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Developing a protocol for *in vitro* assembly of chromatin using genome scale DNA molecules

Master's thesis in Biotechnology

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MASTER'S THESIS 2019, BBTX03

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Abstract

Epigenetics, the study of processes that change gene activity without altering the DNA sequence, has been observed to have a role in cancer, autoimmune diseases and neurodegenerative diseases. Epigenetic modifications can lead to alterations of the structure of the chromatin. To study how such changes affect the physical properties and dynamics of chromatin, *in vitro* assembled chromatin is a necessary tool as all the components are well-defined. In this Master's thesis project, the aim was to develop a protocol for *in vitro* assembly of chromatin using DNA molecules in the kilo-basepair size range. The objectives were to optimize physical and chemical conditions in order to assemble chromatin of high quality, using human derived histone octamers together with lambda DNA and T7 DNA. The assembly was performed using a salt dialysis procedure followed by enzymatic assembly, using the enzymes nucleosome assembly protein 1 (NAP-1) and ATP-utilizing chromatin assembly and remodelling factor (ACF). The chromatin quality was assessed by either enzymatic digestion followed by gel electrophoresis or by using nanofluidic channels combined with fluorescence microscopy. The main goal was to assemble chromatin with periodically spaced nucleosomes. Using lambda DNA and an enzymatic digestion approach, it was possible to visualize DNA fragments on gels from agarose gel electrophoreses that corresponded to mononucleosomes. In one experiment, fragments corresponding to di- and trinucleosomes could also be visualized. The T7 DNA on the other hand yielded bands corresponding to mono- and dinlucleosomes to a higher degree, in comparison to lambda DNA, indicating higher chromatin quality. By using single-molecule nanofluidics in combination with fluorescence microscopy, it was possible to visualize co-localized DNA and histones. Overall, the chromatin assembled was not of the desired quality and it is therefore of interest to develop the protocol further.

Keywords: chromatin assembly, lambda DNA, T7 DNA, nanofluidic channels, fluorescence microscopy

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Hanna Törnkvist, Gothenburg, June 2019

Content

1 Introduction

Up until the 1950s, the focus among scientists when trying to describe diseases and disorders was on genetics and environmental factors (1). It was later discovered that the truth might not lie in these factors solely, but also in epigenetics. Epigenetics can be described as an addition to changes in genetic sequence and includes all processes that change the gene activity without altering the DNA sequence (1, 2). The study of epigenetics and its role in e.g. cancer, autoimmune diseases and neurodegenerative diseases has been of great interest for the last decade (1, 2). Epigenetics involves both modifications of DNA as well as DNA-binding proteins such as histones (1). The modifications can be transmitted from mother cells to daughter cells and some of these modifications have also been shown to be transferred from one generation to another (1, 2). Epigenetic modifications are important for many different cellular functions and if they are not functioning properly, it might lead to disease (2).

DNA can be modified epigenetically via methylation, but there is also another important group of epigenetic modifications, namely the modification of histones (2). Histones can have different epigenetic modifications such as acetylation or microRNAs. In the eukaryotic cell nucleus DNA is found wrapped around histone octamers, forming a DNA-protein complex referred to as chromatin (3). The formation of chromatin leads to a tight packaging of the DNA so that it can fit into the nucleus. Different types of modifications on both DNA and histones can alter the structure of the chromatin, which in turn affects e.g. the gene transcription. The modifications often affect how tightly the DNA is wrapped around the histone octamers and the looser the packaging, the more likely the gene is to be expressed (2). Other DNA processes that are also affected by the level of packaging are e.g. DNA replication and DNA damage repair (4). For these types of processes to function, access to the DNA is crucial and this is highly regulated via spatial and temporal dynamics of the chromatin.

In order to study how epigenetic processes of chromatin are carried out and regulated, *in vitro* assembled chromatin is an essential tool (4). By using *in vitro* assembled chromatin, all components and possible modifications on the DNA and histones are highly defined which enables studies of how the modifications affect the physical properties and dynamics of the chromatin. To get reliable results, it is important that the assembled chromatin is of high quality, i.e. that it has periodically spaced nucleosomes, in order to resemble chromatin found *in vivo* (4).

In multiple studies, short DNA molecules have been used in order to produce nucleosomes, the minimal unit of chromatin, consisting of one histone octamer and 147 base pairs of DNA wrapped around it (4-7). Nucleosomes have been used to study different histone variants, histone modifications, histone point mutations and different DNA sequences. A DNA sequence often used is the 601 DNA (4). The 601 DNA has been shown to both have a high affinity for histone octamers and a strong ability to position the nucleosomes correctly (8). It is therefore a preferred sequence to use when studying structure and function of chromatin. By using the 601 DNA sequence, the dynamics and regulation of chromatin can be studied on mononucleosomes (4). There is an interest in studying these processes on polynucleosomes and further on chromatin, however these studies are usually carried out using trinucleosomal chromatin structures. The polynucleosomes are more difficult to prepare compared to the mononucleosomes and different methods can be applied for the assembly and quality control

(4). A triple 601 DNA sequence is often used for assembly of trinucleosomes. When other DNA sequences are used, it will likely have an impact on the quality, stability and nucleosome positioning of the chromatin.

In this master's thesis project, large DNA molecules in sizes around 4×10^4 base pairs will be used to develop a protocol for the assembly of chromatin. This is in order to find a protocol that works for the assembly of long DNA molecules. Since the DNA molecules used are longer than the 601 sequence mentioned earlier, the assembled chromatin will be more similar to DNA found in eukaryotic cells and could later be used to study epigenetic changes that can occur in eukaryotic cells. It is not yet clear if it is possible to assemble chromatin using this type of DNA and this master's thesis project will therefore focus on finding different settings and trying different physical and chemical conditions regarding the assembly and quality control. The assembly will be performed using salt dialysis followed by enzymatic assembly and the analysis of the chromatin will be performed by gel electrophoresis and nanofluidics. The flowchart of how the different laboratory steps in the project will be performed can be seen in Figure 1.1.

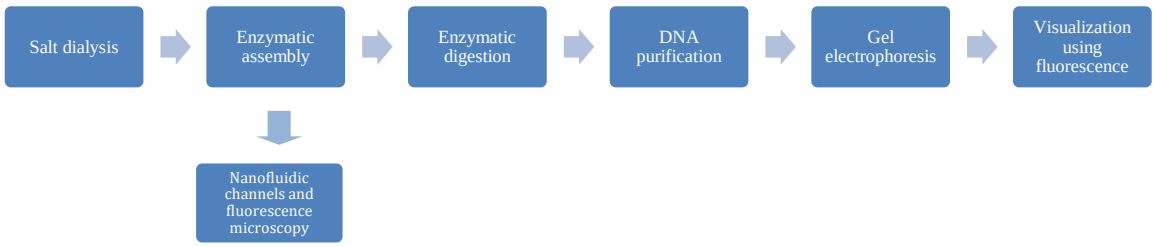

Figure 1.1: Flowchart over the different steps involved in chromatin assembly and analysis.

1.1 Aim of project

The aim of this project is to develop a protocol for *in vitro* assembly of chromatin using genome scale DNA molecules. The assembly will be performed using salt dialysis and enzymatic assembly and the goal is to get chromatin with periodically spaced nucleosomes. The chromatin will be analyzed using gel electrophoresis as well as nanofluidic channels in combination with fluorescence microscopy.

2 Theory

This chapter includes information about chromatin and how it is formed and detailed explanations will be provided about the different laboratory steps that will be performed in order to assemble and analyze the chromatin.

2.1 DNA

In all living cells there are instructions on which proteins that are to be transcribed (3). This leads to both the characteristics of specific species, but also the single individual. These instructions are referred to as the genome of a cell and the genetic information is carried by the series of nucleotides in the DNA molecule. There are four different types of nucleotides present in the cell: adenine, thymine, cytosine and guanine. The DNA molecule consists of two complementary strands of nucleotides bound together by hydrogen bonds, forming a double helix. In eukaryotes the DNA is stored in the nucleus of the cell.

2.2 Chromatin

The human genome consists of approximately 6 billion base pairs of DNA distributed over 46 chromosomes (9). If the DNA helices were to be put after each other in a row, it would reach 2 m (3, 9). To be able to fit in the cell nucleus that has a diameter of 6 µm, the DNA needs to be very densely packed (3, 9). The packaging that is performed is equivalent to fit 40 km of fine thread into a tennis ball (3). This packaging is achieved by proteins called histones which make the DNA more condensed (9). The complex formed of DNA and histones is referred to as chromatin and it is in this form DNA is found in eukaryotic cells (7, 9).

In chromatin, the histones are octamers consisting of four core histone proteins: H2A, H2B, H3 and H4 (10). The DNA is wrapped around the histone octamers with 147 base pairs. This unit of 147 base pairs and one histone octamer is referred to as a nucleosome. The nucleosome is the functional unit of chromatin and is found to have a periodic, repeating structure (9). The fact that chromatin has this periodic spacing of nucleosomes was discovered in 1973, when electron microscopy was used to visualize the structure (11). Chromatin was then referred to having the structure of “beads on a string” (3, 11). Each diploid human cell contains around 30 million nucleosomes and the packaging of DNA into chromatin will turn the DNA into one-third of the length it had before the packaging (3). The structure of DNA wrapped around a histone octamer is shown in Figure 2.1A and a schematic view of the “beads on a string” structure can be seen in Figure 2.1B.

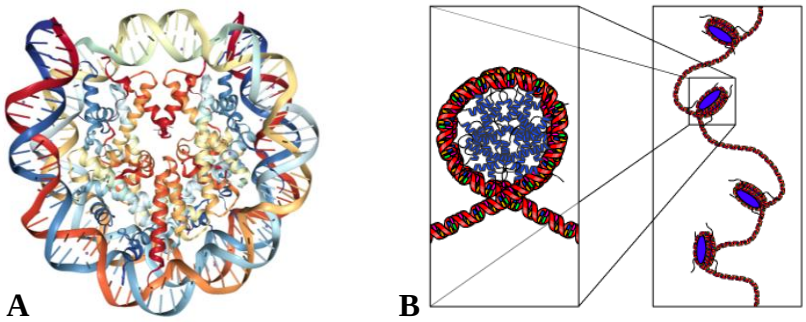

Figure 2.1: A) Structure of a nucleosome. A DNA strand is wrapped around a histone octamer in the center (12). B) Schematic view of the “beads on a string” structure found in chromatin with a nucleosome showed in more detail (13).

Looking more closely into the chromatin formation *in vivo*, it starts with the formation of the histone octamer in which the core histone proteins bind to each other (3, 9). The nucleosome consists of two of each of the core histones H2A, H2B, H3 and H4. In the first step, H3-H4 dimers and H2A-H2B dimers are formed (3). Two H3-H4 dimers then form a tetramer that attaches to the DNA (3, 14). Two H2A-H2B dimers are then incorporated and a nucleosome is formed (14). As mentioned earlier, each histone octamer has 147 base pairs of DNA wrapped around it, which is equivalent to 1.7 turns of DNA (9). The periodically spaced nucleosomes are separated by 20-50 base pairs of linker DNA (7). Eukaryotic cells also have an additional histone protein called H1 (7). This is a linker histone and is not part of the histone octamer. Instead it is located right next to the octamer and 20 additional base pairs are wrapped around it (9). Because of this linker histone, DNA gets wrapped 2 turns around the structure, forming what is referred to as a chromatosome.

The interaction between DNA and the histone octamer is very strong (3). In each nucleosome there are 142 hydrogen bonds between the histone octamer and the DNA. There are also hydrophobic interactions and electrostatic interactions binding the two together. Approximately 50 % of the bonds are between the phosphodiester backbone of the DNA and the backbone of the amino acids of the core histones (3). There are also bonds between the DNA and the side chains of the amino acids. The DNA backbone is negatively charged and approximately one-fifth of the amino acids of the core histone proteins consist of the positively charged amino acids lysine and arginine, which leads to a stronger binding. The many binding possibilities leads to that histones can bind to a very large variety of DNA sequences.

In the cell, there is a balance between assembly and disassembly of chromatin (14). This is important for the regulation of different cellular processes such as DNA replication, transcription, repair and cell cycle progression. In dividing cells, the DNA that has been synthesized needs to be assembled into chromatin and in quiescent cells the assembly of chromatin is necessary to replace old histones with new after they have been degraded. The assembly is also necessary in other cellular processes such as DNA transcription or repair (5). It is crucial that the chromatin is reassembled after repair of the DNA and after transcription as the action of polymerases usually leads to disruption of the chromatin structure.

The histones in chromatin can have a large variety of post translational modifications (10). At least eight different histone modifications have been identified on over 60 different sites. The most studied histone modifications are acetylation, methylation and phosphorylation. The modifications are performed by different enzymes such as methyltransferases and kinases, and most of the modifications are dynamic meaning that enzymes can remove the modification. There are two different mechanisms that have been characterized regarding the functions of the modifications. One of them is to disrupt contacts between either histones in adjacent nucleosomes or between DNA and histones. This can lead to the unravelling of chromatin and it thereby affects the chromatin structure. One of these types of modifications is acetylation which can unfold chromatin by neutralizing the positive charge of lysine in the histone octamer (10). The other type of mechanism is to affect the binding of non-histone proteins to the chromatin. These non-histone proteins can have different enzymatic activities that can modify the chromatin even further and they are involved in regulating different processes such as transcription, repair, replication and condensation. These processes consist

of several steps and for each step there may be a need for a specific remodelling of chromatin and a specific modification that controls it.

2.3 DNA substrates

The genome scale DNA substrates used in this project was lambda DNA and T7 DNA. Lambda DNA comes from the virus bacteriophage λ (15). The DNA is linear, double stranded and consists of 48 502 base pairs. Lambda DNA has sticky ends as the 5' ends have 12 base pairs that are single stranded and self-complementary (16). Lambda DNA can form a circle by annealing of the two single stranded ends and the virus uses this function when the DNA is to be injected into a host (15). Whereas eukaryotic cells package their DNA in the form of chromatin, viruses use another approach where the packaging is achieved by specific viral proteins, forming what is referred to as a capsid particle (17).

T7 DNA is 39 936 base pairs long and originates from the bacteriophage T7 (18). The DNA molecule is double stranded, linear, consists of 50 genes and has blunt ends (18, 19). The bacteriophage T7 is known for having a very fast growth cycle and has evolved an efficient mechanism regarding its DNA replication process (19).

2.4 Chromatin assembly

Salt dialysis and enzymatic assembly are two methods that can be used to assemble chromatin (4) and how these methods work is explained below.

2.4.1 Salt dialysis

Salt dialysis is a method that has been extensively used to assemble chromatin (4). It has shown to generate large quantities of chromatin in a relatively short period of time (20). In the salt dialysis, histone octamers are incubated together with DNA in a buffer with high salt concentration (21). The salt concentration is then lowered by e.g. step salt dialysis, semi gradual dialysis or gradient dialysis (20, 22). In the step salt dialysis, the salt concentration is lowered by transferring the DNA and histone sample through buffers of decreasing salt concentrations (20). In a semi gradual dialysis, buffer of lower salt concentrations are pumped into the original buffer with the use of a peristaltic pump (22). A gradient dialysis is similar to a semi gradual dialysis as a buffer of lower salt concentration is pumped into the original buffer, but in this case the old buffer is pumped out (20). Schematic views of the three different set ups is shown in Figure 2.2. By using a salt dialysis approach, histone octamers will be spontaneously positioned on the DNA as the salt concentration is lowered (21). The salt neutralizes the core histone proteins that are positively charged and thereby prevent unspecific binding to the DNA (6).

The positioning of the nucleosomes during the salt dialysis depends on the DNA sequence. Histones have been shown to, in the absence of other components, have specific DNA sequence preferences (23). Histones in fact have an affinity to DNA that varies with 3 orders of magnitude for different base pair sequences of the 147 base pairs. The specificity depends on the ability for the 147 base pairs to be bent around the histone octamer, the higher the ability to bend, the more likely to bind to a histone octamer. The wrapping of DNA around the histone core is not uniform, but instead kinks of the DNA are observed (3). The bending of DNA leads to that the minor groove needs to get highly compressed and different base pairs in this region have a better ability to be bent since they are more easily compressed. Adenine (A) and thymine (T) are more easily bent than guanine (G) and cytosine (C) (3). A

and T rich regions are therefore often positioned inwards to the histone octamer whereas G and C rich regions are positioned away from the histone octamer. The fact that the histones have DNA sequence specificity leads to that by using salt dialysis, the chromatin might not get periodically spaced nucleosomes (5, 21).

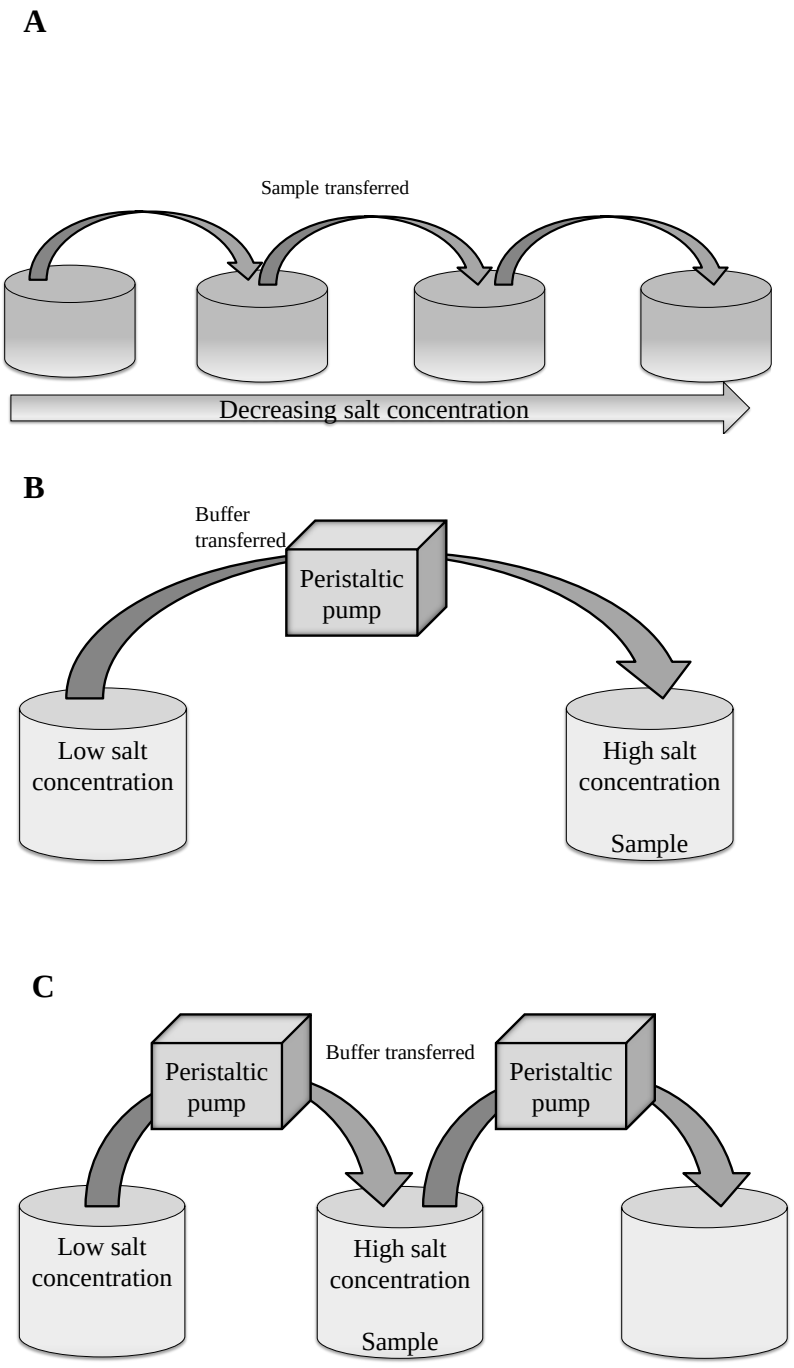

Figure 2.2: A) The setup for a step salt dialysis. The sample with DNA and histone octamers are transferred through buffers of decreasing salt concentrations. B) The setup for a semi gradual dialysis. The sample with DNA and histone octamers are placed in a buffer with high salt concentration and the concentration is then lowered by transferring buffer with low salt concentration. This leads to an increase in volume. C) The setup for a gradient dialysis. The gradient dialysis has the same principle as the semi gradual dialysis, except for the fact that old buffer is pumped out, leaving the volume constant.

2.4.2 Enzymatic assembly

Salt dialysis is a common method to assemble chromatin *in vitro*, however *in vivo* the assembly is carried out with different enzymes (24). Experiments have therefore also been performed where chromatin has been assembled *in vitro* using enzymes (25). The two different groups of enzymes needed are core histone chaperones and ATP-dependent chromatin assembly factors.

The core histone chaperones are acidic proteins that are heterogeneous as a class (24). They bind to histones and have been shown to be involved in nucleosome assembly and disassembly as well as histone trafficking between the cytoplasm and the nucleus (14, 24). Examples of core histone chaperones are nucleosome assembly protein 1 (NAP-1) and chromatin assembly factor 1 (CAF-1) (5, 25). NAP-1 is a polypeptide of 48 kDa, it is conserved among eukaryotes and is highly abundant in the cell, usually existing as a dimer or oligomer (14, 26). The yeast NAP-1 protein is one of the most studied histone core chaperones and it has been used to assemble chromatin *in vitro* (24). However, the exact mechanism of how it assembles chromatin is still unknown. It has been shown that NAP-1 binds with similar affinity to the H2A-H2B dimer and the $(H3\text{-}H4)_2$ tetramer, even if the affinity for H2A and H2B is higher (6, 24). This indicates that its mechanism could be that it performs a sequential deposition of H2A-H2B dimers and $(H3\text{-}H4)_2$ tetramers onto the DNA. The fact that NAP-1 can bind specifically to histones and sequester them has been long known, but how it interacts with chromatin and affects the structure and dynamics has only been assessed in very few studies (26).

Besides being able to assemble chromatin *in vitro*, NAP-1 has also been shown to have an additional function *in vivo* which is to reversibly remove H2A-H2B dimers from the nucleosome and replace them with other dimers (14, 26). This is an ATP-independent process of NAP-1 (26). NAP-1 is also involved in facilitating sliding of nucleosomes along the DNA by the transient removal of H2A-H2B dimers. This is important in order for the nucleosomes to have the thermodynamically most favourable position on the DNA. The role of NAP-1 in histone exchange has in a study been proposed as its main function *in vitro* as well as *in vivo* (24). In the study, they proposed that NAP-1 actually promotes chromatin assembly by removing interactions between histones and DNA that are competitive and non-productive, instead of actually delivering histones to the DNA. NAP-1 is then believed to work by removing H2A-H2B dimers from the DNA, which is crucial for the chromatin assembly process (24).

By using core histone chaperones only, the resulting chromatin will not have periodically spaced nucleosomes (6). To obtain chromatin with periodically spaced nucleosomes, ATP-dependent chromatin assembly enzymes are also necessary (25). The ATP-dependent chromatin assembly enzymes use ATP to position the histone octamers onto the DNA and also to organize the nucleosomes so that they are positioned periodically (5, 25). One example of these enzymes is ATP-utilizing chromatin assembly and remodelling factor (ACF). ACF consists of the two subunits ISWI (an ATPase) and Acf1. Both of these subunits are necessary to assemble chromatin (5).

ACF has been shown to act catalytically together with different histone chaperones, such as NAP-1, to assemble chromatin with periodically spaced nucleosomes (5, 6, 25). Studies have shown that to get an efficient assembly, ACF, NAP-1 and ATP are all necessary and that ACF and NAP-1 work synergistically (6). It is possible for ACF to also work with other core

histone chaperones, such as CAF-1. Compared to NAP-1 that can bind to all four different core histones, CAF-1 has a great preference for H3 and acetylated H4 (6). In the absence of NAP-1 or CAF-1, ACF has been shown to modulate chromatin (6). ACF has a nucleosome mobilization activity and can alter the spacing of the nucleosomes. This process is ATP-dependent. The ability to alter the nucleosome spacing is something that the core histone chaperones do not possess (6). Why ACF performs this alteration of nucleosomes is not fully understood yet, but one idea is that the nucleosomes are moved from a thermodynamically less stable state to a thermodynamically more stable one. ACF thereby works with both the deposition as well as alterations of the nucleosomes (6).

The function of the core histone chaperones are somewhat overlapping with the function of ACF, but still it has been shown that the best result is achieved when both factors are used (6). This is probably due to the very high complexity of chromatin assembly, involving e.g. histone modifications. One idea of how the two types of enzymes work together is based on a two-step mechanism: the ATP-independent process, in which core histone chaperones will deposit the histones onto the DNA, and then the next step, where ACF alters the nucleosomal spacing by the use of ATP. However, as mentioned earlier it has also been found that ACF works synergistically with NAP-1 in deposition of histones which indicates that the interaction between ACF and the core histone chaperone is important for the process (6).

2.5 Analysis of chromatin

After the chromatin has been assembled it can be analyzed by different methods and here the methods used in this project will be explained.

2.5.1 Micrococcal nuclease digestion

Micrococcal nuclease (MNase) is an enzyme that has the ability to digest DNA by making a double stranded cleavage (4, 7). It however only does this in regions of the DNA that are not bound to a protein. Using MNase in the context of chromatin thereby means that it will only digest DNA in the linker regions between two nucleosomes and the DNA that is wrapped around histones forming a nucleosome will be protected from digestion (4). If the chromatin has periodically spaced nucleosomes, digestion with MNase will result in mono- and oligonucleosomes (7). If the fragments are analyzed by gel electrophoresis it will result in a ladder corresponding to mononucleosomes, dinucleosomes, trinucleosomes etc. An example of how the result from such a gel electrophoresis can look like is found in Figure 2.3.

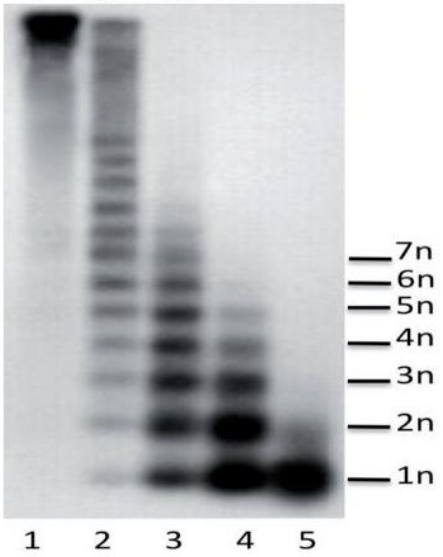

Figure 2.3: An example of how the ladder of nucleosomes can be visualized on a gel electrophoresis after MNase digestion. The five different lanes have been digested with increasing reaction times. Nucleosomes of different sizes are marked as 1n (mononucleosomes), 2n (dinucleosomes) etc. At the top of the gel is undigested DNA (27).

In Figure 2.3, it can be seen that there are bands visible on the gel corresponding to mono-, di- and polynucleosomes. It should be noted that this result is obtained from short DNA sequences in optimal conditions (27). Even if the aim of a project is to get as many and clear bands as possible on the gel, the result shown above would be very hard to reach.

In order for the MNase to be active, Ca^{2+} is necessary and therefore added in the buffer used (25). To stop the digestion ethylenediaminetetraacetic acid (EDTA), can be added. EDTA binds to divalent metal ions such as Ca^{2+}, Fe^{2+}, Pb^{2+} and Hg^{2+} and they together form a strong chelate complex, and thereby prevents enzymatic activity (28).

2.5.2 Gel electrophoresis

Gel electrophoresis is a method that can be applied in order to study both DNA and proteins (3). By using gel electrophoresis the size of DNA fragments or proteins can be determined. For both DNA and proteins, a gel is made in which the sample to be analyzed is added into. A voltage is then applied to the gel which will make the DNA or proteins migrate in the gel. DNA is negatively charged as each nucleotide contains a negative charge from its phosphate group and it will thereby migrate towards the positive electrode (3). The electrophoresis will lead to a separation based on size since smaller fragments will travel faster, as the larger ones will be retarded to a higher degree by the gel. The protein's migration rate in the gel will depend on the net charge, size and shape of the protein (3). If it is desired to separate proteins based on size only, a gel containing SDS can be used. SDS will make the proteins unfold and also give them a negative charge. The charges from the SDS will mask the intrinsic charge of the protein and when a voltage is applied the protein will migrate towards to cathode (3).

Two common gel electrophoreses are polyacrylamide gel electrophoresis (PAGE) and agarose gel electrophoresis (3). PAGE has been shown to be the simplest method and it gives the

highest degree of separation regarding DNA fragments of 20-1000 base pairs in size (29). It can be used in both denaturating and nondenaturating conditions. Agarose gel electrophoresis can be used to separate DNA fragments of 100 base pairs to 25 000 base pairs (30). Depending on the concentration of agarose in the gel, the pore size will be different, and this will affect the migration of the DNA fragments. Agarose gels are formed when agarose polymers bind together non-covalently and form a network (30). In polyacrylamide gels, the gel is formed by a chemical reaction driven by a free radical. When comparing agarose gel electrophoresis to PAGE, some points that differ are: PAGE run vertically and agarose gel horizontally, polyacrylamide gels are of higher concentration, thinner and have better resolution (30). If fragments smaller than 100 base pairs are analyzed, PAGE often provides a more effective separation (29).

When running the gel electrophoresis, loading dyes are often used. The reasons for this are that they give the sample a colour which makes it easier to load onto the gel, it gives the sample a higher density so that it will sink into the wells of the gel and lastly the dye will move with a specific speed in the gel which enables estimation of how far the DNA fragments have migrated in the gel (30).

2.5.3 Nanofluidic channels and fluorescence microscopy

The science of nanofluidics involves using nanoscale devices in order to analyze single molecules (31). An advantage of analyzing single molecules instead of multiple at the same time is that it enables the detection of different phenomena that would otherwise be averaged out. One of the great uses of nanofluidics has been to get sequence information of large and intact DNA molecules (31). Another area in which nanofluidics have been used is to study the interaction of DNA and proteins such as histones (32). The devices used for these types of studies are nanochannels (31). These are channels in the sizes of $\sim 100 \times 100$ nm^2 (32) in which large DNA molecules will be stretched out spontaneously (31). The extension of the DNA molecule in the channel is linear with the contour length (31, 32). The nanochannels are placed next to each other forming an array (31). These arrays are then connected to microchannels which work as loading channels to get the DNA molecule into the device. A schematic view of the channel structure and an image of the device can be found in Figure 2.4. Compared to e.g. optical or magnetic tweezers, the DNA is stretched using nanoconfinement and not by stretching using handles (32). This type of stretching via nanoconfinement leads to a simpler setup, does not require any external force for stretching and it also allows for automation (31).

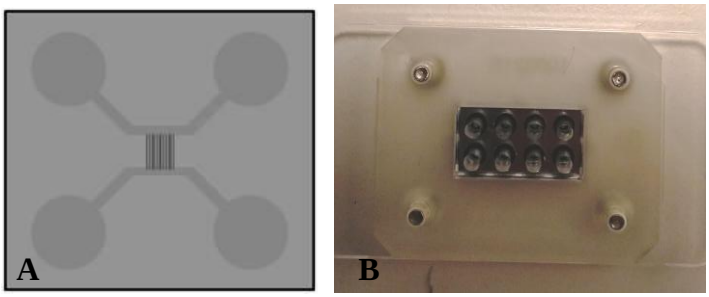

Figure 2.4: A) A schematic view of the micro- and nanochannels connected to the loading chambers. The microchannels are connected to the loading chambers whereas the nanochannels are connected to the microchannels forming an array. B) Image showing the loading chambers in the center which are connected to the micro- and nanochannels. In the device there are two setups of nanochannel arrays and thereby eight loading chambers.

The nanofluidic channels can be combined with fluorescence microscopy which allows for studies of DNA-protein interactions or extraction of sequence data (31, 32). The nanofluidic device is placed on top of the fluorescence microscope and each loading chamber is connected to a tube that allows nitrogen gas to flow into the chamber and make the sample flow into the micro-and nanochannels. A fluorescence microscope is similar to a light microscope, but has some modifications (3). The difference between a light microscope and a fluorescence microscope is that in the fluorescence microscope the light from the light source goes through two different filters: an excitation filter and an emission filter. This leads to that only a specific emission wavelength is detected and the result is an image were the compound glows against a dark background. Because of this, only a very small amount of the fluorescence compound is necessary in order for it to be detected (3). The principle of a fluorescence microscope is shown in Figure 2.5.

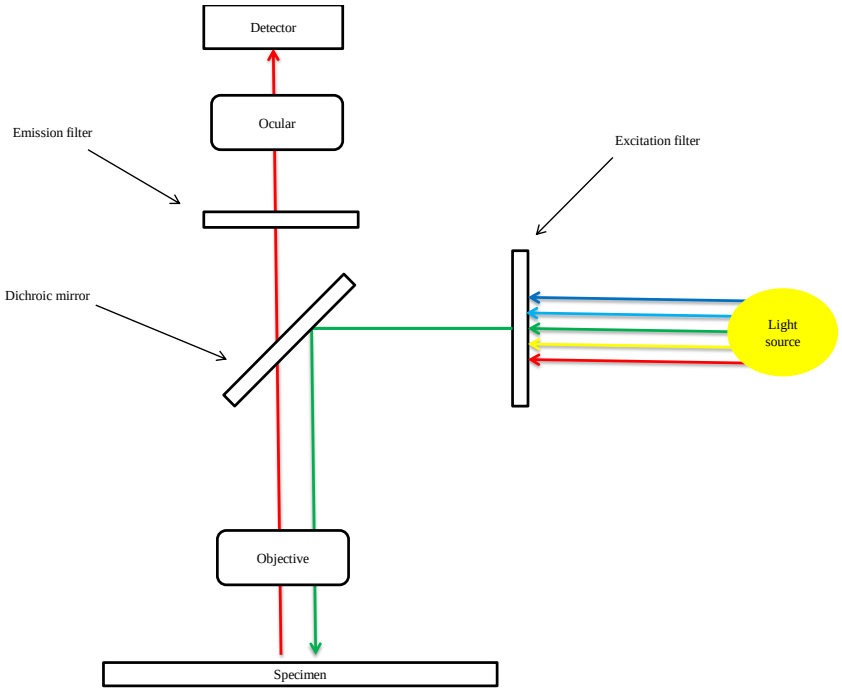

Figure 2.5: The principle of a fluorescence microscope. The components necessary include excitation filter, a dichroic mirror and an emission filter. The specimen will absorb excitation light and emit light of a longer wavelength which is then detected.

In a fluorescence microscope, the light that passes from the light source first goes through an excitation filter as mentioned previously (3). This filter ensures that only the wavelength that will excite the particular fluorophore in the specimen is let through. All other wavelengths are filtered out. The light then passes through a dichroic mirror that reflects the excitation light and transmits emission light. The light then reaches the specimen which can be e.g. a cell, DNA or proteins (3). If the specimen contains a fluorescent molecule that has the potential of being excited by the light of that particular wavelength, it will absorb the light and emit light of a longer wavelength. The emitted light then passes through the dichroic mirror and the emission filter. This filter ensures that the only light that is passed through is the one of the emitted wavelength and that light of other wavelengths, such as the excitation light, is blocked (3). Lastly the emitted light reaches the detector.

When visualizing a DNA molecule using fluorescence, it must be stained with a fluorescent dye (32). One such dye is YOYO-1, which is one of the most commonly used fluorescent dyes when it comes to staining DNA for single molecule fluorescence microscopy experiments (33). YOYO-1 is an oxazole yellow dye that binds to double stranded DNA (34, 35). It consists of two aromatic moieties that are connected with a linker (34). Upon binding, both of the moieties are intercalated in between two base pairs of the DNA strands and leave one position in between them unoccupied. This is referred to as bis-intercalation and leads to an increased length of the DNA molecule (34, 35). YOYO-1 is almost non-fluorescent when in solution, but the fluorescence quantum yield increases to a large degree upon binding to DNA (34). YOYO-1 has its absorption maximum at 489 nm and emission maximum at 509 nm (36). Besides visualization of DNA, fluorescence microscopy is also often used to detect specific proteins within cells (3). A dye commonly used in this application is rhodamine which absorbs light in the green-yellow spectra and emits light in the red spectra. By using

different dyes at the same time, multiple molecules can be detected simultaneously by switching the excitation and emission filters in the fluorescence microscope (3).

The light emitted from the fluorescent dyes is a result of the phenomena that molecules emit light when they return to ground state from an electronically excited state (37). Light consists of an electromagnetic wave, containing electric and magnetic fields. An electron in the ground state molecular orbital can absorb the energy from the electric field in the form of photons and move to an excited state, i.e. an unoccupied higher molecular orbital (37). When a photon is absorbed by the molecule, leading to an electronic transition, it is referred to as electronic absorption. The light required to change the electronic distribution of a fluorophore lies in the visible or ultraviolet region of the spectrum. After the electron has been exited to a higher energy state, it can go through a relaxation and return to the ground state (37). If the molecule is fluorescencent, this relaxation can occur together with the emission of a photon. The different photophysical processes that can occur during an excitation process are often visualized in a Jablonski diagram and a simplified version of this is shown in Figure 2.6.

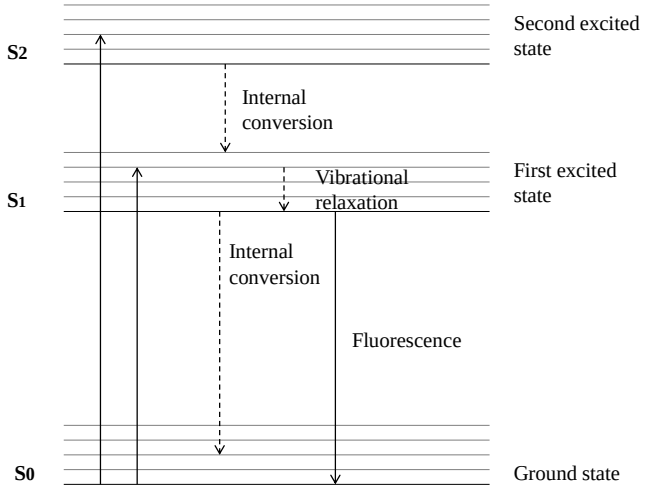

Figure 2.6: A simplified Jablonski diagram illustrating energy levels and different photophysical processes. Electrons have the capacity to absorb photons and move up to the first or second excited state. Energy is then lost via internal conversion, vibrational relaxation and fluorescence. The processes involving the absorption or emission of photons are visualized with solid arrows and the other processes are visualized with dashed arrows.

The emitted photon is of longer wavelength than the absorbed photon (37). This phenomenon is known as Stoke's shift and it occurs since energy is lost due to internal conversion and vibrational relaxation. Internal conversion is the process when electrons move from higher energy states to the first excited state or ground state. Vibrational relaxation is instead when electrons move from a higher vibrational level within an energy level to the lowest vibrational level in the same energy level. Emission always occurs from the lowest vibrational level of the first excited state and the emission spectra of a molecule is thereby, in general, independent of the excitation wavelength (37). There are different parameters that can be used to describe and compare fluorophores. These are extinction coefficient, quantum yield and brightness. The extinction coefficient is the ability of a fluorophore to absorbe light of a specific wavelength, the quantum yield is the efficiency of the fluorophore, i.e. number of

photons emitted per the total number of photons absorbed and, lastly, the brightness is described as the extinction coefficient times the quantum yield.

3 Method

This project aimed to develop a protocol for chromatin assembly and the method used to assemble and analyze chromatin will therefore be repeated multiple times. This chapter explains how each step was performed and what changes that were made between the different runs.

3.1 Chromatin assembly

Chromatin was assembled using different histone:DNA ratios and by using either salt dialysis or enzymatic assembly separately, or in combination.

3.1.1 Histone:DNA ratio

During the chromatin assembly, different ratios of histone octamers and DNA were tested. In theory, there should be one histone for every 167 base pairs (the DNA wrapped around the histone octamer and the linker DNA), which corresponds to a ratio of 1:1. This was the ratio first used in this study. However, other ratios might be more suited to get an optimum assembly. Even if the DNA only can have 1 histone for every 167 base pairs, it might be that an excess number of histones is necessary. Therefore, different ratios were tested. These included (histone:DNA): 0.5:1, 1.5:1, 2:1, 4:1, 6:1, 8:1 and 10:1.

3.1.2 Salt dialysis

The first step in the process of assembling chromatin was to use a salt dialysis. This was performed by first mixing DNA with histone octamers according to the desired ratio. The total volume for each sample was in the first tests set to 25 µl by adding TEK2000 buffer (10 mM Tris-HCl pH 7.8, 0.1 mM EDTA and 2000 mM KCl). The samples were put in dialysis tubes which allows buffer to diffuse into the sample. These tubes were first put in TEK1400 buffer (10 mM Tris-HCl pH 7.8, 0.1 mM EDTA, 1400 mM KCl) for one hour. A peristaltic pump was then used to add TEK10 buffer (10 mM Tris-HCl pH 7.8, 0.1 mM EDTA, 10 mM KCl) at a rate of 1 ml/min for 6.5 hours. The samples were then kept on TEK10 buffer overnight.

In the experiments either TEK2000 or TEK1000 buffer was used. An additional change that was made was the amount of DNA in each dialysis tube. This was varied between 250, 500 and 1000 ng. The total volume in each tube was either 25 or 50 µl.

3.1.3 Enzymatic reactions

During the experiment, some samples were assembled using salt dialysis only, some using first salt dialysis and then an enzymatic assembly and some using only enzymatic assembly. The enzymatic assembly was performed by adding NAP-1 (0.1 mg/ml) to the samples and then incubating them at 37 °C for 30 minutes. ACF buffer (100 mM Tris-HCl pH 7.5, 500 mM KCl and 5mM $MgCl_2$), ATP (8 mM) and ACF (0.01 mg/ml) were then added and the samples were incubated at 37 °C for 3 hours. The concentrations of NAP-1 and ACF were kept constant for all experiments.

3.2 Micrococcal nuclease digestion

The chromatin samples were digested using MNase. The digestion was performed by an addition of MNase buffer (50 mM Tris-HCl pH 7.8 and 5 mM $CaCl_2$), MNase to reach the desired concentration and MQ H_2O to reach a total volume of 100 µl to each chromatin sample. The reaction was then run for the desired time in room temperature or on ice. The amount of assembled DNA in each reaction varied between 83, 100, 125, 167 and 250 ng. The digestion was performed using different concentrations of MNase, performing it on ice or in room temperature as well as using different digestions times. This was done to find optimal conditions for the chromatin to be neither over digested nor under digested. To stop the reaction, 10 µl 0.5 M EDTA was added.

3.3 DNA purification

For most of the experiments it was desired to separate the DNA from the histone octamers. For this purpose, 5 µl 10 % SDS and 2 µl of 20 mg/ml proteinase K were added to the samples that were then incubated at 65 °C for 30 minutes. If it was not desired to separate DNA from the histone octamers this step and the following purification step was not performed. The samples were purified using MinElute Reaction Cleanup Kit (Qiagen). The protocol was modified so that the purified DNA was eluted in 15 µl elution buffer instead of 10 µl. Lastly DNA loading dye was added to each sample before they were loaded into the gel in the gel electrophoresis. In the first experiments, 2 µl of 6X DNA loading dye was added to each sample. This was later changed to 3 µl in order to increase the viscosity of the samples.

3.4 Polyacrylamide gel electrophoresis

Both 5 % and 6 % acrylamide gels were tested as well as different ratios regarding acrylamide and bis-acrylamide (19:1 and 29:1). The 6 % polyacrylamide gels were made by mixing 7.5 ml acrylamide (40 %), 2.5 ml 10X TBE buffer, 40 ml MQ H_2O, 400 µl ammonium persulfate and 40 µl TEMED. The solution was pipetted into glass holders and was allowed to solidify. 0.5X TBE buffer was added and the samples were placed in the wells of the gel. The gel electrophoresis was then run with different voltages for different time periods.

For most polyacrylamide gels, the staining of DNA was performed after the gel electrophoresis and SYBR gold was used. The staining was performed by placing the gel in a larger container with 10 µl of the dye mixed with 100 µl of 0.5X TBE buffer. The gel was shaken in the dye solution for 30 minutes. After the staining, the gel was washed using MQ H_2O for 1.5 hours with an exchange of MQ H_2O every 30 minutes. Regarding the polyacrylamide gels that were stained before the gel electrophoresis, this was performed by adding 4.5 µl of the dye before the gel solidified. The voltages tested varied between 120-200 V and the time between 30-60 minutes. The loading dye was used as an indicator of how long the DNA fragments had travelled in the gel. The electrophoresis was run either in room temperature or in a cold room.

3.5 Agarose gels electrophoresis

Agarose gels were made using 0.8, 1 or 1.5 % agarose. The 1 % gels were prepared by mixing 1 g of MetaPhor agarose with 99 g of 1X TBE buffer. The solution was heated in the microwave and then cooled. The solution was then placed in a frame and allowed to solidify. Just as for the polyacrylamide gels, some agarose gels were pre-stained and some post-stained and SYBR gold was used. In pre-staining, 10 µl of the dye was added to the gel solution before it was put in the frame. If the gel instead were to be post-stained, 10 µl of dye was

added to 100 µl of 1X TBE buffer. The gel was then placed in the buffer and shaken for 30 minutes. The agarose gel electrophoresis was run at 100 V for 60-80 minutes. The electrophoresis was run either in room temperature or in a cold room.

3.6 Nanofluidic channels and fluorescence microscopy

The nanofluidic channels where cleaned by flushing bleach through both the micro- and nanochannels. A lipid buffer (0.11X TBE and 100 mM NaCl) was then flushed through five times before adding a solution of 0.5 % rhodamine liposomes. A buffer consisting of TEK10 and DTT (final concentration of 5 mM) was flushed trough the channels five times in order to remove residual lipids. The channels were at the same time exposed to light in order to bleach the fluorophores of the lipids. Lastly YOYO-1 (final concentration of 1.5 µM) was added to the chromatin (final concentration 4.25 ng DNA/µl) before the chromatin sample was added to the channels. YOYO-1 was added in order to visualize the DNA. It was also desired to visualize the histones and therefore histones labelled with the fluorescent dye tetramethylrhodamine (TMR) were used during the assembly. The chromatin sample was visualized in a fluorescence microscope using two different types of filters, one for YOYO-1 and one for TMR.

4 Results and discussion

This chapter describes the obtained results when developing the protocol and analyzing the chromatin using nanofluidic channels and fluorescence microscopy. There are a number of important factors to take into consideration when aiming to assemble chromatin *in vitro* which will also be covered in this chapter as well as interpretation of the results.

4.1 Protocol development

This chapter will contain a description of the changes that were made for the different runnings when developing the protocol, as well as a visualization of the gel electrophoreses images. If not mentioned otherwise, the chromatin assembly was performed using only salt dialysis and not enzymatic assembly. This was done to save both enzymes as well as time. However, even without enzymatic assembly nucleosomes are supposed to be visible on the gels as chromatin has been assembled to some degree during the salt dialysis. In the first experiments lambda DNA was used and later supercoiled and T7 DNA were used.

4.1.1 PAGE

In the first experiments performed in order to develop a protocol for the assembly of chromatin the histone:DNA ratio was 1:1 and PAGE was used to analyze the DNA fragments after digestion. When performing these experiments, all gels turned out empty regardless of composition of the polyacrylamide gel, the digestion conditions or electrophoresis settings. The reason for this was tested further. One of the initial ideas of why the gels turned out empty was that DNA was lost during the preparation. When performing the salt dialysis there was a large volume increase. When a total volume of 25 µl was used, the sample volume increased from 25 µl to up to 175 µl and no sample got a volume below 100 µl. To reduce the volume, the samples where centrifuged using centrifuge tubes with different pore sizes. Since all gels turned out empty, it might be that the centrifugation step led to that a lot of DNA was lost and therefore the centrifugation step was removed. To deal with the large volume increase, the initial volume in each dialysis tube was instead increased from 25 µl to 50 µl. This change led to smaller volume increases after the salt dialysis. Mostly, the volumes increased to around 100 µl but volumes around 60 µl were also obtained. However, even when the centrifugation step was removed the gels still turned out empty.

To test if the lack of DNA on the polyacrylamide gel instead depended in the gel itself, an agarose gel electrophoresis was performed. The DNA samples were digested using MNase concentrations of 0 and 0.5 U/ml for 3 minutes on ice. The corresponding gel can be seen in Figure 4.1.

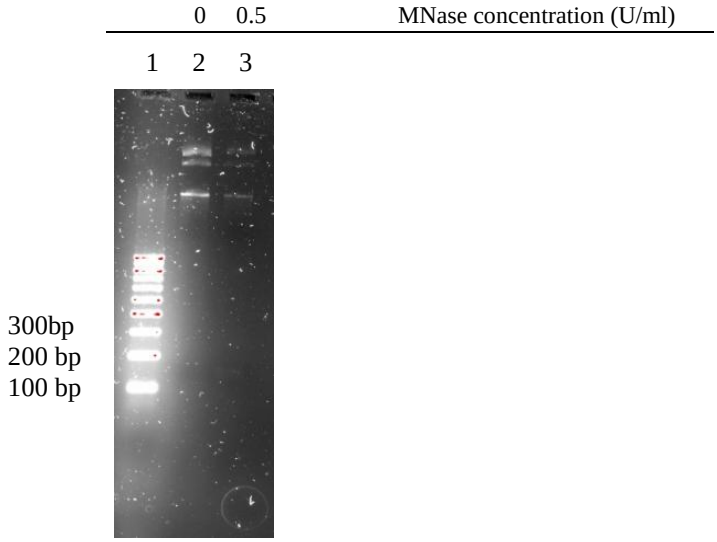

Figure 4.1: Result from agarose gel electrophoresis. Lane 1 corresponds to a ladder with bands separated by 100 base pairs. The samples had been digested for 3 minutes on ice. Each well was loaded with 250 ng lambda DNA. The histone:DNA ratio was 1:1 and the electrophoresis was run at 100 V for 60 minutes. The gel was pre-stained.

It can in Figure 4.1 be observed that some bands corresponding to DNA fragments are visible. These had not been visible on any of the polyacrylamide gels. The bands however correspond to very large DNA fragments, much larger than mono- or dinucleosomes. It is not clear what the bands visible in the gel correspond to but it could be that the initial DNA sample used contained DNA fragments of different sizes. The bands in lane 2 and 3 have the same pattern, but it can be seen that there is less DNA present in lane 3 which is expected as it was digested with MNase and the sample in lane 2 was not.

4.1.2 Comparing PAGE and agarose gel electrophoresis

In order to compare the polyacrylamide gels and agarose gels, samples treated in the same way were run on both type of gel electrophoresis. The result from this is shown in Figure 4.2.

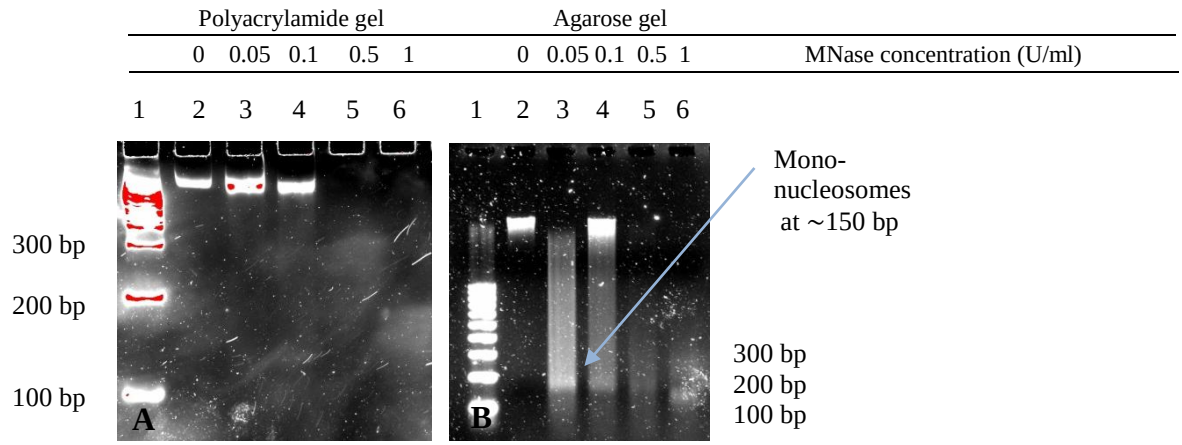

Figure 4.2: A) Result from polyacrylamide gel electrophoresis run at 120 V for 60 minutes. The gel was post-stained. B) Result from agarose gel electrophoresis run at 100 V for 60 minutes. Lane 1 in A) and B) correspond to a ladder with bands separated by 100 base pairs. The gel was pre-stained. The samples had been digested for 3 minutes on ice. Each well was loaded with 100 ng lambda DNA.

In Figure 4.2A it is clear that there is no good separation of the bands in the ladder and only bands on the top of the gel are visible. These bands likely correspond to DNA of different sizes that have not been separated on the gel. In Figure 4.2B samples treated in the same way were used but the result is very different. Lane 2 corresponds to undigested DNA and the following lanes have increasing concentrations of MNase. In lanes 3-6 a smear is visible. This indicates that there have been unevenly spaced nucleosomes in the chromatin and thereby fragments of very different lengths are formed when digesting with MNase. However, in lanes 3-5 there is a band at ~150 base pairs. This means that some of the nucleotides in fact have been positioned with 147 base pair intervals. It is not an unexpected result that not all nucleosomes are evenly spaced as no enzymatic assembly was performed. By comparing Figure 4.2A and Figure 4.2B it becomes clear that there is a problem regarding how the polyacrylamide gels are made or run. On the agarose gel it is observed that mononucleosomes in fact are present, something that could not be visualized on the polyacrylamide gel.

New polyacrylamide gels were made with an acrylamide:bis-acrylamide ratio of 29:1 instead of the previous ratio of 19:1. This was done to see if it would lead to better results than the previous polyacrylamide gels. Different ratios of histone:DNA were also tested, these were 0.5:1, 1:1, 1.5:1 and 2:1. However, independent of the ratio, none of the gels had any bands corresponding to nucleosomes. One of the polyacryamide gels from this experiment is shown in Figure 4.3.

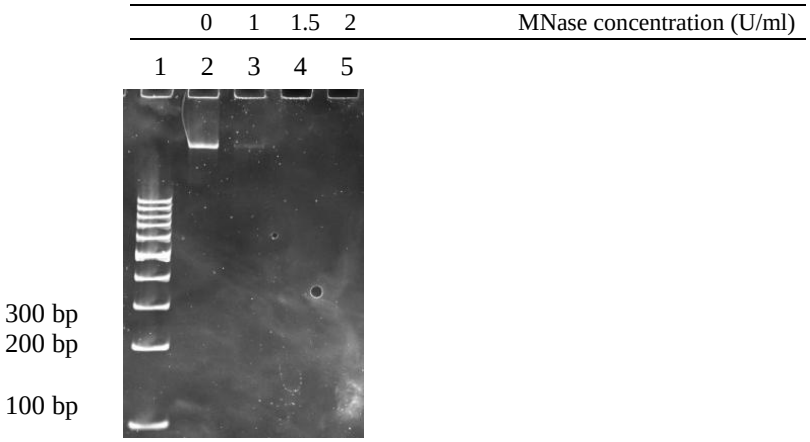

Figure 4.3: Result from polyacrylamide gel electrophoresis. Lane 1 corresponds to the ladder. The samples have been digested for 3 minutes in room temperature. Each well was loaded with 100 ng lambda DNA. The ratio is these samples are 0.5:1 and the electrophoresis was run at 120 V for 40 minutes. The gel was post-stained.

To test the polyacrylamide gels one last time, samples without DNA purification were run on a 5% polyacrylamide gel electrophoresis directly. The electrophoresis was now performed in a cold room instead of at room temperature. Previously, bands corresponding to DNA fragments of different sizes had been visualized, but no bands corresponding to nucleosomes. It was tested if running a gel with lower concentration, for longer time and in cold room would lead to that bands corresponding to nucleosomes were visible. However, except for the ladder, nothing could be visualized on these gels either.

In the beginning of the project, it was thought that PAGE would be the preferred type of electrophoresis to use in order to study the DNA fragments after digestion, as it was known to lead to a better separation and higher resolution compared to agarose gel electrophoresis. However, based on the above results, the PAGE did not work as expected. Whatever changes

that were made regarding the polyacrylamide gels, e.g. changing the composition of the gel, how the staining was performed, running time or voltage, it did not lead to any improvement in the results. The PAGE did not show any bands corresponding to nucleosomes or even a clear smear. At first this was thought to depend on that the chromatin was of poor quality and that the method used for assembly did not work properly. However, as can be seen in Figure 4.2, if samples treated the same way were run on a PAGE and an agarose gel electrophoresis, the agarose gel electrophoresis actually gave good results and both a smear and bands could be visualized. The conclusion was therefore drawn that PAGE was not the most suitable for analyzing the digested chromatin. The reason why PAGE did not work for this application is still not certain. It could be that the settings were still not good or that the DNA fragments were too big in size. Based on these results, it was decided not to move forward with the PAGE, but instead focus on agarose gel electrophoresis.

4.1.3 The effect of enzymatic assembly

To investigate the effect of enzymatic assembly compared to salt dialysis only, two agarose gels were made. One gel contained samples that had only had salt dialysis and in the other one, both salt dialysis and enzymatic assembly had been performed. The result is shown in Figure 4.4.

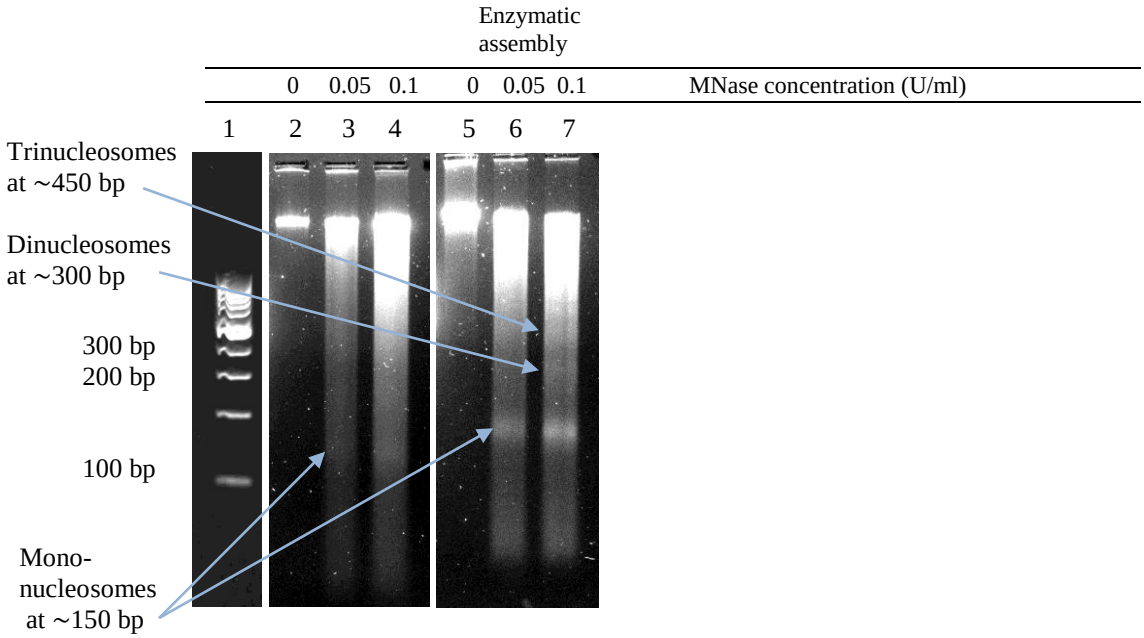

Figure 4.4: Result from agarose gel electrophoreses. Lane 1 is the ladder. The samples had a histone:DNA ratio of 1.5:1. The samples were digested for 3 minutes in room temperature. Each well was loaded with 167 ng lambda DNA. The electrophoresis was run at 100 V for 80 minutes. The gels were pre-stained.

In Figure 4.4 there is a smear for all samples that have been digested with MNase concentrations of 0.05 and 0.1 U/ml. For the samples that had been assembled using salt dialysis only, there is a very weak band at ~150 base pairs, corresponding to mononucleosomes. This band at ~150 base pairs is much clearer for the samples that had been assembled using enzymatic assembly as well. In lane 7, weak bands corresponding to di- and trinucleosomes can also be seen. This indicates that the salt dialysis works for the

assembly of chromatin, but the enzymatic assembly is necessary to get chromatin of good quality, i.e. periodically spaced nucleosomes.

4.1.4 Different digestion times

In Figure 4.4, there was a clear band on the top of the gel in each well. To test if this band corresponds to undigested DNA, a sample containing only undigested DNA without any treatment was added into one of the wells. Up until now, all samples had been digested for three minutes with concentrations of 0, 0.05 or 0.1 U/ml and it was therefore tested to digest them for a longer time. The result is shown in Figure 4.5.

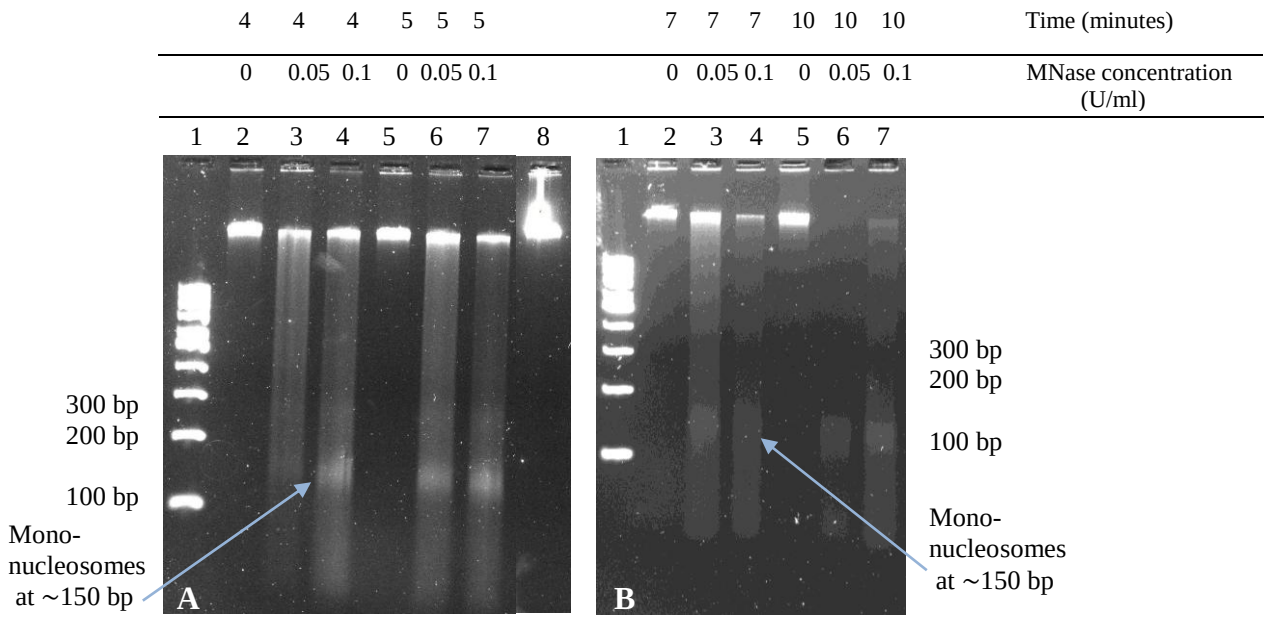

Figure 4.5: Result from agarose gel electrophoreses. Lane 1 in A) and B) correspond to the ladder. Lane 8 in A) contains undigested DNA without any treatment. Each well was loaded with 167 ng lambda DNA. The electrophoresis was run at 100 V for 80 minutes. The histone:DNA ratio was 1.5:1. The gel was pre-stained.

In Figure 4.5A it can be seen that the band on top of each well most likely corresponds to undigested DNA as it is in the same positions as the undigested DNA in lane 8. This means that on most of the gels, there has been undigested, full length DNA left after the digestion.

In both Figure 4.5A and Figure 4.5B there is a smear and a band at ~150 base pairs indicating that longer digestion times than used before could be applied. However, in Figure 4.5B, after 10 minutes there was almost no undigested DNA left. This indicates that a digestion time of 5-7 minutes is probably preferable during these conditions. If using longer digestion times, there is a risk of over digestion and if using lower the DNA will be under digested. However, when digesting samples from another salt dialysis, with a different histone:DNA ratio, another type of DNA or another amount of DNA, the optimal digestion time varied and therefore this type of analysis had to be performed multiple times.

4.1.5 Using higher histone:DNA ratios

The first histone:DNA ratio used in this study was 1:1. However, it is possible that much higher ratios are more suited to get an optimum assembly and therefore the ratios 4:1, 6:1, 8:1

and 10:1 were tested in order to see if any of them would give higher quality chromatin where di- and trinucleosomes could be more clearly detected than before. One of the results from this is shown in Figure 4.6 were a ratio of 10:1 was used.

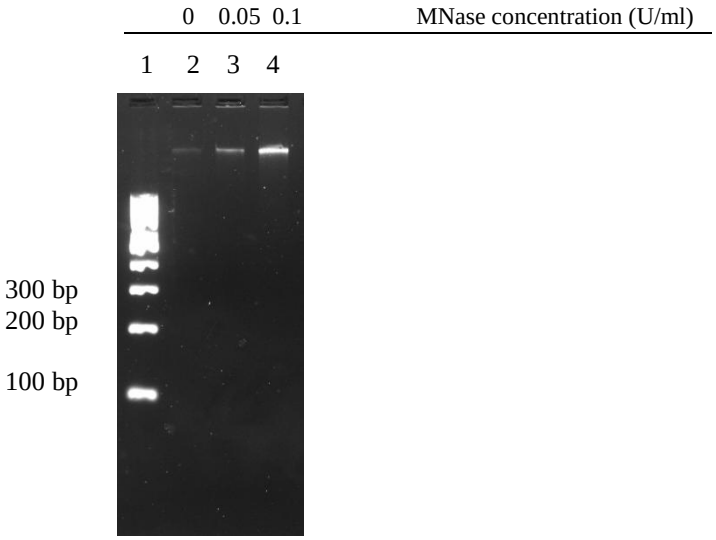

Figure 4.6: Result from agarose gel electrophoresis. Lane 1 is the ladder. The histone:DNA ratio was 10:1. The samples were digested for 7 minutes in room temperature. The electrophoresis was run at 100 V for 80 minutes. The gel was pre-stained.

In Figure 4.6 it can be seen that there is no smear or any bands corresponding to nucleosomes. It can also be observed that the higher the concentration of MNase, the brighter the signal in the wells. This means that there is more undigested DNA the higher the MNase concentration, which is not an expected result. The other three samples with ratios of 4:1, 6:1 and 8:1 were digested as well with different concentrations of MNase and for different periods of time. All these experiments led to very similar results as in Figure 4.6, i.e. no smear or bands corresponding to any nucleosomes could be observed. The amount of undigested DNA also increased as the time or concentration of MNase increased for all samples. This might depend on an error during the dialysis and a new dialysis was therefore performed with the same histone:DNA ratios (4:1, 6:1, 8:1 and 10:1). These were digested with different MNase concentrations for different periods of times, but all agarose gels turned out similar to Figure 4.6. The buffer used during the salt dialysis was at first TEK2000, but this was changed to TEK1000 at the same time as the higher histone:DNA ratios of 4:1, 6:1, 8:1 and 10:1 were used. A new dialysis was now performed were the TEK2000 buffer was again used to see if this could be the reason for why the assembly did not work as expected, but this lead to the same result as in the two previous dialyses.

In the gels where high histone:DNA ratios were used, the histones were from a different batch than the histones used before. Both the old and new batches of histones were run on an SDS-PAGE to test their quality and both of them gave four bands on the gels corresponding to the four monomers, indicating that they were of good quality. From the old batch of histones, there were histones both with and without TMR labelling. As the unlabelled ones had run out, the labelled histones were used in a new dialysis. Samples from this with a ratio of 1:1 and 8:1 was digested and run on an agarose gel electrophoresis, the result is shown in Figure 4.7.

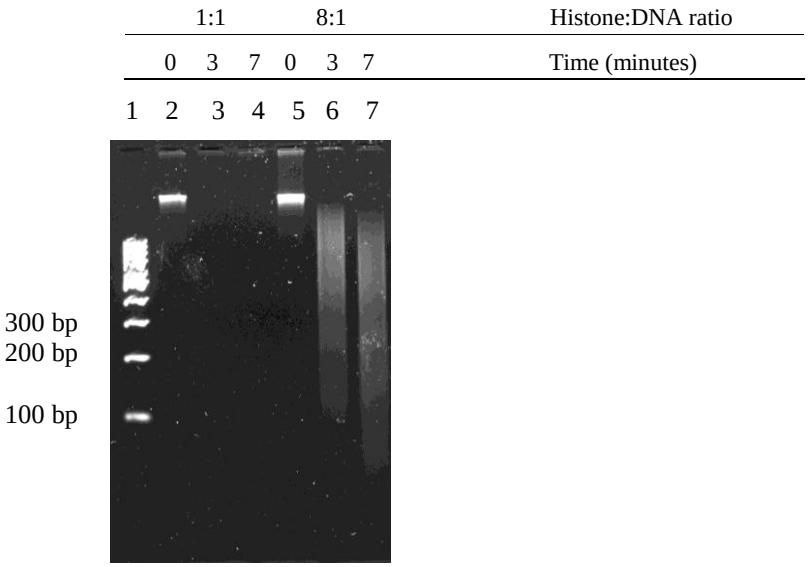

Figure 4.7: Result from agarose gel electrophoresis. 83 ng of lambda DNA was loaded into each well. Lane 1 is the ladder. The samples were digested with an MNase concentration of 0.1 U/ml. The electrophoresis was run at 100 V for 80 minutes. The gel was pre-stained.

For the sample that had a ratio of 1:1, there is no DNA visible except for the control sample that was not digested. For the 8:1 ratio sample, a smear can be seen but no bands corresponding to nucleosomes. This indicates that it is probably not the histones that are the problem. The fact that nothing is visible on the lanes with a 1:1 ratio could be due to that there were very few histones positioned on the DNA and it was thereby easier digested. If there would have been a higher number of histones positioned on the DNA where the ratio was 8:1, but they were not periodically spaced, it could explain the smear.

It was hypothesized that DNA might be lost during the DNA purification step and that this could have an influence on the fact that no bands corresponding to nucleosomes were no longer visible. Therefore samples were added onto the gel without DNA purification. The result is shown in Figure 4.8.

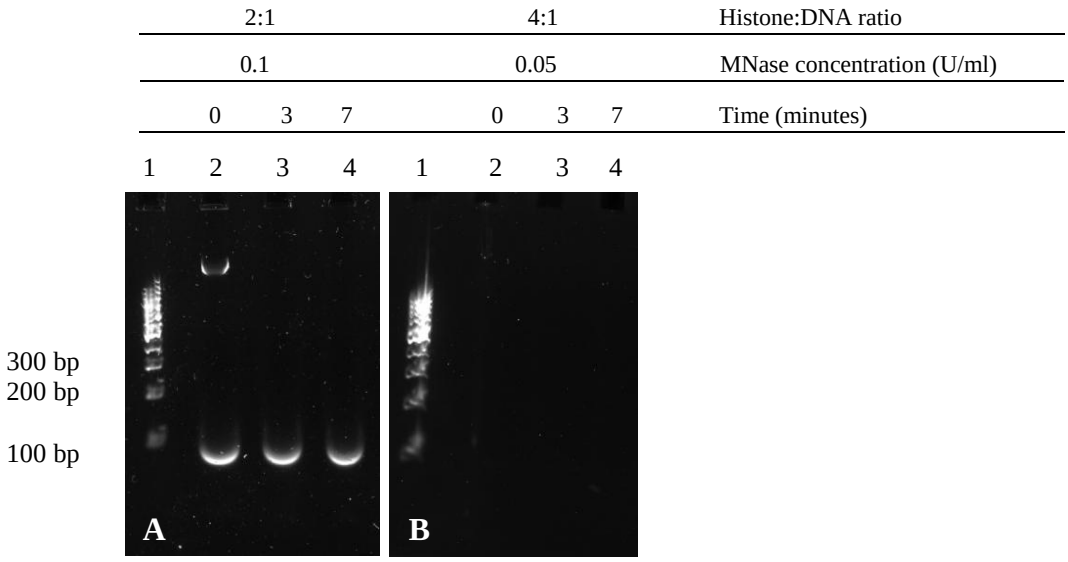

Figure 4.8: Result from agarose gel electrophoresis. In both A) and B) lane 1 is the ladder. No DNA purification was performed and 83 ng of lambda DNA was loaded into each well. The electrophoresis was run at 100 V for 80 minutes. The gel was pre-stained.

The result from Figure 4.8 does not indicate that DNA is lost specifically during the protein purification step as no bands or smears are visible here either. In the sample that had been digested with 0.05 U/ml (Figure 4.8B) no DNA is visible at all, not even in the control sample. In the sample digested with 0.1 U/ml (Figure 4.8A), only the control sample is visualized. In Figure 4.8 there are also clear bands at the very end of the gel, below 100 base pairs; it is not clear what these bands corresponds to.

4.1.6 Using enzymatic assembly only

In order to see the effect of enzymatic assembly only, the dialysis step was not performed. Instead, 500 ng DNA was put together with histones to a ratio of 1:1 in TEK2000 buffer. It was then diluted to reach a salt concentration of 500 mM and NAP-1 and AFC were then added as described earlier. The samples were then digested with an MNase concentration of 0.1 U/ml for 0, 3 and 7 minutes. The result is shown in Figure 4.9.

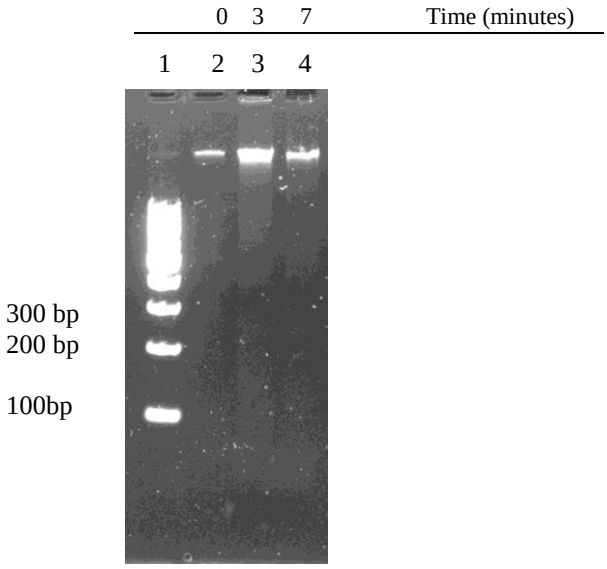

Figure 4.9: Result from agarose gel electrophoresis. 83 ng of lambda DNA was loaded into each well. Lane 1 is the ladder and lane 2-4 corresponds to samples with a ratio of 1:1. The samples were digested with an MNase concentration of 0.1 U/ml. Only enzymatic assembly had been performed and the electrophoresis was run at 100 V for 80 minutes. The gel was pre-stained.

In Figure 4.9, it can be observed that there is a slight smear visible in the samples digested for 3 and 7 minutes. However, there are no bands corresponding to nucleosomes. This indicates that enzymatic assembly solely is probably not enough in order to assemble chromatin with periodically spaced nucleosomes on long DNA. By using salt dialysis only, there has however been bands visible corresponding to nucleosomes. It should be noted that only one experiment was performed where enzymatic assembly only was performed and more tests are necessary to draw any direct conclusions.

4.1.7 Supercoiled DNA and T7 DNA

Since the assembly of chromatin using lambda DNA had not shown any good result since new histones were used, shorter supercoiled DNA of 3000 base pairs was used instead in a new salt dialysis. The result is shown in Figure 4.10.

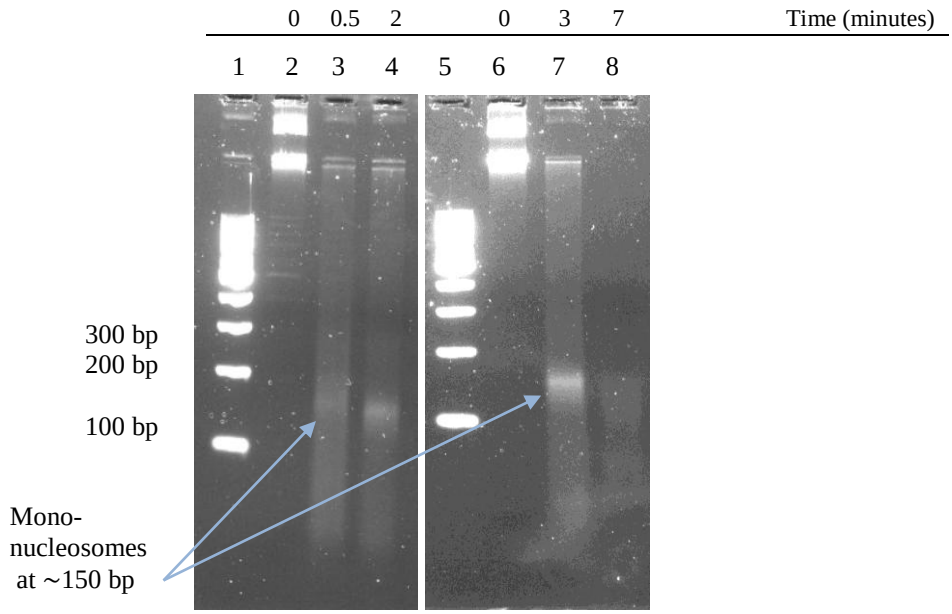

Figure 4.10: Result from agarose gel electrophoresis. 83 ng of supercoiled DNA was loaded into each well. lane 1 and 5 are ladders and the remaining lanes correspond to samples with a ratio of 1:1. The samples were digested with an MNase concentration of 0.1 U/ml. The electrophoresis was run at 100 V for 80 minutes. The gel was pre-stained.

In Figure 4.10 it can be observed that a digestion time between 2-3 minutes is preferable. At 0.5 min the chromatin seems to be under digested and at 7 minutes it is over digested. The samples with digestion times at 2 and 3 minutes both have a clear band at ~150 base pairs which corresponds to mononucleosomes. This indicates that the method for chromatin assembly seems to work much better for shorter DNA fragments than for lambda DNA.

A salt dialysis with higher histone:DNA ratios were made in order to test if the chromatin would be of even better quality in that case. This was performed with supercoiled DNA, but in the salt dialysis a sample of T7 DNA was also added with a histone:DNA ratio of 1:1. The result is shown in Figure 4.11. In wells in Figure 4.10 there are three bands visible at the top of the gel. It was not clear what gave rise to these bands and therefore untreated DNA was also added to the gel in Figure 4.11.

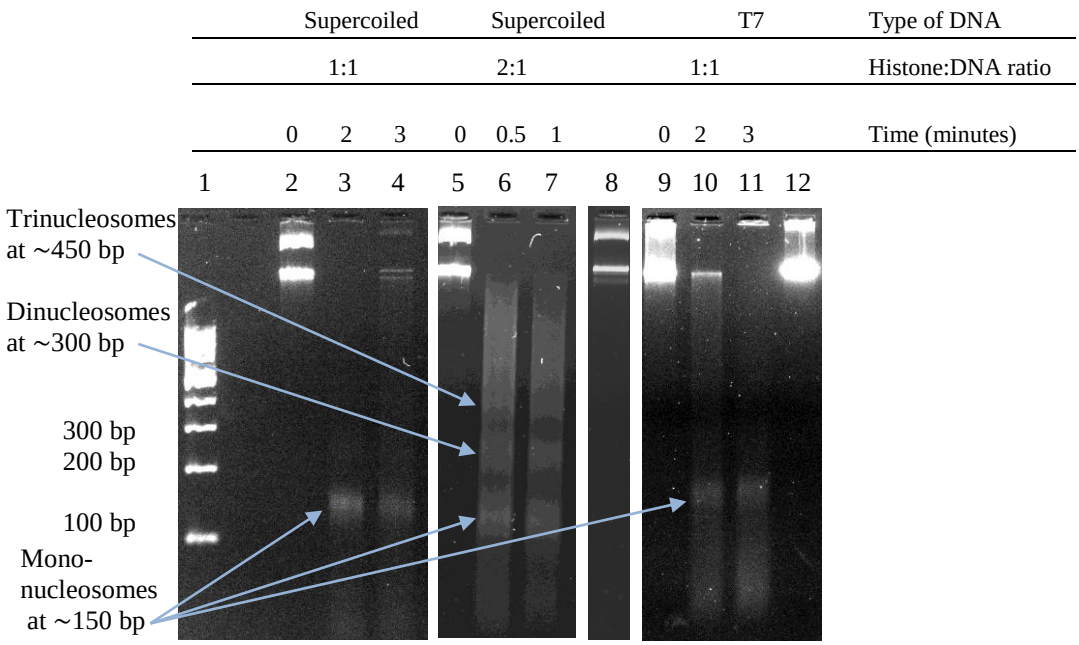

Figure 4.11: Result from agarose gel electrophoresis. 83 ng of DNA was loaded into each well. Lane 1 is the ladder. Lane 8 is untreated supercoiled DNA and lane 12 is untreated T7 DNA. The samples were digested with an MNase concentration of 0.1 U/ml. The electrophoresis was run at 100 V for 80 minutes. The gel was pre-stained.

In Figure 4.11 it can be seen that the supercoiled DNA sample with a histone:DNA ratio of 1:1 and 2:1 has a clear band at ~150 bp corresponding to mononucleosomes. The sample with a ratio of 2:1 also shows a band at ~300 base pairs corresponding to dinucleosomes and at ~450 base pairs corresponding to trinucleosomes. Higher ratios than 2:1 were also run on a gel but none of them showed any bands corresponding to nucleosomes. The T7 DNA with a ratio of 1:1 resulted in a band at ~150 base pairs, which corresponds to mononucleosomes. This indicates that chromatin has been assembled to some extent with the T7 DNA.

In the lane with untreated supercoiled DNA, three bands can be seen that are of the same sizes as the undigested supercoiled DNA. This is what was expected as nothing should have happened to the undigested DNA. Regarding the T7 DNA, the untreated and undigested DNA gave bands at the same position, indicating that they are of the same length. The reason why the supercoiled DNA gives rise to three bands could be due to that there are fragments in three different forms: supercoiled DNA, open circle DNA and lastly linear DNA resulting from circular DNA that has had a double stranded break. Depending on the shape, the DNA will migrate different distances in the gel. The open circular DNA gets the highest degree of retardation by the gel, followed by the linear and the supercoiled DNA travels the fastest. The T7 DNA only contains linear DNA and thereby only one band is visible.

To evaluate the protocol for chromatin assembly further, a salt dialysis was performed with T7 DNA and higher histone:DNA ratios. The ratios used were 1:1, 2:1, 6:1 and 10:1. The result is shown in Figure 4.12.

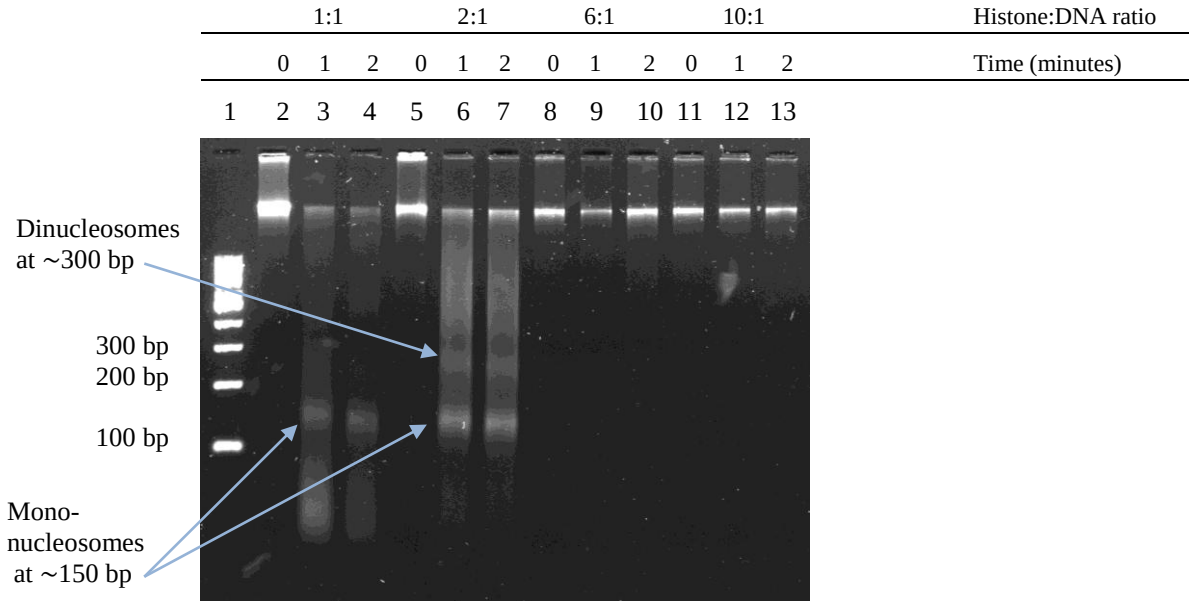

Figure 4.12: Result from agarose gel electrophoresis. 83 ng of T7 DNA was loaded into each well. Lane 1 is the ladder. The samples were digested with an MNase concentration of 0.1 U/ml. The electrophoresis was run at 100 V for 80 minutes. The gel was pre-stained.

The samples with a histone:DNA ratio of 1:1 show a clear band at ~150 base pairs, corresponding to mononucleosomes. This band is even clearer for the sample with a ratio of 2:1, which also has a band at ~300 base pairs, corresponding to dinucleosomes. For the samples with the higher ratios of 6:1 and 10:1 no bands are seen and the signal corresponding to undigested DNA gets stronger for longer reaction times. As there was a lot of undigested DNA left for the samples with a ratio of 6:1 and 10:1, they were digested for longer reaction times as well. This however only led to weaker bands corresponding to undigested DNA and no bands corresponding to nucleosomes.

From the results obtained here and in Chapter 4.1.5, it seems that problems arise during the chromatin assembly when using histone:DNA ratios above 2:1. In the beginning of the project, only histone:DNA ratios of 1:1 were used. This was the starting point in order to test the methods to assemble chromatin and then different ratios were tested and evaluated. It was however hypothesized that using higher ratios of histone:DNA than 1:1 would be preferred. The hypothesis was that a higher amount of histones would lead to chromatin of better quality as there would be smaller risk of histone shortage and that histones would only be positioned at every 167 base pairs. When using lambda DNA and a ratio of histone:DNA that was 1:1 or 1.5:1, there were bands visible that corresponds to nucleosomes, indicating that chromatin had been assembled. The same results were obtained when using supercoiled DNA and T7 DNA at the lower ratios 1:1, 1.5:1 and 2:1. However for all three types of DNA, when higher ratios (4:1 up to 10:1) were used, the obtained results were not as expected. Figure 4.6 and Figure 4.12 show no smear or bands corresponding to nucleosomes and the band corresponding to undigested, full length DNA got brighter the longer the digestion time or the higher the MNase concentration. In practice, this means that the more DNA that was digested, the more undigested DNA was visible on the gel. Different concentrations of MNase, different digestion times as well as different ratios were evaluated, but all led to the same result. The reason for this is still unknown. One explanation to why no bands corresponding to nucleosomes are visible for these higher ratios could be that when there is a high concentration of histones, the DNA gets more densely packed with many histones. This in

turn inhibits the function of the MNase as it cannot reach the free DNA and digest it. However, this does not explain the fact that the signal corresponding to undigested DNA increased with digestion time or concentration.

Chromatin made of T7 DNA was investigated further by comparing chromatin assembled with salt dialysis only with chromatin assembled using enzymatic assembly as well. The result is shown in Figure 4.13.

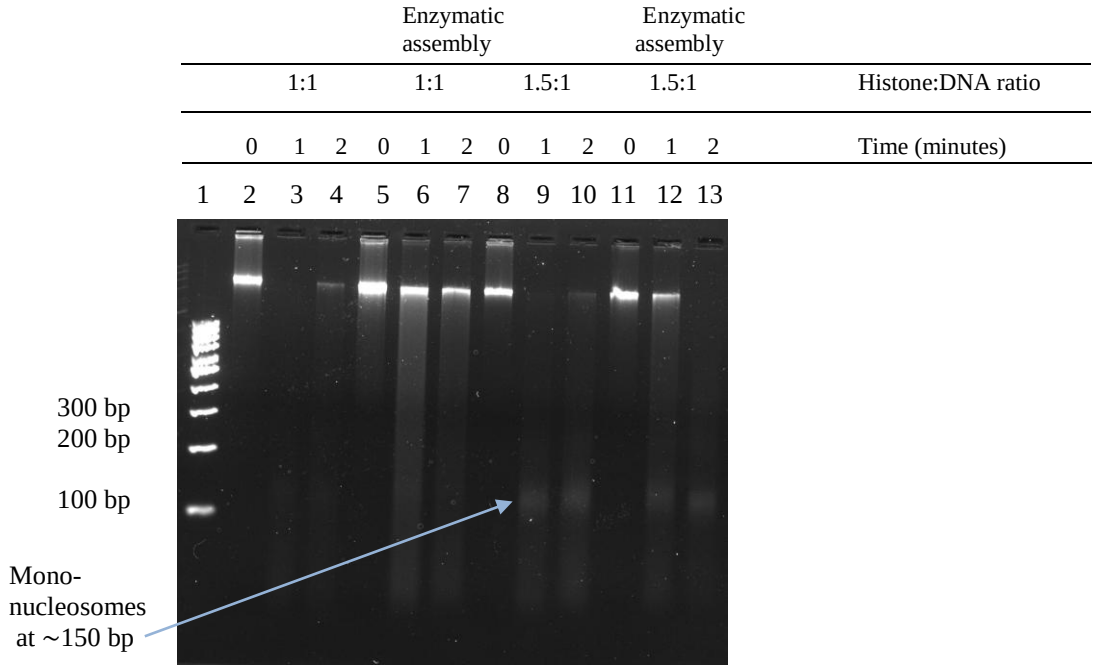

Figure 4.13: Result from agarose gel electrophoresis. 83 ng of T7 DNA was loaded into each well. Lane 1 is the ladder. The samples were digested with an MNase concentration of 0.1 U/ml. The electrophoresis was run at 100 V for 80 minutes. The gel was pre-stained.

Clear bands corresponding to mononucleosomes are present when the DNA ratio is 1.5:1 (lane 8-13), but the signal does not increase when enzymes are added compared to only salt dialysis. For the sample with a histone:DNA ratio of 1:1 (lane 2-7) no bands are visible, but for the samples where enzymes had been added a smear is visible. This indicates that a ratio of 1.5:1 seems to be preferred in order to get periodically spaced nucleosomes. The greater visible smear when enzymes had been added suggests that more nucleosomes have been formed, but they are not periodically spaced. For most samples in Figure 4.13 the amount of undigested DNA is higher when enzymes are added. Since the same digestion conditions are used with and without enzymes, this could be a result of that more histones are positioned on the DNA when enzymes are used. If more histones are positioned on the DNA it will be more difficult for the MNase to reach the DNA and digest it, thereby more undigested DNA would be present.

In the initial protocol the idea was to first perform a salt dialysis followed by an enzymatic assembly in order to assemble the chromatin. The idea behind this was that when using salt dialysis only, the chromatin might not have periodically spaced nucleosomes as the histone octamers have DNA sequence specificity. If an enzymatic assembly is applied however, the

nucleosomes should be periodically spaced as the ACF has a mobilization activity. During the project, most of the digestion reactions were however performed on chromatin that had been assembled using salt dialysis only. In order to save time and resources the enzymatic assembly was not always performed. This was especially the case when different conditions were to be evaluated during e.g. the digestion and gel electrophoresis. In theory, even with just the salt dialysis, chromatin should be assembled but probably with not as high quality.

The effect of enzymatic assembly can be evaluated by comparing samples that had been assembled using salt dialysis only with samples that had been assembled using both salt dialysis and enzymatic assembly. In all other aspects the samples had been treated in the same way. This was performed for lambda DNA in Figure 4.4 and for T7 DNA in Figure 4.13. When doing this analysis with lambda DNA, there were bands corresponding to mononucleosomes in both types of samples. However the bands were much clearer when the chromatin had been assembled using enzymatic assembly and salt dialysis compared to salt dialysis only. The conclusion was therefore that chromatin can be assembled using salt dialysis only, but by using enzymatic assembly as well, there was a higher amount of periodically spaced nucleosomes. This was however not the case when performing the same analysis with T7 DNA. In this case there was no clear difference between the chromatin that had been assembled with salt dialysis only or with enzymatic assembly as well. With both these results in mind, it is hard to draw any direct conclusions regarding the effect of the enzymatic assembly. The same type of comparison was also performed with supercoiled DNA. When the enzymatic assembly was performed, it led to a smear which was not present for the samples with salt dialysis only. It can however be noted that a smear was visible for the control sample that had not been digested, which should not be possible. The relevance of these results can therefore be discussed. An enzymatic assembly was also performed without first doing a salt dialysis, but this did not lead to bands corresponding to nucleosomes on the gel.

These results combined indicate that performing a salt dialysis can be used to make DNA bind to histones and an enzymatic assembly could be added which will increase the chromatin quality to variable extent. To evaluate the effect further it would be preferred to perform more experiments and also to change the conditions during the enzymatic assembly, e.g. change enzyme concentrations and reaction times. During the experiments performed in this project, the concentrations and reactions times were kept constant.

4.1.8 Comparison between lambda DNA, supercoiled DNA and T7 DNA

There were three different types of DNA used to assemble chromatin during this project: lambda DNA, supercoiled DNA and T7 DNA. As can be seen in the results, different types of DNA led to different quality of chromatin. The aim in the beginning of the project was to assemble chromatin using lambda DNA and it was uncertain if it was possible to assemble chromatin from this type of DNA. In the early stage of the project, there were some successful results regarding the assembly. Bands corresponding to mononucleosomes were visualized indicating that chromatin had been assembled and that the nucleosomes were periodically spaced with 147 base pairs around each histone octamer. There was however also a smear visible on these gels. This indicates that there were DNA fragments of many different sizes present and segments of chromatin where the nucleosomes were not periodically spaced. When trying to reproduce these results later on in the project, it turned out non-successful. A smear could then be visualized but no bands corresponding to nucleosomes. This raises the question how good the method actually works for lambda DNA. A theory why the result could not be reproduced was that a new batch of histone octamers was used. However, after

performing the SDS-PAGE it turned out that the histones octamers had the components it should. The new histones also worked well to assemble chromatin of other types of DNA. A theory proposed why it turned out more difficult to assemble chromatin of lambda DNA compared to other DNA fragments is their lengths. Lambda DNA is much longer than the DNA molecules used in previous studies and this increases the complexity as there are more positions where histones should be placed and it is also easier to get histones misplaced in that case.

Supercoiled DNA of 3000 base pairs was assembled into chromatin as a way of testing the method. As this DNA is much shorter than lambda DNA, the hypothesis was that it would more easily be assembled into chromatin. This turned out to be the case. When using supercoiled DNA, bands were visible corresponding to mononucleosomes as well as di- and trinucleosomes. The bands were quite clear and the results were reproducible to a higher extent. This indicated that the method used for the chromatin assembly works at least for shorter DNA molecules. However, there was still a smear present in the gels which means that not all DNA molecules had periodically spaced nucleosomes.

T7 DNA is almost as long as lambda DNA and was therefore used to test if the problem with the method depends on the length of the DNA molecule or some other property of the lambda DNA. When using T7 DNA, clear bands corresponding to both mono- and dinucleosomes were present. The T7 DNA did however not show bands corresponding to both di- and mononucleosomes every time and there was also a smear present. This indicates that the chromatin does not consist of only periodically spaced nucleosomes.

By comparing the results from the three different types of DNA it could be concluded that the protocol can be used to make histone octamers bind to DNA in a somewhat periodical manner for all three types of DNA, but with various success. It seems to work quite well for both supercoiled DNA and T7 DNA where mono-, di-, and trinucleotides have been present. Regarding the lambda DNA, the protocol could probably use some adjustments to obtain chromatin of higher quality and more periodically spaced nucleosomes. An idea was that the method works better for supercoiled DNA than lambda DNA because of the length difference. This however seems not to be the full truth as the method works almost as good for T7 DNA which is almost the same length as lambda DNA. Another possible explanation for the difference in chromatin assembly capability could be that the different DNA molecules have different sequences. As mentioned earlier, the histone octamers have DNA sequence specificity and it could thereby be that the sequence of T7 DNA is preferred over the lambda DNA.

4.2 Nanofluidic channels and fluorescence microscopy

The last part of this master's thesis project was to analyze the assembled chromatin using nanofluidic channels. The DNA used during these experiments was T7 DNA with a histone:DNA ratio of 1.5:1. The initial plan was to assemble chromatin with both salt dialysis and enzymatic assembly before analyzing them in the nanofludic channels, but unfortunately the enzymes needed for this ran out and therefore the chromatin was assembled using salt dialysis only. It should be noted that by using nanofluidic channels and fluorescence microscopy in this way, it is possible to see if there are histones bound to DNA, but not if the nucleosomes are periodically spaced.

The filters were set so that DNA and histones could be visualized separately and by merging the images it was possible to see if the DNA and histones were located at the same position in the nanofluidic channel over time. Examples of the observed molecules are shown in Figure 4.14 – 4.16.

Figure 4.14: Visualization of DNA and histones using nanofluidic channels and fluorescence microscopy. In A) DNA is visualized, in B) histones and in C) the figures of DNA and histones are merged together.

In Figure 4.14A a large DNA molecule is present. In Figure 4.14B however, there are no histones present and when merging the two figures in Figure 4.14C, only the DNA is visible. This indicates that there are no histones bound to the DNA molecule and no chromatin was present.

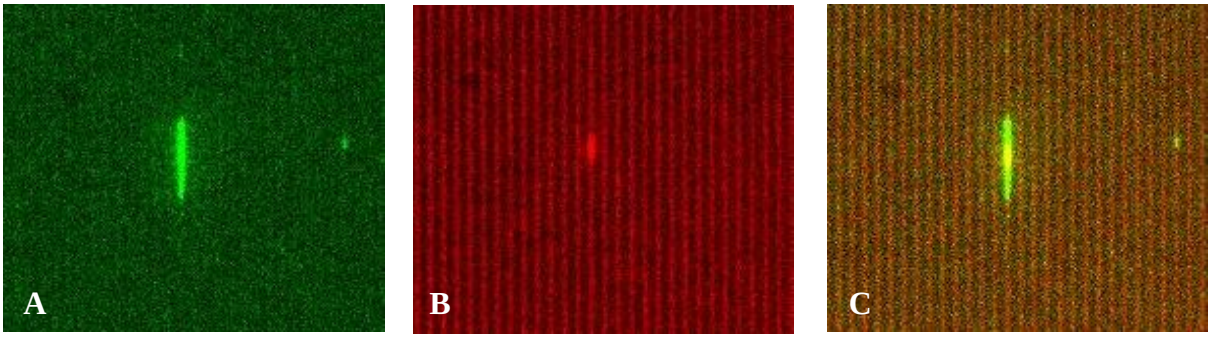

Figure 4.15: Visualization of DNA and histones using nanofluidic channels and fluorescence microscopy. In A) DNA is visualized, in B) histones and in C) the figures of DNA and histones are merged together.

In Figure 4.15A a long DNA molecule is visible and in Figure 4.15B histones are present. When merging these two figures together in Figure 4.15C, it can be seen that they are at the same position. There is a yellow spot in the middle of the DNA molecule, indicating that histones are bound in that position, forming chromatin. The signal also stayed the same over time, meaning that the histones are actually bound to the DNA and not just happen to be at the same location temporally. On the edges of the molecule there is DNA present, but no histones. This means that there is only a part of the DNA molecule where histones are bound.

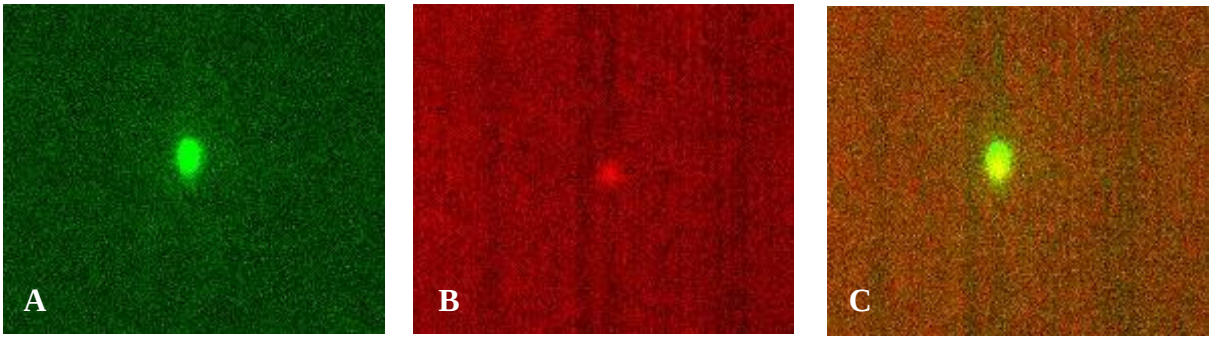

Figure 4.16: Visualization of DNA and histones using nanofluidic channels and fluorescence microscopy. In A) DNA is visualized, in B) histones and in C) the figures of DNA and histones are merged together.

In figure Figure 4.16A, it can be seen that there is a large DNA fragment present and in Figure 4.16B, there are histones present. When merging these two figures in Figure 4.16C the DNA and histones are located in the same position, leading to a yellow colour. Just as in Figure 4.15, the signal also stayed the same over time, meaning that the histones are actually bound to the DNA.

Overall, DNA molecules bound to histones could be observed, but the majority of the DNA molecules did not have histones bound. This is consistent with the results from the analysis using gel electrophoresis. In both cases, it could be concluded that chromatin had been assembled to some degree, but there were also DNA molecules present where the assembly had not worked as desired. It should however be noted that the lack of fluorescence for the histones does not necessarily mean that there are no histones present. It could be that histones are present but not visible due to e.g. bleaching of the fluorophores. The images taken of the DNA and histones were also not taken at the exact same time, meaning that the chromatin could have moved in between the images.

5 Conclusion

By using the protocol developed in this project, it is possible to ensure binding of histone octamers to genome scale DNA molecules *in vitro*. The nucleosomes were periodically spaced to some degree, as both mono-, di- and trinucleosomes have been observed. The assembly worked best when a histone:DNA ratio from 1:1 up to 2:1 was used. It was possible to assemble chromatin of lambda DNA, supercoiled DNA as well as T7 DNA, indicating that the protocol seems to work for different types of DNA and is not limited to lambda DNA.

For each dialysis that was performed the results differed even if the samples were treated in the same way. The volume increase during the dialysis differed as well as the digestion time or MNase concentration that was optimal and the quality of the chromatin. This leads to difficulties of developing a definite protocol. Different conditions are necessary depending on e.g. the type of DNA, the amount of DNA in each digestion reaction and whether the enzymatic assembly is performed or not.

6 Future work

In future studies, it would be of interest to continue developing the protocol further. It is possible that higher quality chromatin could be assembled if the protocol was adjusted regarding the salt dialysis and enzymatic assembly. The optimization of the protocol would also involve the analysis step. In this project, the PAGE did not give the expected results and it would be interesting to investigate the reason for this further. During the chromatin assembly, histone octamers were used and it would be interesting to use the four core histone proteins instead and investigate how it would affect the chromatin assembly. The histone octamers used did not contain the linker histone H1 and it could be investigated further how the addition of this histone would affect the outcome. It could also be of interest to use other methods to determine the quality of the chromatin, such as atomic force microscopy.

Besides developing the protocol further, there is a great range of studies that could be performed once chromatin of high quality is obtained. This involves epigenetic studies, i.e. investigating how the physical properties of the chromatin are affected by different epigenetic changes. By having different post translational modifications on the histone octamers, e.g. the addition of an acetyl group, it would be possible to analyze how this affects the chromatin. This could then be connected to how different cellular processes such as transcription are regulated in eukaryotic cells.

7 Bibliography

1. Moosavi A, Motevalizadeh Ardekani A. Role of Epigenetics in Biology and Human Diseases. Iranian biomedical journal. 2016;20(5):246-58.

2. Weinhold B. Epigenetics: the science of change. Environmental health perspectives. 2006;114(3):A160-A7.

3. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular biology of the cell: Garland Science; 2008.

4. Muthurajan U, Mattiroli F, Bergeron S, Zhou K, Gu Y, Chakravarthy S, et al. In Vitro Chromatin Assembly: Strategies and Quality Control. Methods in enzymology. 2016;573:3-41.

5. Ito T, Levenstein ME, Fyodorov DV, Kutach AK, Kobayashi R, Kadonaga JT. ACF consists of two subunits, Acf1 and ISWI, that function cooperatively in the ATP-dependent catalysis of chromatin assembly. Genes & development. 1999;13(12):1529-39.

6. Ito T, Bulger M, Pazin MJ, Kobayashi R, Kadonaga JT. ACF, an ISWI-Containing and ATP-Utilizing Chromatin Assembly and Remodelling Factor. Cell. 1997;90(1):145-55.

7. Khuong MT, Fei J, Cruz-Becerra G, Kadonaga JT. A simple and versatile system for the ATP-dependent assembly of chromatin. The Journal of biological chemistry. 2017;292(47):19478-90.

8. Lowary PT, Widom J. New DNA sequence rules for high affinity binding to histone octamer and sequence-directed nucleosome positioning11Edited by T. Richmond. Journal of Molecular Biology. 1998;276(1):19-42.

9. Annunziato A. DNA Packaging: Nucleosomes and Chromatin. Nature Education. 2008;1(26).

10. Kouzarides T. Chromatin Modifications and Their Function. Cell. 2007;128(4):693-705.

11. Olins DE, Olins AL. Chromatin history: Our view from the bridge. Nature Reviews Molecular Cell Biology. 2003;4:809–14.

12. PDBID:1KX3, Davey CA, Sargent DF, Luger K, Maeder AW, Richmond TJ. Solvent mediated interactions in the structure of the nucleosome core particle at 1.9 a resolution. J Mol Biol. 2002;319(5):1097-113.

13. Wheeler R. The major chromatin structures. en.wikipedia.org.

14. Park YJ, Luger K. The structure of nucleosome assembly protein 1. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(5):1248-53.

15. Becker A, Murialdo H. Bacteriophage lambda DNA: the beginning of the end. Journal of bacteriology. 1990;172(6):2819-24.

16. Casjens SR, Hendrix RW. Bacteriophage lambda: Early pioneer and still relevant. Virology. 2015;479-480:310-30.

17. Lieberman PM. Chromatin organization and virus gene expression. Journal of cellular physiology. 2008;216(2):295-302.

18. Dunn JJ, Studier FW, Gottesman M. Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. Journal of Molecular Biology. 1983;166(4):477-535.

19. Richardson CC. Bacteriophage T7: Minimal requirements for the replication of a duplex DNA molecule. Cell. 1983;33(2):315-7.

20. Lee K-M, Narlikar G. Assembly of Nucleosomal Templates by Salt Dialysis. Current Protocols in Molecular Biology. 2001;54(1):21.6.1-.6.16.

21. Peterson CL. Salt gradient dialysis reconstitution of nucleosomes. CSH protocols. 2008;2008:pdb.prot5113.

22. Nguyen UTT, Bittova L, Müller MM, Fierz B, David Y, Houck-Loomis B, et al. Accelerated chromatin biochemistry using DNA-barcoded nucleosome libraries. Nature Methods. 2014;11:834.

23. Struhl K, Segal E. Determinants of nucleosome positioning. Nature structural & molecular biology. 2013;20(3):267-73.

24. Andrews AJ, Chen X, Zevin A, Stargell LA, Luger K. The histone chaperone Nap1 promotes nucleosome assembly by eliminating nonnucleosomal histone DNA interactions. Molecular cell. 2010;37(6):834-42.

25. Fyodorov DV, Kadonaga JT. Chromatin assembly in vitro with purified recombinant ACF and NAP-1. Methods Enzymol. 2003;371:499-515.

26. Park YJ, Chodaparambil JV, Bao Y, McBryant SJ, Luger K. Nucleosome assembly protein 1 exchanges histone H2A-H2B dimers and assists nucleosome sliding. The Journal of biological chemistry. 2005;280(3):1817-25.

27. Diagenode. Chromatin assembly kit manual.

28. Wistrand L. EDTA ne.se: Nationalencyklopedin; [2019-04-01].

29. Maniatis T, Jeffrey A, Van deSande H. Chain length determination of small double- and single-stranded DNA molecules by polyacrylamide gel electrophoresis. Biochemistry. 1975;14(17):3787-94.

30. Lee PY, Costumbrado J, Hsu C-Y, Kim YH. Agarose gel electrophoresis for the separation of DNA fragments. Journal of visualized experiments : JoVE. 2012(62):3923.

31. Reisner W, Larsen NB, Silahtaroglu A, Kristensen A, Tommerup N, Tegenfeldt JO, et al. Single-molecule denaturation mapping of DNA in nanofluidic channels. Proceedings of the National Academy of Sciences. 2010;107(30):13294-9.

32. Frykholm K, Nyberg LK, Westerlund F. Exploring DNA-protein interactions on the single DNA molecule level using nanofluidic tools. Integrative biology : quantitative biosciences from nano to macro. 2017;9(8):650-61.

33. Günther K, Seidel R, Mertig M. Mechanical and structural properties of YOYO-1 complexed DNA. Nucleic Acids Research. 2010;38(19):6526-32.

34. Murade CU, Subramaniam V, Otto C, Bennink ML. Interaction of Oxazole Yellow Dyes with DNA Studied with Hybrid Optical Tweezers and Fluorescence Microscopy. Biophysical Journal. 2009;97(3):835-43.

35. Reuter M, Dryden DTF. The kinetics of YOYO-1 intercalation into single molecules of double-stranded DNA. Biochemical and Biophysical Research Communications. 2010;403(2):225-9.

36. Rye HS, Yue S, Wemmer DE, Quesada MA, Haugland RP, Mathies RA, et al. Stable fluorescent complexes of double-stranded DNA with bis-intercalating asymmetric cyanine dyes: properties and applications. Nucleic acids research. 1992;20(11):2803-12.

37. Jain A, Blum C, Subramaniam V. Chapter 4 - Fluorescence Lifetime Spectroscopy and Imaging of Visible Fluorescent Proteins. In: Verdonck P, editor. Advances in Biomedical Engineering. Amsterdam: Elsevier; 2009. p. 147-76.