

# Differential viral accessibility (DIVA) identifies alterations in chromatin architecture through large-scale mapping of lentiviral integration sites

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Alterations in chromatin structure play a major role in the epigenetic regulation of gene expression. Here, we describe a step-by-step protocol for differential viral accessibility (DIVA), a method for identifying changes in chromatin accessibility genome-wide. Commonly used methods for mapping accessible genomic loci have strong preferences toward detecting 'open' chromatin found at regulatory regions but are not well suited to studying chromatin accessibility in gene bodies and intergenic regions. DIVA overcomes this limitation, enabling a broader range of sites to be interrogated. Conceptually, DIVA is similar to ATAC-seq in that it relies on the integration of exogenous DNA into the genome to map accessible chromatin, except that chromatin architecture is probed through mapping integration sites of exogenous lentiviruses. An isogenic pair of cell lines are transduced with a lentiviral vector, followed by PCR amplification and Illumina sequencing of virus-genome junctions; the resulting sequences define a set of unique lentiviral integration sites, which are compared to determine whether genomic loci exhibit significantly altered accessibility between experimental and control cells. Experienced researchers will take 6 d to generate lentiviral stocks and transduce the target cells, a further 5 d to prepare the Illumina sequencing libraries and a few hours to perform the bioinformatic analysis.

## Introduction

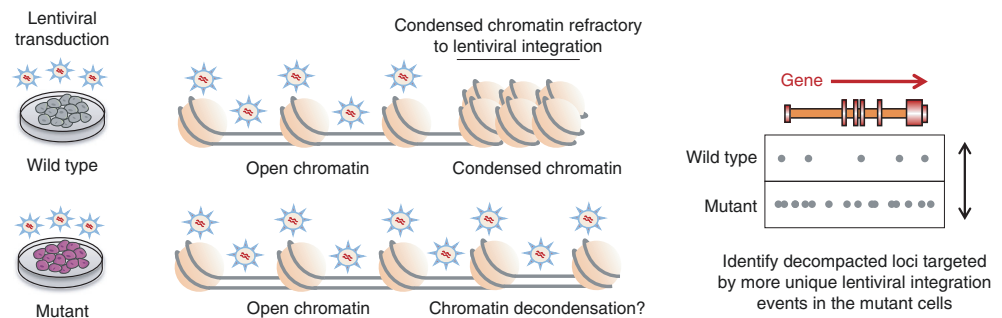
As alterations in chromatin structure play a pivotal role in the epigenetic regulation of gene expression, the ability to examine chromatin structure is critical to understanding the molecular basis of gene regulation. A variety of approaches have been developed for probing chromatin accessibility, including DNase-seq<sup>1</sup>, MNase-seq<sup>2</sup>, FAIRE-seq<sup>3</sup>, NA-seq<sup>4</sup>, RED-seq<sup>5</sup> and ATAC-seq<sup>6</sup>, but, although these methodologies effectively detect regions of accessible, 'open' chromatin, they are less suited to examining changes at 'closed' genomic regions that adopt a more compact chromatin structure.

### Development of DIVA

We have recently characterized the human silencing hub (HUSH) complex, along with its effector proteins SETDB1 and MORC2, as an epigenetic regulator of heterochromatin marked by the repressive H3K9me3 modification<sup>7–10</sup>. Motivated by a desire to examine potential dysregulation of heterochromatin architecture upon loss of HUSH function, we developed DIVA, a genome-wide method to profile chromatin accessibility in cultured cells<sup>9</sup>. Conceptually, DIVA is similar to ATAC-seq, except that, rather than using transposon integration sites to map accessible chromatin, DIVA maps the integration sites of exogenous lentiviruses (Fig. 1). Whereas the Tn5 transposase used in ATAC-seq exhibits a strong preference for integration near transcriptional start sites (TSSs)<sup>11</sup>, HIV-1-derived lentiviral vectors utilized in DIVA also target gene bodies and intergenic regions<sup>12</sup>, thereby allowing the DIVA technique to interrogate the architecture of a broader range of loci.

We used DIVA to compare the chromatin architecture of wild-type and MORC2 knockout HeLa cells, identifying a range of loci showing increased viral accessibility upon loss of MORC2 (ref. <sup>9</sup>). In principle, a similar genetic approach could be used to examine the effect of ablation or overexpression of any epigenetic regulator on chromatin architecture. Similarly, if chemical inhibitors targeting an

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**Fig. 1 | Conceptual overview of DIVA.** DIVA examines the integration preferences of exogenous lentiviruses to infer changes in chromatin accessibility between control (wild-type) and treated (mutant) cells. Adapted with permission from ref. <sup>9</sup>, Springer Nature.

epigenetic modifier of interest are available, DIVA could be used to compare chromatin architecture in vehicle versus drug-treated cells.

### Overview of the procedure

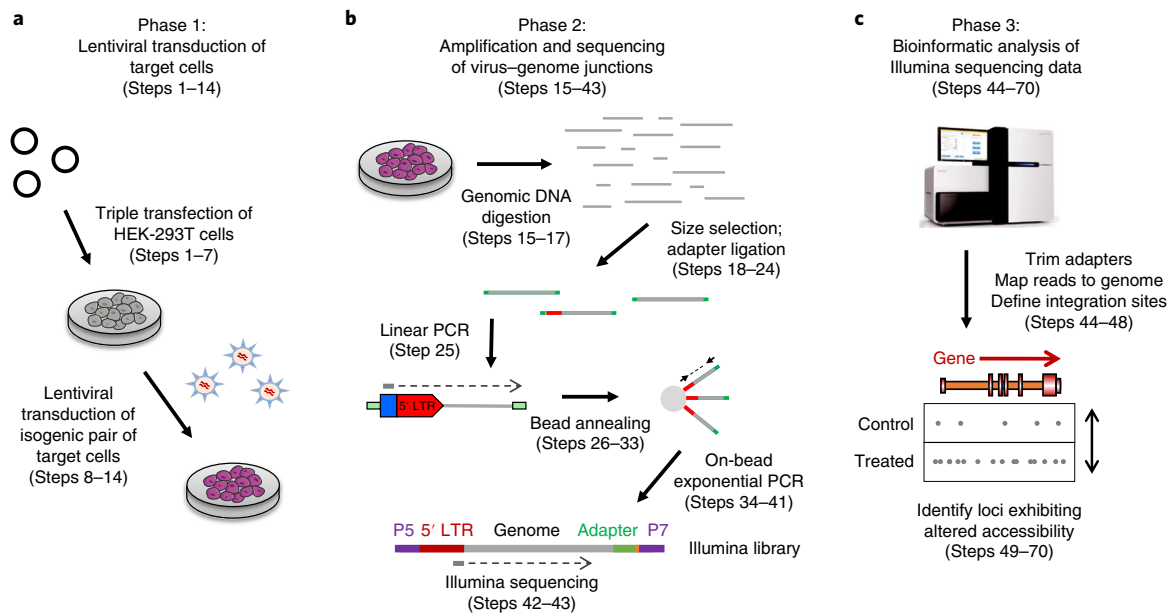
DIVA involves three phases (Fig. 2). In phase 1, lentiviral particles are packaged in HEK 293T cells and used to infect the target cells at a high multiplicity-of-infection (MOI) (Fig. 2a). Following integration of the lentiviruses into the genome of the target cells, the goal of phase 2 is to map the sites of lentiviral integration. Genomic DNA is extracted and digested, adapters are ligated onto the ends of the fragments, and then fragments containing proviral DNA are enriched through a linear PCR reaction. The linear PCR products are subsequently captured and subjected to exponential PCR to generate libraries for Illumina sequencing (Fig. 2b). Finally, in phase 3, the Illumina sequencing data are used to generate a set of unique lentiviral integration sites detected in the population; comparing the pattern of lentiviral integration between control and experimental samples reveals loci exhibiting differential accessibility (Fig. 2c).

### Comparison with other methods

As compared to alternative genome-wide high-throughput chromatin accessibility assays, a major advantage of DIVA is its ability to assay both accessible and inaccessible chromatin simultaneously because of the ability of lentiviruses to integrate across gene bodies and intergenic regions (Fig. 3a). By contrast, ATAC-seq and MNase-seq, plus the related techniques DNase-seq, NA-seq<sup>4</sup> and RED-seq<sup>5</sup>, all employ enzymes that exhibit a strong preference for activity at accessible chromatin regions found at TSSs and hence are less suited to the study of ‘closed’ genomic regions.

DIVA requires a similar sequencing depth to that of other chromatin accessibility assays<sup>6</sup>. 30 million uniquely mapped reads per sample are sufficient to identify a large number of unique lentiviral integration sites and provide coverage across a large mammalian genome; downsampling analysis of our data<sup>9</sup> suggests that ~10 million uniquely mapped reads per condition would be the minimum sequencing depth at which meaningful conclusions could be drawn from a DIVA experiment (Fig. 3b–d). As ATAC-seq involves the purchase of an expensive Illumina kit and often requires deeper sequencing because of mitochondrial DNA contamination, DIVA is likely to be more cost-effective than a typical ATAC-seq experiment. Indeed, as the majority of the reagents required are inexpensive and the depth of Illumina sequencing required is modest, the overall cost of a DIVA experiment is relatively low.

However, DIVA is also subject to some technical limitations. DIVA requires a large number of cells (~6 million) to ensure sufficient coverage of the genome, whereas ATAC-seq can reveal regions of open chromatin using just 500 cells<sup>11</sup> and has even been adapted to analyze single cells<sup>13</sup>. Furthermore, in contrast to the simple and rapid sample preparation required for ATAC-seq, DIVA involves a more complex and time-consuming library generation protocol that also relies on the availability of a biosafety level 2 (BSL-2) tissue culture room. Thus, although ATAC-seq retains clear advantages over DIVA for researchers interested only in interrogating sites of open chromatin, DIVA may be better suited to examining changes in heterochromatic regions, gene bodies and intergenic regions.



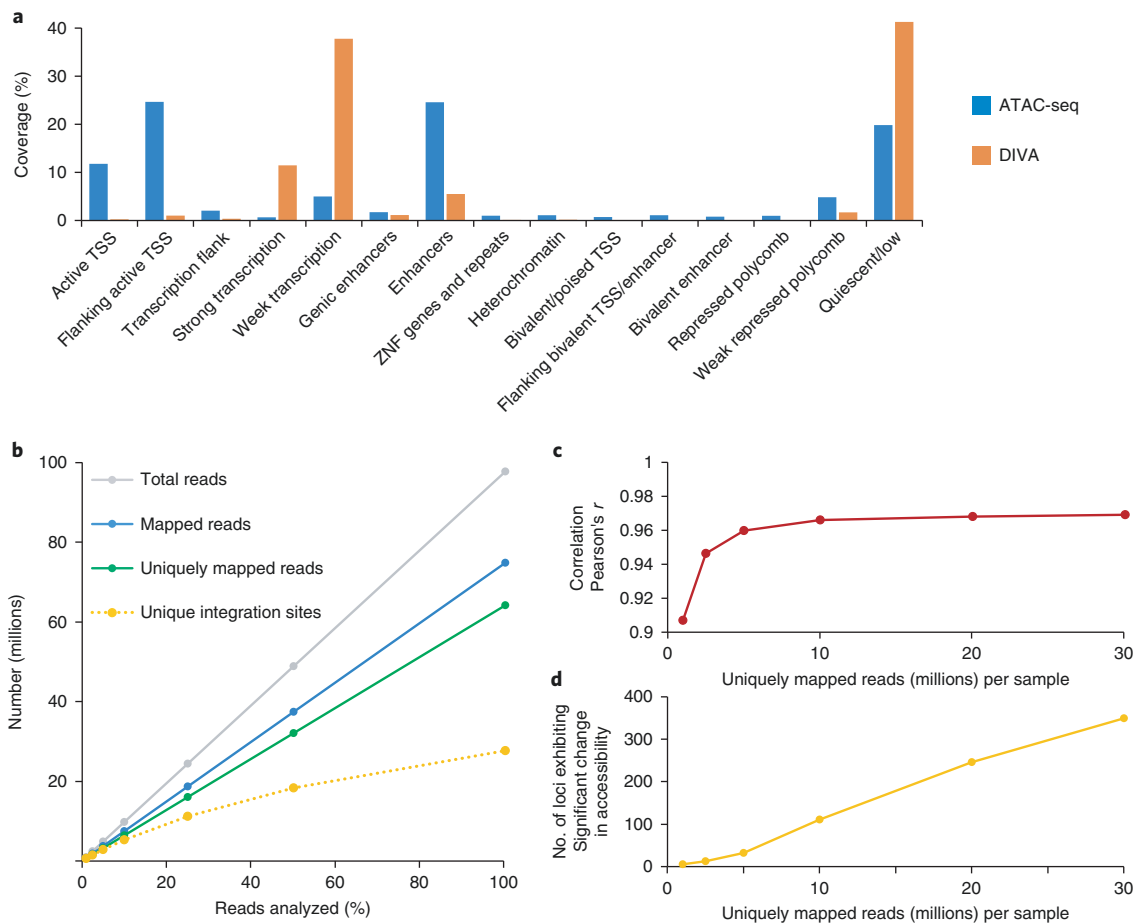
**Fig. 2 | Workflow of a DIVA experiment.** **a**, Lentiviral transduction of target cells. Lentivirus is generated through the triple transfection of HEK 293T cells, and the viral supernatant is used to transduce the matched pair of control and treated target cells. **b**, Mapping of lentiviral integration sites. Genomic DNA harvested from the transduced cells is enzymatically digested and adapter ligated, followed by linear PCR amplification of virus–genome junctions. Biotinylated linear PCR products are captured on streptavidin beads, which are then used as the template for a final exponential PCR to generate a library for Illumina sequencing. **c**, Bioinformatic analysis of Illumina sequence data. Illumina reads are mapped to the reference genome to define a set of unique lentiviral integration sites in each cell type (control versus treated); comparing the pattern of integration sites in control (wild-type) cells versus that in treated (mutant) cells reveals genomic loci exhibiting altered chromatin architecture.

### Limitations of the method

There are a number of limitations to the current version of the DIVA protocol. First, sufficient coverage of a large mammalian genome can be obtained only if millions of independent lentiviral integration sites are mapped. This relies on achieving a high MOI when transducing the target cells with the lentiviral vector, which may not be possible in cell lines or primary cells that are difficult to transduce. Second, DIVA may not be suitable for comparisons between non-isogenic cell lines, in which it is difficult to control for differences in the abundance or activity of other factors besides changes in chromatin architecture that may influence viral integration preferences. For example, differences in the abundance of the LEDGF/p75 protein, a cellular tethering factor for HIV-1-based lentiviruses<sup>14</sup>, could result in altered patterns of viral integration that are not related to altered chromatin accessibility. Finally, DIVA may be less well suited to measuring decreases in chromatin accessibility: whereas increased accessibility is revealed by an increase in the number of viral integration sites detected at a given locus, decreased accessibility must be inferred from the absence of detection. However, provided that sufficient numbers of lentiviral integration sites are mapped across such regions, in principle, a reproducible decrease in the number of lentiviral integration sites as measured by DIVA should be indicative of decreased chromatin accessibility.

### Applications

A critical part of the DIVA protocol is the mapping of the sites of integration of exogenous lentiviral DNA on a large scale. The linear amplification-mediated polymerase chain reaction (LAM-PCR) approach described here was adapted from that originally devised by the Brummelkamp laboratory<sup>15</sup> and has been used extensively to map the integration sites of gene-trap retroviral vectors to analyze forward genetic screens in haploid human cell lines<sup>16–18</sup>. This technique is likely to be applicable in other experimental settings, and, employing appropriately redesigned primers, the LAM-PCR protocol used for DIVA could be readily adapted to locate any exogenously integrated DNA in large mammalian genomes.



**Fig. 3 | Genome coverage and sequencing depth required for DIVA. a**, Comparison of the genomic coverage obtained with DIVA versus ATAC-seq. The bar chart indicates the percentage of either ATAC-seq peaks (blue) or unique lentiviral integration sites identified by DIVA (orange) that occur across 15 types of chromatin. Compared to ATAC-seq, lentiviral integration sites are depleted from transcriptional start sites and enhancers, and are instead enriched in gene bodies and intergenic regions. ATAC-seq peaks are based on data from ref. <sup>11</sup>, DIVA lentiviral integration sites are based on data from ref. <sup>9</sup>, and the chromatin state classifications are based on data from ref. <sup>21</sup>. **b-d**, Sequencing depth required for DIVA. **b**, Downsampling analysis of Illumina sequencing data for wild-type HeLa cells<sup>9</sup>. Although the proportion of uniquely mapped reads scales linearly with the total number of sequencing reads generated, the number of unique integration sites detected plateaus rapidly. Thus, further sequencing of these DIVA libraries would have yielded few additional unique integration sites. **c**, The maximal correlation between wild-type and mutant samples was essentially achieved with 10 million uniquely mapped reads per condition, and did not improve further with the addition of more reads. **d**, Quantification of the number of genomic loci exhibiting a significant change in accessibility as a function of sequencing depth. The number of significant loci continues to increase as the number of mapped reads increases; in our experiment, however, similar biological conclusions could have been drawn with 10 million uniquely mapped reads per cell type.

### Experimental design

#### Cell lines

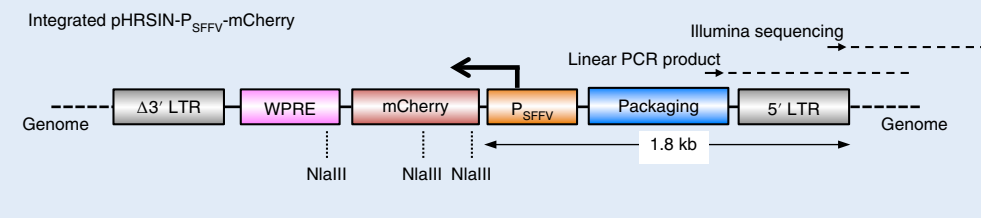
DIVA was developed as a comparative method to reflect differences in chromatin accessibility between a control and a treated cell line. Therefore, we recommend that DIVA be performed in isogenic cell lines (Step 8), as differences in the abundance or activity of cellular factors that affect lentiviral integration preferences could lead to altered integration patterns that are not indicative of changes in chromatin structure.

#### Choice of lentiviral vector

In principle, any standard lentiviral vector could be used to perform a DIVA experiment (Step 3). There are two particular advantages to the pHR SIN-P<sub>SFFV</sub>-mCherry vector that we use<sup>9</sup>: (i) expression of the mCherry fluorescent protein allows the transduction of the target cells to be easily monitored by flow cytometry, and (ii) the arrangement of NlaIII restriction sites in the vector is favorable for efficient LAM-PCR amplification of virus-genome junctions (Box 1).

**Box 1 | Size selection of large DNA fragments containing virus–genome junctions**

During Steps 18–22 of the protocol, a size selection is performed to enrich for large DNA fragments following NlaIII digestion of the genomic DNA. The diagram shows the location of NlaIII recognition sites in the pHRSIN-P<sub>SFFV</sub>-mCherry lentiviral vector when integrated into a genome, plus the primer binding sites for PCR amplification of the virus–genome junction. The distribution of NlaIII sites is such that a genomic DNA fragment containing the 5′ LTR must be at least ~1.8 kb; therefore, selecting for large DNA fragments enriches for the fragments of interest that contain proviral DNA. The distribution of restriction sites in the proviral DNA is therefore an important consideration, should the protocol be adapted to use alternative lentiviral vectors or restriction enzymes. Abbreviations are as follows: LTR, long terminal repeat; P<sub>SFFV</sub>, spleen focus-forming virus promoter; WPRE, woodchuck post-transcriptional regulatory element.

**Choice of restriction enzyme**

This protocol uses a single restriction enzyme, NlaIII, to digest the genomic DNA isolated from the transduced target cells (Step 16). NlaIII cleaves at its 4-bp recognition site, 5′-CATG-3′, which occurs every ~200 bp in the human genome. A proportion of all the lentiviral integration sites in the transduced population will lie either too close or too far from an NlaIII cleavage site to be mapped, however; therefore, to increase the coverage obtained, the genomic DNA could also be digested (in separate reactions) with additional ‘4-cutter’ restriction enzymes that are not affected by CpG methylation, such as MseI or MluCI. Adapting the protocol for additional enzyme(s) would require the design of modified adapter oligonucleotides with the appropriate cohesive ends; in these cases, this would require the removal of the CATG overhang from the 3′ end of the NlaIII-BSA oligonucleotide and addition of a TA (MseI) or an AATT (MluCI) overhang to the 5′ end of the TSA oligonucleotide (Table 1). Care also needs to be taken to ensure that the lentiviral vector used will not itself be cleaved by the restriction enzyme in a manner that would prevent PCR amplification of the virus–genome junction (see Box 1 for further details).

**Production of high-titer lentivirus**

To gain sufficient coverage of the genome, millions of lentiviral integration sites must be mapped in each cell type. It is therefore critical that high-titer lentivirus be produced (Steps 1–7). We recommend using low-passage HEK 293T cells and endotoxin-free DNA vectors. To optimize the conditions for virus production, a lentiviral vector encoding a fluorescent protein can be used. HEK 293T cell seeding density, DNA/TransIT transfection reagent ratio, the number of cells to be transduced and the amount of viral supernatant used for the transduction will all affect the transduction efficiency, and hence all of these parameters may need to be optimized to achieve maximum transduction efficiency for DIVA. Enhanced transduction efficiency can also be achieved by concentrating the viral supernatant, for example, by using Lenti-X Concentrator or by performing the transduction in the presence of Polybrene at a final concentration of 8  $\mu\text{g ml}^{-1}$ .

**Sequencing depth required**

We used a single run of an Illumina HiSeq 2500 machine in rapid run mode to generate the sequencing data for our DIVA experiment in HeLa cells<sup>9</sup>, which yielded ~50 million uniquely mapped reads for each condition. An analysis of the sequencing depth requirements for DIVA are presented in Fig. 3b. This analysis suggests that 30–50 million uniquely mapped reads are sufficient to map the majority of unique lentiviral integration sites present in a DIVA library, whereas 10 million uniquely mapped reads per condition should be considered the minimum required to generate meaningful data. We recommend single-end 50-bp reads to minimize the cost of the experiment (Step 43).

**Data analysis**

This protocol uses the SeqMonk application (<https://www.bioinformatics.babraham.ac.uk/projects/seqmonk/>) to analyze the Illumina sequencing data (Steps 44–70). SeqMonk is a powerful genome

**Table 1 | Oligonucleotides required for DIVA**

Oligo name	Sequence 5'-3'	Purpose	Step used
TSA	CCTATAGTGAGTCGTATTATATGAGTAGTACCATGGGAAC	Top strand adapter	23
NlaIII-BSA	GTTCCCATGGTACTACTCATATAATACGACTCACT ATAGGCATG	Bottom strand adapter	23
Bio-Pack_F	[Biotin]-CTTTCGCTTTCAAGTCCTGTTCG	Linear PCR primer	25
P5-HIV1_F	AATGATACGGCGACCACCGAGATCTACACTCTTCCCTAC ACGACGCTCTCCGATCTGATCTGGTCTAACCAGAGAG	Exponential PCR primers (X = index bases)	34
P7-Adapter_R	CAAGCAGAAGACGGCATACGAGAXXXXXXXGTG ACTGGAGTTCAGACGTGTGCTCTTCCGATCTGT TCCCATGGTACTACTCATATAATACG	Exponential PCR primers (X = index bases)	34
ISP	CTTGTCTTCGTTGGGAGTGAATTAGCCCTTCCA	Illumina sequencing primer	43

browser that allows both the visualization and quantitation of mapped sequence data. Its graphical user interface is ideal for biologists with limited programming experience; experienced bioinformaticians, however, could readily automate these steps using standard tools (e.g., the BedTools suite<sup>19</sup>).

**Level of expertise required**

Successful DIVA experiments can be performed by competent graduate students with relevant experience in cell culture and molecular biology techniques and a basic level of Unix knowledge. As the LAM-PCR protocol to map viral integration sites is relatively challenging, however, we would not recommend it be attempted by users inexperienced in molecular biology techniques. The steps described in this protocol do not require any non-standard reagents or equipment, and hence could be adopted by any laboratory with access to a BSL-2 tissue culture room and a next-generation sequencing facility. Previous experience working with lentiviral vectors would be beneficial, but is not essential, as the technique could be easily mastered after a short induction.

**Materials**

**Biological materials**

- Target cell type of choice plus appropriate culture media. We have used HeLa cells (ATCC, cat. no. CCL-2), cultured in RPMI 1640 (see Reagent setup). **! CAUTION** The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.
- **▲ CRITICAL** DIVA relies on a comparison between two experimental conditions, and therefore a control and a treated cell line are required. For example, we used DIVA to compare wild-type versus mutant HeLa cells<sup>9</sup>.
- HEK 293T cells (ATCC, cat. no. CRL-1573) **! CAUTION** The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

**Reagents**

- RPMI 1640 (Thermo Fisher Scientific, cat. no. 11875093)
- DMEM (Thermo Fisher Scientific, cat. no. 11965092)
- Opti-MEM I Reduced Serum Medium (Thermo Fisher Scientific, cat. no. 31985070)
- FBS (HyClone, cat. no. SH30071.03)
- Penicillin–streptomycin (Thermo Fisher Scientific, cat. no. 15140122)
- Trypsin-EDTA (0.25%; Thermo Fisher Scientific, cat. no. 25200056)
- Lentiviral transfer vector (Box 1). We have used the pHRSIN-P<sub>SFFV</sub>-mCherry vector, which encodes mCherry from the spleen focus-forming virus (SFFV) long terminal repeat (LTR) promoter, allowing transduced cells to be fluorescently marked (Addgene, cat no. 74670).
- Lentiviral packaging plasmids pMD2.G and psPAX2 (Addgene, cat. nos. 12259 and 12260)
- TransIT-293 transfection reagent (Mirus, cat. no. MIR 2700)
- Polybrene (Sigma-Aldrich, cat. no. H9268)
- PBS (Thermo Fisher Scientific, cat. no. 10010023)
- Gentra Puregene Cell Kit (Qiagen, cat. no. 158767)
- NlaIII (NEB, cat. no. R0125L)
- UltraPure water (Thermo Fisher Scientific, cat. no. 10977015)

- QIAquick PCR Purification Kit (Qiagen, cat. no. 28104)
- Agencourt AMPure XP beads (Beckman Coulter, cat. no. A63880)
- Oligonucleotide adapters (Sigma-Aldrich; see Table 1 for oligonucleotide sequences and Reagent setup for preparation)
- 10× NEB buffer 2 (NEB, cat. no. B7002S)
- T4 DNA Ligase (NEB, cat. no. M0202S)
- Biotinylated linear PCR primer (Sigma-Aldrich; see Table 1 for oligonucleotide sequences)
- Accuprime Taq DNA Polymerase System (Thermo Fisher Scientific, cat. no. 12339016)
- dNTPs (NEB, cat. no. N0447L)
- Streptavidin Dynabeads M-280 (Thermo Fisher Scientific, cat. no. 11205D)
- Ethanol (absolute, 200 proof, molecular biology grade; Thermo Fisher Scientific, cat. no. BP2818500)
- 5 M NaCl (Thermo Fisher Scientific, cat. no. 24740011)
- 1 M Tris-HCl, pH 7.5 (Thermo Fisher Scientific, cat. no. 15567027)
- 0.5 M EDTA (Thermo Fisher Scientific, cat. no. AM9260G)
- Tween 20 (Sigma-Aldrich, cat. no. P9416)
- Exponential PCR primers (Sigma-Aldrich; see Table 1 for oligonucleotide sequences)
- Bioanalyzer High Sensitivity DNA Kit (Agilent, cat. no. 5067-4626)
- Lenti-X Concentrator (Clontech, cat. no. 631231)

### Equipment

- Syringes (VWR, cat. no. 309646)
- 0.45-µm Syringe filters (Corning, cat. no. 431220)
- 5-ml Polypropylene round-bottom tubes for flow cytometry (Corning, cat. no. 352002)
- Six-well cell culture plates (Thermo Fisher Scientific, cat. no. 140675)
- Cell culture incubator (37 °C, 5% CO<sub>2</sub>, 90% humidified; Thermo Fisher Scientific, cat. no. 51026332)
- Cell culture hood (Thermo Fisher Scientific, cat. no. 51023607)
- Centrifuge (Eppendorf, cat. no. 51026332)
- Flow cytometer (BD LSRFortessa; BD Biosciences, cat. no. 649225)
- Thermocycler (C1000 Touch Thermal Cycler; Bio-Rad, cat. no. 1851196)
- NanoDrop spectrophotometer (Thermo Fisher Scientific, cat. no. ND-2000)
- Orbital shaker (Eppendorf, cat. no. 5382000023)
- Microcentrifuge (Fisher Scientific, cat. no. 05-400-006)
- 0.2-ml PCR tubes (Fisher Scientific, cat. no. 14-222-252)
- 1.5-ml Microcentrifuge tubes (Eppendorf, cat. no. 022363204)
- Magnetic rack (Thermo Fisher Scientific, cat. no. 15567027)
- Bioanalyzer (Agilent, cat. no. G2939BA)
- Computer running Unix/Linux distribution with at least 4 GB of RAM (see below for software that should be installed)

### Software

- FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>)
- FASTX-Toolkit ([http://hannonlab.cshl.edu/fastx\\_toolkit/](http://hannonlab.cshl.edu/fastx_toolkit/))
- Samtools (<http://www.htslib.org/>)
- Bowtie 2 (<http://bowtie-bio.sourceforge.net/bowtie2/index.shtml>)
- SeqMonk (<https://www.bioinformatics.babraham.ac.uk/projects/seqmonk/>)

### Reagent setup

#### Complete DMEM cell culture medium

To make up complete DMEM cell culture medium, in a cell culture hood, add 50 ml of FBS (10% (vol/vol) final) and 5 ml of penicillin–streptomycin solution to 500 ml of DMEM, and mix thoroughly by inverting. The medium should be stored at 4 °C and discarded if unused after ~1 month.

#### Complete RPMI cell culture medium

To make up complete RPMI cell culture medium, in a cell culture hood, add 50 ml of FBS (10% (vol/vol) final) and 5 ml of penicillin–streptomycin solution to 500 ml of RPMI 1640, and mix thoroughly by inverting. The medium should be stored at 4 °C and discarded if unused after ~1 month.

**70% (vol/vol) ethanol**

Add 0.3 volumes of UltraPure water to 0.7 volumes of the stock 100% (vol/vol) ethanol solution and mix by inverting. The solution can be kept tightly capped at room temperature (20–25 °C).

**Annealing adapters**

Dilute the 10× NEB buffer 2 two-fold by adding an equal volume of UltraPure water, thus generating 5× NEB buffer 2. Resuspend the TSA (top strand adapter; Table 1) and NlaIII-BSA (bottom strand adapter; Table 1) oligonucleotides in the 5× NEB buffer 2 at a final concentration of 100 μM. Then add 50 μl of each oligonucleotide to a PCR tube, mix by pipetting, and anneal by placing the tube in a thermocycler and running the following program:

Step	Denature	Anneal	Hold
1	95 °C, 5 min		
2		Slow cool (0.1 °C s <sup>-1</sup> ) to 4 °C	
3			4 °C

Annealed adapters can then be stored indefinitely at –20 °C.

**PBS + 0.5% (wt/vol) BSA**

Weigh out 0.25 g of BSA, dispense into a 50-ml plastic tube, and add 50 ml of PBS. Mix gently by inverting. Place in a water bath at 37 °C for 30 min to dissolve fully. This solution should be made fresh on each occasion.

**20% (vol/vol) Tween 20**

Add 4 volumes of distilled water to 1 volume of Tween 20 and mix gently by inverting until the solution is homogeneous. The solution can then be stored for months at room temperature when protected from light.

**2× B&W buffer**

In a 500-ml bottle, add 200 ml of 5 M NaCl, 5 ml of 1 M Tris-HCl, pH 7.5, and 1 ml of 0.5 M EDTA. Make up to a total volume of 500 ml with distilled water and mix by inverting. The solution can be stored for months at room temperature.

**1× B&W buffer**

In a 500-ml bottle, add 250 ml of 2× B&W buffer and 250 ml of distilled water and mix by inverting. The solution can be stored for months at room temperature.

**2× B&W buffer + 0.2% (vol/vol) Tween 20**

Pipette 500 μl of 20% (vol/vol) Tween 20 into 50 ml of 2× B&W buffer and mix by inverting. The solution can be stored for months at room temperature.

**1× B&W buffer + 0.1% (vol/vol) Tween 20**

Pipette 250 μl of 20% (vol/vol) Tween 20 into 50 ml of 1× B&W buffer and mix by inverting. The solution can be stored for months at room temperature.

**Equipment setup**

**Software setup**

Follow the online installation instructions to install FastQC, the FASTX-Toolkit and Bowtie 2 programs onto the Linux computer. These need to be added to the PATH environment variable in order to successfully execute the commands in the protocol; detailed instructions on how to do this can be found in the online documentation for each tool. SeqMonk can be installed on a computer running any operating system.

**Procedure**

**Generation of lentivirus stocks ● Timing 4 d**

- 1 After thawing a frozen vial of HEK 293T cells, allow 2–3 d for the cells to recover. Then trypsinize the stocks of HEK 293T cells, and seed  $6 \times 10^5$  cells in each well of a 6-well plate in complete DMEM cell culture medium. Grow overnight (~16–20 h) to achieve 70% confluency.



The following morning, perform a triple transfection of the HEK 293T cells to generate lentivirus. For each well of HEK 293T cells to be transfected, perform the following procedure (Steps 2–6).

- 2 Pipette 200  $\mu$ l of Opti-MEM into a sterile 1.5-ml microcentrifuge tube. Then add 7.5  $\mu$ l of TransIT-293 transfection reagent and mix thoroughly by pipetting. Incubate at room temperature for 5 min.
- 3 During the incubation, pipette 50  $\mu$ l of Opti-MEM into a second sterile 1.5-ml microcentrifuge tube. Then add the following amounts of plasmid DNA: 1  $\mu$ g of lentiviral transfer vector, 0.7  $\mu$ g of psPAX2 and 0.3  $\mu$ g of pMD2.G. Mix by pipetting.
- 4 Add the TransIT-293/Opti-MEM mix from Step 2 to the DNA/Opti-MEM mix from Step 3 and mix gently, but thoroughly, by pipetting. Incubate at room temperature for 20 min.
- 5 During the incubation, aspirate the medium from the HEK 293T cells from Step 1 and replace it with 2 ml of fresh DMEM cell culture medium per well. Pipette slowly onto the side of the well to minimize cell detachment.
- 6 Add the 250  $\mu$ l of DNA/TransIT-293/Opti-MEM mix from Step 4 dropwise to the HEK 293T cells. Rock the plate gently back and forth to evenly distribute the transfection complexes. Return the plate to the 37 °C incubator for 48 h.

**! CAUTION** Users should familiarize themselves with their institutional guidelines for handling lentivirus. If appropriate, cells should be transferred to a BSL-2 tissue culture room at this point.

- 7 *Harvest viral supernatant.* Pipette the medium off of the cells and into a syringe attached to a 0.45- $\mu$ m filter. Insert the plunger and gently force the viral supernatant through the filter into a sterile collection tube.

**■ PAUSE POINT** Viral supernatants can be divided into 1.5-ml aliquots in microcentrifuge tubes and stored at –80 °C for several months with minimal loss of activity.

#### Lentiviral transduction ● Timing 3 d

- 8 *Seed target cells for transduction.* For HeLa cells, seed  $1 \times 10^6$  cells in each well of a 6-well plate in 2 ml of complete RPMI cell culture medium (a total of  $6 \times 10^6$  cells in 12 ml of complete RPMI cell culture medium). Return the plate to the 37 °C incubator for 5 h.

**▲ CRITICAL STEP** Both control and treated cells need be seeded to allow for parallel transduction. Continue to culture some wild-type target cells, as these will be required as a control sample when estimating transduction efficiency at Step 14.

- 9 *Transduce the target cells with lentivirus.* Defrost the viral supernatant from Step 7 and add it to the target cells. For HeLa cells, aspirate the 2 ml of growth medium and replace it with 1.5 ml of viral supernatant.
- 10 (Optional) To maximize transduction efficiency, polybrene (to a final concentration of 8  $\mu$ g ml<sup>–1</sup>) can be added with the virus and/or the cells can be spun following the addition of virus (800g, 60 min, 37 °C).
- 11 3 h later, aspirate and discard the viral supernatant from the cells and replace it with complete RPMI cell culture medium. Return the plate to the 37 °C incubator overnight.
- 12 *Repeat transduction of the target cells.* To increase the overall MOI achieved, 24 h after the initial transduction, repeat the transduction process described in Steps 9–11.
- 13 *Harvest the transduced cells.* 24 h after the second transduction, trypsinize the cells using 0.5 ml of trypsin-EDTA per well, quench with an equal amount of complete RPMI cell culture medium, pool and pipette into a 50-ml conical tube. Pipette a few drops of the cell suspension into a round-bottom 5-ml polypropylene tube for flow cytometry analysis<sup>9</sup> (Step 14). Then pellet the remainder by centrifugation (500g, 5 min, 4 °C), and wash the cells once by resuspending the pellet in 50 ml of PBS and repeating the centrifugation (500g, 5 min, 4 °C). Finally, remove the supernatant and resuspend the pellet in 3 ml of Cell Lysis Buffer from the Genra Puregene Cell Kit.

**■ PAUSE POINT** According to the manufacturer, cell lysates can be stably stored at room temperature for at least 2 years.

- 14 *Verify transduction efficiency by flow cytometry.* If the lentiviral vector used expresses a fluorescent marker, then the transduction efficiency of the target cells can be assayed by flow cytometry. Set up the instrument using untransduced target cells, and then analyze the aliquot of transduced cells from Step 13. If high-level transduction has been achieved, then nearly 100% of the cells should be positive for expression of the fluorescent protein.

#### ? TROUBLESHOOTING

**LAM-PCR amplification of virus-genome junctions** ● **Timing 5 d**

15 *Extract genomic DNA from cell lysates.* Follow the Gentra Puregene Cell Kit manual protocol for genomic DNA extraction from the cultured cell lysates from Step 13. Resuspend the final DNA pellet in 500 µl of DNA hydration solution (from the Gentra kit).

■ **PAUSE POINT** Genomic DNA can be stored stably for years at 4 °C.

16 *Digest genomic DNA with NlaIII.* Measure the DNA concentration using a NanoDrop spectrophotometer. Take half of the DNA out for restriction enzyme digestion and keep the other half at 4 °C as a backup. For each 10 µg of DNA to be digested, set up the following reaction in a 1.5-ml microcentrifuge tube:

Component (add in this order)	Amount	Final concentration
Genomic DNA	10 µg	0.1 µg µl <sup>-1</sup>
10× NEB CutSmart Buffer <sup>a</sup>	10 µl	1×
ddH <sub>2</sub> O	Up to 90 µl	
NlaIII (10 U µl <sup>-1</sup> )	10 µl	1 U µl <sup>-1</sup>
Total volume	100 µl	

<sup>a</sup>10× NEB CutSmart Buffer is supplied with NlaIII.

For example, if a total of 200 µg of DNA was extracted, digest 10 µg of DNA in ten separate digestion reactions. Incubate all tubes at 37 °C overnight.

! **TROUBLESHOOTING**

17 *Purify and concentrate the digested DNA using a spin column.* Follow the QIAquick PCR Purification Kit protocol. To avoid exceeding the binding capacity of the columns, perform a separate purification for each 10 µg of digested DNA. Elute the DNA from each column in 30 µl of UltraPure water, and pool all of the eluted material in a fresh 1.5-ml microcentrifuge tube. Alternatively, a standard phenol/chloroform extraction followed by ethanol precipitation<sup>20</sup> could be used to purify all of the DNA in a single process.

■ **PAUSE POINT** Digested DNA can be stored indefinitely at -20 °C.

18 *Size selection to enrich for large DNA fragments that contain virus-genome junctions.* Vortex the stock solution of Agencourt AMPure XP beads thoroughly. Add 0.5 volumes of beads to the pooled digested DNA solution from Step 17 and mix well by pipetting. Incubate for 5 min at room temperature.

19 Place the tube on a magnetic stand and wait until the beads are collected by the magnet on the back of the tube. Then remove the supernatant and discard.

20 Add 500 µl of 70% (vol/vol) ethanol to wash the beads. Aspirate and discard the ethanol, and then repeat the wash step. Allow the beads to air-dry for a few minutes.

21 Remove the tube from the magnet. Add 100 µl of UltraPure water and pipette to resuspend the beads. Incubate the tube for 5 min at room temperature.

22 Place the tube back on the magnet. Wait for the magnet to collect the beads on the back of the tube (~1 min). Collect the supernatant containing the eluted DNA into a fresh 1.5-ml microcentrifuge tube, and measure the concentration of the sample using a NanoDrop spectrophotometer. Typical recovery would be about one-third of input DNA.

23 *Ligate annealed adapters to the size-selected DNA fragments.* For each 3 µg of DNA, set up the following reaction in a 0.2-ml PCR tube:

Component (add in this order)	Amount	Final concentration
Size-selected, NlaIII-digested genomic DNA	3 µg	0.06 µg µl <sup>-1</sup>
Annealed adapter (50 µM)	5 µl	5 µM
10× NEB T4 Ligase Reaction Buffer <sup>a</sup>	5 µl	1×
ddH <sub>2</sub> O	Up to 48 µl	
T4 DNA Ligase (400 U µl <sup>-1</sup> )	2 µl	16 U µl <sup>-1</sup>
Total volume	50 µl	

<sup>a</sup>10× NEB T4 Ligase Reaction Buffer is supplied with T4 DNA Ligase.

Incubate at 16 °C overnight in a PCR instrument.

- 24 *Clean up the ligated DNA using Agencourt AMPure XP beads.* Pool all of the ligation reactions in a 1.5-ml microcentrifuge tube, add 0.7 volumes of beads and follow the procedure described in Steps 18–22.
- ▲ **CRITICAL STEP** This cleanup step removes unligated adapters from the sample, which otherwise would decrease the efficiency of the subsequent PCR steps.
- **PAUSE POINT** Ligated DNA can be stored indefinitely at  $-20\text{ }^{\circ}\text{C}$ .
- 25 *Amplify virus–genome junctions by linear PCR.* Measure the concentration of adapter-ligated DNA using a NanoDrop spectrophotometer; the total amount of DNA should be roughly equivalent to the total amount of input DNA. To amplify the material, for each 2  $\mu\text{g}$  of adapter-ligated DNA, make up the following mix:

Component (add in this order)	Amount	Final concentration
Adapter-ligated genomic DNA	2 $\mu\text{g}$	0.04 $\mu\text{g } \mu\text{l}^{-1}$
Accuprime PCR Buffer II (10 $\times$ )	5 $\mu\text{l}$	1 $\times$
Bio-Pack_F (Table 1; 0.25 $\mu\text{M}$ )	1 $\mu\text{l}$	5 nM
ddH <sub>2</sub> O	Up to 49.6 $\mu\text{l}$	
Accuprime Taq DNA Polymerase (2 U $\mu\text{l}^{-1}$ )	0.4 $\mu\text{l}$	0.016 U $\mu\text{l}^{-1}$
Total volume	50 $\mu\text{l}$	

\*Accuprime PCR Buffer II is supplied with the Accuprime Taq DNA Polymerase System.

Run the following thermocycler program:

Step	Denature	Anneal	Extend	Hold
1	94 $^{\circ}\text{C}$ , 5 min			
2–200	94 $^{\circ}\text{C}$ , 60 s	55 $^{\circ}\text{C}$ , 45 s	68 $^{\circ}\text{C}$ , 90 s	
201			68 $^{\circ}\text{C}$ , 7 min	
202				4 $^{\circ}\text{C}$

### ? TROUBLESHOOTING

- **PAUSE POINT** PCR products can be stored indefinitely at  $-20\text{ }^{\circ}\text{C}$ .
- 26 *Capture biotinylated PCR products onto streptavidin-coated magnetic beads.* Pool all of the linear PCR reactions in a single 1.5-ml microcentrifuge tube. Estimate the total volume using a pipette.
- 27 *Prepare streptavidin beads.* Vortex the tube of beads thoroughly, and then pipette 5  $\mu\text{l}$  of beads per PCR reaction (i.e., 40  $\mu\text{l}$  of beads total for eight PCR reactions) into a 1.5-ml microcentrifuge tube. Block the beads by collecting the beads on the magnet for  $\sim 1$  min. Aspirate the supernatant, discard and resuspend the beads in PBS + 0.5% (wt/vol) BSA.
- 28 Incubate the tube for 30 min at room temperature, inverting it occasionally to prevent the beads from sinking.
- 29 Collect the beads on a magnet for  $\sim 1$  min, wash once with 1 ml of 1 $\times$  B&W buffer, and discard. Then resuspend the beads in 2 $\times$  B&W buffer + 0.2% (vol/vol) Tween 20; the volume of buffer should be equal to the volume of the pooled PCR products.
- 30 Add the pooled PCR products from Step 26 to the beads from Step 29, and mix thoroughly by pipetting. Incubate for 2 h at room temperature, shaking at 1,100 r.p.m. in an orbital shaker to prevent the beads from sinking.
- 31 To wash unbound material from the streptavidin beads, place the tube on the magnet to collect the beads, and then remove the supernatant. (The initial supernatant can be stored for months at  $-20\text{ }^{\circ}\text{C}$  as a backup; this could be re-amplified in a further linear PCR reaction later if necessary.)
- 32 Add 1 ml of 1 $\times$  B&W + 0.1% (vol/vol) Tween 20 to the beads, resuspend by pipetting, and return the tube to the orbital shaker at 1,100 r.p.m. for 10 min.

- 33 Wash the beads thoroughly by repeating Steps 31 and 32 ten times.  
**■ PAUSE POINT** The beads can be stored in 1× B&W + 0.1% (vol/vol) Tween 20 at 4 °C for several weeks.
- 34 *Generate Illumina sequencing libraries through an on-bead exponential PCR reaction.* Prepare the PCR master mix on ice. In total, perform the same number of independent exponential PCR reactions as the number of linear PCR reactions performed in Step 25. The following mix details the components for one reaction:

Component (add in this order)	Amount (μl)	Final concentration
Accuprime PCR Buffer II (10×)	5	1×
P5-HIV1_F (Table 1; 100 μM)	0.25	0.5 μM
P7-Adapter_R (Table 1; 100 μM)	0.25	0.5 μM
ddH <sub>2</sub> O	Up to 49.4	
Accuprime Taq DNA Polymerase (2 U μl <sup>-1</sup> )	0.6	0.024 U μl <sup>-1</sup>
Total volume	50	

For example, if eight separate linear PCR reactions were performed in Step 25, multiply each of the values above by 8 to make up an 8× master mix of 400-μl total volume.

- 35 Collect the streptavidin beads from Step 33 on the magnet, and perform one final wash with PBS. Then collect the beads on the magnet, aspirate the PBS and resuspend the beads in all of the PCR master mix. Distribute 50 μl of the bead-PCR mix into each tube of an 8-strip of PCR tubes.
- 36 Run the following thermocycler program:

Step	Denature	Anneal	Extend	Hold
1	94 °C, 2 min			
2-11	94 °C, 30 s	55 °C, 30 s	68 °C, 90 s	
12			68 °C, 7 min	
13				4 °C

- 37 Carefully remove the tubes from the PCR machine and place them on ice. Without disturbing the pellet of beads, remove 15 μl of the supernatant from each of the reactions and pool them in a separate 1.5-ml tube kept on ice. This is the ten-cycle sample.
- 38 Return the remaining PCR reaction mix to the thermocycler for a further four cycles of PCR:

Step	Denature	Anneal	Extend	Hold
1	94 °C, 2 min			
2-5	94 °C, 30 s	55 °C, 30 s	68 °C, 90 s	
6			68 °C, 7 min	
7				4 °C

- 39 Again, carefully remove 15 μl of the supernatant from each of the reactions and pool them in a separate 1.5-ml tube kept on ice. This is the 14-cycle sample. Then return the tubes to the thermocycler for a final four cycles of PCR:

Step	Denature	Anneal	Extend	Hold
1	94 °C, 2 min			
2-5	94 °C, 30 s	55 °C, 30 s	68 °C, 90 s	
6			68 °C, 7 min	
7				4 °C

- 40 Remove all of the remaining volume (including the beads) from the PCR reactions and pool in a separate 1.5-ml tube kept on ice. To remove the beads, place the tube on the magnet for 2 min and transfer the supernatant to a fresh tube. This is the 18-cycle sample.
- 41 *Perform a final cleanup of the Illumina libraries.* For each of the samples (10 cycles, 14 cycles and 18 cycles), perform an Agencourt AMPure XP bead cleanup as described in Steps 18–22, using 0.8 volumes of beads. Elute in 20  $\mu$ l of UltraPure water.
- **PAUSE POINT** Sequencing libraries can be stored indefinitely at  $-20^{\circ}\text{C}$ .
- 42 *Validate the Illumina sequencing libraries using an Agilent Bioanalyzer.* Run 1  $\mu$ l of each of the sequencing libraries on a DNA High Sensitivity Chip. The library electropherogram should reveal a broad size distribution between  $\sim 240$  bp and  $\sim 600$  bp, with minimal adapter dimer contamination at  $\sim 120$  bp.
- ? **TROUBLESHOOTING**
- 43 *Submit optimal sample for Illumina sequencing.* Using the Bioanalyzer software, quantify the concentration of the DNA of the correct size (between  $\sim 240$  bp and  $\sim 600$  bp). Choose the sample that has been subjected to the lowest number of PCR cycles, but still exceeds the minimum amount required by your sequencing facility. Submit the final libraries for Illumina sequencing, along with a custom sequencing primer that binds to the extreme end of the lentiviral 5' LTR (ISP, in our case; see Table 1). We generated 50-bp single-end sequence reads using an Illumina HiSeq2500 machine.
- ? **TROUBLESHOOTING**
- **PAUSE POINT** Analysis of sequencing data can be performed at any time.

### Mapping of Illumina reads and identification of genomic loci exhibiting differential accessibility ● Timing <1 d

- 44 Copy the output .fastq sequencing files generated by the Illumina sequencing run to the hard drive of the Linux computer.
- 45 Run FastQC to check the run. Navigate to the folder containing the sequencing files, open a terminal window and type the following command after the \$ sign:

```
$ fastqc inputfile.fastq
```

where *inputfile* is the name of the file to be analyzed.

- 46 Perform an adapter trim to remove adapter sequences from the 3' ends of the reads. Type the following command:

```
$ fastx_clipper -a CCTATAGTGAGTCGTA -l 18 -v -i inputfile.fastq -o outputfile_trimmed.fastq
```

where *inputfile* is the name of the file to be analyzed and *outputfile\_trimmed* is the desired name of the output .fastq file containing the trimmed reads.

- 47 Map to the human genome using Bowtie 2. Type the following command:

```
$ bowtie2 -t -x GRCh38 -U inputfile_trimmed -S outputfile.sam
```

where *GRCh38* is the basename of the Bowtie2 index, *inputfile\_trimmed* is the file of trimmed reads from Step 46, and *outputfile* is the desired name of the output .sam file containing the mapped reads.

- 48 To save disk space, compress the output .sam file to a bam file by typing:

```
$ samtools view -b outputfile.sam > outputfile.bam
```

- 49 *Import the resulting .bam file into SeqMonk.* The following can be performed on any operating system. Navigate to the folder containing the unzipped SeqMonk installation files, and launch the program by running the SeqMonk executable file.
- 50 From the 'File' menu, select 'New Project'. From the resulting dialog box, click the 'Import Genome From Server' button and select the genome build to which the data were mapped (GRCh38, in this example). Then click on 'Start New Project' to initialize the project.

- 51 To load the data, from the 'File' tab, select 'Import Data' and then click on 'BAM/SAM'. Select the location of the relevant .sam or .bam file(s) from Step 47 or Step 48 on your computer, and then click 'OK'.
- 52 Specify import options: set 'Remove duplicate reads' to 'Yes' and 'Min mapping quality' to '20'. Click 'OK'.
- 53 *Collapse the sequencing reads into a set of unique sites of lentiviral integration.* Using SeqMonk's import function, from the 'File' tab, choose 'Import Data' and then 'Visible Data Stores'.
- 54 Set 'Extend reads by (bp)' to '-1,000', and then click 'Import'. This creates a new data track for each sample in which only the first base of each read (i.e., the point of viral integration) is displayed.
- 55 Remove the original data tracks: click on the 'Change Data Tracks' icon, highlight the original data files in the 'Displayed Tracks' window, and click the 'Remove' button, followed by the 'OK' button.
- 56 *Segment the genome into a set of equally sized intervals and quantitate the number of unique lentiviral integration sites in each window.* From the 'Data' tab, choose 'Define Probes' and highlight 'Running Window Generator' from the 'Probe Generator Options' list.
- 57 Set 'Probe Size (bp)' to '10000' and the 'Step Size (bp)' to '5000'. Then click the 'Create Probes' button. This divides the genome into a set of 10-kb intervals which overlap by 5 kb.
- 58 To count the number of integration sites in each window, highlight 'Read Count Quantitation' from the 'Quantitation Options' list. Ensure that the 'Correct for total read count' and 'Log Transform Count' boxes are checked, and click 'Quantitate'.
- 59 *Visualize the data as a scatter plot and allow normalization.* From the 'Plots' tab, select 'Scatter Plot'.
- 60 Select the two relevant datasets to compare, for example, wild-type on the  $x$  axis and mutant on the  $y$  axis. Each dot on the resulting plot represents the quantitation for one 10-kb genomic interval. Dots lying on the diagonal represent intervals containing equal frequencies of viral integration in both conditions.
- 61 The majority of the dots should lie along the diagonal. Because the data have been collapsed into a set of unique integration sites, skewing of the data away from the diagonal may be observed at the top end of the distribution. If this is observed, normalize the data by clicking on the 'Data' tab, selecting 'Quantitate Existing Probes', and then highlighting 'Match Distribution Quantitation' from the list of 'Quantitation Options'. Select the two datasets to normalize from the 'Data Stores to Normalise' list on the right, and then click the 'Quantitate' button. Re-display the scatter plot to verify that the normalization has been successfully applied.
- 62 *Identify loci exhibiting significant changes in lentiviral accessibility.* From the 'Filtering' tab, choose 'Filter by Statistical Test', then 'Intensity Difference' and 'Individual Probes'.
- 63 In the resulting dialog box, select the relevant datasets to compare. The control (e.g., wild-type) dataset should be selected from the first list, called 'From Data Store / Group', and the experimental (e.g., mutant) dataset should be selected from the second list, called 'To Data Store / Group'. Maintain the default parameters on the right, and click 'Run Filter'.
- 64 To visualize the loci exhibiting significant changes in lentiviral accessibility, re-create the scatter plot by following the instructions in Steps 59 and 60.
- 65 Then click on the 'Highlight Sublists' button in the top right corner. From the 'Available Lists' list, highlight the entry that begins 'Intensity Difference  $p < 0.05$ ' and click 'Add', followed by 'OK'. The scatter plot now shows loci exhibiting significantly altered accessibility in the experimental versus the control datasets colored in blue.
- 66 Export the scatter plot by clicking on the 'Save Image' button.
- 67 Finally, a summary of the data can be exported for further analysis. On the list on the left-hand side of the home screen, under 'Probe Lists' click on the icon next to 'All Probes' to expand the display and highlight the list of significant loci by clicking on it.
- 68 Under the 'Reports' tab, select 'Annotated Probe Report'.
- 69 In the resulting dialog box, under 'Annotate with', select 'overlapping' from the left-hand menu and 'gene' from the right-hand menu, and then click 'OK'.
- 70 The 'Annotated Probe Report' can then be saved and further manipulated in standard programs such as Microsoft Excel.

## Troubleshooting

Troubleshooting advice can be found in Table 2.

Step	Problem	Possible reason	Solution
14	Low transduction efficiency observed by flow cytometry	Inefficient viral packaging in HEK 293T cells Target cells refractory to lentiviral transduction	Thaw a fresh aliquot of HEK 293T cells and verify that they are free of <i>Mycoplasma</i> infection. Passage the cells every 2 d and do not allow them to become over-confluent. Prepare fresh plasmid stocks of the lentiviral vectors Verify that high-titer lentiviral stocks have been generated by performing a parallel transduction of a cell line that is readily transducible, for example, HEK 293T or HeLa
16	Inefficient digestion of genomic DNA	Addition of too much DNA to the digestion reaction	If the genomic DNA samples are 'stringy', dilute them further in DNA hydration solution before measuring their concentration. Take duplicate NanoDrop readings of the genomic DNA samples and verify that the readings are similar
25	Low sample volumes after the linear PCR	Evaporation during the PCR reaction	Avoid placing samples in the outer tubes of the PCR strip. Instead add 200 $\mu$ l of water to the outer tubes. Ensure that the PCR strips are tightly capped
42	No peaks detected in the Bioanalyzer trace	Could result from failure at any step of LAM-PCR protocol	Efficient NlaIII digestion of genomic DNA can be verified by agarose gel electrophoresis of the digested DNA; this should generate a smear with a mean fragment size of ~200bp. Efficient adapter ligation can be verified by a simple PCR reaction using the adapter-ligated DNA (from Step 24) as the template with a single primer that will anneal to the adapter ligated onto both ends of the fragments (e.g., P7-Adapter_R; Table 1). Using standard cycling conditions (e.g., 30 cycles of those described in Step 36), the product should appear as a bright smear when observed by agarose gel electrophoresis. If both of these steps have worked correctly, then repeat the LAM-PCR, bead-annealing and exponential PCR steps of the protocol
43	Failed Illumina sequencing run	Low complexity	Correct library preparation should result in a complex library with approximately even representation of each base across the read. Problems with low complexity indicate an excessive amount of adapter artifacts. In this case, it would be advisable to repeat the LAM-PCR procedure, verifying efficient NlaIII digestion by agarose gel electrophoresis and adapter ligation by PCR before the LAM-PCR steps

## Timing

Steps 1–7, generation of lentivirus stocks: 4 d  
 Steps 8–14, lentiviral transduction of target cells: 3 d  
 Steps 15–43, LAM-PCR amplification of virus–genome junctions: 5 d  
 Steps 44–70, bioinformatic analysis of Illumina sequencing data: <1 d

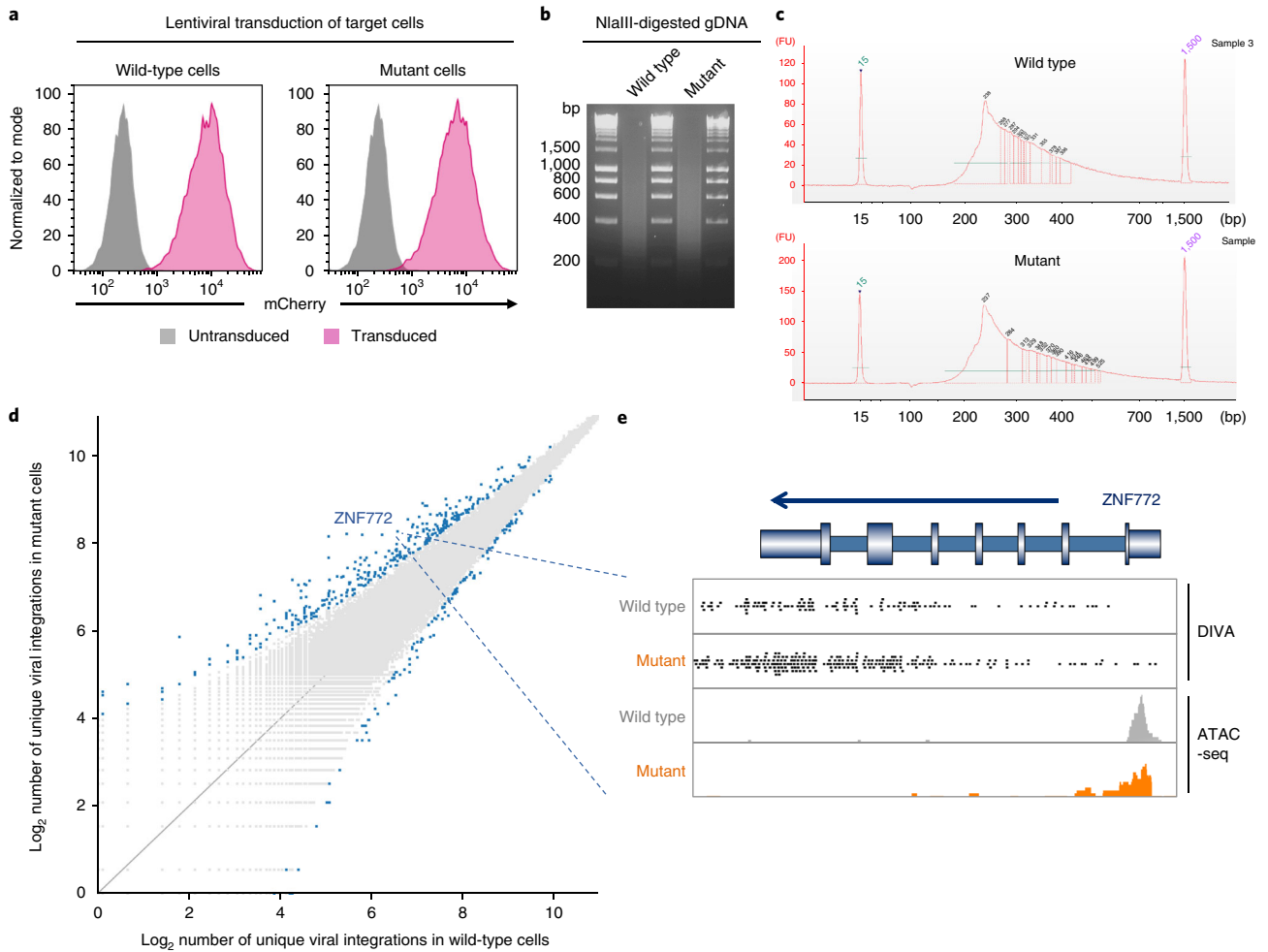
## Anticipated results

### Transduction of target cells

We recommend using a lentiviral vector that encodes a fluorescent marker, which enables the transduction of the target cells to be assayed by flow cytometry (Step 14). If high-efficiency transduction of the target cells has been achieved, then ~100% of the cells should be positive. An example of successful transduction of wild-type and mutant HeLa cells with the pHRSIN-P<sub>SFFV</sub>-mCherry lentiviral vector is shown in Fig. 4a.

### LAM-PCR preparation of Illumina sequencing libraries

For HeLa cells, in Step 15, the total amount of DNA extracted from the entire 6-well plate of transduced cells should be ~200  $\mu$ g. Following successful digestion with NlaIII, the vast majority of the genomic DNA should be fragmented to <1 kb and can be visualized as a smear by agarose gel electrophoresis (Fig. 4b). After size selection for large DNA fragments in Steps 18–22, the amount of DNA recovered should be ~20–30% of the total input digested DNA. Successful LAM-PCR amplification of virus–genome junctions can be assayed by Agilent Bioanalyzer analysis of the final Illumina library; we would expect the Bioanalyzer trace in Step 42 to look similar to that shown in



**Fig. 4 | Anticipated results of a DIVA experiment.** **a**, Successful lentiviral transduction of target cells. Transduction of wild-type and mutant cell lines with high-titer virus should result in ~100% infection; this can be easily monitored by flow cytometry if the virus used expresses a fluorescent protein. **b**, Efficient digestion of genomic DNA with NlaIII as visualized by 2% agarose gel electrophoresis. Molecular weight markers are shown in lanes 1, 3 and 5. A broad smear should be observed with fragments ranging from ~100 bp to ~2 kb in both cell types. **c**, Validation of successful Illumina library generation. Using the pHR SIN-P<sub>EFFV</sub>-mCherry lentiviral vector with NlaIII digestion, Agilent Bioanalyzer analysis of the final exponential PCR product should reveal a trace starting at ~220 bp, peaking at ~240 bp, and then gradually tailing off by ~600 bp. A similar profile should be observed in both cell types. **d**, Results of a successful DIVA experiment. (Left) Each point on the scatterplot indicates the correlation between the number of unique lentiviral integrations detected in the wild-type cells versus that in the mutant cells for a given 10-kb genomic locus. Loci exhibiting a significant change in accessibility between the wild-type and mutant samples are highlighted in blue. Adapted with permission from ref. <sup>9</sup>, Springer Nature. (Right) Raw data for one locus of interest (*ZNF772*), comparing the coverage achieved using DIVA and ATAC-seq. ATAC-seq transposon integrations are concentrated at the transcriptional start site, whereas DIVA lentiviral integration sites are found predominantly across the body of the gene. A greater number of unique integration sites are detected across the body of the gene in the mutant sample, indicating an increase in chromatin accessibility in the mutant cell line.

Fig. 4c, with the trace starting at ~220 bp, peaking at ~240 bp, and then gradually tailing off by ~600 bp.

**Bioinformatic analysis of Illumina sequencing data**

Any abnormalities with the sequencing data should be revealed by FastQC at Step 45. Efficient amplification of virus–genome junctions should result in approximately even representation of each of the four bases across the sequence reads; strong bias in base representation is indicative of adapter artifacts.

On the basis of downsampling analysis of our DIVA Illumina sequencing data<sup>9</sup> (Fig. 3), we found that ~10 million uniquely mapped reads per condition would have been sufficient to draw meaningful conclusions from the experiment. Therefore, for high coverage of a large mammalian genome, we recommend that >10 million uniquely mapped reads in each of the control and treated samples



should be obtained at Steps 43–47. Useful data may still be derived from samples with lower coverage, however, although an increased number of loci exhibiting a significant change in accessibility would probably be uncovered as more uniquely mapped reads are generated (Fig. 3d).

The overall aim of this protocol is to identify genomic loci exhibiting altered chromatin accessibility between control and treated cells. A scatterplot (Steps 59 and 60) summarizing the results of a DIVA experiment is shown in Fig. 4d. At the completion of a successful experiment, the researcher should have derived a list of genomic sites (tabulated in Steps 67–70) that show significantly altered accessibility between the two cell types.

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## Author contributions

R.T.T., I.A.T. and P.J.L. conceived the method. I.A.T. and R.T.T. performed all the experiments and, together with P.J.L., analyzed the data and wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

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