methods and protocols

MDPI

Protocol

Protocol for Generating Monoclonal Cell Lines with Specific Chromosomal Rearrangements

Nikolai A. Lomov [1,*], Viktoriia O. Ivanova [1], Vladimir S. Viushkov [1], Nikolai A. Nikolaev [2,3], Ekaterina V. Morgatskaia [1], Elena A. Zerkalenkova [4], Yegor S. Vassetzky [5] and Mikhail A. Rubtsov [1,6]

1 Department of Molecular Biology, Faculty of Biology, Lomonosov Moscow State University, Moscow 119234, Russia; vyushkovvs@my.msu.ru (V.S.V.)
2 Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, RAS, Moscow 117437, Russia
3 Institute of Translational Medicine, Pirogov Russian National Research Medical University, Moscow 117513, Russia
4 Oncology and Immunology, Dmitry Rogachev National Medical Research Center of Pediatric Hematology, Moscow 117198, Russia
5 CNRS UMR9018 Institut Gustave Roussy, Université Paris-Saclay, 94805 Villejuif, France
6 Department of technological leadership, Institute of translation medicine and biotechnology, Sechenov First Moscow State Medical University, Moscow 119435, Russia
* Correspondence: lomovna@my.msu.ru

Abstract

Chromosomal translocations are a hallmark of hematological malignancies. This protocol enables the expeditious production of monoclonal cell lines with specific rearrangements to serve as models for studying leukemia-associated translocations. The approach employs Cas9 to induce chromosomal breaks leading to rearrangements, followed by hierarchical subcloning and PCR-based screening to isolate and expand single-cell clones. Using the lymphoblastoid cell line RPMI8866 and the *KMT2A::AFDN* translocation as an example, we detail the steps from sgRNA design to validation of clones with the desired translocation.

Keywords: CRISPR-Cas; chromosomal rearrangements; cell models

1. Introduction

Genome editing is widely used for research purposes. However, genome editing events occur with varying efficiency. For instance, a frameshift induction is a relatively probable event, as it relies on the joining of DNA double-strand break ends that are in close proximity. In contrast, chromosomal rearrangements are rarer because they require the joining of ends from different DNA breaks that are located far apart or even on different chromosomes. To address this challenge, we have developed a protocol that enables the efficient derivation of monoclonal lines with specific chromosomal translocations.

In this protocol, we focus on the t(6;11)(q27;q23) translocation involving *KMT2A* (formerly *MLL*) and *AFDN* (formerly *AF6*), common among *KMT2A*-rearranged leukemias [1,2]. However, the proposed approach can be adapted to engineer other chromosomal translocations or deletions. Such cell lines can serve as in vitro models to study the effects of specific chromosomal aberrations on various aspects of tumor cell physiology and their sensitivity to anticancer drugs [3,4]. The protocol describes the generation of a monoclonal cell line, meaning that all cells in the line will contain the desired chromosomal translocation. Isolating a monoclonal line is key to eliminating the heterogeneity of polyclonal populations and create a reliable and reproducible experimental system. The primary technical challenges are the low initial frequency of cells that acquire the translocation and

Check for updates

Academic Editors: Philip Hublitz and Antónia Monteiro

Received: 3 October 2026
Revised: 25 November 2026
Accepted: 26 November 2026
Published: 28 November 2026

Copyright: © 2026 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

the absence of selectable markers to enrich these rare cells. To overcome this, we propose combining a hierarchical subcloning and direct PCR-screening strategy. This approach systematically isolates the rare translocation-positive cells from a bulk population, progressing from transfected cell cultures down to single-cell-derived monoclonal lines.

The protocol is optimized for a lymphoblastoid cell line RPMI8866 (LCL) chosen as an easy-to-manipulate cell line, yet it can be adapted to other immortalized cell lines. However, we do not recommend harnessing primary cells in the proposed protocol due to their limited expansion potential, which hinders the isolation of rare translocation-positive clones. For researchers aiming to model leukemogenesis de novo in non-transformed human models (e.g., primary umbilical cord blood HSPCs) where single-cell cloning is not feasible, we recommend a workflow specifically optimized for those cell types previously published by T Benz et al. [5]; however, this approach aims to obtain a bulk population modestly enriched in translocation-positive cells, in contrast to a homogeneous population carrying the rearrangement. When modeling a translocation intended to result in a fusion gene expression, always verify that the 5′ partner gene (e.g., *KMT2A*) is expressed in the chosen cell line. Otherwise, fusion gene expression is unlikely to occur, rendering the model useless.

We begin by detailing the design of specific single-guide RNAs (sgRNAs) targeting the intronic breakpoint cluster regions (BCRs) of *KMT2A* and *AFDN*. The chosen breakpoints match those found in patient leukemias, so the resulting chimeric *KMT2A::AFDN* mRNA reflects the disease-associated fusion. In this protocol, the *KMT2A::AFDN* translocation serves as a proof-of-concept to demonstrate the efficiency of our approach. The target sequences can be easily modified to engineer other chromosomal translocations (such as *KMT2A::AFF1*, *BCR::ABL1*, or *RUNX1::RUNX1T1*), making this protocol a versatile tool for studying various chromosomal rearrangements.

2. Experimental Design

The key innovation of this protocol lies in a sequential enrichment of translocation-positive cells and direct PCR screening: from a bulk population to 500-cell subpopulations, then to 20-cell subpopulations, and finally to single-cell clones. This approach overcomes the bottleneck of identifying rare translocation events, reducing protocol time and improving the efficiency of monoclonal line derivation. To improve the efficiency of translocation induction, we construct a single plasmid that encodes Cas9, both sgRNAs, and a fluorescent reporter protein, which allows the transfected cells to be sorted by fluorescence-activated cell sorting (FACS). Validation includes FISH, junction sequencing, and confirmation of chimeric mRNA expression. This method provides a robust and adaptable framework, typically yielding validated monoclonal lines within 2–3 months.

This protocol is designed for CRISPR-based editing of immortalized (continuously proliferating) cell lines, because isolating the monoclonal populations requires many cell divisions after the translocation event. The method cannot generate chromosomal rearrangements that are not stable through cell divisions, such as dicentric chromosomes or acentric derivatives, which would be lost during clonal expansion. Keep in mind that Cas9 can have off-target effects; to mitigate potential clonal variations caused by these effects, we recommend obtaining multiple independent clones for your downstream studies.

2.1. Materials

1. RPMI-1640 medium with alanyl-glutamine (PanEco Ltd., Moscow, Russia; Cat. no.: S363p);
2. Fetal Bovine Serum (FBS) Advanced (Capricorn Scientific GmbH, Ebsdorfergrund, Germany; Cat. no.: FBS-11A);
3. Antibiotic-Antimycotic 100X (Biowest, Nuaillé, France; Cat. no.: L0010);

4. DPBS (Capricorn Scientific GmbH, Ebsdorfergrund, Germany; Cat. no.: PBS-1A);
5. Proteinase K (Biolabmix LLC, Novosibirsk, Russia; Cat. no.: EP-1200);
6. dNTPs (Thermo Fisher Scientific, Waltham, MA, USA; Cat. no.: R0182S);
7. Hot start Taq Polymerase (Evrogen JSC, Moscow, Russia; Cat. no.: PK017L);
8. BstV2I (Sibenzyme Ltd., Novosibirsk, Russia; Cat. no.: E297);
9. PciSI (Sibenzyme Ltd., Novosibirsk, Russia; Cat. no.: E497);
10. DNase I, RNase-free (Thermo Fisher Scientific, Waltham, MA, USA; Cat. no.: EN0521);
11. *KMT2A* (*MLL*) gene break apart detection probe (Wuhan HealthCare Biotechnology Co., Ltd., Wuhan, Hubei, China; Cat. no.: FP-026);
12. Plasmid Miniprep 2.0 endotoxin-removing (Evrogen JSC, Moscow, Russia; Cat. no.: BC221L);
13. Animal and bacterial cells DNA isolation kit (Biolabmix LLC, Novosibirsk, Russia; Cat. no.: D-Cells-250);
14. DNA cleanup Mini (Evrogen JSC, Moscow, Russia; Cat. no.: BC023S);
15. RUplus. Column total RNA isolation kit (Biolabmix LLC, Novosibirsk, Russia; Cat. no.: RUplus-50);
16. RNAscribe RT (Biolabmix LLC, Novosibirsk, Russia; Cat. no.: R04-50);
17. Cell line: LCL RPMI8866 (European Collection of Authenticated Cell Cultures, Salisbury, UK; Cat. no.: 95041316).

2.2. Equipment

- CO2 Incubator (BINDER GmbH, Tuttlingen, Germany; Cat. no.: BD 115);
- Class II Biological Safety Cabinets (Labconco Corporation, Kansas City, MO, USA; Cat. no.: MP-Logic Class II, Type A2);
- Inverted microscope (Nikon Corporation, Yokohama, Japan; Cat. no.: Eclipse TS100);
- Eppendorf 5804R centrifuge (Eppendorf SE, Hamburg, Germany; Cat. no.: EP5805000327);
- Eppendorf A-2-DWP Microplate Rotor and Buckets (Eppendorf SE, Hamburg, Germany; Cat. no.: EP5804740009);
- Neon™ Transfection System (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA; Cat. no.: MPK5000);
- Invitrogen™ Neon™ Transfection System 100 µL Kit (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA; Cat. no.: MPK10096);
- Hemocytometer (MiniMed, Moscow, Russia; Cat. no.: 12001714);
- Flow cytometer cell sorter (FACSAria SORP instrument, BD Biosciences, San Jose, CA, USA; Cat. no.: N/A);
- T100 Thermal Cycler (Bio-Rad Laboratories, Singapore; Cat. no.: 1861096);
- 48 well Cell Culture Plates (NEST Scientific/Wuxi NEST Biotechnology, Wuxi, China; Cat. no.: N-748011);
- ml polystyrene culture tube (Greiner Bio-One GmbH, Kremsmünster, Austria; Cat. no.: 120161);

3. Procedure

This protocol describes the production of monoclonal cell lines with specific rearrangements. The approach employs Cas9 to induce chromosomal breaks leading to rearrangements, followed by hierarchical subcloning and PCR-based screening to isolate and expand single-cell clones (Figure 1).

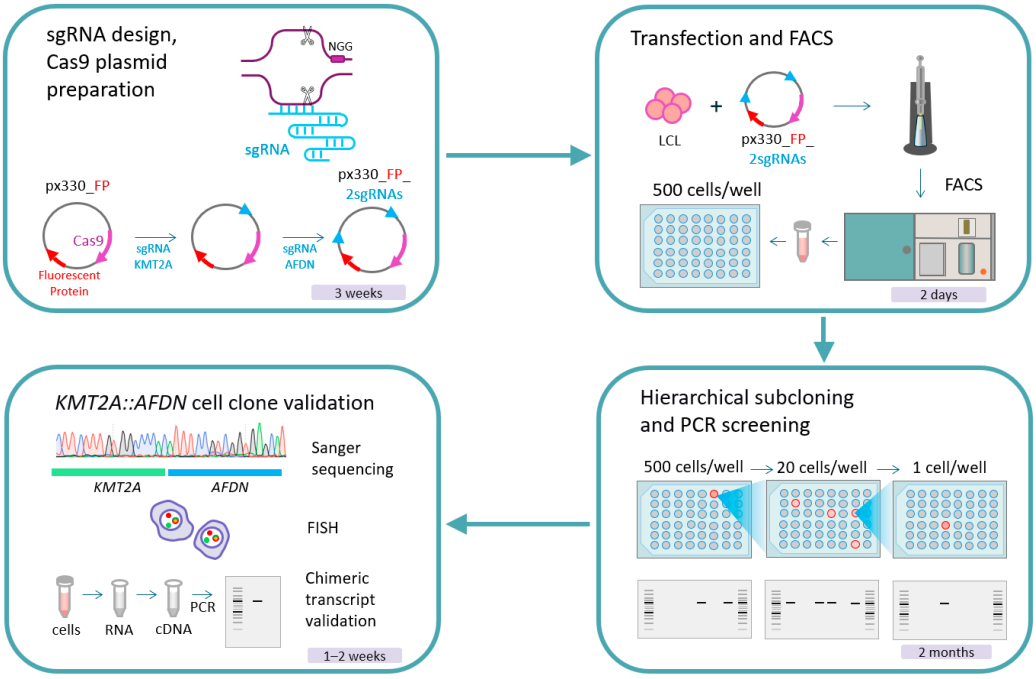

Figure 1. Graphical protocol showing the simplified workflow.

3.1. sgRNA Design

The selection of genomic breakpoints is determined by the specific chromosomal rearrangement being modeled. Because the oncogenic effect of translocation is driven by the expression of chimeric mRNA and protein, it is critical to target Cas9 to the same introns reported in patient-derived rearrangements. To achieve this, use online tools such as CRISPOR, CHOPCHOP, or DeepSpCas9 to identify target introns and design sgRNA [6–8].

During the sgRNA selection, ensure design of good PCR primers around sgRNA target sites for subsequent screening, as intronic regions often contain low complexity or repetitive sequences.

⚠ **CRITICAL** Do not use sgRNAs that target sequences near the 5′ and 3′ splice sites or the branch point of the intron [9–11]. To maximize efficiency, design sgRNAs to target regions as far from the splice sites as possible; a minimum 50 bp from the splice site to the Cas9 cleavage site is recommended. Disruption of splice sites will reduce the efficiency of, or entirely prevent, the chimeric intron splicing. Ensure that the predicted chimeric intron sequence retains a functional branch point.

OPTIONAL STEP Test the selected guides to ensure they are working properly. Multiple approaches for sgRNA efficacy estimation are described elsewhere [12,13].

This protocol uses *KMT2A::AFDN* translocation as an example to demonstrate the workflow. Target introns were chosen based on genomic data from leukemia patients with *KMT2A::AFDN* translocation. Breakpoints are located in the ninth intron of *KMT2A* and the first intron of *AFDN*, respectively [2,14] (Figure 2). For the sgRNA design and exon annotations, the following GenBank sequences were used: *KMT2A* NCBI Gene ID: 4297, transcript NM_001412597.1 and *AFDN* NCBI Gene ID: 4301, transcript NM_001386888.1. The sgRNA sequences were TTCCTAGGCTAGAACATGTG (for *KMT2A*) and ATGCTCACCGACGGAAATCA (for *AFDN*), selected with CHOPCHOP tool [8].

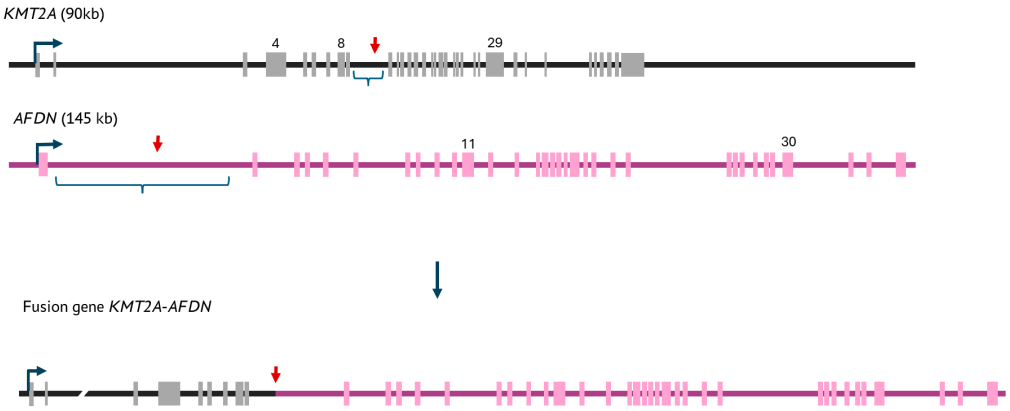

Figure 2. Schematic representation of gene translocation and the formation of the *KMT2A::AFDN* fusion gene. The curly brackets indicate the genomic region evaluated for sgRNA design; these regions correspond to the introns containing the breakpoints typically identified in patients with this translocation. Red arrows highlight the specific sgRNA targets used in this example.

3.2. Primer Design

1. Create a map of the chimeric locus using the reference sequences of the translocation partner genes. Include approximately 1–2 kb sequences of the partner genes adjacent to the breakpoint. Consider that Cas9 introduces a break between the 3rd and 4th base pairs upstream of the protospacer adjacent motif (PAM) sequence (NGG for spCas9—Cas9 from *Streptococcus pyogenes*) [15,16].
2. Design several pairs of primers using the primer designing tool (we recommend NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed on 25 November 2026)), verify primer pair specificity against the Homo sapiens Refseq representative genomes, and set the PCR product size from 400 to 1500 [17,18].

⚠ **CRITICAL** Selected primers should anneal at least 100 bp away from the Cas9 cleavage site because Cas9 can lead to the formation of large deletions in the edited region [19].

3. Design primers for a non-rearranged locus. Amplification using these primers will serve as a positive control for direct PCR efficiency.

OPTIONAL STEP Design multiple primer sets for both the translocation and control loci to select the best combination if PCR screening optimization is required.

4. Design primers for chimeric gene expression analysis. To do this, select which mRNA variants are relevant for the cells and determine which exons should be present in the chimeric mRNA. Design primers for exons of a chimeric transcript according to general primer design guidelines for reverse transcription PCR [20,21]. The forward primer should be in the breakpoint-proximal exon of *KMT2A*. The reverse primer should be in the breakpoint-proximal exon of *AFDN*. As a positive control, design primers for the housekeeping gene expression.

3.3. Construction of an Empty px330_FP_2sgRNA Backbone

To induce a chromosomal rearrangement, construct an expression vector encoding both the Cas9 nuclease and a pair of sgRNAs. As an example, the construction of such vector is detailed below. To maximize delivery efficiency, this single construct co-expresses the Cas9 nuclease, both sgRNAs, and a fluorescent reporter, the latter enabling FACS-based enrichment of transfected cells. To facilitate the targeting of any new gene pair, we developed the universal vector px330_FP_2sgRNA containing sites for type IIS restriction enzymes for the rapid insertion of the recognizing regions of the sgRNAs (Figure S1).

Using two cloning steps, this empty backbone can be programmed to introduce a new chromosomal rearrangement.

The plasmid px330_FP_2sgRNA was constructed via sequential molecular cloning to generate an all-in-one vector for co-expression of Cas9, a fluorescent reporter (we use SantakaRFP ATUM's synthetic non-Aequorea fluorescent protein, but you can use another available fluorescent protein), and two user-defined sgRNAs. All primers used in this protocol are listed in Supplementary Table S1.

3.3.1. Insertion of the SV40 Promoter

A DNA fragment containing the SV40 early promoter was amplified from plasmid phU6_sgRNA (#53188 Addgene) using primers SV40_F_CciNI and SV40_R_SbfI. The primers were designed to introduce a 5′ CciNI site and a 3′ SbfI site, the latter immediately preceded by a BmtI site added for the subsequent cloning step. The PCR product was digested with CciNI and SbfI and ligated in the backbone plasmid pX330-U6-Chimeric_BB-CBh-hSpCas9 (#42230 Addgene) digested with CciNI and SbfI.

3.3.2. Insertion of the Fluorescent Protein (FP) Reporter Gene

A Kozak-SantakaRFP-SV40polyA fragment was amplified from a plasmid OR3_Santaka_red_304 (kindly provided by Prof. Kerstin Bystricky) using primers FP_F_BmtI and SV40polyA_R_BH_SbfI. The primers contain BmtI, SbfI, BstAFI and HindIII sites for subsequent cloning. The amplified fragment and the intermediate plasmid from Step 1 were digested with BmtI and SbfI, purified, and ligated. The resulting construct was designated px330_FP_ gRNA.

3.3.3. Engineering of the sgRNA Donor Plasmid Intermediate (53188-PciSI)

The plasmid phU6_sgRNA (#53188 Addgene) was digested with BstV2I (BbsI isoschizomer). A double-stranded oligonucleotide adapter, formed by annealing oligonucleotides PciSI_53188_F and PciSI_53188_R, was ligated into the linearized backbone. This adapter introduced PciSI (Type IIS) recognition sites, generating the donor plasmid 53188-PciSI.

3.3.4. Cloning of the sgRNA Expression Cassette into the Vector px330_FP_gRNA

The U6 promoter and sgRNA scaffold cassette was amplified from the donor plasmid 53188-PciSI using primers U6_BstAF_F and sgRNA_R_Hind, which contain BstAFI and HindIII sites. The amplified cassette and the plasmid px330_FP_gRNA were digested with BstAFI and HindIII, purified, and ligated.

The resulting plasmid was named px330_FP_2sgRNA (Figure S1) and was then used for cloning the two sgRNA recognition sequences of *KMT2A* and *AFDN* into it with BstV2I and PciSI restriction enzymes, respectively.

3.4. Insertion of Spacer Sequences into the Empty Backbone

Insert spacer sequences into the empty backbone of px330_FP_2sgRNA to direct the sgRNAs to the target genes (Figure S2). The spacer sequences (20 nucleotides) are inserted as a DNA fragment consisting of two complementary oligonucleotides with overhangs suitable for ligation into the empty backbone of px330_FP_2sgRNA digested with BstV2I and PciSI restriction enzymes. The *KMT2A* and *AFDN* gene sequences recognized by the guide RNAs are shown in the "sgRNA design" section. If your target sequence starts with an A, C, or T, add an extra "G" before it to enhance transcription from the U6 promoter. This makes your targeting sequence 21 bases long, though the mismatched "G" generally does not affect Cas9 targeting efficiency [22]. The sequences of the complementary forward

and reverse oligonucleotides used for cloning *KMT2A* and *AFDN* sgRNAs are detailed in Supplementary Table S1. Verify the insertions in the resulting plasmid by Sanger or Nanopore sequencing. For Sanger sequencing, use primer eCMV_R for sgRNA1 (*KMT2A*) and primer sgRNA_R_Hind for sgRNA2 (*AFDN*).

3.5. Plasmid px330_FP_2sgRNA Preparation for Transfection

Prepare the plasmid for transfection: the plasmid solution must be sterile and have a concentration of at least 1 µg/µL. For this purpose, it is recommended to perform several routine minipreps, pool them, and then perform ethanol precipitation, dissolving the DNA pellet in a small volume of elution buffer (supplied with the miniprep kit) prefiltered through a 0.2 µm membrane. Measure the plasmid concentration using a microvolume UV spectrophotometer. Perform all steps, starting with ethanol precipitation, under sterile conditions.

We typically use 4–6 miniprep columns to extract plasmid from 20 to 30 mL of overnight culture. Alternatively, a midi-prep can be performed to obtain the required quantity of plasmid. However, pooling multiple mini-preps is often faster than a single midi-prep protocol.

3.6. Preparing LCL Cell Culture for Transfection

1. Culture the cells in RPMI-1640 medium supplemented with 10% FBS and 1× Antibiotic-Antimycotic (further in the text—cell culture medium) at 37 °C in a humidified 5% CO_2 incubator. Maintain culture at a density of 0.1 to 1 ×10^6 cells/mL by periodic dilutions every 3–4 days.

⚠ **CRITICAL**: Standard aseptic techniques and regular mycoplasma screening should be maintained throughout all cell culture procedures.

2. Prepare cells for transfection.
    a. One day before transfection, determine the cell density by counting with a hemocytometer or an automatic cell counter.
    b. Dilute the culture with fresh, pre-warmed cell culture medium to a concentration of approximately 0.5 × 10^6 cells/mL.

3.7. Transfection of LCLs

1. Prepare reagents and transfection equipment.
    a. Pre-warm cell culture medium to 37 °C.
    b. Add 2 mL of warm medium to wells of a six-well plate.
    c. Use one well for plasmid-transfected cells and another for the mock-transfected control (cells processed identically but without plasmid DNA). We used the Neon Electroporation System, but you can use another electroporator.
    d. Insert a Neon Electroporation Tube into the Pipette Station.
    e. Transfer 3 mL of Neon Buffer E2 (supplied with the kit) into the Neon Electroporation Tube.
2. Prepare cells for transfection.
    a. Determine cell concentration using a hemocytometer.
    b. Transfer 4 × 10^6 cells to a 15 mL conical tube. Use 2 ×10^6 cells per plasmid transfection and 2 ×10^6 per mock transfection.
    c. Pellet cells by centrifugation at 500× *g* for 3 min.
    d. Aspirate and discard the supernatant.
    e. Resuspend the pellet in 5 mL of DPBS and repeat steps c–d.
3. Prepare cell-DNA mixture.

a. Resuspend the cell pellet in 220 µL of Neon Buffer R (supplied with the kit) and transfer the cell suspension to a 1.5 mL tube. We recommend using 110 µL of Neon Buffer R per transfection rather than 100 µL to ensure sufficient cell suspension volume for subsequent steps.
b. Separate cell suspension into halves and add 9 µg of plasmid DNA to one half (plasmid-transfected sample).

4. Perform electroporation.
   a. Aspirate 100 µL of cell-DNA mixture or plasmid-free mock sample into a Neon 100 µL pipette tip (supplied with the kit). Process mock-transfected sample first. It is important to aspirate 100 µL without bubbles.
   b. Insert the Neon Pipette into the Pipette Station and select the protocol on the touchscreen.
   c. Use the following electroporation settings: 1050 V, 30 ms, two pulses.
5. Immediately after electroporation, transfer the cell suspension from the pipette tip into the prepared six-well plate with prewarmed medium. Place the plate in a humidified incubator at 37 °C with 5% CO_2.
6. Incubate the cells for 2 days to enable robust expression of the transgenes.

3.8. Fluorescence-Activated Cell Sorting (FACS)

Sorting of the transfected LCL cells by FACS allows the enrichment of plasmid-containing cells expressing fluorescent protein.

1. Two days after transfection transfer 2 mL of transfected cells into 5 mL polystyrene tubes for sorting. Use mock-transfected (non-transfected) cells to set the sorting gate above the background autofluorescence of the cell line used.
2. Perform cell sorting of Santaka RFP-positive cells using the excitation wavelength of 561 nm and a set of 595LP + 610/20BP emission filters based on fluorescence reporter signal. Sort in "Purity" mode to minimize the possible admixture of non-target cells. The optimal nozzle size for cultured hematologic cell lines (including RPMI8866 cells whose characteristic diameter is approximately 15 µm) is 85 µm. Use larger nozzles for larger cells and cells that show low viability upon hydrodynamic stress.
3. Transfer fluorescence-positive cells into a well of a six-well plate containing 1 mL of prewarmed culture medium. Determine cell concentration and prepare dilution for subcloning into 500-cell subpopulation (see next section).
4. A portion of the cells will be subcloned into small populations (see below), while the rest of the culture should be maintained for use as a positive control (bulk population control) during subsequent PCR screenings, as well as for preparing cryopreserved backups.
5. Culture the sorted cell suspension until positive subpopulations are obtained (see below).
6. When the culture reaches a density of approximately 1×10^6 cells/mL, cryopreserve a backup stock of sorted cells in 10% *v*/*v* sterile DMSO containing cell culture media [23].

3.9. Subcloning

This section describes hierarchical cloning. The same protocol is applied at multiple stages as follows: subcloning of sorted cell population to 500-cell subpopulations, subcloning of 500-cell subpopulations to 20-cell subpopulations, and subcloning of 20-cell subpopulations to single-cell clones (Figure 3).

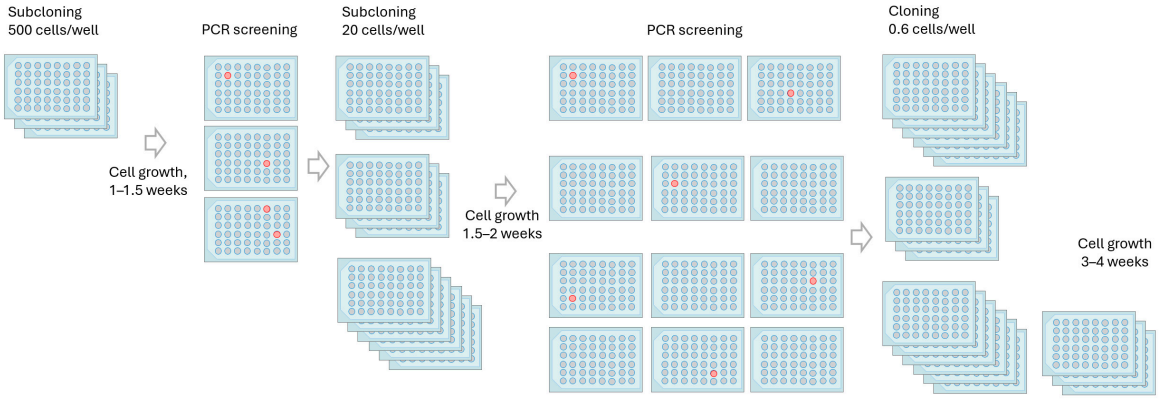

Figure 3. Schematic representation of the subcloning process following FACS. Red wells indicate clones that tested positive by PCR screening.

⚠ **CRITICAL**: Subclone the sorted cell population immediately following cell sorting on the same day. This maximizes the probability that translocation-positive cells across different 500-cell subpopulations are non-clonal.

Generally, most downstream applications require several independent cell clones carrying the rearrangement (i.e., the translocation occurred independently in different cell clones).

Because translocation induction efficiency is typically low, usually no more than one translocation-positive cell falls into each 500-cell subpopulation. Consequently, all 20-cell subpopulations and subsequent single-cell clones derived from a single 500-cell subpopulation will likely originate from the same positive cell. Therefore, obtaining multiple independent positive 500-cell subpopulations is usually crucial for subsequent work.

In cases where a high rearrangement frequency is anticipated, it may be possible to subclone a sorted cell population directly into 20-cell subpopulations, or, in rarer cases, directly to single-cell clones.

1. Determine the cell concentration accurately and prepare serial dilutions in pre-warmed (37 °C) culture medium to obtain a final concentration appropriate for seeding 500, 20, or 1 cell in a volume of 300 µL (per one well of a 48-well plate).
    - Final concentration should be 1667 cells/mL for obtaining 500-cell subpopulations and 67 cells/mL for 20-cell subpopulations. Final concentration for monocloning should be 2 cells/mL. This concentration results in a theoretical average of 0.6 cells per well (300 µL × 2 cells/mL = 0.6 cells/well), ensuring optimal conditions for no more than one cell per well.
    - The number of plates required depends on the rearrangement efficiency. As a starting point, we recommend using three 48-well plates for subcloning each positive well at each step.
    - Since LCLs exhibit poor survival at low densities, single-cell cloning results in high cell mortality and a subsequent lack of monoclonal population growth. Setting up a large excess of subclones is essential to compensate for this loss and guarantee the growth of a few viable monoclonal populations.
2. Fill all wells of several 48-well plates by adding 300 µL of cell suspension containing the required number of cells to each well. Place plates in a humidified CO_2 incubator at 37 °C.

⚠ **CRITICAL**: Mix the cell suspension thoroughly by gentle vortexing or pipetting before each dilution step and immediately before seeding to ensure a homogeneous suspension and accurate cell distribution.

3. Confirm the presence of cells carrying the translocation in the post-FACS population by PCR (see the following section) before proceeding to the subsequent stages of the protocol.
4. Cryopreserve aliquots of post-FACS population as a backup stock and maintain the remaining cells from positive wells into T25 culture flasks as a positive control culture for further PCR screening.

3.10. PCR-Based Screening for Chromosomal Translocations

This step identifies translocation-positive cell populations by direct PCR (i.e., PCR without prior DNA extraction). End-point PCR combined with gel electrophoresis enables direct visualization of the product size.

1. Examine wells microscopically for cell proliferation. Wait for culture to reach a density of approximately 100,000 cells/mL.

⚠ **CRITICAL**: Do not screen wells with lower cell numbers, as an insufficient template may yield false-negative results.

⚠ **CRITICAL**: Use non-transfected cells as a negative control and cells with confirmed translocation (e.g., sorted population after transfection) as a positive control. Use nuclease-free water instead of lysate for the No-template control.

- Before performing direct PCR screening on the rearrangement for the first time, choose a suitable pair of primers and optimize PCR conditions using genomic DNA purified from a 3–5-day post-sort culture. Select the following two pairs of primers: (1) for rearrangement detection and (2) for intact gene detection (as a positive control). A positive control primer pair is essential for subsequent direct PCR to validate that each reaction contains sufficient cells, thus minimizing false negatives. Using purified genomic DNA provides a clean, highly reproducible template that avoids potential enzyme inhibition by cell lysate components during the initial setup. To ensure robust translocation detection, test multiple primer pairs under PCR conditions (annealing temperature, elongation time, etc.) [24]. Successful PCR also serves as confirmation of the efficiency of the selected sgRNAs. If PCR fails, the sgRNAs should be tested individually (multiple approaches for sgRNA efficacy estimation are described elsewhere [12,13]), and the ineffective sgRNA must be replaced.

Once the optimal primer pairs and PCR programs have been selected, proceed to direct PCR. The following protocol was optimized for Hot start Taq polymerase (Evrogen HS Taq Pol).

2. Prepare a lysis premix according to Table 1 (calculations are per one well per two PCR reactions). Prepare a separate premix for each primer pair.

Table 1. Lysis premix, 20 µL for 2 PCRs.

Component	Volume/Concentration per 2 PCR *
10× PCR Buffer	4 µL
10× dNTP mix (2 mM of each dNTPs)	4 µL (to 0.4 mM) of each dNTP
Proteinase K (20 mg/mL)	0.4 µL
PCR-grade Water	to 20 µL

* Scale amounts of reagents proportionally to the number of wells for screening.

3. Prepare a PCR master premix according to Table 2 (volumes are per one reaction):

Table 2. PCR master premix, 10 µL per 1 reaction.

Component	Volume *
10 µM Forward Primer	0.5 µL
10 µM Reverse Primer	0.5 µL
Hot Start Taq Polymerase	0.8 units
PCR-grade Water	to 10 µL

* Scale amounts of reagents proportionally to the number of wells for screening.

4. Prepare cell lysates for direct PCR.
    a. Transfer approximately 10–20 thousand cells to a 200 µL PCR tube.
        - The number of cells per reaction depends on the proportion of cells with translocation in the analyzed sample. If this proportion is low, it is better to take more cells; however, large numbers of cells may inhibit the PCR [25]. If there is a monoclonal population in the well, you can take as few as 500 cells. There is no need to count cells in every well, as direct PCR works efficiently across a wide range of cell numbers (500–100,000 cells; [25]). Instead, precisely count the cells in the wells with the lowest and highest densities, and estimate the cell concentration in the remaining wells by visual interpolation.
    b. Centrifuge the tube at 500 *g* for 3 min using a rotor suitable for PCR tubes or PCR plates.
    c. Carefully aspirate and discard the supernatant. The pellet might be invisible. This is not a problem, as sufficient material for PCR generally will remain. To ensure that the cell pellet remains, you may leave 1 µL of supernatant when aspirating.
    d. Add 20 µL of the lysis premix to the cell pellet. Mix thoroughly by pipetting up and down at least 10 times to resuspend cells in lysis premix.
    e. Incubate cell suspension at 65 °C for 15 min to lyse then 5 min at 95 °C to inactivate Proteinase K.
5. Perform PCR amplification and analyze products.
    a. Divide the lysate into two PCR tubes per 10 µL per sample.
    b. Add 10 µL of PCR premix with translocation-specific primers into one tube, and positive control PCR premix into the second tube.
    c. Perform PCR amplification using the following cycling conditions: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 61 °C for 1 min, and extension at 72 °C for 1.5 min. A final extension step was performed at 72 °C for 5 min.
        - These are the conditions for primers and polymerase that we use (Evrogen HS Taq Pol). Use the parameters recommended by the polymerase manufacturer.
    d. Analyze the PCR products by agarose gel electrophoresis. A positive population is characterized by the presence of bands corresponding to both primer pairs, whereas a negative population displays a single band corresponding only to the intact gene primer pair. The total absence of PCR products indicates a PCR failure, requiring a repeat of the direct PCR for the respective well.

⚠ **CRITICAL**: Separate pre- and post-PCR workflows: open reaction tubes and run electrophoresis exclusively in a designated room to avoid PCR contamination.

- To confirm positive results, genomic DNA can be extracted from selected wells and used as a template in conventional PCR.

3.11. Validation of Cell Lines with Chromosomal Rearrangements

3.11.1. Validate the Translocation Junction by Sanger Sequencing

1. Purify genomic DNA from translocation-positive monoclones and perform PCR amplification of a translocation junction with a high-fidelity DNA polymerase according to the manufacturer's instructions. The primers used at the screening step may also be used at this step.
2. Purify the PCR product using a DNA clean-up kit. Check the result using agarose gel electrophoresis.
3. Analyze the purified product by Sanger sequencing through a commercial vendor. The translocation junction point should lie within the Sanger sequencing range (typically up to 800–1000 bp) from a sequencing primer. If this is not satisfied with the primers used for screening, different primers should be used. Alternatively, use Nanopore sequencing for longer PCR products.
4. Analyze sequencing data for the translocation breakpoint. Compare the resulting sequence to the predicted rearrangement junction sequence.

3.11.2. Validate Translocation by FISH

1. Prepare fixative solutions: 75 mM KCl (10 mL per sample + 10% extra), methanol:glacial acetic acid 6:1 (2 mL per sample + 10% extra), and methanol:glacial acetic acid 3:1 (1 mL per sample + 10% extra).

⚠ **CRITICAL**: All steps involving methanol-containing fixatives must be performed in a fume hood.

2. Transfer 1 × 10^6 cells to 15 mL tube. Centrifuge at 800 *g* for 3 min. Aspirate all but 2 mL of the supernatant. Resuspend pellet.
3. Add 10 mL of 75 mM KCl and mix by gentle inversion. Incubate for 20 min at 42 °C. Centrifuge at 800 *g* for 5 min. Aspirate the supernatant completely.
4. Add 1 mL of fixative "6:1" to the pellet and resuspend thoroughly. Centrifuge at 800 *g* for 5 min. Add another 1 mL of fixative "6:1" and resuspend. Centrifuge at 1100 *g* for 4 min.
5. Resuspend in 1 mL of fixative "3:1". Centrifuge at 1100 *g* for 4 min. Aspirate ~750 µL of supernatant. Resuspend the pellet in the remaining ~250 µL.

⏸ PAUSE STEP Store the fixed samples at −20 °C.

6. After storage, prepare a humid chamber: cover a thermoplate with moist-blotting paper and heat to +75 °C. Cool object slides in distilled water at +4 °C. Mix samples vigorously with a glass Pasteur pipette, add two to four sample drops to cold wet object slide, place it in the humid chamber for up to 10 min and then air-dry.
7. For FISH assessment, mark a spot with a single-point diamond, add 1 µL of ready-to-use FISH probe to the marked place, cover it with a coverslip, and seal with a rubber cement. We use *KMT2A* (*MLL*) gene break apart detection probe. If a commercial break-apart FISH probe for a specific gene translocation is unavailable, commercially manufactured custom probes can be ordered from specialized vendors.
8. Denature at +75 °C 5 min and then hybridize overnight at +37 °C in a humid chamber.
9. The following day, remove the rubber cement and coverslip from the object slide, wash the slide in 0.4× SSC + 0.3% NP-40 for 3 min 30 s at 75 °C, then in 2× SSC + 0.1% NP-40 for 30 s at room temperature, rinse with distilled water, and air-dry in the dark.
10. Add 5 µL DAPI/antifade mixture to the marked place and cover with a coverslip.
11. Count at least 200 interphase nuclei and 20 metaphase plates. FISH analysis should confirm that the translocation is present in every cell of the analyzed clone.

⏸ PAUSE STEP Fixed samples can be stored at −20 °C indefinitely. After probe hybridization, keep object slides in the dark at all times to avoid fluorophore fading.

3.11.3. Validate Chimeric Gene Expression

The protocol described below is suitable for use with the Column total RNA isolation kit (RUplus, Biolabmix) and the RNAscribe reverse transcriptase (Biolabmix). If using different kits, follow the manufacturer's instructions.

1. RNA purification
    a. Pellet up to 3 × 10^6 cells (500 *g*, 3 min), wash once with 1 mL of DPBS, and resuspend the final pellet in 50 µL of DPBS.
    b. Extract total RNA using an RNA isolation kit following the manufacturer's instructions. Ensure the use of RNase-free reagents and plastic throughout the procedure.
    c. Measure RNA concentration at 260 nm.
    d. Assess the quality of the isolated RNA using agarose gel electrophoresis or an Agilent Bioanalyzer.

    ⏸ **PAUSE STEP** Extracted RNA can be stored at −80 °C.

2. DNase I treatment and cDNA synthesis
    a. Perform DNase I Treatment according to the manufacturer's instructions.
    b. Perform cDNA synthesis using a random primer mix according to the manufacturer's instructions.

3. Fusion gene expression analysis via PCR
    a. Prepare PCR reactions using the 0.25 µL of cDNA template, 0.5 µL each of forward and reverse primers (10 µM stocks), following the manufacturer's instructions. Set up reaction and experimental controls.
    b. Include a no-template control (NTC) with deionized water in place of cDNA.
    c. Use cDNA from a non-transfected cell culture as negative control for chimeric gene expression.

Run PCR using the following program: pre-incubation at 50 °C for 2 min and initial denaturation at 95 °C for 5 min, followed by 25–35 cycles of denaturation at 95 °C for 15 s, annealing at 62 °C for 20 s, and extension at 72 °C for 30 s. A final extension step was performed at 72 °C for 5 min.

4. Verify the presence and expected size of the PCR products using agarose gel electrophoresis to detect fusion gene expression. Sanger sequencing is recommended to confirm the correct exon–exon junction in the mRNA.
    - Quantitative PCR (qPCR) is suboptimal for this application, as it requires an additional step to determine primer efficiency. A Ct value from a single clone, even when adjusted for primer efficiency, provides no meaningful information about expression levels without a proper calibrator or reference sample. However, it may still be useful if you wish to compare fusion gene expression levels among different clones.

We do not recommend Western blotting as well due to the technical challenges of detecting high-molecular-weight fusion proteins (e.g., ~320 kDa for KMT2A-AFDN), which become challenging when screening a large number of clones. The scope of this protocol is limited to the generation and genetic validation of the translocation model. Confirmation of the genomic rearrangement and expression of the chimeric mRNA is sufficient to validate

the cell line. Nevertheless, Western blotting can be performed as a downstream analysis to assess fusion protein expression or stability.

4. Expected Results

The expected result of this protocol is the production of several monoclonal lines containing the target chromosomal rearrangement. In our experiment with the *KMT2A::AFDN* translocation, transfection achieved an 11% efficiency. FACS-sorted cell population was then divided into 43 500-cell subpopulations, five of which were positive for the *KMT2A::AFDN* translocation. When we analyzed 57 20-cell subpopulations derived from a single positive 500-cell subpopulation, seven were positive. Out of 41 single-cell clones generated from these seven 20-cell subpopulations, 10 carried the expected rearrangement. We consider the established models to be functionally monoclonal. Due to the low initial frequency of the induced translocation (less than ~10^{-3}), the statistical probability of two independent translocation-positive cells occupying the same well during the single-cloning step is lower than 10^{-6}, thereby guaranteeing the clonality of cell population.

The cell populations were screened by PCR at each step. PCR should yield a band approximately corresponding to the theoretical length of the PCR product for the rearrangement, with no such band in the negative control (PCR from the non-transfected LCL). The size of the PCR product may differ from the theoretical size, as deletions or insertions may occur at the breakpoint during DNA repair. Positive control primers should amplify the target in all samples. If no product is found in this positive control PCR, the direct PCR must be repeated. In our example, primer pairs were KMT2A_F1 and AFDN_R1 for translocation and AFDN_F1 and AFDN_R1 for positive control. Figure 4 shows the expected PCR screening results.

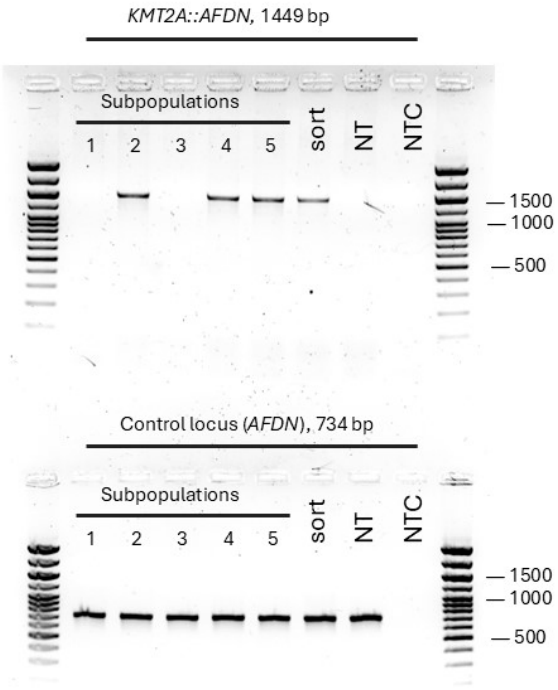

Figure 4. Example of direct PCR screening of 500 cell subpopulations grown from post-sort cells. PCR products amplified with primer pair KMT2A_F1 and AFDN_R1 specific for the *KMT2A::AFDN* translocation (expected size: 1449 bp) and with primer pair AFDN_F1 and AFDN_R1 specific for the control locus (expected size 734 bp). "Sort" represents DNA from the sorted cell suspension (positive control for the presence of the translocation); "NT" is non-transfected cells, "NTC" is a no template control. Sizes of 3 fragments of DNA ladder are indicated in base pairs.

If no translocation-positive wells are identified among the 500-cell subpopulations, the transfection, sorting, and splitting sorted cells into 500-cell subpopulations should be performed again. However, in this case increase the number of 500-cell wells. If translocation is detected in the mother 500-cell subpopulation, but is absent in the 20-cell subpopulations, or among single-cell wells, repeat the subcloning of this subpopulation by slightly increasing the number of 20-cell wells or number of cells per well slightly (e.g., from 0.6 cell per well to one cell per well) during cloning.

Sequencing the PCR product should confirm the expected fusion gene sequence (Figure 5), although deletions or insertions can frequently occur at the breakpoint.

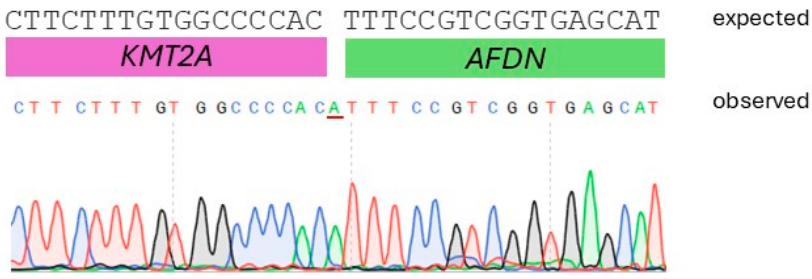

Figure 5. Sanger sequencing chromatogram for the translocation breakpoint in a cell clone. Read from primer KMT2A_F1 is shown. The expected sequence is shown in black; a single nucleotide insertion at the breakpoint is underlined in red.

FISH should also reveal the rearrangement in all cells of the analyzed clone (Figure 6). When using the *KMT2A* (*MLL*) gene break apart detection probe, the 5′ and 3′ signals should be separated, indicating a break in the *KMT2A* allele. In cells heterozygous for the rearrangement (the most frequent allelic status in both patients and cell models), one *KMT2A* allele remains intact, and the green and red signals colocalize, appearing as a yellow signal.

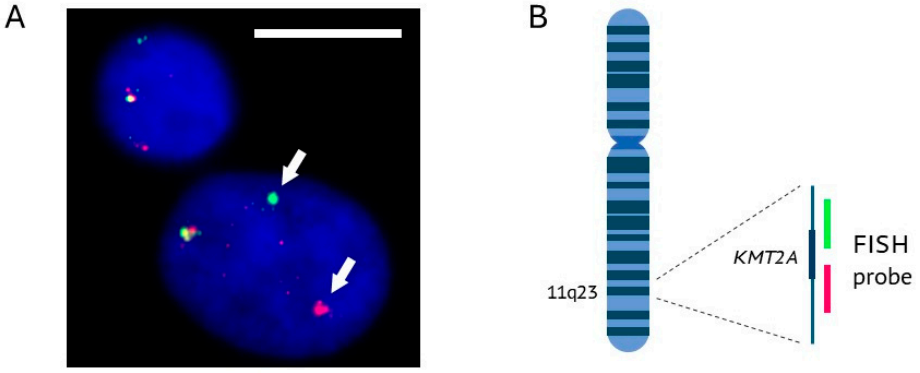

Figure 6. FISH validation of the *KMT2A::AFDN* translocation. (**A**) DAPI-stained nuclei after hybridization with the *KMT2A* FISH probe. Green and red signals correspond to the 5′ and 3′ ends of *KMT2A*, respectively. Co-localized signals indicate an intact *KMT2A* allele; separated signals (indicated by arrows) confirm the translocation. The scale bar is 10 µm. (**B**) Schematic of the *KMT2A* FISH gene break-apart detection probe design (FISH probe; green for: 5′- *KMT2A*; red for: 3′-*KMT2A*).

Cell clones with the *KMT2A::AFDN* rearrangement should demonstrate expression of the chimeric gene (Figure 7). Expression of the chimeric gene formed by the reciprocal rearrangement (*AFDN*::*KMT2A*) is also often observed. No PCR bands of chimeric genes should be observed in the non-transfected cells.

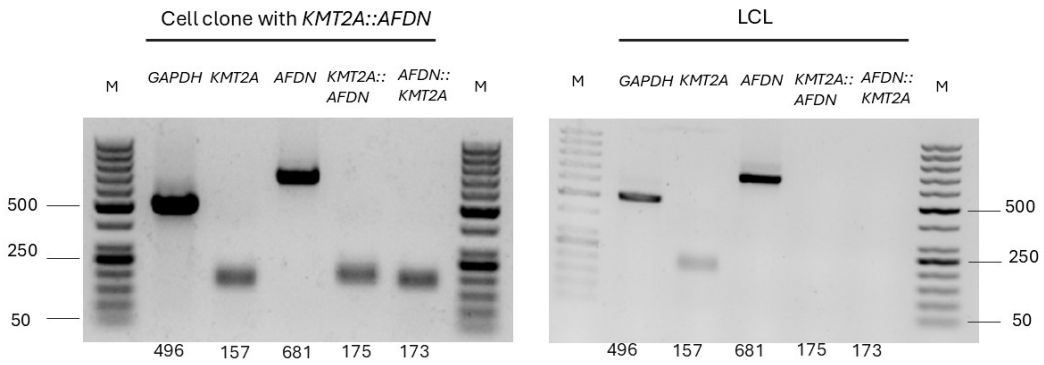

Figure 7. PCR validation of the KMT2A::AFDN chimeric gene expression. Agarose gel showing PCR products from cDNA samples for LCL and one of the *KMT2A::AFDN* clones. Expression of the *GAPDH* housekeeping gene (primer pair GAPDH_f/GAPDH_r, expected size 496 bp) and intact *KMT2A* (primer pair KMT2A_F3/KMT2A_R, expected size 157 bp) and AFDN (primer pair AFDN_F2/AFDN_R2, expected size 681 bp) genes are shown as positive control. The sizes of some marker bands (bp) are indicated. The lengths of the expected products (in bp) are indicated below the corresponding electrophoresis lanes.

If the absence of off-target edits is essential for the downstream applications of these cells, we recommend performing in silico prediction combined with targeted sequencing of the top off-target sites (as, for example, described by Atkins et al. [26]).

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/mps9060173/s1, Table S1: List of primers used in the study. Figure S1: Map of the empty backbone of px330_FP_2sgRNA plasmid expression vector. Figure S2. Map of the px330_FP_2sgRNA plasmid expression vector targeting the *KMT2A* and *AFDN* genes. Figure S3: FACS gating strategy. File S1: Complete nucleotide sequence of the pX330_FP_2sgRNA-based construct carrying TagRFP as a fluorescent reporter.

Author Contributions: N.A.L. and V.S.V. developed the methodology and managed the project, V.O.I., V.S.V., E.V.M. and N.A.N. performed the experiments, E.A.Z. performed FISH analysis, N.A.L., V.O.I., E.A.Z., V.S.V. and Y.S.V. wrote the manuscript, M.A.R. provided valuable advice and financial support. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Russian Science Foundation, grant number 24-15-00093 to M.A.R.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Acknowledgments: The project was supported in part by the M.V. Lomonosov Moscow State University Program of Development (cell sorting), and in part under the state assignment of Lomonosov Moscow State University (plasmid assembly). We thank Kerstin Bystricky for a gift of the OR3_Santaka_red_304 plasmid. We thank Daria Potashnikova for her technical support with FACS.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Zhang, R.; Huang, H.; Zhang, Y.; Xia, Y.; Huang, J.; Jiang, C.; Wang, L.; Lu, H.; Pan, Z.; Wang, G.; et al. Outcomes of Acute Myeloid Leukemia with KMT2A (MLL) Rearrangement: A Multicenter Study of TROPHY Group. *Blood Cancer J.* **2025**, *15*, 84. [CrossRef] [PubMed]
2. Meyer, C.; Larghero, P.; Almeida Lopes, B.; Burmeister, T.; Gröger, D.; Sutton, R.; Venn, N.C.; Cazzaniga, G.; Corral Abascal, L.; Tsaur, G.; et al. The KMT2A Recombinome of Acute Leukemias in 2023. *Leukemia* **2023**, *37*, 988–1005. [CrossRef] [PubMed]
3. Torres, R.; Martin, M.C.; Garcia, A.; Cigudosa, J.C.; Ramirez, J.C.; Rodriguez-Perales, S. Engineering Human Tumour-Associated Chromosomal Translocations with the RNA-Guided CRISPR-Cas9 System. *Nat. Commun.* **2014**, *5*, 3964. [CrossRef] [PubMed]
4. Ginestet, V.; Schwager, A.; Vassetzky, Y.; Shmakova, A. Advances in Modeling Chromosomal Aberrations to Recapitulate Genomic Rearrangements. *Trends Mol. Med.* 2026, *epub ahead of print*. [CrossRef] [PubMed]
5. Benz, T.; Larghero, P.; Meyer, C.; Müller, M.; Brüggmann, D.; Hentrich, A.-E.; Louwen, F.; Erkner, E.; Fitzel, R.; Schneidawind, C.; et al. Protocol for CRISPR-Cas9-Mediated Induction of KMT2A Rearrangements in Cell Line and Umbilical Cord Blood Hematopoietic Stem and Progenitor Cells. *STAR Protoc.* **2025**, *6*, 103481. [CrossRef] [PubMed]
6. Kim, H.K.; Kim, Y.; Lee, S.; Min, S.; Bae, J.Y.; Choi, J.W.; Park, J.; Jung, D.; Yoon, S.; Kim, H.H. SpCas9 Activity Prediction by DeepSpCas9, a Deep Learning-Based Model with High Generalization Performance. *Sci. Adv.* **2019**, *5*, eaax9249. [CrossRef] [PubMed]
7. Concordet, J.-P.; Haeussler, M. CRISPOR: Intuitive Guide Selection for CRISPR/Cas9 Genome Editing Experiments and Screens. *Nucleic Acids Res.* **2018**, *46*, W242–W245. [CrossRef] [PubMed]
8. Labun, K.; Montague, T.G.; Krause, M.; Torres Cleuren, Y.N.; Tjeldnes, H.; Valen, E. CHOPCHOP v3: Expanding the CRISPR Web Toolbox beyond Genome Editing. *Nucleic Acids Res.* **2019**, *47*, W171–W174. [CrossRef] [PubMed]
9. Baeza-Centurión, P.; Miñana, B.; Faure, A.J.; Thompson, M.; Bonnal, S.; Quarantani, G.; Clarke, J.; Lehner, B.; Valcárcel, J. Deep Indel Mutagenesis Reveals the Regulatory and Modulatory Architecture of Alternative Exon Splicing. *Nat. Commun.* **2025**, *16*, 8117. [CrossRef] [PubMed]
10. Taggart, A.J.; Lin, C.-L.; Shrestha, B.; Heintzelman, C.; Kim, S.; Fairbrother, W.G. Large-Scale Analysis of Branchpoint Usage across Species and Cell Lines. *Genome Res.* **2017**, *27*, 639–649. [CrossRef] [PubMed]
11. Wang, N.; Hu, Y.; Wang, Z. Regulation of Alternative Splicing: Functional Interplay with Epigenetic Modifications and Its Implication to Cancer. *Wiley Interdiscip. Rev. RNA* **2023**, *15*, e1815. [CrossRef] [PubMed]
12. Germini, D.; Tsfasman, T.; Zakharova, V.V.; Sjakste, N.; Lipinski, M.; Vassetzky, Y. A Comparison of Techniques to Evaluate the Effectiveness of Genome Editing. *Trends Biotechnol.* **2018**, *36*, 147–159. [CrossRef] [PubMed]
13. Lomov, N.A.; Viushkov, V.S.; Petrenko, A.P.; Syrkina, M.S.; Rubtsov, M.A. Methods of Evaluating the Efficiency of CRISPR/Cas Genome Editing. *Mol. Biol.* **2019**, *53*, 982–997. [CrossRef]
14. Türkmen, S.; Timmermann, B.; Bartels, G.; Gröger, D.; Meyer, C.; Schwartz, S.; Haferlach, C.; Rieder, H.; Gökbuget, N.; Hoelzer, D.; et al. Involvement of the MLL Gene in Adult T-Lymphoblastic Leukemia. *Genes Chromosom. Cancer* **2012**, *51*, 1114–1124. [CrossRef] [PubMed]
15. Cong, L.; Ran, F.A.; Cox, D.; Lin, S.; Barretto, R.; Habib, N.; Hsu, P.D.; Wu, X.; Jiang, W.; Marraffini, L.A.; et al. Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* **2013**, *339*, 819–823. [CrossRef] [PubMed]
16. Jinek, M.; Chylinski, K.; Fonfara, I.; Hauer, M.; Doudna, J.A.; Charpentier, E. A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science* **2012**, *337*, 816–821. [CrossRef] [PubMed]
17. Untergasser, A.; Cutcutache, I.; Koressaar, T.; Ye, J.; Faircloth, B.C.; Remm, M.; Rozen, S.G. Primer3–New Capabilities and Interfaces. *Nucleic Acids Res.* **2012**, *40*, e115. [CrossRef] [PubMed]
18. Dieffenbach, C.W.; Lowe, T.M.; Dveksler, G.S. General Concepts for PCR Primer Design. *PCR Methods Appl.* **1993**, *3*, S30–S37. [CrossRef] [PubMed]
19. Adikusuma, F.; Piltz, S.; Corbett, M.A.; Turvey, M.; McColl, S.R.; Helbig, K.J.; Beard, M.R.; Hughes, J.; Pomerantz, R.T.; Thomas, P.Q. Large Deletions Induced by Cas9 Cleavage. *Nature* **2018**, *560*, E8–E9. [CrossRef] [PubMed]
20. Bong, D.; Sohn, J.; Lee, S.-J.V. Brief Guide to RT-qPCR. *Mol. Cells* **2024**, *47*, 100141. [CrossRef] [PubMed]
21. Yuryev, A. PCR Primer Design Using Statistical Modeling. *Methods Mol. Biol.* **2007**, *402*, 93–104. [CrossRef] [PubMed]
22. Ran, F.A.; Hsu, P.D.; Wright, J.; Agarwala, V.; Scott, D.A.; Zhang, F. Genome Engineering Using the CRISPR-Cas9 System. *Nat. Protoc.* **2013**, *8*, 2281–2308. [CrossRef] [PubMed]
23. Whaley, D.; Damyar, K.; Witek, R.P.; Mendoza, A.; Alexander, M.; Lakey, J.R. Cryopreservation: An Overview of Principles and Cell-Specific Considerations. *Cell Transplant.* **2021**, *30*, 0963689721999617. [CrossRef] [PubMed]
24. Roux, K.H. Optimization and Troubleshooting in PCR. *Cold Spring Harb. Protoc.* **2009**, *2009*, pdb.ip66. [CrossRef] [PubMed]

25. Lomov, N.A.; Viushkov, V.S.; Zamalutdinov, A.V.; Sboeva, M.D.; Rubtsov, M.A. Direct ENIT: An Easy and Reliable Tool for gRNA Efficacy Verification by Tracking Induced Chromosomal Translocation. *MethodsX* **2020**, *7*, 101104. [CrossRef] [PubMed]
26. Atkins, A.; Chung, C.-H.; Allen, A.G.; Dampier, W.; Gurrola, T.E.; Sariyer, I.K.; Nonnemacher, M.R.; Wigdahl, B. Off-Target Analysis in Gene Editing and Applications for Clinical Translation of CRISPR/Cas9 in HIV-1 Therapy. *Front. Genome Ed.* **2021**, *3*, 673022. [CrossRef] [PubMed]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.