Epigenetic Markers in T-cell Acute Lymphoblastic Leukemia at Single-Cell Level

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Abstract

T-cell acute lymphoblastic leukemia (TALL) is a genetic disease. Also, epigenetic regulation through DNA methylation is likely to be important for the characteristics of the disease. Alterations in DNA methylation patterns may be associated with the development of the disease and enable survival and proliferation of leukemic cells. Therefore, DNA methylation markers are in the spotlight of current cancer research with potential to reveal valuable information about drug responsiveness or resistance in cancer cells, prognosis and molecular mechanisms of the disease. However, heterogeneity of DNA methylation profiles between individual cells within a leukemic cell population restrains identification of such markers. Especially rare cell types present within a leukemic cell population are difficult to identify using conventional methods. Single-nucleus methylcytosine sequencing (snmC-seq) is a new method to study DNA methylation patterns at single-cell level. This approach overcomes the heterogeneity problem by identifying methylomes for each cell individually. My goal was to determine whether sensitivity of TALL cells to dexamethasone drug can be improved by using combination of dexamethasone and decitabine, an epigenetic drug which inhibits DNA methylation. Furthermore, my goal was to implement and pilot a

new method (snmC-seq) enabling further characterisation of DNA methylation markers in CCRF-CEM cell line and TALL patient cells at single-cell level. Results from the drug treatment response with several concentrations of Dex and Dec on CCRF-CEM cells showed that a Dex and Dec combination treatment caused less proliferation due to apoptosis compared to cells treated with Dex alone after 72 h of treatment. DNA libraries using snmC-seq were successfully constructed from non-treated and dexamethasone-treated CCRF-CEM TALL cells and TALL patient sample at diagnosis. This method can be applied to study the role of different methylation patterns in single cells of diverse diseases.

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List of Abbreviations

5mC	5-methylcytosine
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
Dex	Dexamethasone
Dec	Decitabine
GC	Glucocorticoid
NOPHO	Nordic Society of Pediatric Hematology and Oncology
SnmC-seq	Single-nucleus Methylcytosine Sequencing
TALL	T-cell Acute Lymphoblastic Leukemia
TET	Ten-Eleven-Translocation
TCR	T-cell Receptor
WGBS	Whole Genome Bisulfite Sequencing

1. Introduction

1.1. Background and Motivation for the Study

Acute lymphoblastic leukemia (ALL) is the most common pediatric malignancy both in the Nordic countries and worldwide. Success in cancer therapy and treatment responsiveness in ALL combined with developing knowledge in cancer genomics have improved significantly during the last decade. Still, relapse signifies poor prognosis among children. Another common type of childhood leukemia is acute myeloid leukemia (AML). AML is less common among children compared to ALL, but relatively common among adults. Both ALL and AML are treated in Finland with chemotherapy, which implies the use of drugs to eliminate fast-growing cells, such as cancer cells. More severe diagnoses for adults today require stem cell therapy and, in some cases, even a stem cell transplant (Lohi, Taskinen & Wartiovaara-Kautto, 2019). Leukemia is a genetic disease caused by chromosomal alterations or mutations in the DNA that affect the regulation of gene expression in the cells with hematopoietic origin. Not only somatic mutations but also alteration of DNA methylation can disrupt transcriptional regulation (Figueroa et al., 2013). ALL can originate from T-cell linage or B-cell linage and those are further divided into multiple subtypes defined by immunophenotype and gene expression profile, including mutations affecting tumour suppressors (Iacobucci, Mullighan, 2017). The treatment strategy for ALL during the last decades has included glucocorticoids (GCs) in combination with other drugs. High concentrations of the GC dexamethasone (Dex) is administrated to the patients during a long-term period in therapy. Harmful and unwanted side effects are a common consequence of the treatment and long-term treatment of Dex is associated with resistance (Masanori Nojima et al., 2009). Resistance to GC treatment in TALL is a crucial problem and is associated with poor outcome. GC resistance may be caused by a defective GC receptor (*NR3C1*) ineffectual in causing apoptosis. The full understanding of the mechanism of resistance is unknown (Miller et al., 2014). In 1983, the first study of DNA methylation in GC

resistance of TALL discovered that a change of the DNA methylation state could affect the sensitivity of GC (Bourgeois, Ryden & Gasson, 1983). Further studies have shown that GC resistance can be reversed by addition of decitabine (Dec) (Miller et al., 2014). Thus, a GC-resistant cell line treated with Dec would achieve GC-sensitive cells and show a decreased cell viability. One cannot assume that every drug-treated cell would convert to GC-sensitive, since multiple mechanisms in the heterogeneous environment are responsible for the switch to drug-sensitivity (Miller et al., 2014).

Just like all cancers, leukemia becomes more heterogeneous during the course of the disease in patient specific manner. Metastasis development and biochemical mechanisms driving cancer progression might differ from person to person. The tumour contains a mix of cells harbouring different molecular characters with different levels of drug sensitivity, which is the major underlying factor for resistance and relapse (Dagogo-Jack, Shaw, 2018). In leukemia several different cancer cell clones are surrounded by normal cells in the blood and bone marrow. These clones may carry different mutations or DNA methylation alterations. New knowledge of the heterogeneity of leukemia would facilitate development of personalized treatments and targeted therapies leading to better outcomes for patients (Allison, Sledge, 2014). Quantitative methods used to study DNA methylations in ALL have encountered problems with cellular heterogeneity of different leukemic cell populations that are present in the sample. Bulk analysis is difficult when the aim is to identify the methylation status, due to the diversity of cells. The results are, therefore, untrustworthy (Nordlund, Syvänen, 2018). With single-cell sequencing it is possible to study cell-to-cell variability on a DNA methylation level and eradicate heterogeneity. Single-nucleus methylcytosine sequencing (snmC-seq) is among the latest assays that shows promising results in identification of cellular identity of a mixture of cell lines (Luo et al., 2017).

The goal of my thesis was to determine whether a drug treatment combination with Dex and Dec of CCRF-CEM TALL cell line would convert the cells to become sensitive to Dex and prevent the proliferation or survival of the leukemic cells. Also, my goal was to implement the new snmC-seq method which enables characterization of DNA methylation markers in leukemic cells at single-cell level associated with drug resistance in TALL cells. CCRF-CEM is an uncloned cell line

and is therefore heterogenous. The cell line consists of both GC-resistant and GC-sensitive cells harbouring different genotypes and phenotypes (Medh et al., 2003).

1.2. T-cell Acute Lymphoblastic Leukemia

Normal development of T-cells begins when haematopoietic stem cells in the bone marrow differentiate into thymic progenitors and are transported through the blood vessels into the thymus. The complex process of T-cell differentiation takes place in thymus and results in an effective immune defence system, T-cells. The mature T-cells exit the thymus and circulate in the bloodstream, lymph nodes and spleen. (Figure 1A). In leukemia, the thymic progenitor cells stop to differentiate in the thymus and immature cells, or blasts, start to accumulate in the bone marrow and bloodstream and other organs (Figure 1B). The malignant cells are rapidly proliferating, and the differentiation of hematopoietic stem cells occurs all along. Normally, 5% of the T-cells generated in thymus exit as mature cells. The differentiation process in thymus includes several complex steps where genetic rearrangements of the T-cell receptor (*TCR*) genes occur. The loss of 95% of T-cells is a natural result of this quality control, ending in apoptosis for instance when the TCR is not appropriately developed (Karrman, Johansson, 2017).

The amount of blast cells in the bone marrow of a healthy person is typically 1-2%. If the percentage of blast cells is raised to 25%, the person is diagnosed with ALL (Karrman, Johansson, 2017). Loss of a certain blood cell type gives rise to different symptoms. For instance, loss of red blood cells causes anaemia which, in turn, leads to fatigue and a fast heartbeat. Loss of platelets gives rise to bleeding and skin that easily bruises. Loss of neutrophils causes infection (Shuvani Sanyal, 2017).

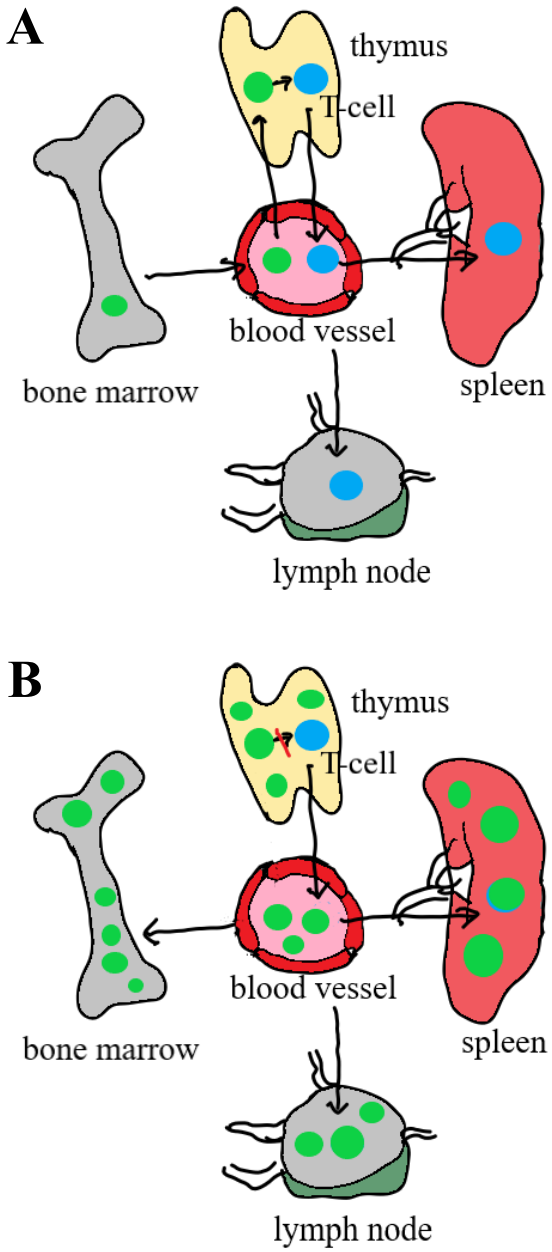


Figure 1 Normal and malignant T-lymphoblasts.

(A) Normal T-cell differentiation. Hematopoietic stem cells in the bone marrow differentiate into early thymic progenitor cells and are transported to the thymus to mature into T-cells. T-cells are circulating in the blood system, lymph nodes and spleen. (B) Malignant T-cell differentiation. Genetic and epigenetic alterations disrupt the differentiation of immature T-cells in the thymus. The malignant T-cells are circulating in the blood system, bone marrow and lymph nodes as blasts giving rise to leukemic symptoms.

1.2.1. Incidence

Certain factors in children are associated with a worse prognosis of leukemia. For instance age is considered a negative factor (infants or ≥ 10 years old children) (Inaba, Greaves & Mullighan, 2013). According to the Nordic Society of Pediatric Hematology and Oncology (NOPHO), a total of 200-250 patients are annually diagnosed with ALL in the Nordic countries and the Baltic States together (Lohi, Taskinen & Wartiovaara-Kautto, 2019). The aim of the TALL recovery is to extinguish all the cancer cells and provide recovery to the child. Normal hematopoiesis is restored and a healthy functioning status is maintained (Pui, Robison & Look, 2008). According to the World Health Organization (WHO), 300 000 children are annually diagnosed with cancer worldwide. In high-income countries, such as Finland, 80% of the children with leukemia are cured. Tragically, only 20% of the children with leukemia in low-income countries are cured. This is due to the lack of diagnosis, side effects, relapse, abandonment of treatment and lack of technologies (World Health Organization, 2018). The way to overcome this problem is to find specific biomarkers that will ease the identification of leukemia at an early stage. A more personalized treatment would increase the survival rate and focus on the severe cases of ALL and relapse status.

1.2.2. Diagnosis and Classification

Current classification for diagnosis of ALL was published in 2001 by WHO. The guidelines combine genetic information with the study of morphology, cytochemistry and immunophenotype by flow cytometry (Pui, Robison & Look, 2008). Chromosome instability has harmful consequences on immature cells; as in cancer overall, cancer-cells escape cell death and ignore stimuli from the environment. Single-cell whole genome sequencing provides an unbiased map of copy number variation and reveal heterogeneity. Development of TALL involves chromosome instability, which drives the

evolution of leukemia (Bakker et al., 2016). Aberrant DNA methylation is associated with chromosomal instability. The methylation status is therefore essential in the research on cancerogenesis (Gaudet et al., 2003).

1.2.3. Current Treatments

Most drugs developed for ALL therapy during the 1950s are still in use today. The standard treatment for TALL is based on chemotherapy and consists of a combination of drugs, which always includes a GC. Other drugs used in combination may be a vincristine (a plant alkaloid which inhibits microtubule structures) or an asparaginase (cytotoxic enzyme) (Pui, Robison & Look, 2008). Both genetic and epigenetic novel therapeutic approaches are available for TALL. The most promising genetic treatment is γ-secretase inhibitors affecting the NOTCH1 pathway. NOTCH1 mutations have been detected in over 50% of TALL patients. Epigenetic drugs include DNA methyltransferase inhibitors, such as Dec (Pui, Robison & Look, 2008). The aim of epigenetic drug treatment is to demethylate the genome to reverse e.g. the silencing of tumour suppressor genes (Gaudet et al., 2003).

Response to treatment is highly associated with the leukemic-cell biology of the patient. Therefore, the correct treatment and dose of drugs for a child with leukemia requires a specific treatment based on a correct diagnosis. Techniques such as flow cytometry provide information about the patient's leukemic-cell levels on how low or how high the risk is for the patient to fall into relapse, which is crucial in order to provide the best treatment (Pui, Robison & Look, 2008).

1.3. Glucocorticoids

Glucocorticoids are steroid hormones that naturally are produced by the adrenal glands. GCs have been used as an effective anti-inflammatory drug and during 50 years it has been used in the treatment of leukemia (Buentke et al., 2011). GCs as a therapeutic agent are synthetically made and are injected or taken orally. Two GC drugs that are used in the clinics for the treatment of TALL are dexamethasone and prednisolone. According to "WHO Model List of Essential Medicines for Children", the concentration of Dex administrated by injection is 4 mg/ml and in oral form 2 mg / 5 ml (World Health Organization, 2017).

Cancer cells differ in their energy metabolism compared to normal cells. In normal cells, glucose is metabolized in the presence of oxygen in the mitochondria through oxidative phosphorylation. Cancer cells have higher rates of glycolysis, despite adequate oxygen levels. Instead of oxidative phosphorylation, the cell uses glycolysis as the main energy source where an increase in the conversion from pyruvate to lactate in the cytosol decrease the pH levels in the cell. This hallmark of cancer is called the Warburg effect. Previous research has shown that treatment of ALL with Dex inhibits glycolysis (Buentke et al., 2011).

GCs bind to the glucocorticoid receptor in the cytoplasm via passive transport. The attachment is followed by dimerization and releasing of the heat shock proteins 70 and 90. The dimer complex is later transported into the nucleus to regulate transcription of downstream genes, for instance the c-myc proto-oncogene affecting cell survival (Spenerova et al., 2014). Long-term use of GCs will reduce the number and the production of white blood cells through G1-phase cell-cycle arrest and apoptosis (Buentke et al., 2011). Addition of 1 μM of Dex to CCRF-CEM cells has shown to induce apoptosis already after 20 h, but most striking decline in cell number is noticed after 48 h of drug treatment. Several studies support the fact that CCRF-CEM is sensitive to 100 μM and 1 μM of Dex and induce apoptosis at several time points up to 72 h of treatment (Bindreither et al., 2014, Noh et al., 2004, Y S Yuh, E B Thompson, 1989). Focusing on epigenetics, analysis of promoter methylation in the GC receptor has shown higher levels of methylation in Dex-sensitive cells compared to Dex-resistant cells (Mata-Greenwood et al., 2015). The information about different methylation patterns between Dex-resistant and Dex-sensitive TALL cells do not solve the remaining

question about the underlying mechanism of resistance. The methylation status must therefore be further studied.

1.4. Decitabine

Decitabine is mostly used in combination with other drugs as a hypomethylation agent. Dec is a DNMT inhibitor with the function to reactivate e.g. silencing of tumour suppressor genes by DNA methylation of CpG sites in their promoter regions (Hagemann et al., 2011). As an analogue to cytosine, Dec is incorporated into DNA and binds to the DNMT to block methylation. Another advantage by using Dec in TALL therapy is that the drug induces apoptosis (Gerson et al., 2018).

1.5. Epigenetic Markers

Epigenetic regulation through DNA methylation, expression of non-coding RNAs and posttranslational modification of histone proteins are underlying mechanisms required for regulation of gene expression and control the organization and stability of the genome (Huang, Litt & Blakey, 2015). These epigenetic mechanisms mentioned can be altered and deregulated in cancer such as leukemia. Each genetic subtype of ALL has different gene expression and CpG methylation signatures (Nordlund, Syvänen, 2018). DNA methylation regulates gene expression, embryonic development, genomic imprinting, X chromosome inactivation and cell differentiation (Peirs et al., 2015). DNA methylation is maintained by DNA methylation transferase enzymes (DNMT). The mechanism of DNMT action is to transfer a methyl group from the cofactor S-adenosyl-L-methionine to cytosines, given rise to binding sites for binding domain proteins which can force the methylated DNA to repress transcription (Brandes, McCabe & Vertino, 2009). A specific protection mechanism in normal tissue removes erroneously positioned DNA methylations by DNMTs. The removal is mediated by Ten-Eleven-Translocation (TET) enzyme oxidation of methylcytosines.

Insufficiency in this methylation protection mechanism may results in aberrant methylation pattern. In cancer, DNA hypermethylation has been associated with loss of TET (Pfeifer, 2018). The TET protein family contains of several proteins. TET2 is frequently mutated in hematopoietic malignancies occurring in early tumour development (Rasmussen, Helin, 2016).

1.5.1. CpG Sites and DNA Methylation in TALL

A CpG island is a short region in the DNA with a high frequency of cytosine nucleotides followed by guanine nucleotides, forming CpG dinucleotides. This is stated as a CpG site (Yang et al., 2016). In 1987, Gardiner-Garden and Frommer defined following characteristics of a CpG island: a DNA region with more than 50% of CpG dinucleotides, a CpG region with more than 200 bp and an observed/expected CpG frequency exceeded to 0.6 (Gardiner-Garden, Frommer, 1987). CpG islands are found all over the genome, for example in promoters and repetitive sequences (Jang et al., 2017). It is estimated that the human genome contains approximately 28 million CpG sites (Nordlund, Syvänen, 2018). The special thing about CpG sites, compared to other dinucleotides in the DNA is that more than 80% of the CpG sites are methylated (Antequera, 2003), expect those CpG sites that are located in CpG islands (Cedar, Bergman, 2009). DNA methylation takes place on the 5^{th} carbon of the pyrimidine ring of cytosine (5mC), within a CpG dinucleotide (Brandes, McCabe & Vertino, 2009). The majority of the genes in the human genome have methylated CpG sites at their promoter region (Yang et al., 2016). One hallmark of cancer is abnormal DNA methylation, such as hypermethylation or hypomethylation in specific regions causing silencing of suppressor genes (Degerman et al., 2014). Hypermethylation in CpG islands in promoter regions of the genome is a mark of TALL and signifies poor outcome. Hypermethylation has been associated with single gene mutation and disruption of specific signalling pathways, for instance resist of cell death, activation of invasion and proliferation signalling. There are indications that

DNA methylomes of leukemia contain large hypomethylated blocks that trigger chromosomal instability, for instance enhance transcriptional elongation or affect splicing (Figure 2) (Van der Meulen et al., 2014).

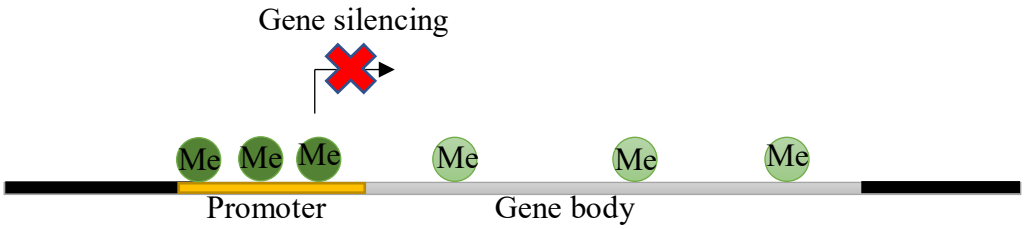


Figure 2 DNA methylation in TALL.

Hypermethylation in promoter regions leads to alteration in gene expression and silences genes. Hypomethylation in the gene body can affect the chromosomal instability.

Hypermethylation in promoter regions occurs early in the tumour growth. The full understanding of the emergence of the event is unknown. Hypermethylation of *CDKN2A* and *CDKN2B* gene promotors have been most frequently observed in TALL patients. A recent study showed that hypermethylation in the promoter region of *CDKN2B* gene is significantly more harmful than deletion (Jang et al., 2019).

1.6. DNA Methylation Analysis in TALL

Earlier studies of DNA methylation in TALL have aimed to analyse specific candidate genes affected by methylation, rather than the whole genome. The first genome-wide study of DNA methylome of TALL was performed in 2013 by using microarray based assay (Figueroa et al., 2013). Comparison of gene expression profiles with DNA methylomes showed 65% correlation (Figueroa et al., 2013). Ever since numerous epigenome-wide studies have been reported, most of which have been performed with whole genome bisulfite sequencing (WGBS) by using bulk cells. Single-cell analysis of transcriptomes have proven that bulk-cell

transcriptomic profiles may lead to ambiguous results and cell subpopulations cannot be distinguished (Ye, Huang & Guo, 2017). Bulk sequencing at RNA level is adapted to study variations between different conditions or between patients. A bulk sample contains approximately 1000 cells per cell type. The result reflects an average of the gene expression and observes all cells as homogenous (Chen, Ning & Shi, 2019). Therefore, for heterogeneous samples such as cancer tissues would be important to use single-cell approaches to study cell-to-cell variability. These methods, however, have emerged only recently. In particular, DNA methylome analysis have not yet been widely used.

New paths in cancer research have been opened by characterization of intra-tumour cellular heterogeneity. With single-cell technologies it is now possible to identify rare cell types and measure the mutation rates. Traditional single-cell methods provide a small amount of information from the samples. Only a limited number of genes or proteins can be profiled at the same time. One of the major problems is to have enough material of nucleic acid to reach the threshold level to get a significant result. Another problem is how to handle each single cell sample independently. The aim for method development is to reduce the costs and to improve the throughput. Also, the library preparation has to be improved so that the information will not be lost during the destruction of the cells (Saadatpour et al., 2015).

1.7. Single-nucleus Methylcytosine Sequencing

Single-nucleus methylcytosine sequencing (snmC-seq) is one of the most recent techniques applied to discriminate the cellular identity of a mixture of different cell lines. In comparison to the first published methods, it has improved the read mapping rates and reduced artefactual reads, for instance adapter dimers (Luo et al., 2017). Compared to traditional methods, snmC-seq is based upon independent novel adapter chemistry. The first step in library preparation is to utilize single-stranded DNA, instead of double stranded DNA. This enables post-bisulfite adapter tagging, leading to maximal recovery of the library (Figure 3). Bisulfite

conversion is a harsh chemical method where DNA is denatured and fragmented during the process. Broken library molecules result in a smaller sample size for further DNA library construction. As little as 100 pg input DNA is needed to produce DNA libraries for snmC-seq. Bisulfite conversion of single-stranded DNA converts unmethylated cytosine residues to uracil. The following step is to amplify the DNA with PCR where the converted uracil nucleotides converts to thymine. Final DNA libraries are sequenced to measure genome-wide levels of DNA methylation in individual cells (Luo et al., 2017).

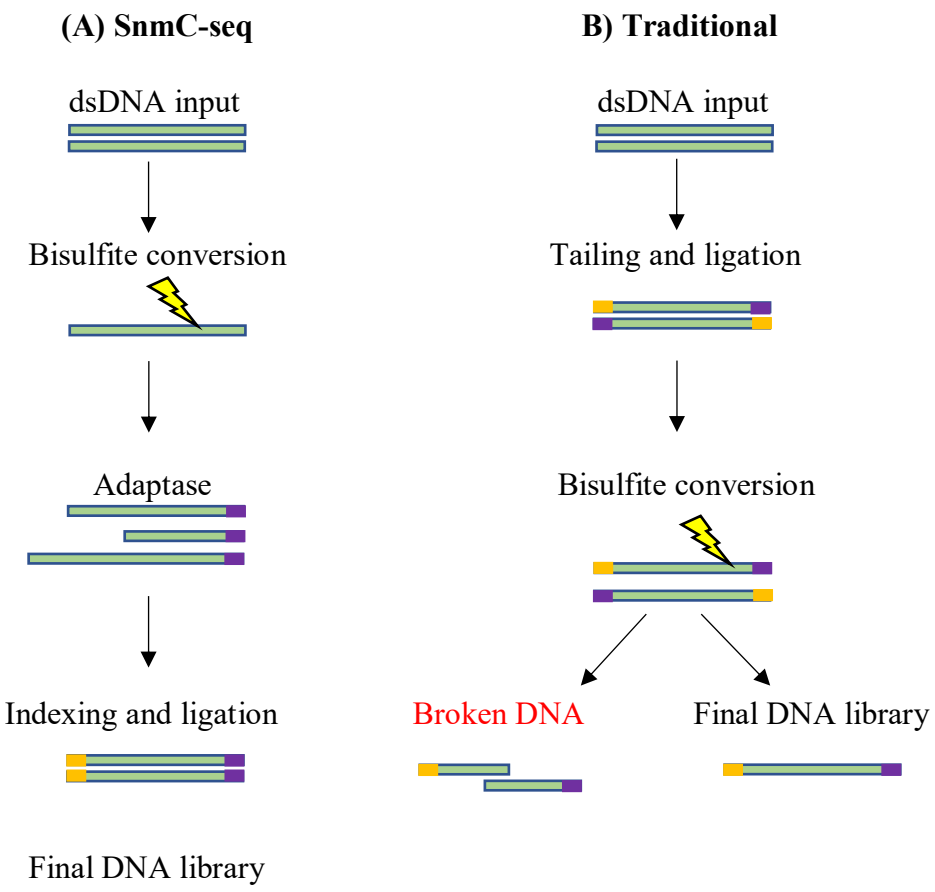


Figure 3 DNA library workflow of snmC-seq and traditional methods.

A) SnmC-seq is based on bisulfite conversion prior to adaptase reaction, indexing and PCR. B) A large amount of DNA is lost during bisulfite conversion in traditional methods. This is due to bisulfite conversion of dsDNA, leading to broken DNA fragments with loss of adapters. A higher amount of input DNA is required to get enough final DNA library sample.

1.8. Bisulfite Conversion

Bisulfite sequencing is a widely used technology to analyse the methylation status of a DNA sequence. The development of the bisulfite sequencing technique has led to revolution in our understanding of DNA methylation (Smallwood et al., 2014). And also, the importance in disease development, such as in cancer (Hu, Ting & Li, 2015). Due to loss of DNA sample during bisulfite treatment, the approach is continuously updated. The aim of bisulfite treatment is to obtain methylation status of single nucleotides, by which unmethylated cytosine residues are converted into uracil. Methylated cytosines will be unaffected by bisulfite and will remain intact. After PCR amplification, uracil is further modified into thymine. Sequencing data is analysed by comparing the DNA library of which nucleotides were converted to thymine or not, determinates the methylation status.

Bisulfite conversion is set-up by several steps which requires reagents, such as bisulphite (sulphonation) and desulphonation solution (Darst et al., 2010). Unmethylated lambda DNA is added to the DNA sample in the first step of the conversion process. Lambda DNA enables determination of bisulfite conversion rates, as a reference sequence (Thermo Fisher Scientific, 2019). In the next step, bisulfite is added to the DNA, which deaminates and removes the amine group from cytosine and replaces it with oxygen. Methylated cytosine residues are not susceptible to bisulfite and will not be deaminated and converted into uracil (Figure 4). Desulphonation is the next step at which desulphonation solution is added to ensure successful PCR amplification, resulting in fully conversion of uracil to thymine and bisulphites are removed during a final cleaning step (Patterson et al., 2011).

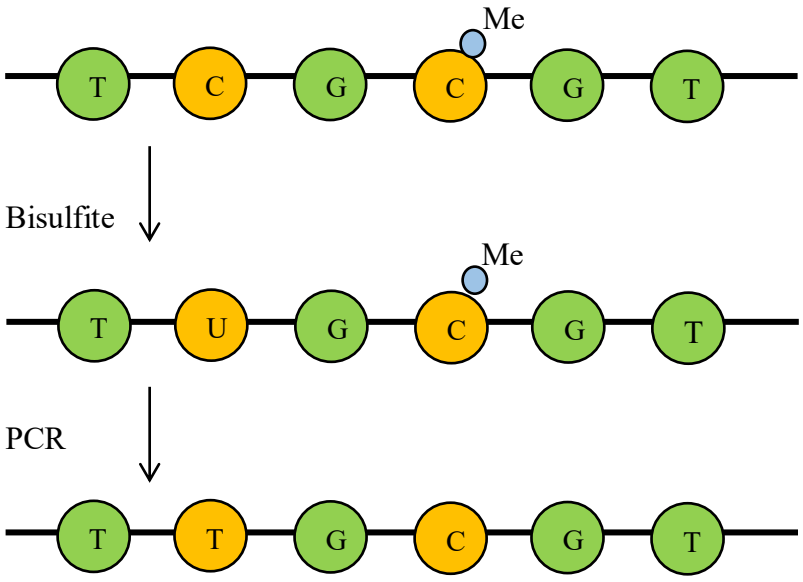


Figure 4 Bisulfite conversion.

Conversion of unmethylated cytosine into uracil is the chemical process DNA undergoes during bisulfite conversion. Methylated cytosine residues remain unaffected. PCR amplification results in conversion of uracil into thymine.

1.9. Research Question, Hypothesis, and Goals

Glucocorticoids in combination with Decitabine on CCRF-CEM have in earlier studies shown an increased sensitivity to GCs. I will test this hypothesis in a cytotoxicity assay to see if a combination of Dex and Dec together will induce a higher rate of apoptosis in CCRF-CEM cells, compared to the use of each drug individually. In addition, my aim is to implement single-nucleus methylcytosine sequencing (snmC-seq) to show if the library preparation method is applicable. The method will be later on utilised to determine cellular heterogeneity in samples collected from children with ALL and to reveal molecular mechanism of leukemia and drug responses.

2. Experimental Design

CCRF-CEM TALL cell line was used to show whether a drug treatment combination of Dex and Dec increases the sensitivity to Dex. And if so, is it a result of apoptosis? A cytotoxicity assay was performed based on common WST-1 staining assay to generate proliferation curves. CCRF-CEM cells in cell culture were treated as follows: control (no drug), 1 μM Dex, 1 μM Dex + 0.1 μM Dec, 1 μM Dex + 0.05 μM Dec, 100 nM Dex, 100 nM Dex + 0.1 μM Dec, 100 nM Dex + 0.05 μM Dec, 0.05 μM Dec and 0.1 μM Dec in triplicate. Also, each experiment with three equal replicates of each drug treatment. The cells were treated for 0, 24, 48 and 72 hours. Cell viability assessment was performed beside the WST-1 staining to measure the viability of CCRF-CEM cells during drug treatment. Library preparation for snmC-seq is based on the methods described in the article "Single-cell methylomes identify neuronal subtypes and regulatory elements in mammalian cortex" (Luo et al., 2017) and their updated version "Robust single-cell DNA methylomes profiling with snmC-seq2" (Luo et al., 2018). The snmC-seq workflow of the library preparation is illustrated in Figure 5. The steps included were flow cytometry, bisulfite conversion, indexing using random priming, SPRI bead purification, adaptase and indexing PCR. DNA libraries were constructed from a TALL patient sample at diagnosis and CCRF-CEM cell line as in vitro models. The CCRF-CEM cell samples included a control (no drug) sample and a 1 μM of Dex-treated cell sample. The quality and quantitation of the final single-cell DNA bisulfite libraries were measured with Qubit and Bioanalyzer.

2.1. Materials

2.1.1. T-ALL Cell Culture and Culture Conditions

CCRF-CEM acute lymphoblastic leukemia cells were from the American Type Culture Collection (LGC Standards, 2016). These human lymphoblasts were originally derived from peripheral blood of a 4-year-old girl with leukemia in 1964. Cells were growing in cell culture medium and maintained in 5% CO_2 at 37 ºC.

2.1.2. Patient Sample

T-ALL patient blood sample was collected at diagnosis in 2015. The peripheral blood mononuclear cells (PBMCs) were frozen and delivered from University of Kuopio by collaborator Dr. Merja Heinäniemi.

2.2. Experimental Procedure

2.2.1. Cytotoxicity Assay

Drug testing was applied based on WST-1 cell cytotoxic assay to generate proliferation curves and trypan blue staining for cell viability assessment. The aim was to examine the optimal concentration and treatment durability of Dex treatment for the single-cell sequencing. A combination of Dex and Dec was performed between the selected concentrations to study the viability effect on

CCRF-CEM cells. Dex (Sigma Aldrich, Cat. No. 3K27) and Dec (Abcam, Cat. No. ab120842) were dissolved in ethanol and added to the cell culture medium of CCRF-CEM. Dex was added at the concentrations 100 nM and 1 µM and Dec at the concentrations 0.05 µM and 0.1 µM. The final volume in each well of a 96-well cell culture microplate (Greiner bio-one, Cat. No. 655180) was 100 µl containing 1 x 10^4 cells. The cells were treated for 0, 24, 48 and 72 hours and all experiments were carried out in triplicate. At the end of each experiment, one well from each condition was counted using trypan blue to calculate the cell viability. Next, 10 µl of WST-1 (Sigma-Aldrich) was added to each well and the plate was incubated for 30 min in 5% CO_2 at 37 ºC. The absorbance was measured with Thermo Multiskan Ascent Plate Reader at 450 nm according to the instructions by the manufacturer of WST-1 reagent.

After assessed Dex concentration, time point of treatment durability and cell density, 5 ml of cells suspension was from each condition placed in cell culture bottles (Greiner bio-one, Cat. No. 690160). One bottle was marked "control" and contained non-treated CCRF-CEM cells. The other bottle was marked "drug treatment" and contained 1 µM of Dex-treated cells. The bottles were prepared in duplicates.

2.2.2. Single-Cell Sorting and Cell Lysis

Leukemia cells grow in suspension and were harvested after 48 h of Dex treatment by centrifugation at 250 x g for 4 min. The supernatant was discarded, and pellets were resuspended in 500 µl of PBS followed by filtering by using a falcon tube with cell-strainer cap (Falcon, Cat. No. 352235) to remove big cell clumps. Similarly, the cryopreserved leukemia cells collected from patient were thawn and prepared for sorting. The cells were sorted using by flow cytometry where single nuclei were sorted into each well of a 384-well plate (ThermoFisher Armadillo PCR Plate, Cat. No. AB2384). The 384-well plate was preloaded with 1 µl of M-Digestion Buffer (Cat. No. D5021-9), 0.1

µl of 20 µg/µl Proteinase K (Cat. No. ZRC191291) and 0.9 µl of Nuclease-Free H_2O (L/N 1808054) per well to lyse the cells.

Flow cytometry was performed using Sony's Cell sorter SH800S available in the Cell imaging and Flow cytometry core facility at Turku Bioscience Centre. The sorting was performed with the pressure of 20 PSI and wavelengths of 488 nm and 561 nm. The cells were dyed with 5 µl of propidium iodide (PI) shortly before sorting. PI stains dead cells and it was strived to only sort live single cells that are intact and to ensure that sufficient amount of DNA will be in the wells. The 384-well plate was adjusted based on the water drop position on an empty and sealed 384-well plate. One cell mode was selected to ensure the single-cell sorting. Single cells were then sorted into each well of the plate following fluorescence-activated nuclei sorting where stained dead cells were removed.

2.2.3. Bisulfite Treatment

Bisulfite conversion was carried out using Zymo EZ-96 DNA Methylation-Direct™ Kit (Deep-Well Format, Cat. No. D5023). The 384-well plate containing sorted cell nuclei was shortly spinned down and heated at 50 °C for 20 min to ensure break of the cell membrane. To convert unmethylated cytosine to thymine, 25 µl of CT Conversion Reagent (Cat. No. D5003-1) was first added to each well. The cells were transferred from the 384-well plate to a 96-well plate (Conversion Plate, Cat. No. C2005). A total of 90 cells were taken from the 384-well plate to the new 96-well plate. The remaining 6 wells were negative controls that only contained M-Digestion Buffer, Proteinase K, Nuclease-Free H_2O and CT Conversion Reagent. Negative control for unmethylated cytosines was accomplished by adding 1 µl of 57 fg/µl Lambda unmethylated DNA (Promega, Cat. No. D1521) to each well containing DNA. The plate was sealed with a cover foil and shortly spinned and then placed in a thermocycler at 98 °C for 8 min, 64 °C for 3.5 h and 4 °C for ∞. Each well of a Zymo-Spin I-96 Binding Plate (Cat. No. C2004, on top of a collection plate

Cat. No. C2002) was preloaded with 150 µl of M-Binding Buffer (Cat. No. D5006-3). Bisulfite conversion reactions were transferred from the 96-well plate to the I-96 Binding Plate. The plate was then centrifuged at 5000 x g for 5 min. Next, 400 µl of M-Wash Buffer (Cat. No. D5040-4) was added to the wells and the plate was again centrifuged at 5000 x g for 5 min. To convert cytosine to uracil, 200 µl of M-Desulphonation Buffer (Cat. No. D5002-5) was added to each well and the plate was incubated at room temperature for 15 min, followed by centrifugation at 5000 x g for 5 min. Each well was then washed with 400 µl of M-Wash Buffer twice. At the last step, 12 µl of M-Elution Buffer (Cat. No. D5007-6) was added to each well and the plate was incubated at room temperature for 5 min. The plate was then placed over a 96-well PCR plate (Eppendorf twin. Tec microbiology PCR Plate, semi-skirted, Cat. No. 4655270) and was centrifuged at 5000 x g for 3 min. Lastly, 9 µl of eluted DNA was collected in the PCR plate.

2.2.4. Indexing Using Random Primers

Library preparation of bisulfite-converted DNA was carried out by using the Swift Biosciences ACCEL-NGS® ADAPTASE™ MODULE for Single-Cell Methyl-Seq Library Preparation, version 1.0, according to the instructions by the manufacturer. Indexing by using random primers (Table 1) is the first barcoding step to produce individually barcoded libraries. This step is performed by incorporation of a truncated P5 adapter to the 5' end of the bisulfite-converted DNA. First, 1 µl of each 5 µM indexed random primer (P5L-AD002-N9, P5L-AD006-N9, P5L-AD008-N9 and P5L-AD010-N9) was added to each well of a 96-well PCR plate with bisulfite converted nuclei. The plate was placed in a thermocycler at 95 °C for 3 min and immediately after placed on ice for 2 min. A total of 10 µl of enzyme mix [2 µl of Blue Buffer (Enzymatics Qiagen, LOT 120817), 1 µl of 10 mM dNTP Kapa (Biosystems, Cat. No. KN1009), 1 µl of DNA polymerase Klenow exo- (50U/µl, Enzymatics, LOT 22021819) and 6 µl of H_2O] was added to each well. Klenow

exo- is a DNA polymerase required for random primed labelling and filling in overhangs of dsDNA. The plate was placed in a thermocycler with following program:

4 °C for 5 min
Ramp up to 25 °C at 0.1 °C/sec
25 °C for 5 min
Ramp up to 37 °C at 0.1 °C/sec
37 °C for 60 min
4 °C for ∞

An enzymatic step was necessary to remove excess primers and nucleotides from single-stranded DNA before PCR. 2 µl of Exonuclease I 3' → 5' (20U/µl, Biolabs, Cat. No. M0293L) and 1 µl of 1U Shrimp Alkaline Phosphatase (Biolabs, Cat. No. M03715) were added. The plate was mixed and placed in a thermocycler at 37 °C for 30 min and 4 °C for ∞. The indexed random primers were purchased from Integrated DNA Technologies (IDT).

Table 1 P5L Indexed Random Primers

#Primer	Barcode
P5L-AD002-N9	/5SpC3/ TTCCCTACACGACGCTCTTCCGATCT**CGATGT** (N1:25252525)(N1)(N1)(N1)(N1)(N1)(N1)(N1)(N1)
P5L-AD006-N9	/5SpC3/ TTCCCTACACGACGCTCTTCCGATCT**GCCAAT** (N1:25252525)(N1)(N1)(N1)(N1)(N1)(N1)(N1)(N1)
P5L-AD008-N9	/5SpC3/ TTCCCTACACGACGCTCTTCCGATCT**ACTTGA** (N1:25252525)(N1)(N1)(N1)(N1)(N1)(N1)(N1)(N1)
P5L-AD010-N9	/5SpC3/ TTCCCTACACGACGCTCTTCCGATCT**TAGCTT** (N1:25252525)(N1)(N1)(N1)(N1)(N1)(N1)(N1)(N1)

2.2.5. SPRI Bead Purification

SPRI bead purification was performed in a horizontal laminar flow hood to minimize contamination. The volume ratio of beads to DNA is a depending factor in size-selection. The lower ratio of beads, the higher is the DNA fragments after the purification. To remove DNA fragment lower than 400 bp, 0.8x of beads was used. First, 17.6 µl of SPRI beads (Beckerman Coulter, LOT 17385200) was added to each well of the 96 well plate. The nuclei were pooled and transferred to strips where one strip contained four nuclei, one of each indexed random primer. The strips were incubated at room temperature for 5 min and after that, placed on a magnetic separator. The supernatant was removed, and the samples were washed with 180 µl of ethanol (80%) thrice. The beads were air dried at room temperature and 10 µl of Low EDTA TE (Swift Biosciences™, Cat. No. 90296) was added to each strip to resuspend the beads. The eluted samples were transferred to a new 96-well PCR plate and

placed in a thermocycler for DNA denaturation at 95 °C for 3 min and immediately after placed on ice for 2 min.

2.2.6. Adaptase Reaction

Adaptase reaction enables A-tailing and adaptor ligation following to PCR amplification. The adaptor ligation contains P5 and P7 primers (Table 2 and Table 3). Ligation contributes to pooling of the samples, which all nuclei contain a unique set of primers for identification and detection of the final library. First, 10.5 µl of Adaptase master mix (containing 4.25 µl of Elution Buffer, 2 µl of Buffer G1, 2 µl of Reagent G2, 1.25 µl of Reagent G3, 0.5 µl of Enzyme G4 and 0.5 µl of Enzyme G5) was added to each well and mixed by pipetting. The plate was placed in a thermocycler at 37 °C for 30 min and 4 °C. A total of 30 µl of PCR mix [0.3 µl of P5L indexing primer 100 µM, 5.0 µl of P7L indexing primer 10 µM, 1 µl of Kapa Hifi enzyme 1 U/µl (Kapa Biosystems, Cat. No. KE2502), 1.5 µl of dNTPs (Biolabs, Cat. No. N0447S), 10 µl of 5x Kapa Hifi Fidelity Buffer 2 mM Mg (Kapa Biosystems, Cat. No. KB2500) and 9.8 µl of H_2O] was added to each well and the PCR plate was placed in a thermocycler with the following program:

95 °C for 2 min
98 °C for 30 sec
6 cycles of (98 °C for 15 sec, 64 °C for 30 sec, 72 °C for 2min)
72 °C for 5 min
4 °C for ∞

Table 2 P5L Amplification Primers

Flow cell attachment, read 1 sequencing primer

#Primer	Barcode
P5L_D501	AATGATACGGCGACCACCGAGATCTACACTATAGCCT ACACTCTTTCCCTACACGACGCTCT
P5L_D502	AATGATACGGCGACCACCGAGATCTACACATAGAGGC ACACTCTTTCCCTACACGACGCTCT
P5L_D503	AATGATACGGCGACCACCGAGATCTACACCCTATCCT ACACTCTTTCCCTACACGACGCTCT

Table 3 P7L Amplification Primers

Flow cell attachment, read 2 sequencing primer

#Primer	Barcode
P7L_D701	CAAGCAGAAGACGGCATACGAGATCGAGTAAT GTGACTGGAGTTCAGACGTGTGCTCTT
P7L_D702	CAAGCAGAAGACGGCATACGAGATTCTCCGGA GTGACTGGAGTTCAGACGTGTGCTCTT
P7L_D703	CAAGCAGAAGACGGCATACGAGATAATGAGCG GTGACTGGAGTTCAGACGTGTGCTCTT
P7L_D704	CAAGCAGAAGACGGCATACGAGATGGAATCTC GTGACTGGAGTTCAGACGTGTGCTCTT
P7L_D705	CAAGCAGAAGACGGCATACGAGATTTCTGAAT GTGACTGGAGTTCAGACGTGTGCTCTT
P7L_D706	CAAGCAGAAGACGGCATACGAGATACGAATTC GTGACTGGAGTTCAGACGTGTGCTCTT

2.2.7. Library Quantification and Sequencing

After PCR amplification, the PCR products were cleaned up by using 0.8x SPRI beads. The concentrations of the final libraries were measured fluorometrically by using Qubit dsDNA High Sensitivity kit (Thermo Fisher Scientific) and fluorometer. The assay uses a fluorescent dye which binds specifically to the DNA and affects the fluorescence. The fluorescence is measured, and concentrations calculated based on the differences in the fluorescence between unbound and bounds die molecules. Bioanalyzer HS DNA kit (Agilent Technologies) was used to identify the features of the final DNA libraries and to monitor size distribution with electrophoresis on Bioanalyzer Instrument.

Libraries are later pooled together and resolved on 2% agarose gel and purified to remove adapter primers. Lastly, the final libraries are sequenced by using Illumina Nova-Seq 6000 next-generation sequencer. The raw data is processed and further analysed in collaboration with the Medical Bioinformatics Centre at Turku Bioscience.

2.3. Statistical Analyses

All experiments were performed 3 times and results are presented as proliferation curve and cell viability with grouped columns, in which the mean ± standard error of the mean (SEM) is calculated. Two-way repeated measures ANOVA followed by Tukey's multiple comparisons test was used for the statistical analyses. Two-way ANOVA was calculated to know whether the drug treatment of Dex, Dec and the combination of the two drugs shown an effect on TALL by comparing each measured sample mean with every other mean after 72 h of treatment. It was performed using GraphPad Prism 8.2.1. (GraphPad Software for Windows, La Jolla California USA). The statistical analysis of the cell viability was performed with R 3.6.1. (R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria). The result is significant when the p-value was <0.05.

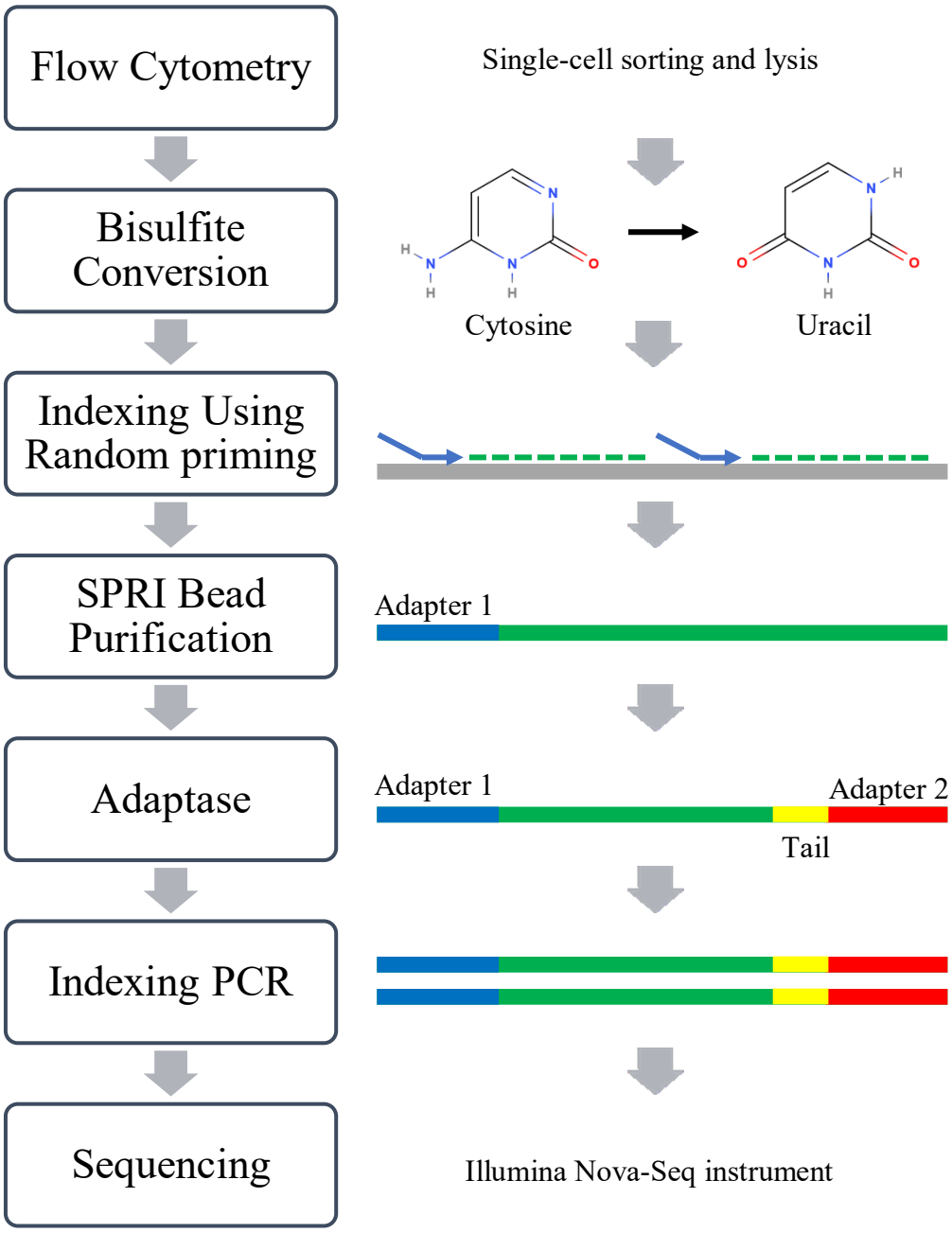


Figure 5 SnmC-seq workflow.

Library preparation for a methylation-profiling assay contains several steps. Sorted cells are bisulfite converted where cytosine nucleotides convert to uracil. The ssDNA is indexed using random primers followed by SPRI bead purification. A second primer is added during adaptase reaction and the DNA is amplified with PCR. The final library molecule is sequenced with Illumina Nova-Seq instrument.

3. Results

3.1. Effects of Dexamethasone and Decitabine on Proliferation in CCRF-CEM

To determine the effect of the two commonly used ALL drugs Dex and Dec on CCRF-CEM cell line, the cells were treated with various concentrations of the drugs both separately and in combinations. Dex was added at the concentrations 100 nM and 1 µM. Dec was added at 0.1 µM and 0.05 µM. Eight samples were tested with different treatments and the experiment was repeated three times at the time points 0 h, 24 h, 48 h and 72 h. To determine the proliferation, the absorbance was measured with WST-1 staining assay. Results are represented in Figure 6. Dex is a drug that causes G1-cycle arrest inducing apoptosis and Dec is a hypomethylating agent, which also induces apoptosis. All combined treatments: 1 µM Dex + 0.1 µM Dec, 1 µM Dex + 0.05 µM Dec, 100 nM Dec + 0.1 µM Dec and 100 nM Dex + 0.05 µM Dec showed a significant decrease in absorbance compared to the control sample at 72 h ($*p < 0.05$). Results even showed a significant decrease in the drug combinations in 100 nM Dex + 0.1 µM Dec, 1 µM Dex + 0.05 µM Dec and 1 µM Dex + 0.1 µM Dec compared to 100 nM Dex alone ($*p < 0.05$). This indicates that CCRF-CEM is more sensitive to Dex in combination with Dec than to Dex alone.

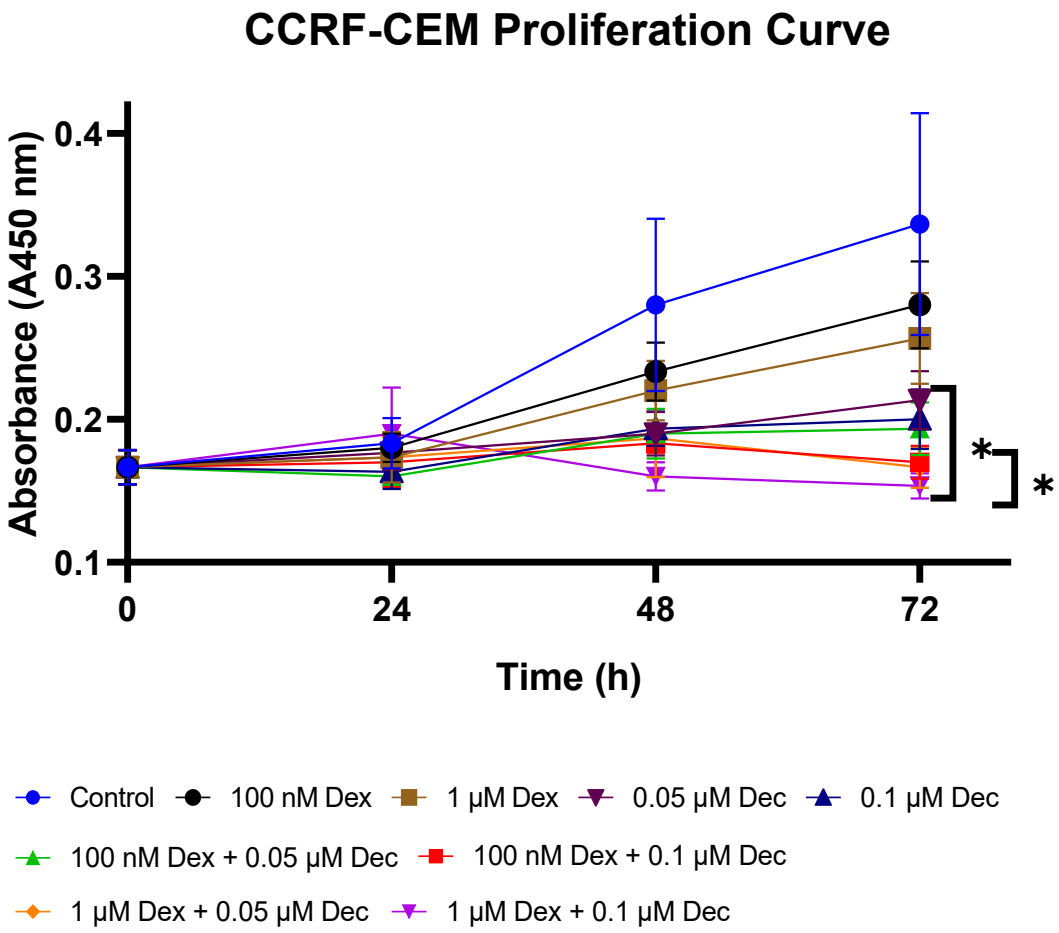


Figure 6 CCRF-CEM proliferation curve based on WST-1 assay.

Effects of Dex and Dec on CCRF-CEM after 72 h of treatment. Each sample represents the normalized value with error bars SEM. All combined treatments are significantly lower than the control sample. Also, 100 nM Dex was measured with a higher absorbance compared to the same concentration but in combination with Dec (*$p < 0.05$).

3.2. Cell Viability in CCRF-CEM

To examine the cell viability in CCRF-CEM drug treatment, cell counting was performed with trypan blue staining. The results are represented as percentages in Figure 7. Significant cytotoxic responses were observed in the samples containing 0.05 µM Dec, 0.1 µM Dec, 1 µM Dex + 0.05 µM Dec and 1 µM Dex + 0.1 µM Dec after 72 h (**$p < 0.005$). Meaning that these samples had a higher rate of

apoptosis compared to the control. Results indicate that Dec induces apoptosis and the apoptosis rate is higher in combination with 1 µM Dex and Dec. The grouped columns are in triplicate and error bars are shown as SEM.

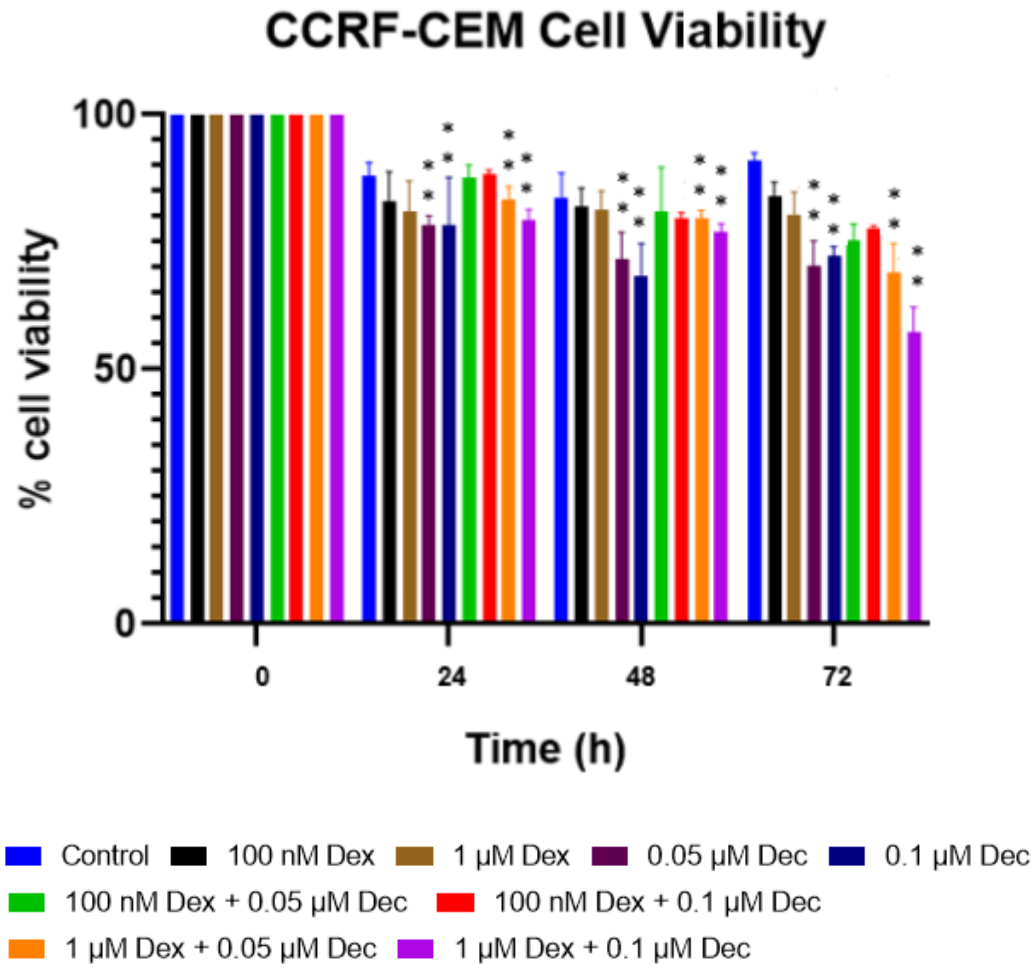


Figure 7 CCRF-CEM cell viability.

The cell viability is shown as percentage and non-treated cells as 100% cell viability. Cell viability was measured beside the cytotoxicity assay using trypan blue staining when cell counting the samples at 24 h, 48 h and 72 h after drug treatment. Samples treated with 0.05 µM Dec, 0.1 µM Dec 1 µM Dex + 0.05 µM Dec and 1 µM Dex + 0.1 µM Dec had a significantly lower percentage of viable cell compared to the non-treated control sample (**$p < 0.005$).

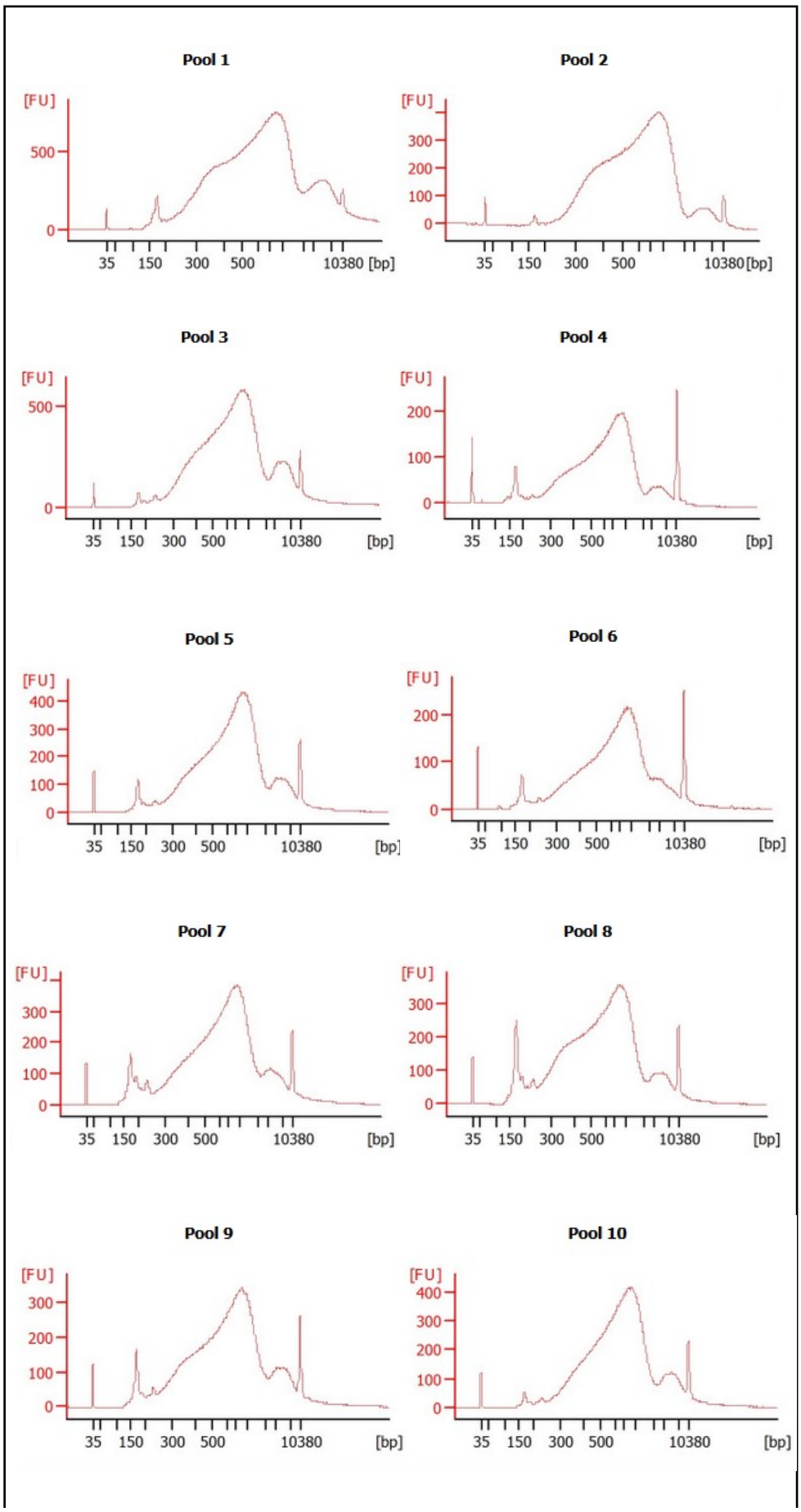
3.3. Single-cell Bisulfite Libraries

The last step of library preparation of snmC-seq measures the quality and quantitation of the final single-cell DNA bisulfite libraries to ensure that the library preparation had been successful, there is enough input DNA for sequencing and that the samples did not denature during the library preparation. Results of the measured concentrations of the pooled samples are represented in Table 4. The quantification was measured with Qubit and the concentrations (ng/µl) are listed as Pool 1 – Pool 10 patient samples and Pool 11 – Pool 14 CCRF-CEM cells. The concentrations ranges from 3.08 – 18.62 ng/µl. The library preparation was performed in the same way for all samples, which expects that the results would be very similar. The wide range of concentrations between the samples indicates that there is not the same amount of DNA in each sample. Thus, all samples are still qualified for sequencing.

Table 4 Qubit measurements of final libraries.

Sample	Concentration (ng/µl)
Pool 1	18.62
Pool 2	12.64
Pool 3	9.68
Pool 4	3.16
Pool 5	6.88
Pool 6	3.08
Pool 7	6.40
Pool 8	7.52
Pool 9	5.98
Pool 10	9.74
Pool 11	3.56
Pool 12	11.00
Pool 13	11.38
Pool 14	4.08

Bioanalyzer was conducted to quantify and assess the size distribution features of the final libraries. Results of the patient samples are represented in Figure 8 and CCRF-CEM cell line in Figure 9. The electropherograms are plotted as migration in time against fluorescence unit (FU). The lower marker is at 35 bp peak and the higher marker at the 10 380 bp peak. As expected, the size distribution of the libraries is between 300 bp to 1000 bp for all the samples. The average size is approximately 550 bp - 580 bp and the percentage of the total is 75-80%. The smooth shape of the library curve indicates that the samples are pure. The FU values have been set low enough for the samples to align to read the peaks, which indicates that the runs were of good quality. The small peaks between the lower marker and the library (35 bp – 300 bp) are adapters, which are removed before sequencing. As a conclusion, the library preparation from single cells for DNA methylation analysis was successful.


Pool 1
[FU]
500
0
35 150 300 500 10380 [bp]
Pool 2
[FU]
300
200
100
0
35 150 300 500 10380 [bp]
Pool 3
[FU]
500
0
35 150 300 500 10380 [bp]
Pool 4
[FU]
200
100
0
35 150 300 500 10380 [bp]
Pool 5
[FU]
400
300
200
100
0
35 150 300 500 10380 [bp]
Pool 6
[FU]
200
100
0
35 150 300 500 10380 [bp]
Pool 7
[FU]
300
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100
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35 150 300 500 10380 [bp]
Pool 8
[FU]
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35 150 300 500 10380 [bp]
Pool 9
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Pool 10
[FU]
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35 150 300 500 10380 [bp]

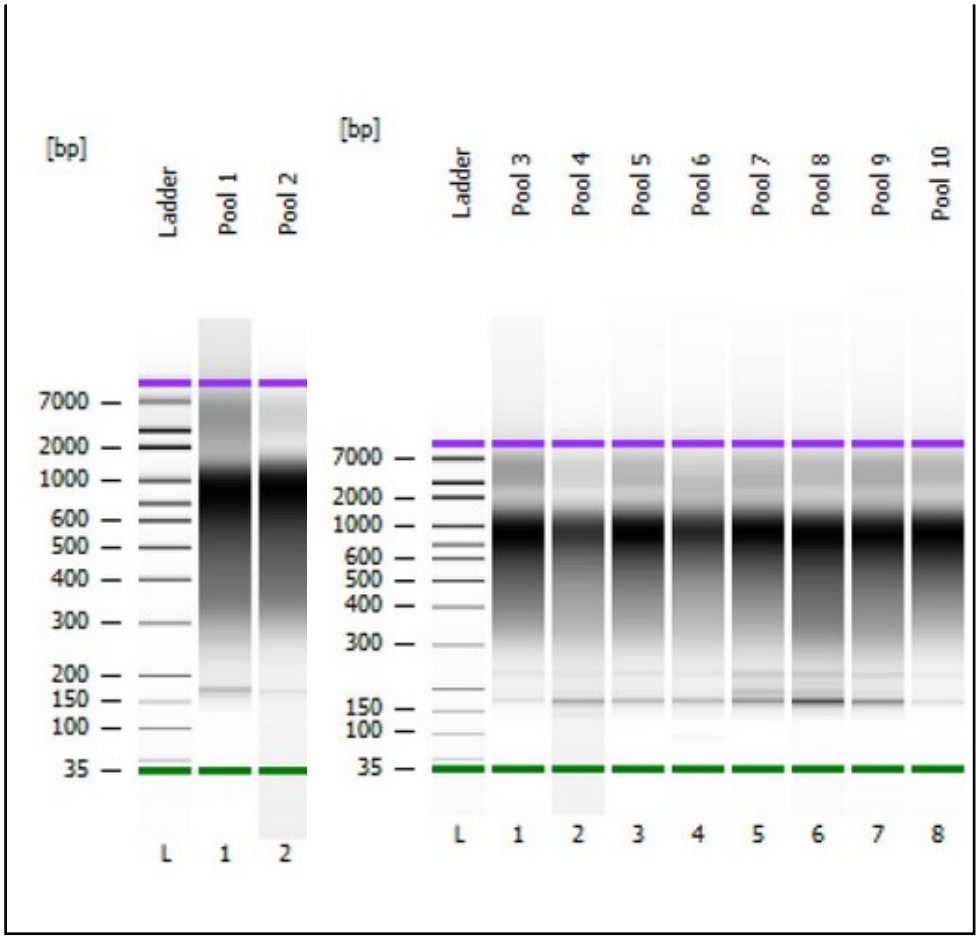

Figure 8 Bioanalyzer results from final DNA libraries of patient sample.

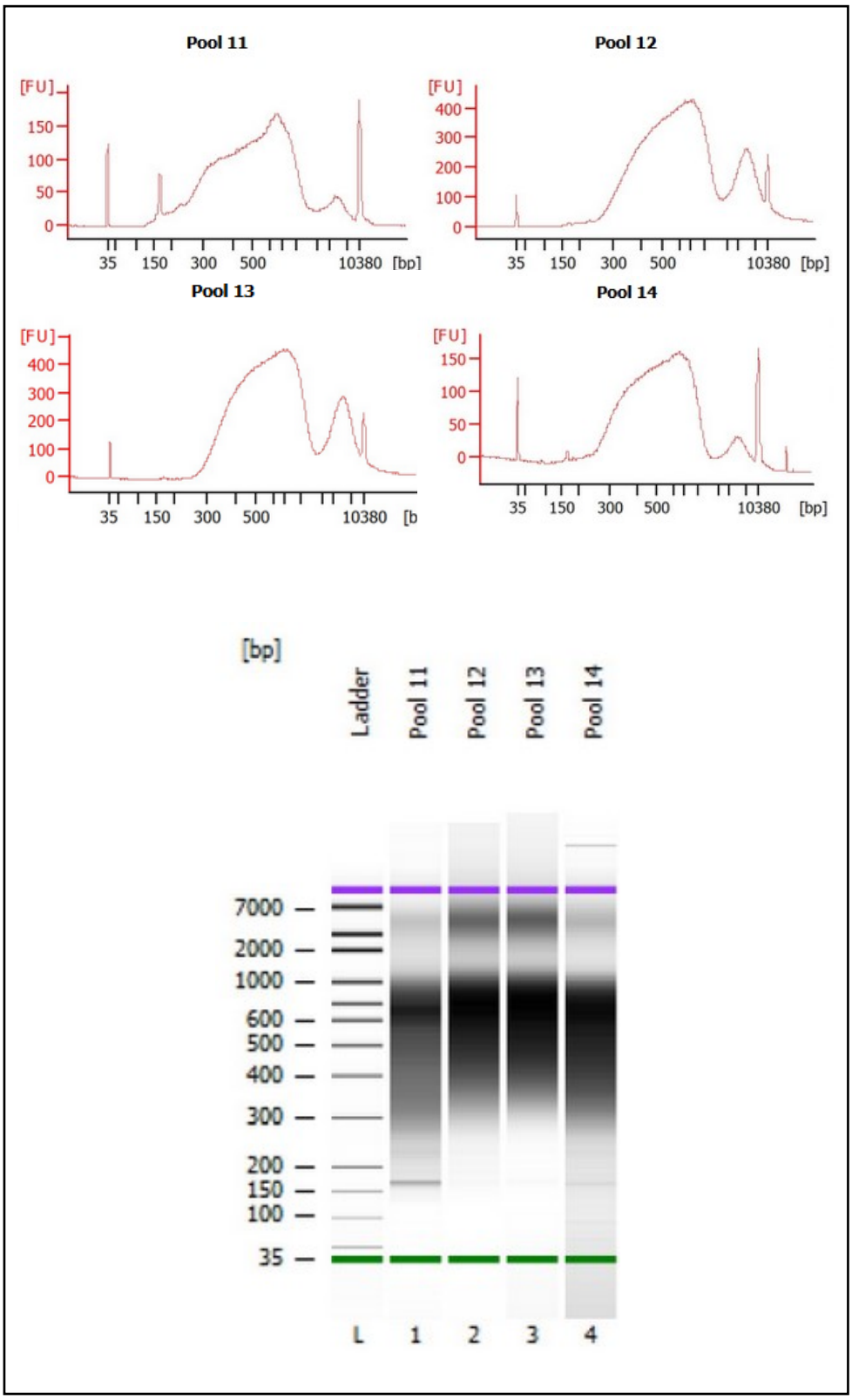

Figure 9 Bioanalyzer results from final DNA libraries of CCRF-CEM.

4. Discussion

4.1. Drug Treatment Assay

The finding of the drug treatment assay is that treatment of CCRF-CEM TALL cell line with dexamethasone and decitabine showed less proliferation compared to Dex treatment alone after 72 h. The results based on WST-1 staining show the proliferation for each timepoint and drug composition (Figure 6). The proliferation curve does not show what happens with nor within the cells during the treatment. Are the cells dying in apoptosis or have their proliferation stopped? Results on cell viability show a trend that the cells die gradually depending on longer treatment period and higher concentrations of the two drugs (Figure 7). Earlier studies have shown that glucocorticoid-resistant CCRF-CEM cells become more sensitive to glucocorticoids when glucocorticoid-treatment is combined with Dec, and causes apoptosis (Miller et al., 2014). My data support similar results. The following questions are, how are cell signalling and gene expression affected by Dex and Dec and can DNA methylation markers for resistance be identified in the individual cells, which do not respond to the drug treatment. Cell death and alteration in DNA methylation caused by the hypomethylating agent Dec are affected by multiple complex systems, whose mechanisms are not known.

Cytotoxicity assay for visualization of a cellular-growth curve is a widely used method to determine the best timepoint for the effects of a drug tested on a cell line. Incubation for 30 min with WST-1 is a suitable incubation time for CCRF-CEM, since the colour change could be noticed with the naked eye already at that timepoint. DNA methylation occurs already within 24 h of stimulation with Dex and Dec, whereas changes on the gene expression level are detected later. As expected, no significant effect was noticed on the cell proliferation after 24 h. Further analysis to identify which methylations sites that are affected by Dex treatment is possible with the new single-nucleus methylcytosine sequencing method (snmC-seq). To my knowledge, single-cell analysis to identify methylomes has never earlier been tested on CCRF-CEM.

4.2. DNA Library Preparation

The DNA library preparation based on snmC-seq succeeded and generated 14 pools of DNA libraries. All libraries were constructed equally. Bioanalyzer results showed pure libraries between 300 bp and 1000 bp (Figure 8 and 9). The amount of DNA corresponded to the DNA concentrations measured with Qubit (Table 4). As all libraries were equally constructed, it is expected that all concentrations would be similar as every pool contains DNA from four nuclei. The concentrations ranged from 3.08 to 18.62 ng/µl of DNA. Reasons for this may be that the sorted single-cells did not end up in the digestion buffer during flow cytometry, which led to three instead of four nuclei in one pooled sample; or, the DNA dried for too long during the SPRI purification, or in any other step that exceeded for too long, resulting in loss of DNA. Results from Qubit and Bioanalyzer indicate that the samples are pure and are qualified to be sequenced. There was no difference in the library preparation nor results between the patient sample and the CCRF-CEM cell line.

The library preparation protocol followed instructions from commercially available kits. The use of the kits individually may be applied for other purposes than snmC-seq. The Swift-kit exploits the underlying technique from snmC-seq. The next step beyond the scope of my study is to sequence the DNA libraries, which will provide further evidence if the method is applicable to study cell-to-cell variability, understand the cell heterogeneity and reveal molecular mechanisms of TALL and drug responses. Previously, this method has proven to work even in one of the most challenging tissues, in the original article to identify neuronal subtypes. Blood samples from TALL patients are valuable and difficult to come by. Cells from patients are very sensitive and may easily die during maintenance. Cell cultures are easier to manipulate and provide an unlimited resource for research. These factors were the reason for the set up for this snmC-seq pilot study. Results from this experiment indicate that patient samples on a larger scale can be prepared with the snmC-seq library preparation method in the future.

4.3. SnmC-seq

Sequencing of the snmC-seq DNA libraries produces genome-wide data of the methylation status of CpG sites at single nucleotide resolution in a single nucleus. The methylome profiles exhibit the different cell type distribution in the tissue and identification of the cell-to-cell variability. The subtypes in the cancer tissue can be identified in a cluster diagram, depending on the sum of methylated CpG sites. The aim is to identify the driving forces of the disease and responses to drug treatment at the methylation level. With snmC-seq it is also possible to visualize hypermethylation, hypomethylation and marker genes for each cell type. The advantages of this method are the high coverage rates compared to similar methods, and fewer duplicates in the sequencing. Pooling the samples reduces the cost and eases the identification of common sequences. The unique index for each nucleus makes it possible to identify each cell during data analysis. DNA methylation alone does not affect gene expression, rather the combination of epigenetic modifications and the chromatin conformation. Further studies on chromatin interactions combined with single-cell analysis could provide more information about the driving forces of leukemia.

4.4. Future Prospects

To my knowledge, single-cell sequencing with the aim to identify methylomes has earlier never been accomplished with CCRF-CEM. A further goal would be to find methylated regions affected by treatment of 1 μM of Dex. Another experiment with a GC-resistant CCRF-CEM cell clone would provide more information about the mechanism of resistance in TALL. Importantly, the next step also includes extension of the study to more patient samples in order to determine the mechanisms of TALL and drug resistance in each individual and in individual cancer cells. The epigenomic state is known to be a driving force in cancers by silencing tumour suppressor genes. The field of epigenetics and the understanding of its role in cancer development has exploded in recent years. Methods for library

preparation and sequencing techniques are continuously developed and improved. About 80% of all TALL patients are cured in Finland. The challenges that remain are how to earlier recognise the patients with relapse and how to stratify TALL patients to specific treatment groups for individualized treatment. As GC-resistance is a problem in relapse of TALL, the mechanism of resistance needs to be further studied and one key method is single-cell sequencing.

5. Epigenetiska markörer i T-cell akut lymfatisk leukemi på singelcellnivå

T-cell akut lymfatisk leukemi (TALL) är den vanligaste barncancerformen i Norden. Årligen diagnostiseras 200 nya fall i Norden och Baltikum, varav 80 % tillfrisknar. Leukemi kännetecknas av genetisk och epigenetisk heterogenitet med uppvisad resistens mot kemoterapi och aggressiva återfall. Heterogenitet innebär att tumören består av en mix av celler med olika molekylära egenskaper. DNA-metylering är en epigenetisk markör för reglering av genuttrycket och är länken mellan arvet och livsstilen. Till exempel, en del transkriptionsfaktorer binder inte till DNA:t när det är metylerat, medan andra faktorer endast binder då DNA:t är metylerat. I DNA:t är 80 % av CpG-dinukleotiderna metylerade. Metylering sker på cytosinets femte kolatom via katalys av metyltransferaset DNMT. Metyleringarna är olikt fördelade över genomet beroende på celltyp och sjukdomsförloppet. Avvikande DNA-metylering orsakar kromosominstabilitet och promotor-hypermetylering av tumörsuppressorgener. Dessa faktorer kan driva utvecklingen av leukemi och även andra cancerformer. Behandling av TALL består vanligen av en läkemedelskombination med en glukokortikoid. Dexametason (Dex) är en glukokortikoid och är ett anti-inflammatoriskt läkemedel som binder till glukokortikoidreceptorn i cytoplasman, som bland annat inaktiverar transkriptionen av gener för inflammatoriska proteiner. Decitabine (Dec) är ett epigenetisk läkemedel. Molekylen är analog till cytosin som genom replikation kan inkorporeras i DNA:t, binda till DNMT och inhibera DNA-metyleringen. Tidigare studier av CCRF-CEM TALL-cellinje har visat att glukokortikoid-resistens kan återställas med behandling av

Dex och Dec i kombination (Miller et al., 2014). Målet är att visa ifall en kombination av Dex och Dex påverkar celltillväxten och viabiliteten av CCRF-CEM cellinje, jämfört med enskild behandling av läkemedlen. Utifrån tillväxtkurvan med WST-1-analys bestäms tidpunkten och läkemedelshalten av Dex på CCRF-CEM för vidare analys av metylomen.

Tidigare sekvenseringsstudier av TALL har gjorts på tumörvävnadens hela genom. Resultatet på hel-genom sekvensering reflekterar ett medelvärde på genexpressionen och observerar alla celler som homogena, vilket gör det svårt att analysera variationen mellan celltyperna (Nordlund, Syvänen, 2018). Med den nya metoden metylcytosinsekvensering av enskilda cellkärnor (snmC-seq) undviker man heterogenitetsproblemet genom att först sortera cellerna med flödescytometri (Luo et al., 2017). Varje enskild cell sekvenseras för att kunna urskilja den cellulära identiteten och profilera metylomen. Vid flödescytometri färgas cellerna med propidiumjodid (PI) för att identifiera döda celler så att endast levande celler sorteras. PI binder till dsDNA och upptäcks med fluorescens. Utöver singelcellsortering baserar sig snmC-seq på bisulfitkonvertering där ometylerade cytosin omvandlas till uracil, som sedan kan analyseras vid dataanalys med Lambda DNA som referensgenom. Metylcytosin påverkas inte av bisulfit utan de förblir intakta. I slutskedet av framställningen av DNA-bibliotek konverteras uracil till tymin efter polymeraskedjereaktion. Målet är att testa snmC-seq på TALL-patientprov vid diagnos och CCRF-CEM-cellinje ifall framställningen av DNA-bibliotek fungerar enligt protokollet. Kommersiella kit används för bisulfitkonvertering och framställning av DNA-bibliotek.

Effekten av leukemiläkemedlen Dex och Dec analyserades med WST-1 för att åstadkomma en tillväxtkurva för dos-respons samverkan. Den uppmätta absorbansen reflekterar antalet levande celler. En kombination av Dex och Dec visar på signifikant lägre celltillväxt jämfört med behandling av Dex enskilt efter 72 h (Figur 6). DNA-metylering sker inom 24 h men som förväntat ser man ingen effekt på celltillväxten under det första dygnet. Tillväxtkurvan beskriver inte vad som händer på cellnivå, hur signaleringen och genexpressionen påverkas av Dex-behandling. För att ta reda på ifall cellerna dör i apoptos eller slutar att dela på sig räknades cellerna med trypanblått i samband med WST-1. Trenden på cellviabiliteten visar på högre apoptos ju längre behandlingen pågår, och ju högre läkemedelskoncentrationer som använts (Figur 7).

Totalt framställdes 14 pooler DNA-bibliotek på både patientprov och 1 µM Dex-behandlade CCRF-CEM-celler med snmC-seq. Kvantifierings- och kvalitetskontroll av de färdiga DNA-biblioteken gjordes med Bioanalyzer, en automatiserad elektroforesmetod och Qubit. Koncentrationerna på DNA:t varierade mellan 3,08 – 18,62 ng/µl, vilket är inom ramen för Illumina-sekvensering. Bioanalyzer-elektroferogrammen visar att DNA-fragmentens storlek är mellan 300 bp – 1000 bp. Framställningen av DNA-biblioteken lyckades. Ytterligare bekräftande av metoden fastställs först efter sekvensering och dataanalys av DNA-biblioteken. Syftet med att testa snmC-seq är att i framtiden kunna applicera metoden på patientprover för att analysera heterogeniteten och mekanismen för resistens mot kemoterapi.

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7. Appendices

Appendix 1 – Metadata

Table S1 Metadata of human TALL patient sample.

Sample	Source	Area	FACS date	Labeling	FACS channel (nm)	FACS count	Bisulfite conversion method	Library type	Library pooled	P5L primer	P7L primer
Pool_1_AD002_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	29.11.2018_1	D501	D701
Pool_1_AD006_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	29.11.2018_1	D501	D701
Pool_1_AD008_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	29.11.2018_1	D501	D701
Pool_1_AD010_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	29.11.2018_1	D501	D701
Pool_2_AD002_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	29.11.2018_2	D501	D702
Pool_2_AD006_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	29.11.2018_2	D501	D702
Pool_2_AD008_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	29.11.2018_2	D501	D702
Pool_2_AD010_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	29.11.2018_2	D501	D702
Pool_3_AD002_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_3	D501	D704
Pool_3_AD006_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_3	D501	D704
Pool_3_AD008_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_3	D501	D704
Pool_3_AD010_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_3	D501	D704
Pool_4_AD002_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_4	D501	D705
Pool_4_AD006_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_4	D501	D705
Pool_4_AD008_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_4	D501	D705
Pool_4_AD010_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_4	D501	D705
Pool_5_AD002_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_5	D501	D706
Pool_5_AD006_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_5	D501	D706
Pool_5_AD008_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_5	D501	D706
Pool_5_AD010_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_5	D501	D706
Pool_6_AD002_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_6	D502	D701
Pool_6_AD006_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_6	D502	D701
Pool_6_AD008_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_6	D502	D701
Pool_6_AD010_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_6	D502	D701
Pool_7_AD002_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_7	D502	D702
Pool_7_AD006_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_7	D502	D702
Pool_7_AD008_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_7	D502	D702
Pool_7_AD010_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_7	D502	D702
Pool_8_AD002_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_8	D502	D703
Pool_8_AD006_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_8	D502	D703
Pool_8_AD008_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_8	D502	D703
Pool_8_AD010_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_8	D502	D703
Pool_9_AD002_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_9	D502	D704
Pool_9_AD006_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_9	D502	D704
Pool_9_AD008_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_9	D502	D704
Pool_9_AD010_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_9	D502	D704
Pool_10_AD002_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_10	D502	D705
Pool_10_AD006_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_10	D502	D705
Pool_10_AD008_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_10	D502	D705
Pool_10_AD010_indexed	Patient - Diagnosis	Blood sample (mononuclear cells)	23.11.2018	PI	488	1	Zymo EZ-96 direct	snmC-seq2	16.1.2019_10	D502	D705

Table S2 Metadata of human CCRF-CEM cell line

Sample	Source	Area	Treatment	FACS date	Labeling	FACS channel (nm)	FACS count	Bisulfite conversion method	Library type	Library pooled	P5L primer	P7L primer
Pool_11_AD002_indexed	Cell Line	Blood sample	Control 1	8.3.2019	PI	488	1	Zymo EZ-96 direct	smmC-seq2	14.3.2019_11	D503	D701
Pool_11_AD006_indexed	Cell Line	Blood sample	Dexamethasone 1 (1 µM)	8.3.2019	PI	488	1	Zymo EZ-96 direct	smmC-seq2	14.3.2019_11	D503	D701
Pool_11_AD006_indexed	Cell Line	Blood sample	Control 2	8.3.2019	PI	488	1	Zymo EZ-96 direct	smmC-seq2	14.3.2019_11	D503	D701
Pool_11_AD010_indexed	Cell Line	Blood sample	Dexamethasone 2 (1 µM)	8.3.2019	PI	488	1	Zymo EZ-96 direct	smmC-seq2	14.3.2019_11	D503	D701
Pool_12_AD002_indexed	Cell Line	Blood sample	Control 1	8.3.2019	PI	488	1	Zymo EZ-96 direct	smmC-seq2	14.3.2019_12	D503	D702
Pool_12_AD006_indexed	Cell Line	Blood sample	Dexamethasone 1 (1 µM)	8.3.2019	PI	488	1	Zymo EZ-96 direct	smmC-seq2	14.3.2019_12	D503	D702
Pool_12_AD006_indexed	Cell Line	Blood sample	Control 2	8.3.2019	PI	488	1	Zymo EZ-96 direct	smmC-seq2	14.3.2019_12	D503	D702
Pool_12_AD010_indexed	Cell Line	Blood sample	Dexamethasone 2 (1 µM)	8.3.2019	PI	488	1	Zymo EZ-96 direct	smmC-seq2	14.3.2019_12	D503	D702
Pool_13_AD002_indexed	Cell Line	Blood sample	Control 1	8.3.2019	PI	488	1	Zymo EZ-96 direct	smmC-seq2	14.3.2019_13	D503	D703
Pool_13_AD006_indexed	Cell Line	Blood sample	Dexamethasone 1 (1 µM)	8.3.2019	PI	488	1	Zymo EZ-96 direct	smmC-seq2	14.3.2019_13	D503	D703
Pool_13_AD006_indexed	Cell Line	Blood sample	Control 2	8.3.2019	PI	488	1	Zymo EZ-96 direct	smmC-seq2	14.3.2019_13	D503	D703
Pool_13_AD010_indexed	Cell Line	Blood sample	Dexamethasone 2 (1 µM)	8.3.2019	PI	488	1	Zymo EZ-96 direct	smmC-seq2	14.3.2019_13	D503	D703
Pool_14_AD002_indexed	Cell Line	Blood sample	Control 1	8.3.2019	PI	488	1	Zymo EZ-96 direct	smmC-seq2	14.3.2019_14	D503	D704
Pool_14_AD006_indexed	Cell Line	Blood sample	Dexamethasone 1 (1 µM)	8.3.2019	PI	488	1	Zymo EZ-96 direct	smmC-seq2	14.3.2019_14	D503	D704
Pool_14_AD006_indexed	Cell Line	Blood sample	Control 2	8.3.2019	PI	488	1	Zymo EZ-96 direct	smmC-seq2	14.3.2019_14	D503	D704
Pool_14_AD010_indexed	Cell Line	Blood sample	Dexamethasone 2 (1 µM)	8.3.2019	PI	488	1	Zymo EZ-96 direct	smmC-seq2	14.3.2019_14	D503	D704

Appendix 2 – Methods for cell culture

Thawing frozen cells

1. Thaw the frozen cells in a 37 ºC water bath until all the ice have thaw. The thawing procedure must proceed rapidly to ensure that as many cells as possible survive. Transfer the cells to a 15 ml Falcon tube. Add 2 ml of cell culture medium (pre-warmed to 37ºC).
2. Centrifuge the Falcon tube at 300 x g for 5 min.
3. Discard the supernatant and resuspend the pellet in 2 ml of cell culture medium.
4. Add 18 ml of growth medium to a cell culture bottle. Add the cell suspension (2 ml) to the bottle.
5. Mark the bottle with your name, cell line name and date.
6. Study the cells under a microscope to ensure the survival and the shape of the cells.
7. Store the cell culture in a heating cabinet at 37ºC and CO_2 at 5%

Maintenance of cell culture

The cell culture medium was changed 1:10 every Monday and Thursday. The cell condition was studied under microscope and the cells were stored in a heating cabinet at 37ºC and CO_2 at 5%.

Freezing of cells

1. Centrifuge the cells at 300 x g for 5 min.
2. Remove the supernatant and resuspend the pellet with 2 ml of freezing medium.
3. Divide the cell suspension into two cryo tubes, 1 ml of cells in each.
4. Immediately place the tubes in a storage container and place it in -80 °C overnight. Remove the tubes to -130 °C to store it for years.

Cell culture medium

- 176 ml RPMI
- 20 ml FBS
- 2 ml L-Glutamine
- 2 ml Penicillin-Streptomycin

Freezing medium

- 150 µl DMSO
- 1850 µl FBS