Epigenetic Reprogramming During Gametogenesis

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Purpose

The specific aim of this study is to assess the regulatory mechanisms of the DNA methyltransferase 3 (Dnmt3) gene prosequent to genetic reprogramming in developing male gametes. The gene codes for the Dnmt3 protein, which transfers methyl groups to CpG sites within DNA, and as such is a gene-regulatory protein itself. Alternatively to direct methylation, DNA can be regulated via Histone modifications, including methylation, acytelation, the addition of phosphate groups, and other regulatory moieties added to the Histone tail. Specifically the H3 protein of the Histone complex will be investigated for the presence of tri-methylation at the 9^{th} lysine residue. This state is indicative of gene silencing. Theoretically the Dnmt3 gene would be silenced by H3L9 tri-methylation until just prior to genetic reprogramming in the developing gamete when the

The ChIP assay was utilized in which protein associated with DNA is covalently bonded to the DNA via treatment with formaldehyde. This effectively complexes the nucleosome, allowing for retrieval of the DNA-protein complex via chromatin immunoprecipitation. Alternatively the chromatin can be sheared and dissociated to retrieve the protein via immunoprecipitation for Western blot analysis, or for the retrieval of DNA via ethanol precipitation. Both methods where employed in this study.

Optimization

The enzymatic shearing conditions were tested to ascertain the optimal treatment time and concentration with micrococcal nuclease. Because the male gamete progenitor cells (PGCs) are impractical to culture we are using SLN cells, which are supportive feeder cells that can be used to model PGCs. We aimed to test the cell number at 2.5 X 10^5 SLN cells for optimization, since PGCs are difficult to amass in large numbers because they are collected directly from the embryos. The optimal shearing condition can be applied to PGCs in the ChIP assay.

ChIP Assay Optimization with 1.0 X 10^6

Initially 1.0 X 10^6 SLN cells were used as an assessment of technique (figure 1). The results showed that the enzymatic treatment time and the DNA yield were not correlated. Furthermore there was difficulty loading the samples as the electrophoretic bands have different sizes in thickness. Therefore the procedure was repeated but using fewer number of samples this time to make it more manageable (figure 2).

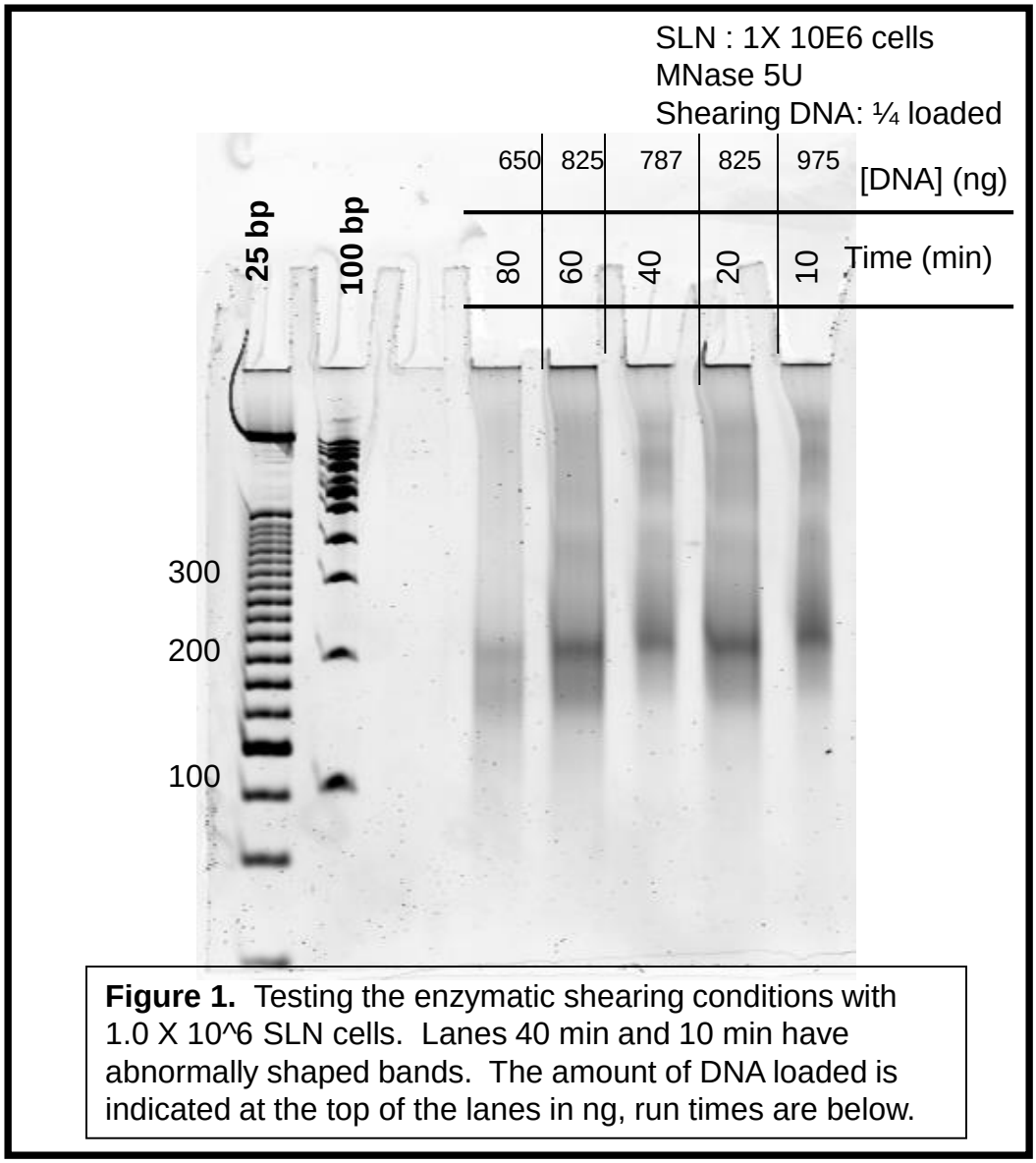


Figure 1. Testing the enzymatic shearing conditions with 1.0 X 10^6 SLN cells. Lanes 40 min and 10 min have abnormally shaped bands. The amount of DNA loaded is indicated at the top of the lanes in ng, run times are below.

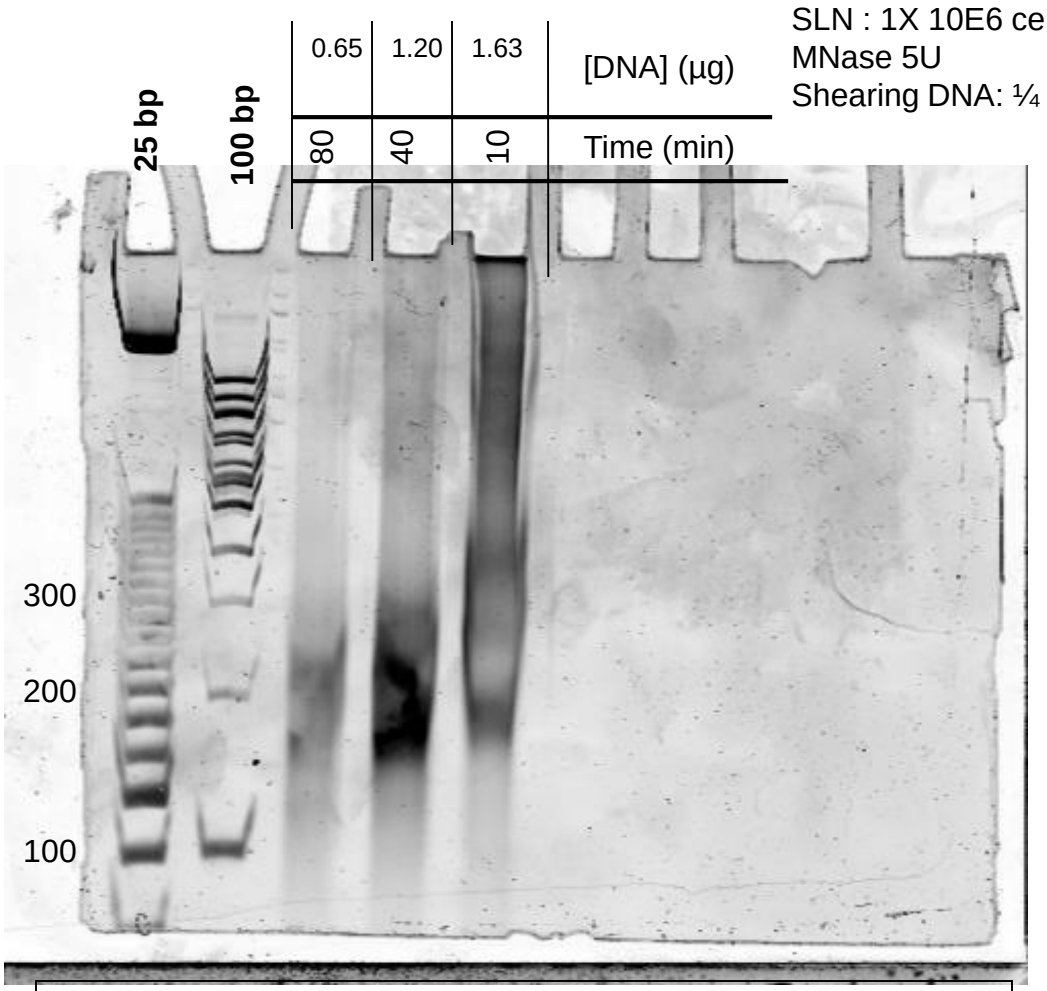


Figure 2. Experiment repeated with 1.0 X 10^6 SLN cells but only using 3 samples. The amount of DNA loaded is indicated at the top in µg, run times below.

The results show that there is a sufficient yield of DNA, in fact the lane with the 80 min sample may have some quenching due to oversaturation of the fluorescent signal. Although an optimal enzymatic shearing time can be determined from this experiment, it was repeated for verification (figure 3).

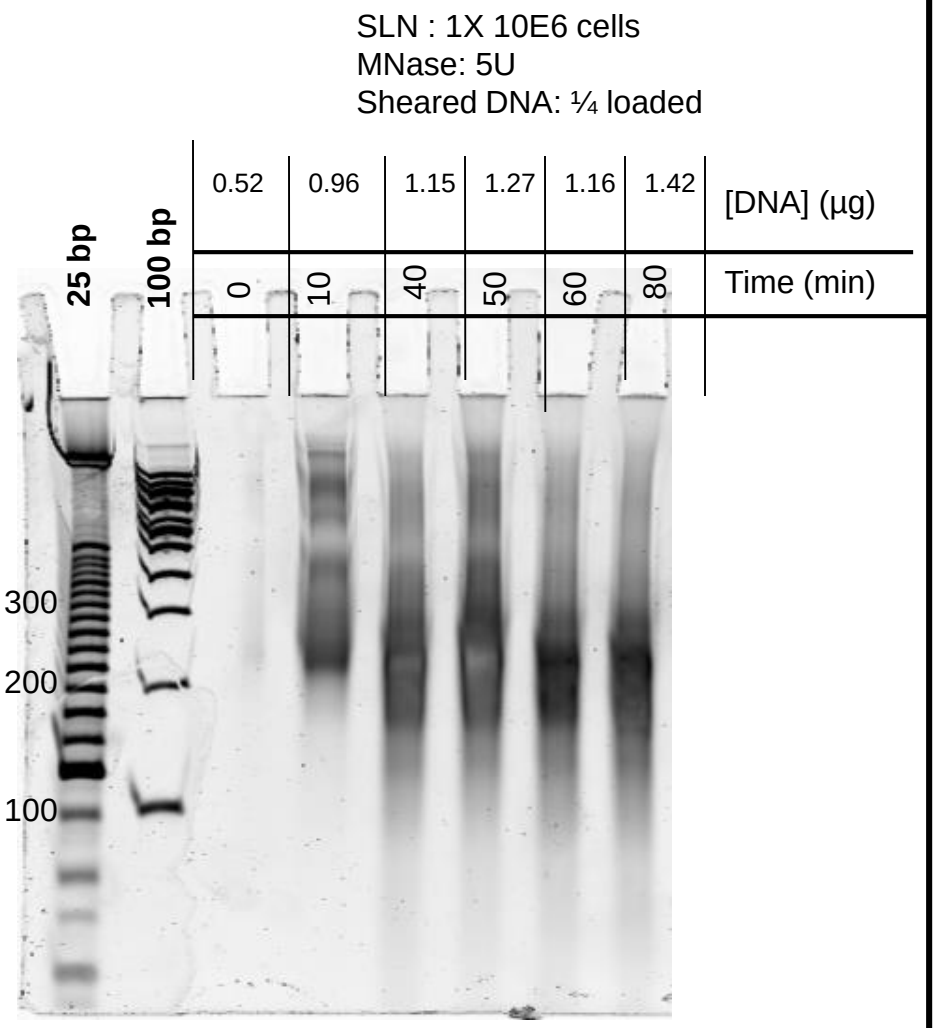


Figure 3. Enzymatic shearing conditions repeated at 1.0 X 10^6 SLN cells to verify that the technique is acceptable and reproducible. DNA amounts loaded are indicated at the top of the lanes in µg while treatment times are indicated in minutes. There may be quenching occurring at the 50 min treatment time. No DNA band is detectable at the 0 min treatment.

The results were faithfully reproduced, and by including 3 more samples the optimal treatment time could be more precisely determined. Furthermore in this experiment the termination of electrophoresis was closely monitored to insure it was stopped at the exact moment that the loading dye exited the acrylamide gel.

Assessing the Enzymatic Shearing Conditions Using 1.0 X 10^5 SLN Cells:

Since it is difficult to collect 1.0 X 10^6 PGCs the enzymatic shearing conditions were assessed using a reduced number of SLN cells. A quantity of 1.0 X 10^5 SLN cells was therefore used. The same procedure was followed except that the MNase was reduced from 5U to 0.5U. No meaningful results were obtained (figure 4). The experiment was therefore repeated using a fewer number of samples (figure 5).

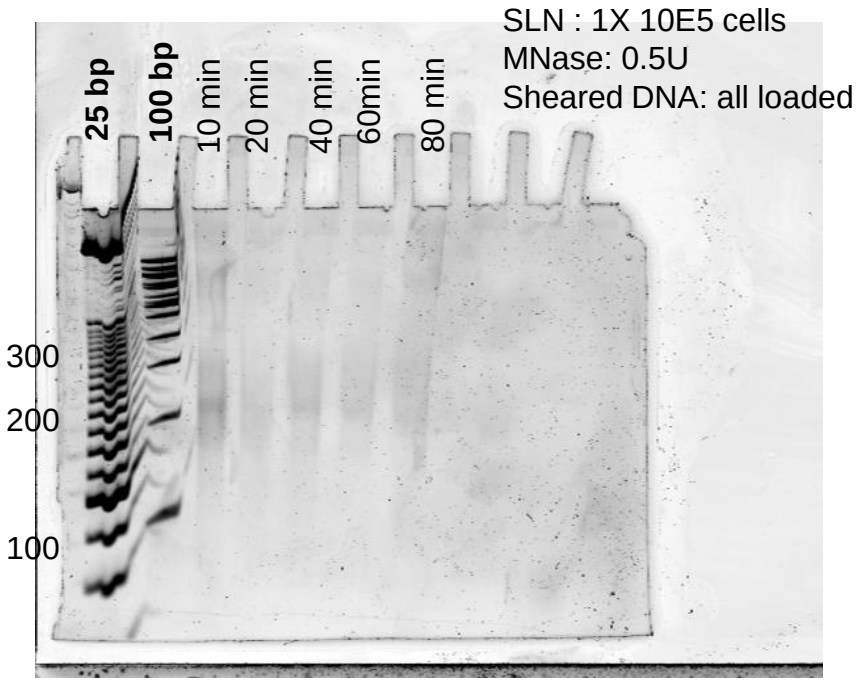


Figure 4. Enzymatic shearing conditions assessed at 1 X 10E5 SLN cells. Almost no DNA is observed.

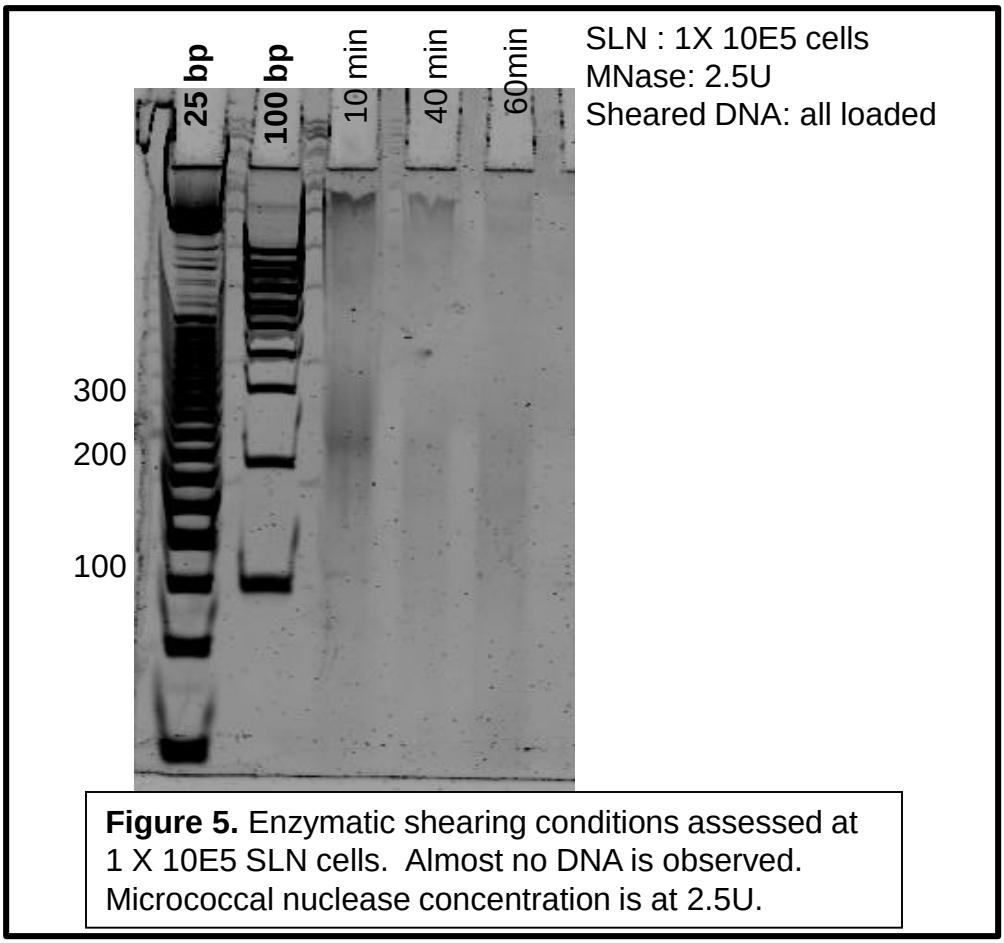


Figure 5. Enzymatic shearing conditions assessed at 1 X 10E5 SLN cells. Almost no DNA is observed. Micrococcal nuclease concentration is at 2.5U.

The yield of DNA is extremely low and no meaningful results can be obtained from these data. The background flourescence is high in this image as well, which obfuscates visualization since it is a low amount of DNA that is being worked with. A revised protocol was implemented and the experiment was repeated with the same number of cells (figure 6).

Optimization of Enzymatic Shearing Conditions with a Revised Protocol:

The revised protocol uses a longer incubation time for de-crosslinking the DNA from the histones as well as a lower incubation temperature. The amount of Proteinase K is also increased by 10X. Furthermore a more rigorous lysis procedure was implemented in which the samples would be aspirated 20-30 times every 15 minutes to facilitate disruption of the plasma membrane (figure 7). Almost no DNA is observed. However the problem was most likely due to the multiple stops during the experiment, especially a stop after cell lysis which was already known to be problematic.

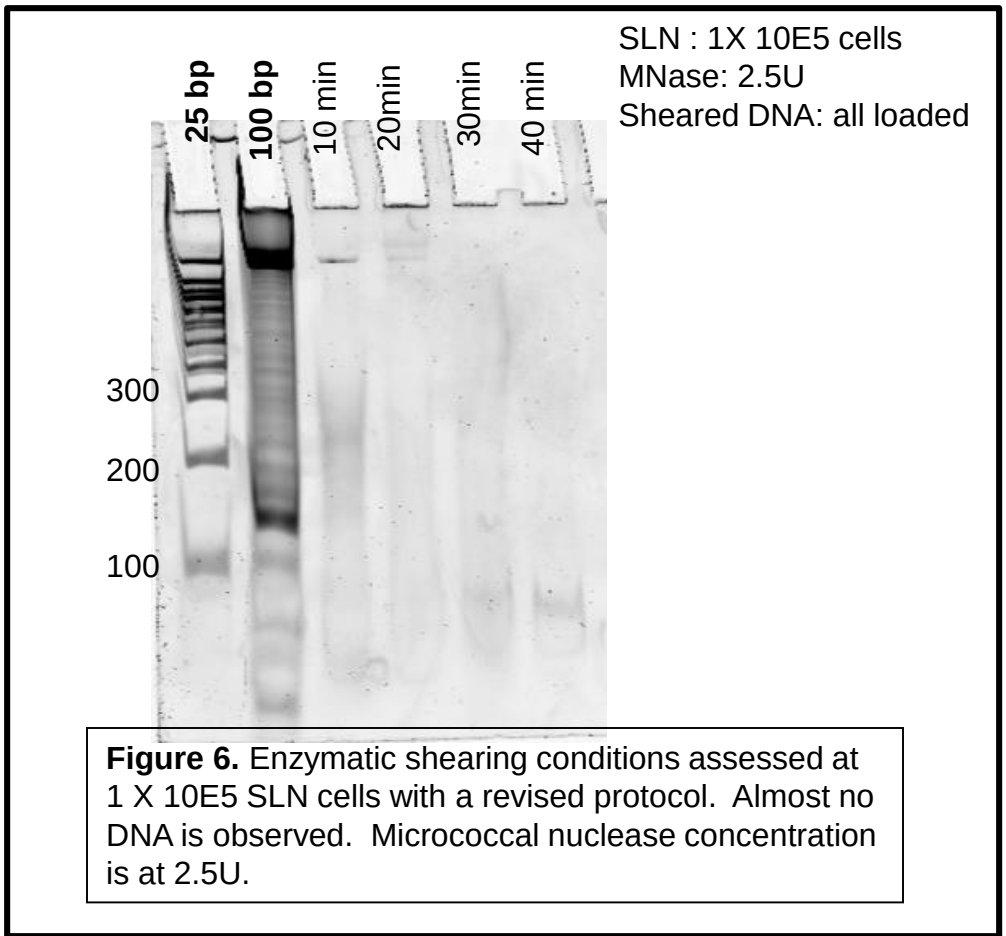


Figure 6. Enzymatic shearing conditions assessed at 1 X 10E5 SLN cells with a revised protocol. Almost no DNA is observed. Micrococcal nuclease concentration is at 2.5U.

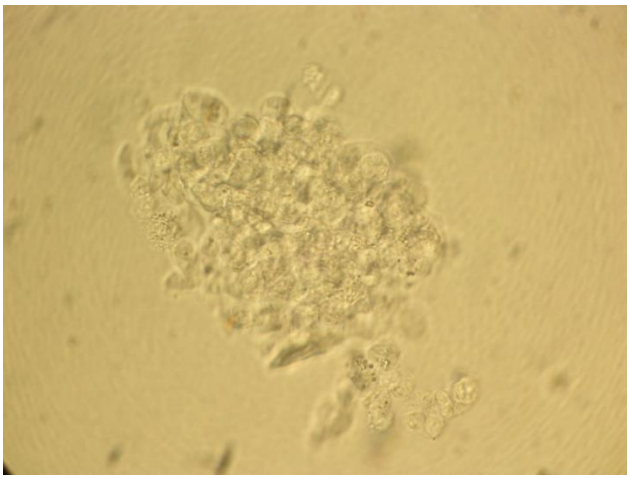


Figure 7. Aggregate of SLN nuclei after cell lysis. Image obtained using confocal light microscopy.

The plasma membrane is sufficiently disrupted and only SLN nuclei are observed aggregating together.

Assessing the Shearing Conditions with the Revised Protocol and a SLN cell concentration of 5.0 X 10E5 Cells/ mL:

Since it was found that 2.5 X 10E5 SLN cells could be harvested the optimization experiment was performed with 5.0 X 10E5 SLN cells (figure 8).

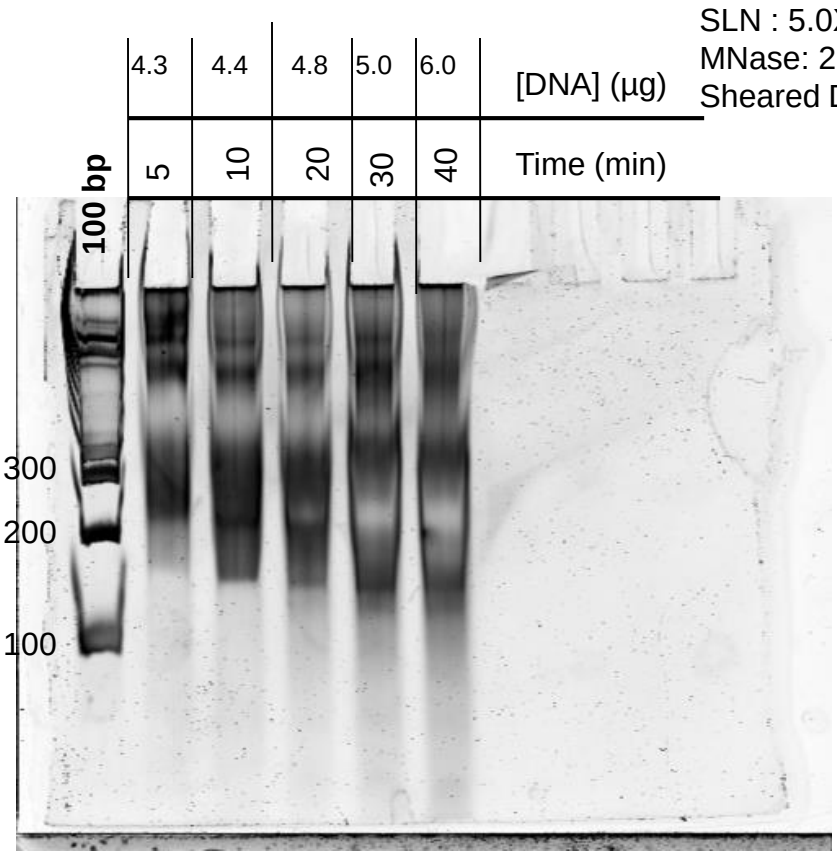


Figure 8. Enzymatic shearing conditions tested at 5.0 X 10E5 SLN cells with the revised protocol. DNA amounts loaded are indicated at the top of the lanes in µg while treatment times are indicated in minutes. There may be quenching occurring at the 30min and 40min treatment times.

There is a high yield of DNA, so much so that quenching is observed in the last two samples due to oversaturation of the signal. DNA concentrations are correlated to treatment times. The Ladder has an aberrant migration pattern which may be due to degradation of the DNA ladder.

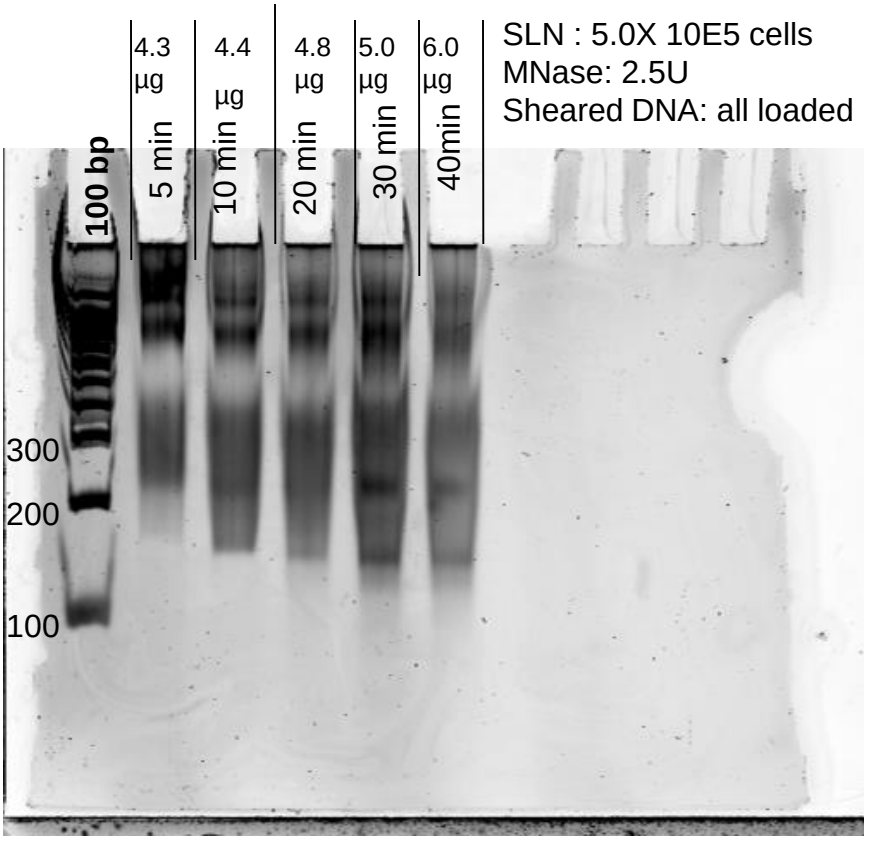


Figure 9. This is the same gel as Figure 8 except that it has been restained with ethidium bromide so that the 30 and 40 min samples could be better visualized. Bands are observed at the lower intensity.

With the lower senstivity of ethidium bromide the previously quenched bands could be observed (figure 9). This indicates that 2µg is an optimal loading concentration for the DNA.

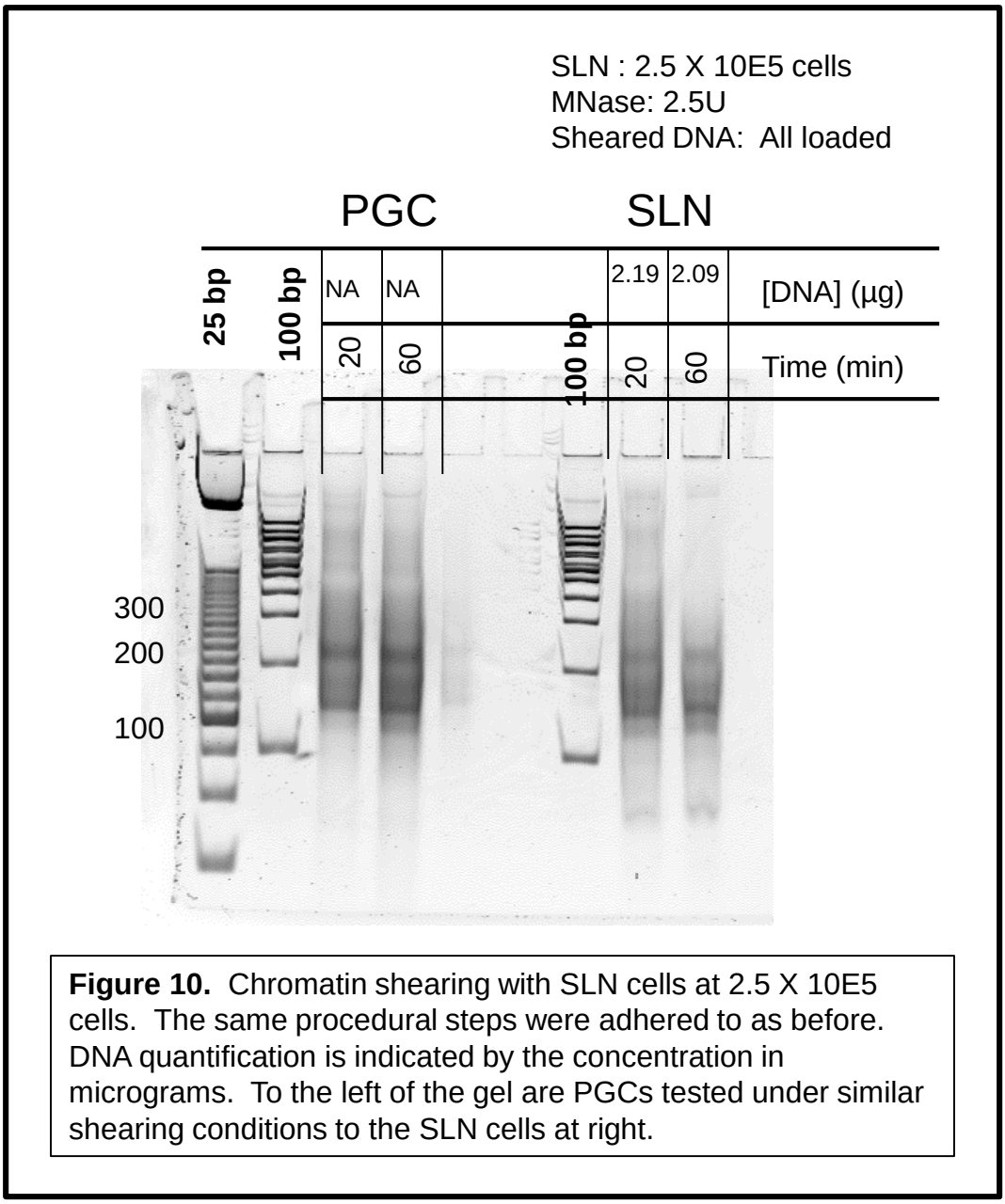


Figure 10. Chromatin shearing with SLN cells at 2.5 X 10E5 cells. The same procedural steps were adhered to as before. DNA quantification is indicated by the concentration in micrograms. To the left of the gel are PGCs tested under similar shearing conditions to the SLN cells at right.

Assessing the incubation time of micrococcal nuclease with 2.5 X 10E5 SLN cells. Incubation times were tested at 20 minutes and 60 minutes. The sharpest band is observed for the 60 minute treatment time. PGCs were similarly tested however there is little difference between the incubation times.

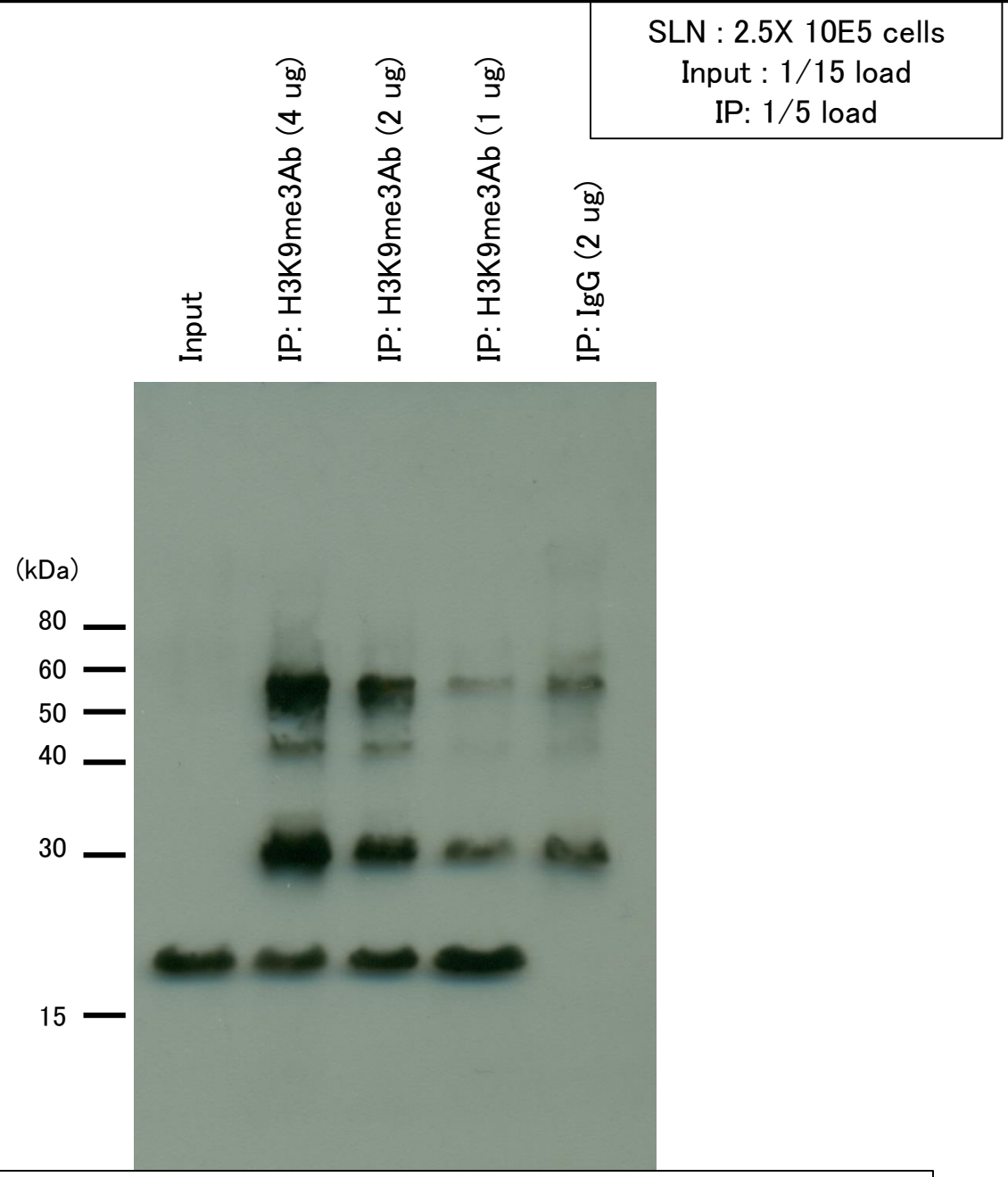


Figure 11. Western blot analysis with the H3K9 antibody tested with serial dilutions as indicated in parentheses. IgG shows the location of the light and heavy chains, while the input indicates the location of the H3 subunit, at approximately 16 kDa. No difference in IP strength is observed with different ab dilutions.

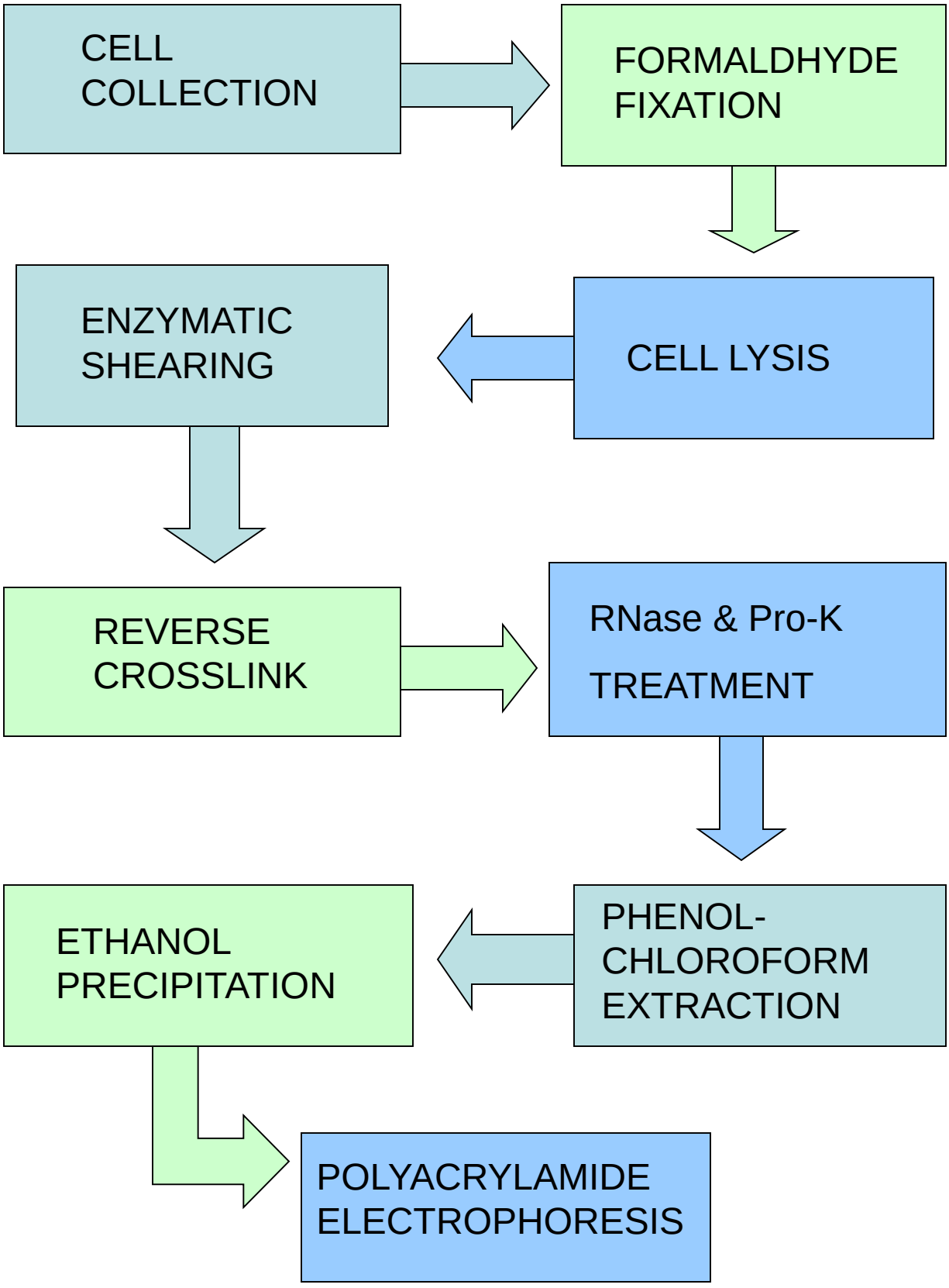
Western blot analysis was performed with the H3K9 trimethylation antibody, which immunoprecipitates H3 histone subunits with trimethylated lysine residues at the 9th position in the histone polypeptide tail. Several dilutions were used in this assay, however no distinction was observed at these concentrations.

Summary

The lowest number of SLN cells tested for optimization was 2.5 X 10E5 cells. Since this is the projected estimate for the number of PGCs that will be collected for the ChIP assay it is the most pertinent optimization data for incubation time and MNase concentration. At 2.5 X 10E5 cells the optimal shearing condition is with 2.5 U of Micrococcal nuclease, at 37°C for 60 minutes.

Western blot analysis shows that the H3K9me3 ab is functional and can successfully IP the H3 subunit. This indicates that it will be operational for the ChIP assay when PGCs are immunoprecipitated to interogate the Dnmt3 promoter.

SLN Cell Number	Incubation Time	MNase concentration
2.5 X 10E5	60 min	2.5 U
5.0 X 10E5	80 min	2.5 U
1.0 X 10E6	60 min	5.0 U


CELL COLLECTION
FORMALDHYDE FIXATION
CELL LYSIS
ENZYMATIC SHEARING
REVERSE CROSSLINK
RNase & Pro-K TREATMENT
PHENOL-CHLOROFORM EXTRACTION
ETHANOL PRECIPITATION
POLYACRYLAMIDE ELECTROPHORESIS