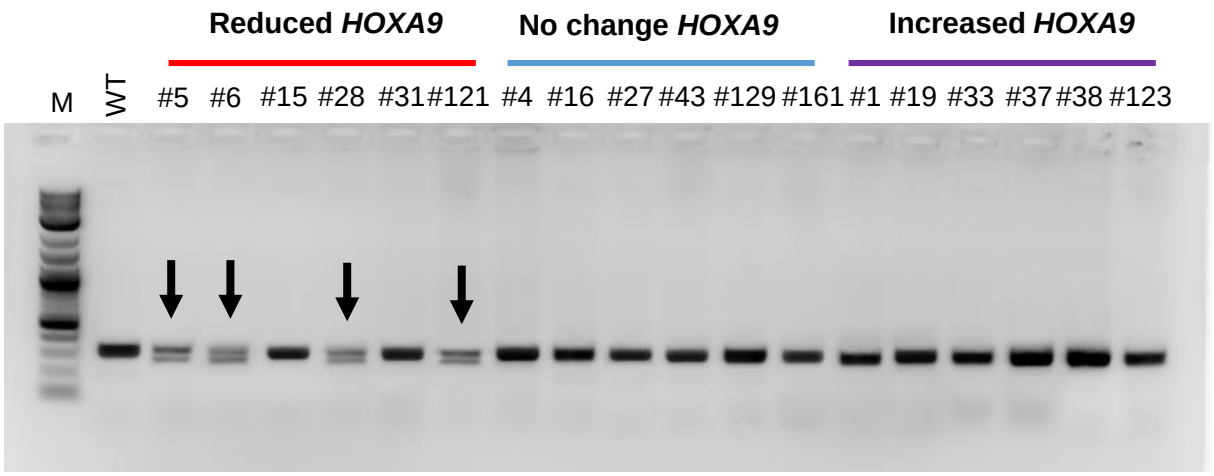


Figure 5. Indel mutations of integrated sgRNAs positive clone were confirmed with the PCR-based genotyping and nuclease assay.

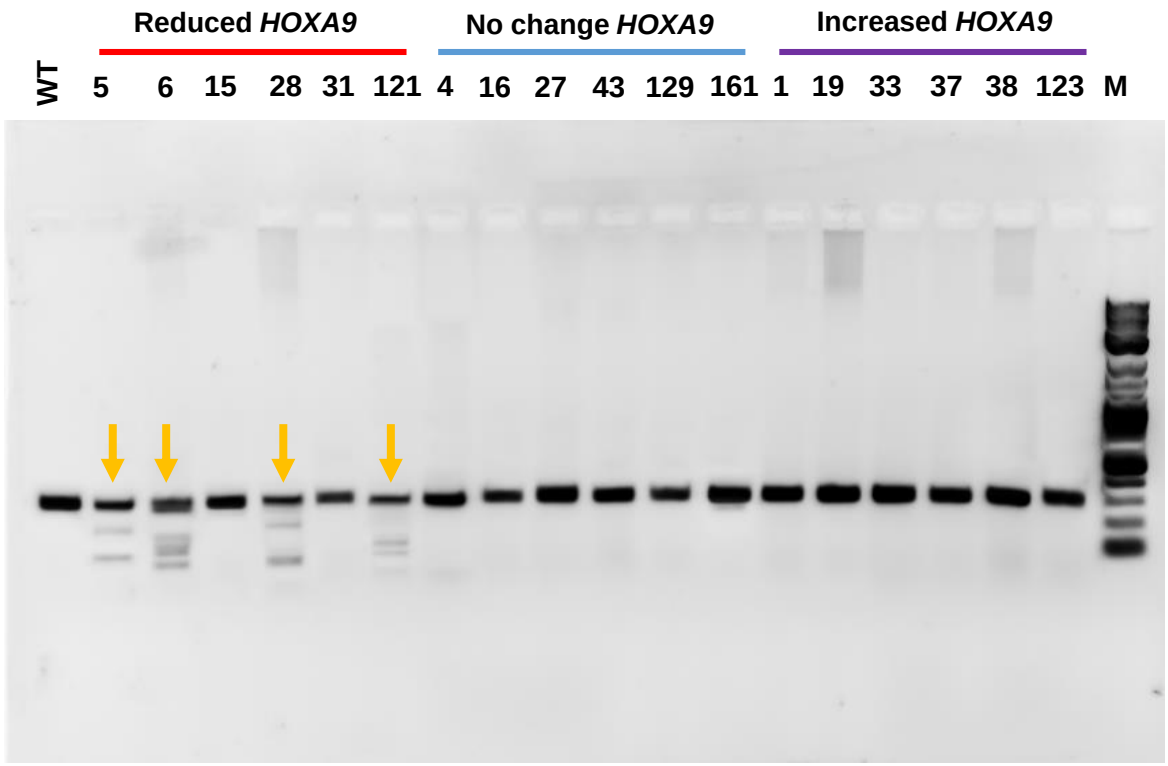
Figure 5

A



Representative sgRNA library infected clones

B



Representative sgRNA library infected clones

Lane 1: WT(reference) PCR products (homo-duplexes)
Lane 2-19: WT/test PCR products mixed (hetero-duplexes)
Land 20: marker (M)

Table 1. sgRNAs pool library targeting information. Click here to access/download;Table;table 1.xlsx

Gene/Motif	sgRNA sequence	Gene/Motif	sgRNA sequence
ABCC1	AAAATGTGATTGGCCCCAAG	HOXD89-F-3	AGCTTTACGAGATCAGAAAG
ABCC1	AACCTGACAGCATCGAGCGA	HOXD89-F-4	AATTAAATTCAGTGGACTGG
ABCC1	AGTACACGGAAAGCTTGACC	HOXD89-F-5	AAAAATTAAATTCAGTGGAC
ABCC1	CAAGTTCGTGAATGACACGA	HOXD89-F-6	TAAATTCAGTGGACTGGAGG
ABCC1	CCAGCCGAAAGAGAGTTCCA	HOXD10-F-1	CGCTCTTACTGATCTCTAGG
ABCC1	GCGCCACCGGCATGGCGCTC	HOXD10-F-2	TAACGCTCTTACTGATCTCT
ABCC1	TAGAAGTAGCCCTGCCAGTC	HOXD10-F-3	AGAGCGTTAACCTCACCGAC
ABCC1	TCTGCTTCGTCAGTGGCATG	HOXD10-F-4	GATCTCTAGGCGGCGCTCGC
ACAD11	AGAACGTTCAGCCATATATG	HOXD10-F-5	TGATCTCTAGGCGGCGCTCG
ACAD11	CCACTCCGATAGCTGTTGCA	HOXD10-F-6	CTAGGCGGCGCTCGCGGGTG
ACAD11	CCTTGGCCAAAAGTAGAACA	HOXD10-11-F-1	AACGTGAGCGCGCCCTCGTG
ACAD11	CTGAGCCAATGTTTCTACCG	HOXD10-11-F-2	GGGCCTGGAGATCCACACGA
ACAD11	TGCAGTGATACTTCTGTCAT	HOXD10-11-F-3	GGGGCCTGGAGATCCACACG
ACAD11	TTACGTAATGGAACATGTGC	HOXD10-11-F-4	TCTTGGTCAAACGCGGCTTC
ACAD11	TTGTTTGTACAGACTCGGAA	HOXD10-11-F-5	GCGCCCTCGTGTGGATCTCC
ACKR3	AAGACAGCGATAATGGAGAA	HOXD12-13-R-1	GCCAATGCCGCCCAATGCCC
ACKR3	ACTGGACGCCGAGATGGCTC	HOXD12-13-R-2	GAGCGCGCTCGCCATCTCCT
ACKR3	CATCTCGGCGTCCAGTGACC	HOXD12-13-R-3	GCCGCCCAATGCCCAGGAGA
ACKR3	CCAACAATGAGACCTACTGC	HOXD12-13-R-4	GGAGCGCGCTCGCCATCTCC
ACKR3	CCGTTTCCTTACCTCCGGGC	HOXD12-13-R-5	CGGCTGCGCCCCGATAGGCA
ACKR3	GCACTGCTACATCTTGAACC	HOXD12-13-R-6	CTCGCCATCTCCTGGGCATT
ACKR3	GCATTATATACACTGCAGAA	HOXD12-13-R-7	GCTCGCCATCTCCTGGGCAT
ACKR3	GGTCCACGCTCATGCACGTG	HOXD13-1-R-1	GACATCTAGCGCCAGGCGTG
ACSL3	ATGATTACTGCAATATCTGA	HOXD13-1-R-2	TTGCAGGGACATCTAGCGCC
ACSL3	CGAGTGGATGATAGCTGCAC	HOXD13-1-R-3	GGACATCTAGCGCCAGGCGT
ACSL3	GAAAGTTCGAAGCTTGCTAG	HOXD13-1-R-4	GGGACATCTAGCGCCAGGCG
ACSL3	GCAATGGTTTGCTATGAGGT	HOXD13-1-R-5	GGCTCCACTTCCCGGCCCGG
ACSL3	GTGGTGAAGAGTAACCAATG	HOXD13-1-R-6	TTCGGCTCCACTTCCCGGCC
ACSL3	TAACATACCCATGCTGGCCT	HOXD13-1-R-7	GGCGCGCAGTTCCCCACGCC
ACSL3	TATCTAAAGTATCACATCCA	HOXD13-1-R-8	CAGTGTTCGGCTCCACTTCC
ACSL3	TCACATAGTAACATTATTGC	HOXD13-2-R-1	ATGCCTTTATTGCTGTCGTT
ACTC1	CGATGGACGGGAAGACAGCG	HOXD13-2-R-2	TCACAGCAGCCGAAACCGCG
ACTC1	CTACAACTCACCAATGAAGG	HOXD13-2-R-3	AAACCGCGAGGAAAACAGAT
ACTC1	CTGGGCTTCATCACCTACGT	HOXD13-2-R-4	AACCGCGAGGAAAACAGATG
ACTC1	GGTACGGCCAGAAGCATACA	HOXD13-2-R-5	ACCGCGAGGAAAACAGATGG
ACTC1	GTGCTATCCCTGTATGCTTC	Non-Targeting Control 1	ACGGAGGCTAAGCGTCGCAA
ACTC1	GTGTGACATTGATATCCGCA	Non-Targeting Control 2	CGCTTCCGCGGCCCGTTCAA
ACTC1	TCTTCATGAGGTAGTCAGTG	Non-Targeting Control 3	ATCGTTTCCGCTTAACGGCG
ACTC1	TGGTACGGCCAGAAGCATAC	Non-Targeting Control 4	GTAGGCGCGCCGCTCTCTAC
ADGRE2	ACCGTCACAAGTCTCCATGG	Non-Targeting Control 5	CCATATCGGGGCGAGACATG
ADGRE2	AGACAAGGCCCACCACAGAA	Non-Targeting Control 6	GCGTGCGTCCCGGGTTACCC
ADGRE2	CAGACTCACCCCTGGAGTCC	Non-Targeting Control 7	CGGAGTAACAAGCGGACGGA
ADGRE2	CGAGAAAGACGAGAAAGACG	Non-Targeting Control 8	CGAGTGTTATACGCACCGTT
ADGRE2	CTGTTGCAGCATTCTGTGTC	Non-Targeting Control 9	CGACTAACCGGAAACTTTTT
ADGRE2	GAGAGCGAGAACACGTGTCA	Non-Targeting Control 10	CAGGAGTCGCCGATACGCGT
ADGRE2	GCACATCGTAGTGGGCCATG	Non-Targeting Control 11	TTCACGTCGTCTCGCGACCA

ADGRE2	TCCACCAGCACTCACACGGT	Non-Targeting Control 12	CGCTAGTACGCTCCTCTATA
ADGRG6	AACCCATTGGTAACCTACTG	Non-Targeting Control 13	CTATCTCGAGTGGTAATGCG
ADGRG6	AGCCAATATTACCAACATTG	Non-Targeting Control 14	AATCGACTCGAACTTCGTGT
ADGRG6	AGCGTATCATCCCTGTTACC	Non-Targeting Control 15	ACGTTCGAGTACGACCAGCT
ADGRG6	CAATAATGAATCGTATTTCC	Non-Targeting Control 16	GGTCACCGATCGAGAGCTAG
ADGRG6	CTAACAGAATCGATAAACAA	Non-Targeting Control 17	CGTATTCGACTCTCAACGCG
ADGRG6	TATCTGAATGATATAACCGG	Non-Targeting Control 18	GAATCGACCGACACTAATGT
ADGRG6	TCCTTAAGGACACGGCAACT	Non-Targeting Control 19	ACTTCAGTTCGGCGTAGTCA
ADGRG6	TTTGACCTGTTCCACAATGT	Non-Targeting Control 20	CGCCTAATTTCCGGATCAAT
ADK	AAAGTCGAATATCATGCTGG	Non-Targeting Control 21	CGTGGCCGGAACCGTCATAG
ADK	ACAGCAGAGATGTCAAGCAG	Non-Targeting Control 22	ACATAGTCGACGGCTCGATT
ADK	GAGCCACTTTAATTGAATTC	Non-Targeting Control 23	CGCCGGGCTGACAATTAACG
ADK	GCTTGACATCTCTGCTGTAG	Non-Targeting Control 24	CGTCGCCATATGCCGGTGGC
ADK	GTAGTAATGAGCATCCACAT	Non-Targeting Control 25	CGGGCCTATAACACCATCGA
ADK	TCTGGAGAAAAACTGGATGT	Non-Targeting Control 26	CGCCGTTCCGAGATACTTGA
ADORA2A	AAGCAGTTGATGATGTGTAG	Non-Targeting Control 27	CGGGACGTCGCGAAAATGTA
ADORA2A	ATGCTAGGTTGGAACAACTG	Non-Targeting Control 28	TCGGCATACGGGACACACGC
ADORA2A	CTCCACCGTGATGTACACCG	Non-Targeting Control 29	ATCGTATCATCAGCTAGCGC
ADORA2A	CTCCTCGGTGTACATCACGG	Non-Targeting Control 30	CGACGCTAGGTAACGTAGAG
ADORA2A	GAAGGGATTCACAACCGAAT	Non-Targeting Control 31	CATTGTTGAGCGGGCGCGCT
ADORA2A	GCGGCGGCCGACATCGCAGT	Non-Targeting Control 32	CCGCTATTGAAACCGCCCAC
ADORA2A	TAGCCATTGGGCCTCCGCTC	Non-Targeting Control 33	TTTACGATCTAGCGGCGTAG
ADORA2A	TGGCTTGGTGACCGGCACGA	Non-Targeting Control 34	GGTTAGAGACTAGGCGCGCG
ANKDD1A	ACGCACGTGGTTTCTGGCCC	Non-Targeting Control 35	CCTCCGTGCTAACGCGGACG
ANKDD1A	ACTTACATGATCAACCACGT	Non-Targeting Control 36	TTATCGCGTAGTGCTGACGT
ANKDD1A	GGCTGTGCTGCAGCGACTTG	Non-Targeting Control 37	CGCGGCCCACGCGTCATCGC
ANKDD1A	GGGGAACACTGCCCTTCATC	Non-Targeting Control 38	AGCTCGCCATGTCGGTTCTC
ANKDD1A	GTAGCCACTTACATTGTCCA	Non-Targeting Control 39	AACTAGCCCGAGCAGCTTCG
ANKDD1A	TCGACGCCATCGAGCAACAG	Non-Targeting Control 40	CGCAAGGTGTCGGTAACCCT
ANKDD1A	TGCGGTAGGGGCCCTCACAG	Non-Targeting Control 41	CTTCGACGCCATCGTGCTCA
ANKDD1A	TGTGCTGGCGTTCATAATGG	Non-Targeting Control 42	ATAGCCGCCGCTCATTACTT
ANKRD32	ACTATGAATTATATAGTCCT	Non-Targeting Control 43	GTCGTCCGGGATTACAAAAT
ANKRD32	AGAGACCATGTATAGAACCC	Non-Targeting Control 44	TATCGCTTCCGATTAGTCCG
ANKRD32	AGGAAAGTGGATACTAACCA	Non-Targeting Control 45	GTACCATACCGCGTACCCTT
ANKRD32	CATGGCTATTAAGACAGATG	Non-Targeting Control 46	TAAGATCCGCGGGTGGCAAC
ANKRD32	CCATCTTAAATCCTGTCATC	Non-Targeting Control 47	GTTCGCTTCGTAACGAGGAA
ANKRD32	GCATGAAGAACGCATACAGG	Non-Targeting Control 48	GACCCCCGATAACTTTTGAC
ANKRD32	GCTTATCAGTTCTAACAAGG	Non-Targeting Control 49	ACGTCCATACTGTCGGCTAC
ANXA8L1	ACCTTGAAGTCTGAGCTCAG	Non-Targeting Control 50	TGGTTCCGTAGGTCGGTATA
ANXA8L1	ACTTACCCAGGCTTTCCACC	Non-Targeting Control 51	CGCTAGGTCCGGTAAGTGCG
ANXA8L1	CATGGCGTCATGCAGCTCCT	Non-Targeting Control 52	AGCACGTAATGTCCGTGGAT
ANXA8L1	CCAAGAGAAGCAACACGCAG	Non-Targeting Control 53	AAGGCGCGCGAATGTGGCAG
ANXA8L1	CCCTCTACAAAGCCATGAAG	Non-Targeting Control 54	ACTGCGGAGCGCCCAATATC
ANXA8L1	CTTGCCGAACTGAGCCTTGA	Non-Targeting Control 55	CGTCGAGTGCTCGAACTCCA
ANXA8L1	TCTGTATGGCGGATACATAA	Non-Targeting Control 56	GCCGTGTTGCTGGATACGCC
ANXA8L1	TGTGAGCAGCTTTGTGGACC	Non-Targeting Control 57	TACCCTCCGGATACGGACTG
APOOL	AGCGGTGGTGCAGTATATAT	Non-Targeting Control 58	CCGTTGGACTATGGCGGGTC

APOOL	GCAGCTAGTGAAACCAGAGC	Non-Targeting Control 59	AAGAGTAGTAGACGCCCGGG
APOOL	GCGGTGGTGCAGTATATATG	Non-Targeting Control 60	CGGCTCGTTCTACGCACTGA
APOOL	TCAGTCCGTAATAATTGCTA	Non-Targeting Control 61	TCCAGCGCGAGCTTACTCGT
APOOL	TGTAACAACCAGTTGCAGTG	Non-Targeting Control 62	CAATCGGCGACGTTTTAAAT
APOOL	TGTACATGCAGCCAAACAAG	Non-Targeting Control 63	GTACCCCTATGGCCGTTCTA
APOOL	TTGTAGACATGGCGGCCATC	Non-Targeting Control 64	TACCCACGCGTATTCCATCT
APOOL	TTGTTACATTGGCTGGTGCA	Non-Targeting Control 65	CTTGTTGCGTATACGAGACT
AQP3	ATCTTTGCTACCTACCCCTC	Non-Targeting Control 66	GCGAACCCCGTAGCCAGGCT
AQP3	CAAGCTGCCCATCTACACCC	Non-Targeting Control 67	CCGGGAGATTAACGTTAATT
AQP3	CAGCACACACACGATAAGGG	Non-Targeting Control 68	ATCTCGGGTCGACTGCGGAT
AQP3	GATGGTGAGGAAACCACCGT	Non-Targeting Control 69	CGCCGGGACCGTTAGGGAAT
AQP3	TACAACAACCCCGTCCCCCG	Non-Targeting Control 70	GCAAACCCGAGTGACACGTC
AQP3	TGCCCCGGCTGAGCACAACC	Non-Targeting Control 71	GTGCGTGAGTATTAACGCTC
ARHGEF37	ATACAATCTGGACATCCCCG	Non-Targeting Control 72	TGGCCACGAATTCCGCCGCC
ARHGEF37	ATTTAGAAGAGAGGTTCCAG	Non-Targeting Control 73	GTAAGGCCCGCGTACGAGCT
ARHGEF37	CAGATTCCTCCATGATCTGC	Non-Targeting Control 74	CTCCTTACGTCGGGCATTAA
ARHGEF37	CCGGGAGCTCATCGACACTG	Non-Targeting Control 75	ATTCCTTCGGCGCTCTGCGT
ARHGEF37	GACGAGCCATCCTCCAGGTC	Non-Targeting Control 76	ATGCGCTTTAATCGCCGTTC
ARHGEF37	GAGGAACAAGTGCAGCTAGT	Non-Targeting Control 77	TTAGCCCTCGATTGGTTGCG
ARHGEF37	GCACATTCGTGACCCTCCAG	Non-Targeting Control 78	AACGCTGTCGTACGTGTATA
ARHGEF37	TGCAGCCTCCAAGTACACCA	Non-Targeting Control 79	TAACGCGCATATCTGAACAC
ARID1B	AAGTTGCTTCCGTTCCCGTG	Non-Targeting Control 80	CGCTAGGTTATTTCGTGGCC
ARID1B	CAAAGTTGCTTCCGTTCCCG	Non-Targeting Control 81	CGGCCGCATCCTGTTATATT
ARID1B	CAGCAGAGCAGTCCGTACCC	Non-Targeting Control 82	CTGGATCGCCCGCAGAAATA
ARID1B	CTGCCCATGCCATACAACTG	Non-Targeting Control 83	ATTAGCCGTTGCCATATCAA
ARID1B	GGAAGCAACCAGTCTCGATC	Non-Targeting Control 84	ACCCGATAATAGCTACTGGT
ARID1B	GTAATTATTAAACTCCGGGA	Non-Targeting Control 85	CCCGCCGAAGACCCTGCTTG
ARID1B	GTCCGACCCTGGATGCCAAT	Non-Targeting Control 86	CTTACGCGCCTGGTCAAAAG
ARID1B	TGAGTGCAAGATCGAACGTG	Non-Targeting Control 87	CGCATAAGTCGATAGACACA
ATF1	AAGTATCTGCTGTCCATCAG	Non-Targeting Control 88	GTCATCAGCGATTTGACGAG
ATF1	ATCTGTCTTAGTTGTCTGAG	Non-Targeting Control 89	CGAATCGGAACTTTGTACCG
ATF1	CAACTGTAAGGCTCCATTTG	Non-Targeting Control 90	AGGTCAAGCCGACCTCGAAC
ATF1	CCCATCTATCAGACTAGCAG	Non-Targeting Control 91	TGCGCCTTACTCGTTAACTG
ATF1	GCGCCGTGCTAGGATCCCGT	Non-Targeting Control 92	ATCTGAGCGTTTTCGGCCGC
ATF1	TTATCTTCTGAAGATACACG	Non-Targeting Control 93	TGACGCGATAGAGTTGGCTT
ATF1	TTGTACGACCACCTGATTGC	Non-Targeting Control 94	GGAATTACGACTAACCGATT
ATG2A	CACTGCACAGTGCGCGTGTC	Non-Targeting Control 95	GGGTGCCCACTAATAGCCGC
ATG2A	CCTCTGCACACGGACCTCGA	Non-Targeting Control 96	TGCAGTCGCGCTGAGCGTCA
ATG2A	GGAACGTGGTGTGGCCGTCG	Non-Targeting Control 97	GGATTGAATGGCTAACGCGG
ATG2A	GGAGTCAATGGAGTCACCGC	Non-Targeting Control 98	GACGTAGCCTTCCGAAATAT
ATG2A	GGCGGCTGATGCACGTCCAC	Non-Targeting Control 99	GGTTCGAGACCTACTTAAGT
ATG2A	GGTCTTCGGCACCTAGCGGG	Non-Targeting Control 10	CGGCTTTGTTGCCCGTAAGC
ATG2A	GTACCTGTCCGACAAGTGTG	Non-Targeting Control 10	TAGGCGCCCGTAGCATTGGA
ATG2A	TTATACCGAACATGGCTACA	Non-Targeting Control 10	GCGGCGTCTGGGAATCGTTC
ATXN2L	AACTTACCACAACAGCTGTA	Non-Targeting Control 10	TTCAATCACCTCACGGTAAG
ATXN2L	AAGACACTCACAGGTGACTG	Non-Targeting Control 10	CGGTTTACATCTGCCCATCG
ATXN2L	CAAACTGGCAGCCCCCCGGT	Non-Targeting Control 10	GGGTATAGACGCGATCCTCA

ATXN2L	CCACAATGTCCTCCCGACGA	Non-Targeting Control 10	ACAGCGCTCTCGTGTACTAT
ATXN2L	CTAGCTCTTACCATCTGTGG	Non-Targeting Control 10	ACTAGCCTGTTCGCGAGTAG
ATXN2L	CTTCAAGACGCTAAGCTCAA	Non-Targeting Control 10	GACCGCGTGAGATAACGTCA
ATXN2L	TCCTCCTTTAAGATCCGGGG	Non-Targeting Control 10	AAAACATCGACCGAAAGCGT
BCLAF1	ACCTAGAAGATCTATATGAC	Non-Targeting Control 11	ACACCGAAGCACCTGTACGT
BCLAF1	AGACGACCTTATGGGTACAG	Non-Targeting Control 11	CCTACGCGGTAGGGAACTTT
BCLAF1	ATTCATCGATAGACTCAGAT	Non-Targeting Control 11	AAGCACTAGTCCGTATGATG
BCLAF1	GCTTGATAGGGGTAATACCA	Non-Targeting Control 11	AGGCGCCAACATTGACCGTA
BCLAF1	TAAAGAGACTGGATATGTAG	Non-Targeting Control 11	CGTCGGGTAGCTATTTCTTT
BCLAF1	TACCTGTTAGAATCATCAAG	Non-Targeting Control 11	TACTGGAGTTTGCGACTCGG
BCLAF1	TTCCTCTTGATGATTCTAAC	Non-Targeting Control 11	AACATCTCGTTAGGGGTATC
BIN1	ACCTGGCCTCCGTCAAAGGT	Non-Targeting Control 11	GTCAGGTAATAGTCGGACTC
BIN1	AGTCACGCATTGCCAAGCGG	Non-Targeting Control 11	TTCGAGGTCCGGACAGGTCG
BIN1	CATCACTCCTACCTGAGATG	Non-Targeting Control 11	AGCTGCGCGCTACTGGATCA
BIN1	CCAGCTGCTTGTTGAAATTC	Non-Targeting Control 12	GCAAAAACCCAACGCTATTC
BIN1	CTGCTCAAACTGCTCATCCT	Non-Targeting Control 12	GCCGCCGATTTCATAAGTAA
BIN1	TGAGGCAAACAAGATCGCAG	Non-Targeting Control 12	GTTCCGTGAGGGTTACTTCA
BPGM	AAGAAATCTACAACGACCGG	Non-Targeting Control 12	TGTCTTTAAACACGCCATCG
BPGM	ACTCAACAGCGAAGGAATGG	Non-Targeting Control 12	ACAAAATGCCGTGCGTCAAT
BPGM	CTTGGATCAACTGCCACGGT	Non-Targeting Control 12	ACGCTCAGCACCCGCTATGC
BPGM	GATGAGGCCCAACAGCACGC	Non-Targeting Control 12	CGAAACCCTCTTAAGTTAAC
BPGM	GGATCGCCTCTTGGTCACCC	Non-Targeting Control 12	CCATTCTCAACCGGTCCAAT
BPGM	GGCCTTGATCGGTCTCAACA	Non-Targeting Control 12	GTTATTGACCCGTCGGGAGT
BPGM	TCCTTTAAGCTTTCCGACCG	Non-Targeting Control 12	GGTTTCACTTCGAGACCGGC
BRD8	AAGAGGAGGCTGAAGTAAAG	Non-Targeting Control 13	CCCCAACTTTCGCGACTCCG
BRD8	AGGAGGTGATTATCCACTTG	Non-Targeting Control 13	CGGCACACCAATGCGTTCGT
BRD8	ATAAGTACCTATATCTCTCC	Non-Targeting Control 13	ATCGATATACCGCCATAAAA
BRD8	CAGGAGTCAGGACTTAGATG	Non-Targeting Control 13	GGGACGCGAAAGAAACCAGT
BRD8	CTGTTGAAGATGTTATTGTT	Non-Targeting Control 13	TGTCATTAGCGTAACGATAT
BRD8	GATATTGCTGTGTCTTACAC	Non-Targeting Control 13	AGGGCGAGCAGCAGAGTACG
BRD8	TCTTGCTTGACCGTCATTTC	Non-Targeting Control 13	CGTCCAGAAGAACGGCCCCT
BRD8	TGGGACACAGACTCTACAGG	Non-Targeting Control 13	GATGGCGCGCAGTTGAGTCA
C10orf91	ACGCAGAGAAAGCGCTCTCG	Non-Targeting Control 13	GCGATCGGAGTGCCACGATA
C10orf91	ATGCGTCCAGCAAGCTCCCA	Non-Targeting Control 13	GTTACCTGCTACGAAAACGA
C10orf91	CATGTGTACTGAGTTATCCT	Non-Targeting Control 14	ATACCAGATGCGTCCGCTTG
C10orf91	CCATGTGTACTGAGTTATCC	Non-Targeting Control 14	AGGATCGTGTACCGGGGACG
C10orf91	CGCATGACCAGGATTCTGGG	Non-Targeting Control 14	CGACAACGTGCAGGTGTATC
C10orf91	GAAATGTGGAGTTTCCTCCC	Non-Targeting Control 14	TTATGTGAGCACGCCATTAC
C10orf91	TGCAGCTACCTCTCAGCTCC	Non-Targeting Control 14	CGACGGTAATGCACCTACTA
C10orf91	TTCCCCGCTTCAGGCTTCGA	Non-Targeting Control 14	CAGCGCCGAAACTCTTTCCG
C10orf91	TTTCTCTACAGCGTGTCCAT	Non-Targeting Control 14	TCGTAAACACACGACCAAGT
C10orf95	AGCAACGCAGCTACAAAGTG	Non-Targeting Control 14	ACTACTCCGGCAAATACTCG
C10orf95	AGCCCCGCTCTGGATCCCGC	Non-Targeting Control 14	CTAATCACGACCTCACCCTA
C10orf95	AGCTGGCCGCCGCCCAAACA	Non-Targeting Control 14	TTGCGTCAGCGCTGCACATC
C10orf95	CCTACGCCACGACCCTGCGC	Non-Targeting Control 15	CGGTGTGCCCCCAAATATTG
C10orf95	GAAGCGGTGGTATTCCCGTG	Non-Targeting Control 15	TATACTGCGGATCAATCTGA
C10orf95	GGGTCGTGGCGTAGGCCGGA	Non-Targeting Control 15	ACGATCGGTAATGGTCTGTT

C10orf95	GGGTGACGCGCACGTCGGCG	Non-Targeting Control 15	GGGCCTACGATCAGAGGTGT
C15orf41	ACATGCCAAACATCATACTT	Non-Targeting Control 15	AGTTGAATGGACCTCGACTA
C15orf41	CAATTGAAAGTTATTACCAG	Non-Targeting Control 15	GAGTAATTTCGAACGTATTG
C15orf41	GAGGTTTCTACAGGAACACG	Non-Targeting Control 15	TTCCACGGTAAAATCGGTCA
C15orf41	TCTGCTGAGCATCTTCTCCC	Non-Targeting Control 15	CCGGCAAGAAACTATACTTG
C15orf41	TGCTTCCGAAGTATGATGTT	Non-Targeting Control 15	CCGCTGTCTCACTAATCTCA
C15orf41	TGCTTGATGCAGTCCACTAG	Non-Targeting Control 15	TGCTACCTTCGGGACCACCA
C15orf41	TGGTAGGCGGCACAGACACT	Non-Targeting Control 16	CTTAGCTGACCGACAAGGTG
C16orf59	AAGCAGCTTCCCAGAACTCG	Non-Targeting Control 16	CCCTTCTGGCGGGCCAAACA
C16orf59	AAGGCTGTACGAGTTCGAAG	Non-Targeting Control 16	TCTGACGATTAATGCTTCTA
C16orf59	AGGGCCAGAAACTAATGGAG	Non-Targeting Control 16	CAGACGGTTGGTAAGGACGC
C16orf59	CACCGGCGCGAGCAGCCCGC	Non-Targeting Control 16	GGGACTGATATATGGCGAAC
C16orf59	CGGGCTTTGAAGCCACCTCC	Non-Targeting Control 16	CAGGTTTGCACGCATAGCTA
C16orf59	GGGTCCTCTCCATTAGTTTC	Non-Targeting Control 16	GGCCGTCGTATTCCCCCAAG
C16orf59	TCAGACACGAGACCCACCAA	Non-Targeting Control 16	CTCCCATTGATCTACGATGG
C16orf59	TTGCAATTGTCGCTGTGCGC	Non-Targeting Control 16	TTTCGTGCCGATGTAACACA
C1orf86	AACCAGGGGCGGCCGCCAGA	Non-Targeting Control 16	GCCTATCGGCATTCCCACTG
C1orf86	CCGACAGTGAAGACTTCAGT	Non-Targeting Control 17	CAACGACGGGCCTAGTCTCA
C1orf86	CGCGGCTGGGGTTGAGCCGC	Non-Targeting Control 17	GATATCCCGCGAAAAAATCT
C1orf86	CGGCGGCTCAACCCCAGCCG	Non-Targeting Control 17	CGCCTCTCACGTGTAGGCTT
C1orf86	GCAGCCGGTAGGAACGGCCC	Non-Targeting Control 17	GGGCGCTAAGATATATGCCC
C1orf86	GGAGCTGATCCTGGATCACG	Non-Targeting Control 17	CGTTGGGCATAGCGAACACT
C1orf86	GGGGCCCGGGCCGTTCCTAC	Non-Targeting Control 17	GCGGGGCGGTGACTTTCAAG
C1orf86	TCACCCCCCAGGAGAAACCA	Non-Targeting Control 17	AAGGGCGTGCCCTGCGTTGT
C2orf82	CACCGTCGCGCCAGGACCCG	Non-Targeting Control 17	GATCCAGGAGTGATCGAGTA
C2orf82	CATCGTGATCGCCGCCCTGC	Non-Targeting Control 17	AGCATTTGCGCGGCAACTGT
C2orf82	CCAGCAGCAGCGCCATGCGC	Non-Targeting Control 17	TTGTCCCTGAGAAAACGCGG
C2orf82	CTCTTACCTGTGAGCACCGC	Non-Targeting Control 18	GTCCTCATCCGGTCAGGCTG
C2orf82	CTTACCTGTGAGCACCGCAG	Non-Targeting Control 18	TATAGCTGTTTCGAAGGCGC
C2orf82	GCCCACGCTGTGGAACGAGC	Non-Targeting Control 18	TGAATCGTAACCTCGCCATT
C2orf82	GCCGTCGGGAGAAGGCCCCG	Non-Targeting Control 18	AGGACTAGTGTCGCACTCAG
C2orf82	GGCCGGCTCGTTCCACAGCG	Non-Targeting Control 18	GGCACTCCGAAAGACCTTAT
C9orf41	ACCTAGCATAGCTATTTCCC	Non-Targeting Control 18	GACCGCAAAGTGGTCCGAAG
C9orf41	ATGCTAGGTTATGCTTGTCA	Non-Targeting Control 18	GTTGCGAGTTACTATTGGTT
C9orf41	ATGTATGCAATCATTCACAA	Non-Targeting Control 18	TCTAAAGCCGTCCTGATGTT
C9orf41	GCCAGCATCTACATTTGACA	Non-Targeting Control 18	GCCGTGGTATCAAGTCGGTA
C9orf41	GGGCTCCTTACCCGTAGTAG	Non-Targeting Control 18	CGCAATCCCTTAGGATAGCC
C9orf41	TACTCAAGCCAGGTGGAATT	Non-Targeting Control 19	CTAGAGGGGTATAGCAACAA
C9orf41	TCCATGTCAAATGTAGATGC	Non-Targeting Control 19	GAAAACACGATGACGTCTCT
CABIN1	AGTGATTAGGTTATCCAAAC	Non-Targeting Control 19	GACGCCCTAATGCCCATCGT
CABIN1	CTGGAGAACCTAACCAACGG	Non-Targeting Control 19	GGATATTGAGTAAACCCGAT
CABIN1	GGGGGATCCGGATGAGCCTC	Non-Targeting Control 19	TGACTCGGGCAATATCGGTT
CABIN1	GTAATCGTGGTCAATCGGAG	Non-Targeting Control 19	GATCTAGTCCTCTAATCGAT
CABIN1	GTACTTCATCTGCAAAGCTT	Non-Targeting Control 19	GGTACCTGAACAACGGCACC
CABIN1	GTAGTGCAGCAAGTAAACGG	Non-Targeting Control 19	TGGCGGCCCAAACTTAACAC
CABIN1	TGAAATGATAATCAGCCAGG	Non-Targeting Control 19	GGGCGGTCAGGTCGCTCCGA
CACNA1A	CGTCAGTTTCATCCTCGGCG	Non-Targeting Control 19	TCCGGAGGCTCAAACCAAGT

CACNA1A	CTCACCAGCCGTTCAGACAT	Non-Targeting Control 20	CCCGTGGCGTGCGCACCTGT
CACNA1A	GAATTGCATCGTCCTCGCAC	Non-Targeting Control 20	GGCTGGTTGACGACTCCTGA
CACNA1A	GACACAGAACCATACTTCAT	Non-Targeting Control 20	GCCATTCTAGTCCCGGCATA
CACNA1A	GCGCTCCAGCCACGTACGAG	Non-Targeting Control 20	TGAATCGAATACAAACGATG
CACNA1A	GTTTGACCTACGGACGCTGA	Non-Targeting Control 20	CCAATGATAAGCCCGAACGG
CACNA1A	TCCACAAAGGCTCCTACTTG	Non-Targeting Control 20	AGCGATTCACGTATTAGATG
CACNA1A	TCTCACCTTGTACGACGGTG	Non-Targeting Control 20	ATGCTGCAGCTTTACGATCA
CALB1	AGCCGAGTATACAGACCTAA	Non-Targeting Control 20	GTGTATGATGCTTCGACTTA
CALB1	CAGTATGGGCAAAGAGATGA	Non-Targeting Control 20	ACAGCCCTCACGAGCCCGAA
CALB1	CCAGATCTCGAAAAACTGTG	Non-Targeting Control 20	GCTGTTGTAACGGTAGATAT
CALB1	CCAGCAGCTGAAGTCCTGTG	Non-Targeting Control 21	CATTGCACGCCACAGCATTG
CALB1	CGAAAGAAGGCTGGATTGGT	Non-Targeting Control 21	CCAGCAATACCCCGGTATGG
CALB1	TACCTTCATGAATTCCTCAC	Non-Targeting Control 21	TCGAGATGCGCAGCAGATGA
CALB2	ACAGGAAATGGGTATATTGA	Non-Targeting Control 21	ACGGGGTGAAACCATGTCGT
CALB2	ATGTCAAAGAGTGACAACTT	Non-Targeting Control 21	AGCTAGCGATGGCTCTAAGT
CALB2	CTCCAGCGCCGAGTTTATGG	Non-Targeting Control 21	GGTCCGCGCACAAGAGCAGG
CALB2	GAACTGGGACGCCGTCAGCT	Non-Targeting Control 21	TCCTCGATAGCTGGAATCCA
CALB2	GCTGACGGCGTCCCAGTTCC	Non-Targeting Control 21	TACGGATCACCAAATCTTAG
CALB2	GGCAAGGAAAGGCTCTGGCA	Non-Targeting Control 21	ACCGCTCATATAGGTAAAAA
CALB2	GGGACGCCGTCAGCTCGGCC	Non-Targeting Control 21	AGTATTGTGGTGTCGTCAAC
CALB2	TGGAAGCACTTTGACGCAGA	Non-Targeting Control 22	GCTCGCAAGTATTTAAGGAC
CASC4	ACAATAATACCTTTCCCATG	Non-Targeting Control 22	GCCAGGGTTCTTGGTCCCGA
CASC4	CAAGAAACAGATCGACCAGA	Non-Targeting Control 22	GTCGCTGCGCCAGTGAGAAC
CASC4	CAAGCAATCATATTCCACAT	Non-Targeting Control 22	CAGGCTGCGCTTCGCAAGCT
CASC4	CATATTCTAACCTCTTCACA	Non-Targeting Control 22	GATTGTGGTCGCTCAAAACC
CASC4	CATCATTTGATTGAATCTTT	Non-Targeting Control 22	CTTAGGATTCCGAGGTATCT
CASC4	GAACAACATATCGTATCAGA	Non-Targeting Control 22	GAACTGGCAAACAGGCGTGG
CASC4	GGGCCTCGGGAAGAGATGCG	Non-Targeting Control 22	ATAGCAGGACGAGGTTCCTT
CASC4	TGCTCCAGTAGTTGAAGGCG	Non-Targeting Control 22	GCACGCTGTACAGACGACAA
CCDC115	ACGAACGGTGTTGAACGCCC	Non-Targeting Control 22	GAGAGCGTTAGCGTGGGATG
CCDC115	AGCTGGTGTCCACGCCCCAG	Non-Targeting Control 23	TTCAATTCACCGAGGGCGCA
CCDC115	AGTTCCTCACAGTCTACGTC	Non-Targeting Control 23	ATGTCTAGACCTAATCGTTT
CCDC115	ATGTGGGAAGCATACTGCAG	Non-Targeting Control 23	GCTGAACGCCGACAGGACGG
CCDC115	CCGGTTCTGGGGTCTTAGTG	Non-Targeting Control 23	GCCCAGACGCCCTAGAATAG
CCDC115	GCCTCCAGAACCGCATTGAC	Non-Targeting Control 23	GGGATGCGTCTTGCTAAACC
CCDC115	GGCTTCGACCCCAGTCAATG	Non-Targeting Control 23	ATCGTTGCTGACAGGATCTA
CCDC115	GGGGGCTCACCTGCTTCGCG	Non-Targeting Control 23	TAGTCTCACCTGATGGCGTG
CCDC121	AACTGAGCGAGCCAGACAGG	Non-Targeting Control 23	GTTATCCTGTCGAAGTAAAG
CCDC121	AATTTGTTCTGCATATCTGG	Non-Targeting Control 23	CAGCGGTGCTATTTGGTCTT
CCDC121	AGCACCGAACGAATAAACTA	Non-Targeting Control 23	CGCACATCTAAAGTTACTAC
CCDC121	CCAGCGGAAACAGCTACTGG	Non-Targeting Control 24	GTAGGGTACAGCGTCAGCTT
CCDC121	CTGAGACAGCTTCAAAGACA	Non-Targeting Control 24	GAAATGCTATGCTTCGGTTC
CCDC121	TAATCAGTGCCTAAATAGAC	Non-Targeting Control 24	AATGCGAGTGTATCCGCAGT
CCDC121	TCGGGCTTATTCGGTAGCCG	Non-Targeting Control 24	TTTATGCATTTAATACGCCG
CCDC121	TTGGAACATCCTGTCTATTT	Non-Targeting Control 24	TCCGTCTGCTTCATGAGCGG
CCL19	AAGTTCCTCACGATGTACCC	Non-Targeting Control 24	CTAACGGACTGCAGAACGGA
CCL19	ACCCCAGGTTCACCACACTG	Non-Targeting Control 24	CATGGCCTACGGTGTCTTTG

CCL19	ACCCTCCATGGCCCTGCTAC	Non-Targeting Control 24	CTGGCCGAATCTCACTATGT
CCL19	AGTTCCTCACGATGTACCCA	Non-Targeting Control 24	GGGGCTTACGTGAAGGGCGG
CCL19	CCCACAACTCACACTACAGC	Non-Targeting Control 24	ACACCCATTCTCATAACGGA
CCL19	GAGCTGGCGGCCCCTCAGTG	Non-Targeting Control 25	GGCCACGAAGGGCGAAAAGG
CCL19	GGGAAGTCCAGAGAACCAGC	Non-Targeting Control 25	TAACCGATACTCCCCACATT
CCL19	TGCAGCCATCCTTGATGAGA	Non-Targeting Control 25	GAGAGTGCGCCTTGATAGTA
CCL3L3	AGCCATGGTGCAGAGGAGGA	Non-Targeting Control 25	GGATTTGTCGCTTGCCACAC
CCL3L3	ATTCTGTGGAATCTGTCGGG	Non-Targeting Control 25	ATTGCTCTGTCGCATCAATC
CCL3L3	CACAGCTTCCTAACCAAGAG	Non-Targeting Control 25	CTCAGTGGATACGATTTGCT
CCL3L3	CCCCTCAGGCACTCAGCTCC	Non-Targeting Control 25	ACTACTGGCTATCCGCGCCA
CCL3L3	GAGGACGGCAAGGGCAGCAG	Non-Targeting Control 25	ACCCAATGTGGCGGAGCCGA
CCL3L3	TAGTCAGCTATGAAATTCTG	Non-Targeting Control 25	TAGGAGCTGTATCTAGTGGC
CCL3L3	TGCCGTCCTCCTCTGCACCA	Non-Targeting Control 25	CCAATCTTGAACGTCATGTT
CCL3L3	TGGACTCACGTGGTGCAGAG	Non-Targeting Control 26	ACCCATATATGCTGCCGCAC
CCL5	AAGGAGTATTTCTACACCAG	Non-Targeting Control 26	CATAGGTCCCTAGCAACTCC
CCL5	ACTGCCCCGTGCCCACATCA	Non-Targeting Control 26	TTCGTAGGAACTAAACTGTA
CCL5	AGGTACCATGAAGGTCTCCG	Non-Targeting Control 26	CGGTGCTGTGAAAGCCGAGC
CCL5	CTGAGACTCACACGACTGCT	Non-Targeting Control 26	ACGGTTATGGTCTCATGGGG
CCL5	GCAATGTAGGCAAAGCAGCA	Non-Targeting Control 26	AACTAGAATAGGCGGGCTTG
CCL5	GTAGAAATACTCCTTGATGT	Non-Targeting Control 26	TAATCACATTGCTTAACCGG
CCL5	TCAAGACCAGGACTTACATG	Non-Targeting Control 26	CGCCCGTTTATGTGGCTACC
CCL5	TCCCGAACCCATTTCTTCTC	Non-Targeting Control 26	GAGTACAGCGATTCCTCATG
HOXB4-EX1-1	GTGCACCGTGCAGCGCTACG	Non-Targeting Control 26	TTTCTAGTTACTACTGGACG
HOXB4-EX1-2	ACCGCCCGGTCTGTCCCCTC	Non-Targeting Control 27	CACGCACAATCCTTCACGCA
HOXB4-EX1-3	GCCCGAGGGGACAGACCGGG	Non-Targeting Control 27	TGCCGCTATACTAAAACCTT
HOXB4-EX1-4	CGAGGGGACAGACCGGGCGG	Non-Targeting Control 27	GTTTACTCATATCCAGTCAC
HOXB4-EX1-5	TGGCGGCGCAGGAGCCCGAG	Non-Targeting Control 27	TCGGCTCCTGAAGCCAGTAT
HOXB4-EX1-6	AGCCGGAGGCGGGCTTCGGG	Non-Targeting Control 27	TCGATGTAGCCCCGCCCAAG
HOXB4-EX1-7	CACCGCCCGGTCTGTCCCCT	Non-Targeting Control 27	AGACCCCGTAGGCAGGACGT
HOXB4-EX1-8	GGAGCCCGAGGGGACAGACC	Non-Targeting Control 27	TCCCAAGGGTTTAAGTCGGG
HOXB4-EX1-9	GTGGCGGCGCAGGAGCCCGA	Non-Targeting Control 27	CGTGCCTTTACATTCACTTT
HOXB4-EX1-10	AGCGCTGGCCGGGCTCCGGG	Non-Targeting Control 27	GCTGTTCCGAAGTTGAGAAT
HOXB3-EX3-1	GGTGCCGGGACCGCACTTTG	Non-Targeting Control 27	ACTAGAGTCATGATCAGCGA
HOXB3-EX3-2	ACTAGCAACAGCAGTAATGG	Non-Targeting Control 28	CTGCCCCAGGCGTAATCCTC
HOXB3-EX3-3	GTGCCGGGACCGCACTTTGG	Non-Targeting Control 28	GTCCCGTGATTTTAGCCAGG
HOXB3-EX3-4	AGCAACAGCAGTAATGGGGG	Non-Targeting Control 28	GGTCTCACCTGCACCCCGAA
HOXB3-EX3-5	GGGGGCGGGCCCAGCAAAAG	Non-Targeting Control 28	TAGTCAACATTCGCAAGAGG
HOXB3-EX3-6	GCAACAGCAGTAATGGGGGC	Non-Targeting Control 28	GTAGCTGCTGTAAATCGCAT
HOXB3-EX3-7	CTGTTGCTAGTGGCACTGGT	Non-Targeting Control 28	CGAAACCTCCTAACTGAGAG
HOXB3-EX3-8	CCCATTACTGCTGTTGCTAG	Non-Targeting Control 28	ATAAGCCACACTACCCGCCT
HOXB3-EX3-9	CACTAGCAACAGCAGTAATG	Non-Targeting Control 28	TACGTAAGTGACGACAGGAA
HOXB3-EX3-10	AGCTCAACGGCAGCTGCATG	Non-Targeting Control 28	CTTTATCTGGCGTGGGGTAT
DPY30-EX4-1	TGATCCAGGTAGGCACGAGT	Non-Targeting Control 28	CCCCTATGCAGACTACAATT
DPY30-EX4-2	GTTGTGCCTATCTTATTACA	Non-Targeting Control 29	CTGGTGACCGACAATTACAC
DPY30-EX4-3	CACAACTGTCTGATCCAGGT	Non-Targeting Control 29	ACGTGGGGACATATACGTGT
DPY30-EX4-4	AGAAAAGTCATCAAAGCAGA	Non-Targeting Control 29	GTTCCCCGGGAAGTCTATGC
DPY30-EX4-5	AGGCACGAGTTGGCAAAGAC	Non-Targeting Control 29	ATTTCCCTACGGAGATATCC

DPY30-EX4-6	TTTGCCAACTCGTGCCTACC	Non-Targeting Control 29	ATCAAGTCAGGTTATGCGGG
DPY30-EX4-7	TAGGCACAACTGTCTGATCC	Non-Targeting Control 29	GGATACCTGGGCCGACTTTC
DPY30-EX4-8	GCAAGTCCCTGTAATAAGAT	Non-Targeting Control 29	CGCAGGCTAGATGACACCAG
DPY30-EX4-9	AGTTGTGCCTATCTTATTAC	Non-Targeting Control 29	TTCGGAACTTACTCAGGGTA
DPY30-EX4-10	GGGACTTGCTGTGCTTGCAA	Non-Targeting Control 29	AAGCGGGCACACATGACAAG
WDR5-EX3-1	TCCGTGAAATTCAGCCCGAA	Non-Targeting Control 29	GTAAAGAAGCGGAAAGGTCC
WDR5-EX3-2	AATTCAGCCCGAATGGAGAG	Non-Targeting Control 30	TACGTCATTAAGAGTTCAAC
WDR5-EX3-3	ATTCGGGCTGAATTTCACGG	Non-Targeting Control 30	CGATGGATCCCTAGTTCCTG
WDR5-EX3-4	CGGAGGACACTGCTTTGGTG	Non-Targeting Control 30	GCTGCGGCGAGATCACATAA
WDR5-EX3-5	TTTCACGGAGGACACTGCTT	Non-Targeting Control 30	CAGAGCCTTGCGCAATTTTG
WDR5-EX3-6	CTTGCCAGCCACTCTCCATT	Non-Targeting Control 30	CCGCGCATTTCAGAGCACAA
WDR5-EX3-7	GCTCTAAAGTTCACCCTTGC	Non-Targeting Control 30	ACCTATTGTCCCTTCAAGCT
WDR5-EX3-8	CAGCCCGAATGGAGAGTGGC	Non-Targeting Control 30	TTGCAAAGCTGATCGGCTGT
WDR5-EX3-9	TTGCCAGCCACTCTCCATTC	Non-Targeting Control 30	AAAATTATCGGAAACGGTAG
WDR5-EX3-10	TCCATTCGGGCTGAATTTCA	Non-Targeting Control 30	AGTCATAACTGAGTGAATCG
HOXA5-EX1-1	AACTCCCTAAGCAACTCCAG	Non-Targeting Control 30	TAGTTACAGACTCAGCGGGT
HOXA5-EX1-2	CAGCAGAGAGGGGGTTGGCA	Non-Targeting Control 31	CACTTACACATGAGGCGGTA
HOXA5-EX1-3	AAGCAACTCCAGCGGCGCCT	Non-Targeting Control 31	ATAGAAGTGTGACCGCTGGG
HOXA5-EX1-4	CCCACATCAGCAGCAGAGAG	Non-Targeting Control 31	GTATTAAGATGCGTCTTAGA
HOXA5-EX1-5	TGGCACGGCGTCCGGAGCCG	Non-Targeting Control 31	ACTGAGTGGGTAACACGCAT
HOXA5-EX1-6	CCACATCAGCAGCAGAGAGG	Non-Targeting Control 31	CCTAAGGGGTACCACCATGG
HOXA5-EX1-7	GATGTGGGTGCTGCCGGCGT	Non-Targeting Control 31	TCCCCGAGACCATCTTAGGG
HOXA5-EX1-8	CACCCACATCAGCAGCAGAG	Non-Targeting Control 31	TACCCTGGATTGTCCTTGCG
HOXA5-EX1-9	GCTGGCAGGGGCGTCCTCCT	Non-Targeting Control 31	ACGCCATATTTCTGGCTCTA
HOXA5-EX1-10	CGCACTCGCCTGCTCGCTGC	Non-Targeting Control 31	CATCTGTAGGGTTGCAAGCC
HOXA10-EX1-1	AGATCGAAACCGCGCCCCGG	Non-Targeting Control 31	TAGCTCGAGTCATTTCTCTA
HOXA10-EX1-2	GAGATCGAAACCGCGCCCCG	Non-Targeting Control 32	TTTAACTGTCCCGGTGTGCA
HOXA10-EX1-3	AGCCTCCGGCTCGGCCGATG	Non-Targeting Control 32	CCTCGTCCAGATTCCGGCGG
HOXA10-EX1-4	GCCCGCGCTAGCCTCCGGCT	Non-Targeting Control 32	TGGATCGGCAGTGGTACTGG
HOXA10-EX1-5	GGGGGGCGGCGGCGAATCGA	Non-Targeting Control 32	AAATACAAGCTATAGCGATA
HOXA10-EX1-6	CTCCCGCCCGCGCTAGCCTC	Non-Targeting Control 32	CATGAGCGCATTGAATAATA
HOXA10-EX1-7	CCGGCTCGGCCGATGCGGCC	Non-Targeting Control 32	GACTTTGGTTGAGCTTCAAT
HOXA10-EX1-8	GCCGAGCCGGAGGCTAGCGC	Non-Targeting Control 32	GTTGGCATATTGGCCCAGAC
HOXA10-EX1-9	GCCGCTGCCGCAAGCCAGCG	Non-Targeting Control 32	GGAACCCTCCCTGCGATAGA
HOXA10-EX1-10	GGCGCGCAGCAACTCGGGGC	Non-Targeting Control 32	CGACCCGGAGGATGAGATGT
HOXA45-R-1	AAGATAAATCTGCACACCCT	Non-Targeting Control 32	TATTTTGACTTGACGCAGGC
HOXA45-R-2	TCACAGTCAATTCACCCGCT	Non-Targeting Control 33	CGGGATGGTCCCTGCCGAGA
HOXA45-R-3	CACAGTCAATTCACCCGCTT	Non-Targeting Control 33	TAGATTGGCCCCACAAAGCG
HOXA45-R-4	GTTGGGAGAGCTGGCCCAAG	Non-Targeting Control 33	GAACCCAACCTTTTACCGCA
HOXA45-R-5	TGTACTAAAGCGTGCTCTGC	Non-Targeting Control 33	GTACACACTTATGCCATCAC
HOXA45-R-6	TTGGGAGAGCTGGCCCAAGC	Non-Targeting Control 33	TTCCTGCCCGAACTGCAGAA
HOXA67-F-1	TCCCGGCGACGGCCACGGCG	Non-Targeting Control 33	CGGCTGAGGCACCTGGTTTA
HOXA67-F-2	TGCCACGCCGTGGCCGTCGC	Non-Targeting Control 33	AGGTTGAATACCCCTTACTA
HOXA67-F-3	GCCGGTCCCGGCGACGGCCA	Non-Targeting Control 33	CCTGCGCGTAGAACAGTGGT
HOXA67-F-4	CGCTCGCTGCTGCCACGCCG	Non-Targeting Control 33	AATCGCAGGTATCCCAGAGC
HOXA67-F-5	GCCACGCCGTGGCCGTCGCC	Non-Targeting Control 33	ACAAACGACCTTGAGCAGGG
HOXA67-F-6	GCTGCAGCTGGCGCCGGTCC	Non-Targeting Control 34	GTACATTCCAGTATTCACGC

HOXA67-F-7	GCTGGCGCCGGTCCCGGCGA	Non-Targeting Control 34	GGCTGGTTGACCTTCCCGCT
HOXA67-F-8	ATTATTTATTGCGACCGTGC	Non-Targeting Control 34	GATGTGATCTATGGTTGCGA
HOXA79-R-1	GAGGCTGCAGTACCAAACGG	Non-Targeting Control 34	ACGTCAACTGCTGGAGTGGG
HOXA79-R-2	AACGGCGGCCAGCAGATGGC	Non-Targeting Control 34	ATTTAAACCGTTACACAGTC
HOXA79-R-3	ACCAAACGGCGGCCAGCAGA	Non-Targeting Control 34	CACGCCAACTAAAACTGCAG
HOXA79-R-4	GGAGCCACACTGCCATCTGC	Non-Targeting Control 34	CCTAGAGGTCCCAAGGCGTG
HOXA79-R-5	GCGGCCAGCAGATGGCAGTG	Non-Targeting Control 34	CCGTTGATCCCCAGGCGTGC
HOXA79-R-6	CGGCGCGGAAGCCTCTTGCA	Non-Targeting Control 34	CCTCGATGGTCACCTGTAGC
HOXA1011-N-1	GGAAGTGCGCCATCTCGTGG	Non-Targeting Control 34	GTGCGCATGGGCTGATGTTA
HOXA1011-N-2	ATCGGAAGTGCGCCATCTCG	Non-Targeting Control 35	AGACTCGTATTGTCATATTA
HOXA1011-N-3	GGCGCGCAGCCGCCACGAGA	Non-Targeting Control 35	GGATCTAGCTACCTCAAAAG
HOXA1011-N-4	CTGGAACTCCGGCCCAACCT	Non-Targeting Control 35	AGAACCCAGACGCCAGCGGT
HOXA1011-N-5	CCGGCGGCTTTGACATTGAT	Non-Targeting Control 35	GGGACATCCTTGCCGTCTCA
HOXA1113-F-1	GGAGGCTTGTCAACGCGAGG	Non-Targeting Control 35	AGCATTCTCACCAAGACCGA
HOXA1113-F-2	TAGCTGGATTAGTAGATCAA	Non-Targeting Control 35	GAGTGTAAGCTAACACTCTG
HOXA1113-F-3	TTAGCTGGATTAGTAGATCA	Non-Targeting Control 35	ATACAATACTTTGGCGCATA
HOXA1113-F-4	TTGGTTGAAGAATTACAAGG	Non-Targeting Control 35	CTCCCTGCCGGCCGGGTTAG
HOXA1113-F-5	GCTCATGAATTGGCCTTAGC	Non-Targeting Control 35	GAACCTCCCCGAATATCTGG
HOXA13-F-1	GAATGCTAGACTTCAAAAAG	Non-Targeting Control 35	ATCTTCAGGGTAACTACGAA
HOXA13-F-2	CTAGACTTCAAAAAGCGGCA	Non-Targeting Control 36	TTCTAAGCCACGTGTGGTAC
HOXA13-F-3	GCTAGACTTCAAAAAGCGGC	Non-Targeting Control 36	AGAAACTGAACTATCCTACT
HOXA13-F-4	TAGACTTCAAAAAGCGGCAG	Non-Targeting Control 36	TCAATTCTCACTCACGACCA
HOXA13-F-5	CTGCTCCTCGGGCCGAGACT	Non-Targeting Control 36	CGAAGTCTTTCTTAGATGGT
HOXA13-F-6	GGAAACCGAGTCTCGGCCCG	Non-Targeting Control 36	ATGCGAAACGACATTTATTA
HOXA13-F-7	CGGCAGGGGAAACCGAGTCT	Non-Targeting Control 36	CATGATAGATCAGTCTTCCC
HOTTIP-1	GGCTGGAGATCCTACTTGAG	Non-Targeting Control 36	AGTGGGGCGCTAAGTGGGGG
HOTTIP-2	CCAAAATAGAGTGAAATAGC	Non-Targeting Control 36	CCCAATGGCTTCTGCGTGAC
HOTTIP-3	CAAGAAAAAGGGGCTCTTTG	Non-Targeting Control 36	CTTTTTTTATTTATCGATCG
HOTTIP-4	GTAGGATCTCCAGCCTGCAG	Non-Targeting Control 36	TGTAGCTAAGTGAGTATGCC
HOTTIP-5	GACTGGTTCTTGGGCAAAGA	Non-Targeting Control 37	AGTAGACGGACGGTGAGCTG
HOTTIP-6	CAGGCTGGAGATCCTACTTG	Non-Targeting Control 37	TCTACGTGTAGTTGTACATA
HOTTIP-7	GTTGCATTTCCCAGGCACAG	Non-Targeting Control 37	GGTTTTATAAGGGTGGGCCT
HOTTIP-8	AGAGGAAAGGCTTCTTGGAC	Non-Targeting Control 37	TCGGAAGCAAACTTCTGGAG
HOTTIP-9	TAGGATCTCCAGCCTGCAGA	Non-Targeting Control 37	TTAGCCAGTAGTGCATATGA
HOTTIP-10	ACAAGAAAAAGGGGCTCTTT	Non-Targeting Control 37	GGGACTGTAGGAACATCCGC
HOTAIRM1-1	AGCTGCTGCGGCGACTGCAA	Non-Targeting Control 37	AAGAATTAGGCACGGTTACT
HOTAIRM1-2	CTAGGCGGCGGCAGCTGCTG	Non-Targeting Control 37	TTTTTCTCACCCGATGAATC
HOTAIRM1-3	GCGGGGCGGGCAGCGGAGTC	Non-Targeting Control 37	AAACCCTATGCCCAAATGAG
HOTAIRM1-4	CGCAGCAGCTGCCGCCGCCT	Non-Targeting Control 37	CATTAGTCTGATACCTGTGC
HOTAIRM1-5	CTCCCGGAGGCCTGGCGGGG	Non-Targeting Control 38	GGTGCTTAGCTCTGCGCACA
HOTAIRM1-6	TCCCAGCCCCCACCTCCCGG	Non-Targeting Control 38	ATGCCTTAGACTTAACCTCG
HOTAIRM1-7	CCAGTTCATCTTTCATTGAA	Non-Targeting Control 38	CCAGTGCCCTTTTGTCGCAA
HOTAIRM1-8	CAAAGGCCGATTTGGAGTGC	Non-Targeting Control 38	AGCGATCTGGACACTCTCCA
HOTAIRM1-9	GCCCGCCCCGCCAGGCCTCC	Non-Targeting Control 38	AGTCTTAAAGACCCTAAGCT
HOTAIRM1-10	GCCTCCCAGCCCCCACCTCC	Non-Targeting Control 38	AGGTAAGCCCCTTAGAACTG
HOXB45-R-1	GGGGCTCCTCGGGAGCAGAA	Non-Targeting Control 38	GTGTAAATCTGTCCAAGTAG
HOXB45-R-2	CTCTAGCCCTGTGAGCACAG	Non-Targeting Control 38	GACCTATGCCAGAAAGTTCG

HOXB45-R-3	GGGCTCCTCGGGAGCAGAAG	Non-Targeting Control 38	ATGCGCAGCTCCAGAATTTT
HOXB45-R-4	AGGGGCTCCTCGGGAGCAGA	Non-Targeting Control 38	GGTCCCTCAGGGTGCAACTT
HOXB45-R-5	AGCGGCCCCTTCTGCTCCCG	Non-Targeting Control 39	GCCCCAAGCTAGAACTCAGC
HOXB45-R-6	GAAGGGGCCGCTGTGCTCAC	Non-Targeting Control 39	CCATTCCGTAAGGGCTTGGA
HOXB45-R-7	AGCTTGGAGCAGGGGCTCCT	Non-Targeting Control 39	GGTCTGCTCCAATGGGAACC
HOXB45-R-8	GCTTGGAGCAGGGGCTCCTC	Non-Targeting Control 39	GAGCAATCCAAAGTTAACGG
HOXB56-R-1	CCGCGCTCCCGTCGGTCGCC	Non-Targeting Control 39	TTCTTAGAAGTTGCTCCACG
HOXB56-R-2	TCCCGTCGGTCGCCGGGAGG	Non-Targeting Control 39	ATCTCTATACTGTCACTCGC
HOXB56-R-3	GAGCAGAGCGCGCCACCTCC	Non-Targeting Control 39	GAACGTAGAAATTCCCATTT
HOXB56-R-4	CCCGCGCTCCCGTCGGTCGC	Non-Targeting Control 39	CATCATAAATGTACAACGGG
HOXB56-R-5	CGCCACCTCCCGGCGACCGA	Non-Targeting Control 39	TCCCTCCTAGTCAAGAAGAG
HOXB56-R-6	GCCACCTCCCGGCGACCGAC	Non-Targeting Control 39	CGACTGACCCCTGGGTGAAG
HOXB56-R-7	CCGGCGACCGACGGGAGCGC	Non-Targeting Control 40	GGGTGGTCATTCTCTACTTG
HOXB6-F-1	GCCGCGTGTCTCCGAACGGA	Non-Targeting Control 40	AGTGAGTGACAACCAGATCG
HOXB6-F-2	GCTGCCATCTACCGTCCGTT	Non-Targeting Control 40	TATGACCCTGTTACATTGCC
HOXB6-F-3	GGCAGCAGACCGCATAATTT	Non-Targeting Control 40	TGAGCATGTCGGGAGTAACT
HOXB6-F-4	TGGCAGCAGACCGCATAATT	Non-Targeting Control 40	TGGGGACGTTTATCAATATA
HOXB6-F-5	ACCGTCCGTTCGGAGACACG	Non-Targeting Control 40	CGTCCCTTCGTCTCTGCTTA
HOXB89-R-1	GGAGCAAGGGTGCCATCTAG	Non-Targeting Control 40	GTTTTTGGTTAATTGCCTAC
HOXB89-R-2	TTCGCAGAGCAGCCGCTAGA	Non-Targeting Control 40	CATTAGCAGCCCAGCGCCCA
HOXB89-R-3	GGGAGTTTCACATGGAGCAA	Non-Targeting Control 40	ATCAGCCCATTTCTGCGCAC
HOXB89-R-4	CTAGCGGCTGCTCTGCGAAA	Non-Targeting Control 40	GTGAAACAGAGGGTCCATCA
HOXB89-R-5	CCGCTCCAGGGAGTTTCACA	Non-Targeting Control 41	CGTAGTAAATATCTAGCTAA
HOXB89-R-6	AGGGAGTTTCACATGGAGCA	Non-Targeting Control 41	ATTAAACGACACCTTATTCT
HOXB9-1-N-1	CGAGACAGAGACCAACCTCT	Non-Targeting Control 41	CCCTCAGGAGCTACTAAGGT
HOXB9-1-N-2	AACGCCAGGGCGCCGCCTAG	Non-Targeting Control 41	GAGGGGGCTTCAAACATGTG
HOXB9-1-N-3	GACAGAGACCAACCTCTAGG	Non-Targeting Control 41	TCGCAAGGAAGCCAGCTAAG
HOXB9-1-N-4	CCAACCTCTAGGCGGCGCCC	Non-Targeting Control 41	CGGAGCTTAGCGTGGGGGCG
HOXB9-1-N-5	TCAGCGCGGACTCAACGCCA	Non-Targeting Control 41	GCTCCCATCCATAGTAAAAA
HOXB9-1-N-6	CCAGGGCGCCGCCTAGAGGT	Non-Targeting Control 41	TGACTAGCTCTTACATATTC
HOXB9-1-N-7	TTCAGCGCGGACTCAACGCC	Non-Targeting Control 41	CCTTATGGAATCAGACCGTT
HOXB9-1-N-8	CGAGAGAATCTTGTTCAGCG	Non-Targeting Control 41	ATAGCGGATGTCCTTGGAAA
HOXB9-2-F-1	TACCGTGGACAGACACTAGA	Non-Targeting Control 42	ACGCATGCTTCCCAAAGCGT
HOXB9-2-F-2	AACACTCGGCTTTCTGAGCG	Non-Targeting Control 42	AGTGTATCTTCCACCTGTCT
HOXB9-2-F-3	TTACCGTGGACAGACACTAG	Non-Targeting Control 42	AGTATGAGACTCATAGGGTG
HOXB9-2-F-4	TCTAGTGTCTGTCCACGGTA	Non-Targeting Control 42	GAAACGAGAAGTTTGTACTA
HOXB9-2-F-5	CACCCTCTAGTGTCTGTCCA	Non-Targeting Control 42	GTTGATCGAAAATGGGAGAA
HOXB9-2-F-6	GTGTCTGTCCACGGTAAGGC	Non-Targeting Control 42	TAGGGGATTAGCTGACAGTC
HOXB9-2-F-7	ACGTTGGACCCGCCTTACCG	Non-Targeting Control 42	GCTAAGGTCATGTTTGCAAT
HOXB9-2-F-8	GTCCCGGGCCTGGAAACACT	Non-Targeting Control 42	GACACTATCCAACCCAAGAG
HOXB9-3-F-1	GGCCAAACACTGACCCCTGC	Non-Targeting Control 42	GAGTTATTTATTCTCTCGAG
HOXB9-3-F-2	GGGCGCCGCCTTCCCTCGGG	Non-Targeting Control 42	CAGTCGTTTCTATGGGATCT
HOXB9-3-F-3	CCTTCCCTCGGGCGGCCGGC	Non-Targeting Control 43	AAAATCGATGGGCTGAATCT
HOXB9-3-F-4	TTCCCTCGGGCGGCCGGCAG	Non-Targeting Control 43	GACGCCTTGCCCGGCTCACA
HOXB9-3-F-5	GCCGCCTTCCCTCGGGCGGC	Non-Targeting Control 43	ATTTAGTAATGCACACCCAG
HOXB9-3-F-6	CTTCCCTCGGGCGGCCGGCA	Non-Targeting Control 43	TAGTTCTAATCGTTCCTTGA
HOXB9-3-F-7	GACCCCTGCCGGCCGCCCGA	Non-Targeting Control 43	CACCCTTATATTCAGTAACT

HOXB9-3-F-8	GGCCGGCAGGGGTCAGTGTT	Non-Targeting Control 43	TGCCCACTTAGCAACACTCT
HOXB9-3-F-9	TGACCCCTGCCGGCCGCCCG	Non-Targeting Control 43	TGCCTCTCCCTTACCCGGAC
HOXB9-3-F-10	GCCCGAGGGAAGGCGGCGCC	Non-Targeting Control 43	AGAGCATGATGACCCGTGAC
HOXB13-F-1	TAGGACCATTAAAAAGACGT	Non-Targeting Control 43	GGTGTCACCACCGCTTACCA
HOXB13-F-2	TTAGGACCATTAAAAAGACG	Non-Targeting Control 43	ACGCTCTCCTGGCAACAAGT
HOXB13-F-3	GGTGAGCCTCTGTCGGAAGG	Non-Targeting Control 44	GGCGTTAATTAAACTGTTTT
HOXB13-F-4	GTGGTGAGCCTCTGTCGGAA	Non-Targeting Control 44	CAGGGTTGCGCAGAGGACTC
HOXB13-F-5	TGGTGAGCCTCTGTCGGAAG	Non-Targeting Control 44	AAGTGACGGTGTCATGCGGG
HOXB13-F-6	CTGGAGTGGTGAGCCTCTGT	Non-Targeting Control 44	TGTCAGTAGTCAGGACCCCG
HOXB13-F-7	AGTGGTGAGCCTCTGTCGGA	Non-Targeting Control 44	CATTAAACCTTGCCCCACAA
HOXB13-F-8	GCCCGCAGGTTCTCCTGGAG	Non-Targeting Control 44	CGGCACTAGAAGTTTTTGAA
HOXBLINC-1	AAGCGCCTCTCAGCGAAGGG	Non-Targeting Control 44	CCAGTTATAATTAGGGGTTT
HOXBLINC-2	CAGCTGTAAAGAAAAATGCT	Non-Targeting Control 44	TAACCCAGAAGCCCATTCAG
HOXBLINC-3	GAAGAGGCGGCTGGGTGTGA	Non-Targeting Control 44	GCAGTACTACTGAGTTTTTC
HOXBLINC-4	CCCTTCGCTGAGAGGCGCTT	Non-Targeting Control 44	CGACCCATGGATGTGAACCC
HOXBLINC-5	TGGTGTAATAAAAGTCCTTT	Non-Targeting Control 45	GACAGTGAAATTAGCTCCCA
HOXBLINC-6	TGCCCGTCATTAAATATCCG	Non-Targeting Control 45	TGTTCTACTTTCGAAGTTAA
HOXBLINC-7	TGCTGGGAGACCAAGCAGAT	Non-Targeting Control 45	GGGAGTTGATTGTTTCGAGA
HOXBLINC-8	GAGGCGGCTGGGTGTGAAGG	Non-Targeting Control 45	TAGAATTTGACCAAAGGCAC
HOXBLINC-9	GGGAGGAGGAAGAGGCGGCT	Non-Targeting Control 45	CTTCTAGCTGGTTCATTGCT
HOXBLINC-10	ACAGCTGTAAAGAAAAATGC	Non-Targeting Control 45	CCCTGTGAAGGAGGCGTAAG
HOXC5-F-1	AGGATGCAATTCCCCCACAT	Non-Targeting Control 45	CAAGCATTTAGACACCTGTC
HOXC5-F-2	AACAAGCCCACAGCGACACC	Non-Targeting Control 45	CGGCCAAAGAATTAGAAGTT
HOXC5-F-3	AATTCCCCCACATAGGCACC	Non-Targeting Control 45	TGAACGGTGAAGAGATAGGG
HOXC5-F-4	GACACCTGGTGCCTATGTGG	Non-Targeting Control 45	AGCCGGCTTGTGACAGTGAA
HOXC5-F-5	CGACACCTGGTGCCTATGTG	Non-Targeting Control 46	AGGGGCAGGGCTATCTTATG
HOXC5-F-6	ATAGGCACCAGGTGTCGCTG	Non-Targeting Control 46	GTAAACTTTGTCTGGAGTAT
HOXC5-F-7	GCGACACCTGGTGCCTATGT	Non-Targeting Control 46	GAATAGATTTGTCAGTTAGG
HOXC5-F-8	TAGGCACCAGGTGTCGCTGT	Non-Targeting Control 46	AGTTCTGTTCGATAGATGCC
HOXC5-F-9	AGCGACACCTGGTGCCTATG	Non-Targeting Control 46	GTGATAATGATGTATTCTCG
HOXC89-F-1	CATTGGACCAAATGGACGCG	Non-Targeting Control 46	GTTTTCAGTTGCCCAACAGC
HOXC89-F-2	TGGACCAAATGGACGCGAGG	Non-Targeting Control 46	CGCGCAGAAGGCAAGCAGGG
HOXC89-F-3	AGCGCCACCTCGCGTCCATT	Non-Targeting Control 46	ATTTTCGAAAGCTTAGGCCA
HOXC89-F-4	CCGGACTGCATTGGACCAAA	Non-Targeting Control 46	GTTTCGAAACTTGAAGTAAG
HOXC89-F-5	CTGTTGCTCAATGTTAGAGG	Non-Targeting Control 46	TTCTAAGCGCCCTGGGGACA
HOXC89-F-6	GCGCTGTTGCTCAATGTTAG	Non-Targeting Control 47	ATCCTAGGTACAAAAGGACG
HOXC89-F-7	TTGCTCAATGTTAGAGGCGG	Non-Targeting Control 47	GTATTACTGATATTGGTGGG
HOXC10-F-1	GCCATCTAGCAGCTGCCTCG	Non-Targeting Control 47	CTTAAGGCGAGAAAAATTAG
HOXC10-F-2	GGCAGGCGGAGCGCGCAGAG	Non-Targeting Control 47	GGATGTTTCTGTGCGCACAT
HOXC10-F-3	GCTCCGGTGCCCCTACCCCG	Non-Targeting Control 47	TCAGTATCGGCTGCTGGTAA
HOXC10-F-4	TTGTTCGCGGGGAAGGGCTC	Non-Targeting Control 47	CACCATAGAACCTGAAATAC
HOXC10-F-5	TAGCAGCTGCCTCGGGGTAG	Non-Targeting Control 47	AGCTGAAAATATACGTATTC
HOXC10-F-6	GCGCCATCTAGCAGCTGCCT	Non-Targeting Control 47	GGATTAATTCGCTAAATGAT
HOXC10-F-7	CTAGCAGCTGCCTCGGGGTA	Non-Targeting Control 47	ATAAGCTACTCTGAGTTCCT
HOXC10-F-8	TCTAGCAGCTGCCTCGGGGT	Non-Targeting Control 47	GTGAACTGCAATCTTATTAT
HOTAIR-1	TCAGGTCCCTAATATCCCGG	Non-Targeting Control 48	ATGCAAGACAGCCTCCCAGC
HOTAIR-2	TGAGGGTCTAAGTCCCGGGT	Non-Targeting Control 48	TGTAGTCTGGGGTAGACTCC

HOTAIR-3	TCCGGGATATTAGGGACCTG	Non-Targeting Control 48	CTGCCCTCTTGAAATAGCCA
HOTAIR-4	ACCAACACCCCTGCTCCTGG	Non-Targeting Control 48	AGGGATCGTTAGGAAGGGAA
HOTAIR-5	GCCGCCAGGAGCAGGGGTGT	Non-Targeting Control 48	CACATAACATGAGGTATCAG
HOTAIR-6	TAAGAGAGCACCAGGCACTG	Non-Targeting Control 48	CTTCCTGCGTGGCTTTAAAC
HOTAIR-7	TGTTGGTCTGTGGAACTCCC	Non-Targeting Control 48	ATAGCTAAAGTTGATGTGTA
HOTAIR-8	AGCACCAGGCACTGAGGCCT	Non-Targeting Control 48	AGGGAAACCTCTATGGGTAA
HOTAIR-9	AACTCCCAGGCCTCAGTGCC	Non-Targeting Control 48	CCAGAGCCTTGGTTTATATC
HOTAIR-10	CAGACCAACACCCCTGCTCC	Non-Targeting Control 48	TGTAGATATAGGGTGTCTAC
HOXD48-F-1	GAGGAAATCGCGCCCCCTCC	Non-Targeting Control 49	GCGAATGCCTGAAAGTATAA
HOXD48-F-2	CGCTTTCTCCGCGCTCCCGG	Non-Targeting Control 49	TTGCAATGCTGCTATAGAAG
HOXD48-F-3	GCTTTCTCCGCGCTCCCGGA	Non-Targeting Control 49	AAGGCAATTTACTGGATCCT
HOXD48-F-4	CGAGGAAATCGCGCCCCCTC	Non-Targeting Control 49	CTGCACTGTGGAGACGCCCG
HOXD48-F-5	CTTTCTCCGCGCTCCCGGAG	Non-Targeting Control 49	GGAGAGGAAAATCGGCACAG
HOXD48-F-6	TTTCTCCGCGCTCCCGGAGG	Non-Targeting Control 49	TCAGGATCAGGGTGTATGGC
HOXD48-F-7	TCGCGCCCCCTCCGGGAGCG	Non-Targeting Control 49	GGGAGGTGGCTTTAGGTTTT
HOXD48-F-8	CCTCGCTTTCTCCGCGCTCC	Non-Targeting Control 49	AGGATGGATTGAGCAGCGGT
HOXD48-F-9	GCTCCTACAAGCGCAGCACG	Non-Targeting Control 49	AACAGGAAACGTGACTAAAG
HOXD89-F-1	GGAGCAACAGCGCTCTCTAG	Non-Targeting Control 49	GCAAAAGTGGCATAAAACCG
HOXD89-F-2	AGTGGACTGGAGGTGGCATT	Non-Targeting Control 50	TGACACATTGGCTGGGTGTT

Table 2. Sanger sequencing results of sgRNAs presented in the selected HOXA9-decreased, HOXA9-unchanged, and HOXA9-

Click here to access/download;Table;Table 2.xlsx

Clone #	sgRNAs	Targets	Genome locus
#5	**ACCAAACGGCGGCCAGCAGA**	**HOXA7/9**	**chr7: 27200761-27200780**
	ACGTTCGAGTACGACCAGCT	Non-target	
#6	**CGGCGCGGAAGCCTCTTGCA**	**HOXA7/9**	**chr7: 27200725-27200744**
	GCTCCGGTGCCCCTACCCCG	HOXC10/11	chr12:54378732-54378751
#15	GTAGGATCTCCAGCCTGCAG	HOTTIP	chr7: 27240871-27240890
	GCTGCCATCTACCGTCCGTT	HOXB6/7	chr17:46680110-46680129
	GCCAGCATCTACATTTGACA	C9orf41	Chr9:77631311-77631330
#28	**AACGGCGGCCAGCAGATGGC**	**HOXA7/9**	**chr7: 27200757-27200776**
#31	CAAAGGCCGATTTGGAGTGC	HOTAIRM1	chr7:27135844-27135863
	TAAGAGAGCACCAGGCACTG	HOTAIR	chr12: 54361157-54361176
	AGCTCGCCATGTCGGTTCTC	Non-target	
	AGAGCGTTAACCTCACCGAC	HOXD9/10	chr2:176983838-176983857
#121	**GCGGCCAGCAGATGGCAGTG**	**HOXA7/9**	**chr7: 27200753-27200772**
#207	AACGGCGGCCAGCAGATGGC	HOXA7/9	chr7: 27200757 -27200776
#420	CGGCGCGGAAGCCTCTTGCA	HOXA7/9	chr7:27200725 -27200744
#429	TAGGATCTCCAGCCTGCAGA	HOTTIP	chr7: 27240872 -27240891
#479	GTAGGATCTCCAGCCTGCAG	HOTTIP	chr7: 27240871 27240890
#4	GTCGTCCGGGATTACAAAAT	Non-target	
#16	TTATACCGAACATGGCTACA	ATG2A	chr11: 64678504-64678523
#27	GTCGTCCGGGATTACAAAAT	Non-target	
#43	AGAGCGTTAACCTCACCGAC	HOXD9/10	chr2 : 176983838-176983857
#129	AGGTAAGCCCCTTAGAACTG	Non-target	
#161	CCTCGTCCAGATTCCGGCGG	Non-target	
#193	AAGACACTCACAGGTGACTG	ATXN2L	chr16: 28836715-28836734
#222	CCCCAACTTTCGCGACTCCG	Non-target	
#299	TGTTGGTCTGTGGAACTCCC	HOTAIR	chr12:54361131-54361150
	ACCCAATGTGGCGGAGCCGA	Non-target	
#323	CGACACCTGGTGCCTATGTG	HOXC5/6	chr12:54426519-54426538
#363	TTGCTCAATGTTAGAGGCGG	HOXC8/9	chr12:54399922-54399941
#468	CTCCTCGGTGTACATCACGG	ADORA2A	chr22: 24829387 24829406
#519	TGGTGAGCCTCTGTCGGAAG	HOXB13-up	chr17:46802040-46802059
	AACTCCCAGGCCTCAGTGCC	HOTAIR	chr12:54361144-54361163
#1	ACCCTCCATGGCCCTGCTAC	CCL19	chr9: 34691124-34691143
#19	GGCCAAACACTGACCCCTGC	HOXB9/13	chr17: 46755965-46755984
	ACCCAATGTGGCGGAGCCGA	Non-target	
#33	AAGAGGAGGCTGAAGTAAAG	BRD8	chr5:137506062-137506081
	ACCCAATGTGGCGGAGCCGA	Non-target	
#37	AAGGAGTATTTCTACACCAG	CCL5	chr17: 34205559 -34205578
#38	TACCTGTTAGAATCATCAAG	BCLAF1	chr6: 136596978-136596997

#123	AGTGGACTGGAGGTGGCATT	HOXD8/9	chr2: 176991910-176991929
	CTGCTCAAACTGCTCATCCT	BIN1	chr2: 127834229-127834248
#474	GCTTGACATCTCTGCTGTAG	ADK	chr16: 75960555-75960574

Note: HOXA9-decreased, unchanged and increased clones are highlighted in red, blue and purple

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Lipofectamine 3000 reagent	Thermo Fisher Scientific	L3000-008
Proteinase K	Thermo Fisher Scientific	25530049
Puromycin	Thermo Fisher Scientific	A1113802
Stbl3 cells	Life Technologies	C737303
HEK293T	ATCC	CRL-3216
MOLM-13	DSMZ	ACC 554
lentiCRISPRv2	Addgene	52961
pMD2.G	Addgene	12259
psPAX2	Addgene	12260
pGEM®-T Easy Vector Systems	Promega	A137A
T4 ligase	New England Biolabs	M0202S
QIAquick Gel Extract kit	QIAGEN	28706
QIAuick PCR purification kit	QIAGEN	28106
SingleShot™ SYBR® Green One-Step Kit	Bio-Rad Laboratories	1725095
QIAGEN Plasmid Maxi Kit	QIAGEN	12163
Dulbecco's Modified Eagle Medium	Thermo Fisher Scientific	11965084
RPMI 1640	Thermo Fisher Scientific	11875093
Fetal bovine serum (FBS)	Thermo Fisher Scientific	10-082-147
Penicillin/streptomycin/L-glutamine	Life Technologies	10378016
Lenti-X Concentrator	Clontech	631232
Trypan Blue Solution	Thermo Fisher Scientific	15250061
Polybrene	Santa Cruz Biotechnology	sc-134220
Phosphate Buffered Saline (PBS)	Genessee Scientific	25-507
TAE buffer	Thermo Fisher Scientific	FERB49
Surveyor® Mutation Detection Kits	Integrated DNA Technologies	706020
Biorad Universal Hood II Gel Doc System	Bio-Rad	170-8126
Centrifuge 5424 R	Eppendorf	5404000138
Digital Dry Baths/Block Heaters	Thermo Fisher Scientific	88870002
TSX Series Ultra-Low Freezers	Thermo Fisher Scientific	TSX40086V
Forma™ Steri-Cult™ CO2 Incubators	Thermo Fisher Scientific	3308
Herasafe™ KS, Class II Biological Safety Cabinet	Thermo Fisher Scientific	51022484

Sorvall™ Legend™ XT/XF Centrifuge Series	Thermo Fisher Scientific	75004506
Fisherbrand™ Isotemp™ Water Baths	Thermo Fisher Scientific	FSGPD02
Thermo Scientific™ Locator™ Plus Rack and Box Systems	Thermo Fisher Scientific	13-762-353
CFX96 Touch Real-Time PCR Detection System	Bio-Rad	1855195
MiniAmp™ Thermal Cycler	Applied Biosystems technology	A37834
Thermo Scientific™ Owl™ EC300XL2 Compact Power Supply	Thermo Fisher Scientific	7217581
Thermo Scientific™ Owl™ EasyCast™ B1 Mini Gel Electrophoresis Systems	Thermo Fisher Scientific	09-528-178
VWR® Tube Rotator and Rotisseries	VWR International	10136-084
VWR® Incubating Mini Shaker	VWR International	12620-942
Analytical Balance MS104TS/00	METTLER TOLEDO	30133522
DS-11 FX and DS-11 FX+ Spectrophotometer	DeNovix Inc.	DS-11 FX

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Author(s): Huacheng Luo, Amin Sobh, Christopher Vulpe, Yi Qiu, Suming Huang

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Jan 14, 2019

Editor*, JoVE*

Dear Editor,

We sincerely appreciate all of the editor's and reviewers' constructive comments and suggestions. We have revised the manuscript and point-to-point answered all of the comments raised by the editor and reviewers.

In summary, our manuscript should be intriguing for a large number of readers who are interested in understanding the role of non-coding elements in gene regulation. It will fit well with the scope of *JoVE* for publication. Thank you very much for your consideration.

Sincerely,

Suming Huang, PhD
Professor of Pediatrics and Pharmacology
Four Diamonds Epigenetics and Gene Regulation Research Endowed Program

Comments and Answers

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Answer: We appreciate the Editor's comment. We have corrected spelling and grammar issues, and polished the manuscript with the help of language experts.

2. Please revise lines 98-100, 223-225, 255-257, 302-305, and 309-312 to avoid previously published text.

Answer: We have modified these sentences and paragraphs to avoid previously published text.

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Answer: We have uploaded our figures individually in the PDF format.

5. Figure 2: Please include a space between numerical values and their corresponding units (48 h, 72 h). Please replace commercial language with generic terms (Surveyor, Lipo3000).

Answer: We have corrected these issues in Figure 2 (now Figure 3).

6. Figure 3: Please line up the panels better. Please explain what different colors and arrows represent. Please define the error bars in the figure legend. Please indicate the units for numbers in panels B and C.

Answer: We revised Figure 3 (now Figure 4) according to the suggestion. We have added the error bar to Figure 4B (new), and explained the color and arrow markers in the legend of Figure 4.

7. Tables 2-4: Please remove commercial language (SingleShot, iTaq™ Universal SYBR® Green, iScript, Bio-Rad® CFX96™, SYBR®). Please abbreviate liters to L to

avoid confusion. Please include a space between numerical values and their temperature units (50 °C, 95 °C, etc.). Please change the time unit “sec” to “s”.

Answer: We have corrected these issues and removed Tables 2-4, and put the useful information into Protocol section.

8. Please shorten the Summary to no more than 50 words.

Answer: We have shorted the summary of this manuscript to less than 50 words.

9. Please rephrase the Abstract to more clearly state the goal of the protocol.

Answer: We have modified the Abstract section, and highlighted changes in yellow.

10. Please revise the Introduction to include all of the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
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11. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

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Answer: We have removed the commercial language, trademark symbols, registered symbols, and company names from this manuscript.

14. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Answer: We have revised this protocol text to avoid to use the personal pronouns in this manuscript.

15. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Answer: We have revised this protocol text to avoid the use of “could be,” “should be,” and “would be” in this manuscript. We also have adjusted the “Note” section of this manuscript. We have moved the discussion about the protocol to the “Discussion” section.

16. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

Answer: We have revised the “Note” part in this manuscript.

17. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

Answer: We have added more details and steps in the protocol section, and highlighted changes in yellow.

18. 2.4: Please describe how to transform the lentiviral vector library into electro-competent cells and specify the electro-competent cells.

Answer: We have described the details of electro-competent cell transformation, and highlighted changes in yellow.

19. 2.5: Please describe how to extract plasmid DNA from the combined colonies.

Answer: We have given the details on plasmid DNA extraction from the combined colonies, and highlighted changes in yellow.

20. 3.1, 4.1: At what temperature are the cells cultured?

Answer: HEK293T and MOLM13 cells were cultured in a 37 °C incubator.

21. 4.3: Please describe how this assay is done.

Answer: We have described the time-course assay in the manuscript, and highlighted in yellow .

22. 5.5: What happens after centrifugation, supernatant discarded? Please specify.

Answer: Spin down the mixes at 1000 g for 2 h in 33 °C, and gently discard the supernatant without disturbing the cell pellet. We specified this in the text with a yellow highlight.

23. 5.9: Please mention how puromycin selection is carried out.

Answer: We have mentioned the puromycin selection in the step 4 procedure of the Protocol section.

24. 6.2: Please specify the temperature of the incubator.

Answer: We specified this in the text with a yellow highlight.

25. 7.1: Please describe how this is done.

Answer: This assay was carried out from step 7.2 to 7.13.

26. 7.5: Is the medium removed using a pipet?

Answer: Yes, this protocol is to thoroughly remove and discard as much supernatant as possible with a pipet, and without disturbing the cell pellet.

27. Please specify all volumes and concentrations used throughout. We need these details to film.

Answer: We have revised these issues in the manuscript.

28. Please include single-line spaces between all paragraphs, headings, steps, etc.

Answer: We have revised these issues in the manuscript.

29. After you have made all the recommended changes to your protocol (listed above),

please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Answer: The essential steps of the protocol have been highlighted with underline marker.

30. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.

Answer: The essential steps of the protocol have been highlighted with an underline marker.

31. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Answer: The essential steps of the protocol have been highlighted with an underline marker.

32. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Answer: We have revised the discussion section, with changes highlighted in yellow.

33. References: Please do not abbreviate journal titles.

Answer: We have revised these issues in the manuscript.

34. Table of Equipment and Materials: Please remove trademark (™) and registered (®) symbols and sort the items in alphabetical order according to the name of material/equipment.

Answer: We have removed trademarks and commercial symbols, trademark and registered symbols from the manuscript.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Luo et al design a genome-wide CRISPR/cas9 KO library targeting CTCF binding regions at HOX loci along with controls and demonstrate efficacy of their protocol

Major Concerns:
1. The title is misleading and gives the impression that this library targets CTCF regulatory elements across the entire genome rather than those associated with four HOX gene loci.

Answer: We appreciate the reviewer's constructive comment. We have changed to a more specific title for the manuscript.

2. The strategy was to target single guides that I assumed resulted in deletions at the target site rather than dual guides designed to excise regulatory elements by targeting 5' and 3' regions. It would be useful to provide a schematic at the start of a target region in relation to a HOX gene locus and again detail precisely where the sgRNAs were in relation to the target region and then show sequencing results where the deleted bands are shown.

Answer: We appreciate the reviewer's thoughtful comment. We have provided a new figure to explain this question in Figure 1. Also we employed the Indel mutation kit to screen the mutation clones.

3. There are several incidental grammatical errors that should be proofed by a professional copy editor.

Answer: We have revised the grammatical errors in the manuscript.

4. The utility of this library is limited. A library covering genome-wide CTCF sites would have had much greater utility

Answer: The method is focused on determining the role of the CTCF binding site in genome organization and gene regulation in all *HOX* loci. *HOX* genes play critical roles in embryonic development, organ development, hematopoiesis, and leukemogenesis. Thus, the method can be widely used to define CTCF boundary function in these developmental processes. We are also trying to explore genome-wide CTCF sites through next generation sequencing according to CTCF ChIP-seq data in the future research. As a non-coding sequence, the consensus CTCF binding sites are widely presented in the genome, and it will be quite a challenge to distinguish, as well as specifically target each CTCF binding site and monitor its biological effect for selection.

Reviewer #2:
Manuscript Summary:
Luo et al. nicely describe a targeted CRISPR/Cas9 screening approach to interrogate the relative impact of CTCF binding site disruption surrounding HOX gene loci. This is a well-written and easy-to-follow protocol that highlights an approach that could be taken

in many different cell types and contexts to target CTCF sites in lineage transcription factor loci. Screens with focused subsets of sgRNAs are much more likely to succeed in yielding interpretable results and the authors have nicely demonstrated this for the HOX genes in the MOLM13 cell line. In all, I feel that this manuscript is quite suitable for publication in JOVE.

Major Concerns:
None.

Minor Concerns:
Minor copy-editing only.
"SURVEYOR" misspelled in line 336.

Answer: We have revised this issue in the manuscript.

Reviewer #3:
Manuscript Summary:
The topic is excellent. Using of CRISPR/Cas to probe chromatin binding regions is an excellent application of the technology. Moreover, the selection of HOX genes offers an excellent biomedical application for the screen.

Vineeta Bajaj <vineeta.bajaj@jove.com>

Fwd: FW: Reuse the published figures in published in Blood 132: 837-848

Stephanie Weldon <stephanie.weldon@jove.com> Tue, Jan 8, 2019 at 1:12 PM

To: Vineeta Bajaj <vineeta.bajaj@jove.com>
Cc: Adria Gottesman-Davis <Adria.Gottesman-Davis@jove.com>

Dear Vineeta,

The author of JoVE59382 just contacted me with figure permissions (forwarded below). Also, there seemed to be some confusion in our department as to who should be contacted with this kind of information; is the review editor the person I should be providing this to? If not, who should I pass it to in the future?

Thanks!
Stephanie

Stephanie R. Weldon, PhD
Science Editor
JoVE
1 Alewife Center | Suite 200 | Cambridge | MA | 02140
Phone: 617-401-7677 Ext: 850 | Fax: 866.381.2236
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---------- Forwarded message ---------
From: **Huang, Suming** <shuang4@pennstatehealth.psu.edu>
Date: Tue, Jan 8, 2019 at 1:04 PM
Subject: FW: Reuse the published figures in published in Blood 132: 837-848
To: stephanie.weldon@jove.com <stephanie.weldon@jove.com>
Cc: Luo, Huacheng <hluo1@pennstatehealth.psu.edu>

Hi Stephanie,

I obtained the permission for reusing the figures in JoVE from the Blood journal. See below email. Thanks.

Suming

Suming Huang, PhD.

Professor of Pediatrics and Pharmacology

Four Diamonds Endowed Chair in Epigenetics & Gene Regulation Research Program

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Tel: (717) 531-7295 (O), (717) 531-0003 ext. 320230 or 320231 (L)

From: Blood Permissions [mailto:bloodpermissions@hematology.org]
Sent: Tuesday, January 08, 2019 12:49 PM
To: Huang, Suming <shuang4@pennstatehealth.psu.edu>
Subject: RE: Reuse the published figures in published in Blood 132: 837-848

Hi Dr. Huang,

Thank you for contacting Blood and for your contributions to our journal!

This is approved as you may reuse your Blood paper for this new one.

Kind regards,

Pat Baggott

Patrick Baggott

Sr. Manager, Publications

American Society of Hematology

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From: Huang, Suming <shuang4@pennstatehealth.psu.edu>
Sent: Tuesday, January 08, 2019 11:25 AM
To: Blood Permissions <bloodpermissions@hematology.org>
Subject: Reuse the published figures in published in Blood 132: 837-848

Hello,

We are invited by the protocol based journal JoVE to published a detailed protocol of the sub-genomic CTCF sgRNA screening library that we developed. This HOX loci specific sgRNA screening library was used to identify the critical chromatin boundary in the HOXA locus in MLL rearranged AML and published in Blood last year (Blood, 132(8): 837-848, 2018; doi: 10.1182/blood-2017-11-814319). I would like to ask for the permission to reuse the figures 2B, 2C, 2D, S2A, and supplementary Table 1. Thank you for the consideration and I look forward to hearing from you.

Best regards,

Suming

Suming Huang, PhD.

Professor of Pediatrics and Pharmacology

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