Methodology article

Rapid high-throughput analysis of DNasel hypersensitive sites using a modified Multiplex Ligation-dependent Probe Amplification approach

Thomas Ohnesorg¹, Stefanie Eggers¹, Wouter N Leonhard², Andrew H Sinclair¹ and Stefan J White*¹

Address: ¹Murdoch Children's Research Institute and Department of Paediatrics, University of Melbourne, Royal Children's Hospital, Parkville, VIC, Australia and ²Center for Human and Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands

Email: Thomas Ohnesorg - thomas.ohnesorg@mcri.edu.au; Stefanie Eggers - stefanie.eggers@mytum.de; Wouter N Leonhard - w.n.leonhard@lumc.nl; Andrew H Sinclair - andrew.sinclair@mcri.edu.au; Stefan J White* - stefan.white@mcri.edu.au

*Corresponding author

Abstract

Background: Mapping DNasel hypersensitive sites is commonly used to identify regulatory regions in the genome. However, currently available methods are either time consuming and laborious, expensive or require large numbers of cells. We aimed to develop a quick and straightforward method for the analysis of DNasel hypersensitive sites that overcomes these problems.

Results: We have developed a modified Multiplex Ligation-dependent Probe Amplification (MLPA) approach for the identification and analysis of genomic regulatory regions. The utility of this approach was demonstrated by simultaneously analysing 20 loci from the ENCODE project for DNasel hypersensitivity in a range of different cell lines. We were able to obtain reproducible results with as little as \( 5 \times 10^4 \) cells per DNasel treatment. Our results broadly matched those previously reported by the ENCODE project, and both technical and biological replicates showed high correlations, indicating the sensitivity and reproducibility of this method.

Conclusion: This new method will considerably facilitate the identification and analysis of DNasel hypersensitive sites. Due to the multiplexing potential of MLPA (up to 50 loci can be examined) it is possible to analyse dozens of DNasel hypersensitive sites in a single reaction. Furthermore, the high sensitivity of MLPA means that fewer than \( 10^5 \) cells per DNasel treatment can be used, allowing the discovery and analysis of tissue specific regulatory regions without the need for pooling. This method is quick and easy and results can be obtained within 48 hours after harvesting of cells or tissues. As no special equipment is required, this method can be applied by any laboratory interested in the analysis of DNasel hypersensitive regions.

Background

Open chromatin is a characteristic of genomic loci with regulatory functions. These regions are preferentially digested by DNasel [1], and the identification of DNasel hypersensitive sites is frequently used to identify and analyse regulatory regions such as promoters, enhancers and...
silencers [2,3]. However, currently available methods have significant limitations. A commonly used approach involves Southern blotting, but this is time consuming, usually requires radioactivity and is limited to short stretches of DNA. Several PCR-based methods have been described [4,5], but these do not readily allow multiplexing. Recent reports of large scale analysis of DNaseI hypersensitive sites have used either microarrays [6-9] or deep sequencing [9,10]. Whilst valuable for genome wide analysis, the costs involved are a limiting factor for many applications (such as comparing different developmental stages or tissues). Another disadvantage of those methods is that they usually require many millions of cells. For ex vivo studies, this might require extensive pooling of tissues, meaning that these methods are not suitable for all applications.

Multiplex Ligation-dependent Probe Amplification (MLPA) was originally developed to detect deletions and duplications in genomic DNA [11], and has become popular in diagnostic settings for a range of disorders [12,13]. It has since been modified for several other applications as well, including methylation analysis [14], mRNA expression analysis [15], identifying copy number variation in normal populations [16,17], genotyping of mouse models [18,19] and measuring the efficiency of Cre-mediated recombination in mouse models [20]. The principle advantages of this method are the sensitivity and multiplexing potential. It can be used to analyse up to 50 genomic loci with as little as 20 ng genomic DNA in a single reaction. Furthermore, the only equipment that is required is a thermocycler and DNA sequencer, readily available to most researchers.

We describe here a quick and straightforward protocol for analysing DNaseI hypersensitive sites. This is demonstrated by the analysis of 20 different loci in a single reaction, based on data published by the ENCODE consortium [21].

**Results**

**Probe design**

Figure 1 outlines the protocol used in this study. To examine the general feasibility of our approach, we designed 11 probes to cover randomly chosen DNaseI hypersensitive sites in HeLa cells as published by the ENCODE consortium [21]. In addition, nine probes were designed in regions that showed no evidence of DNaseI hypersensitivity. To be able to cover larger genomic regions and to give greater flexibility in probe design we employed the recently developed extension MLPA [20]. All 20 probes were combined in a single mix, and could be differentiated from each other on the basis of length (the final product length range was 94-207 bp).

**Nuclei isolation and DNaseI treatment**

We tested different methods for the isolation of nuclei, with the Ne-Per Kit (Thermo Scientific) found to be most suitable. Although intended for the isolation and separation of nuclear and cytoplasmic proteins, it proved to be useful for the isolation of nuclei. Another obstacle to overcome was the isolation of the genomic DNA. This approach requires efficient recovery of very long as well as very short fragments of genomic DNA. Unfortunately, DNA obtained using the standard method for this task, phenol-chloroform purification, is not suitable for use with MLPA, as traces of phenol are known to interfere with the enzymatic reactions. Instead we used the High Pure PCR Purification Kit (Roche), which, according to the manufacturer, is suitable for the isolation of fragments of up to 50 kb. As expected, in our experiments the recovery of DNA from the untreated and therefore most intact sample, was often least efficient, however, the quality and quantity of the DNA was still sufficient for MLPA analysis. Figure 2 shows a typical example of digested DNA obtained after treating isolated nuclei with increasing amounts of DNaseI and purification.

**Analysis of DNaseI hypersensitive sites in HeLa cells**

We next used the same probe mix to analyse the DNaseI hypersensitivity of DNA within intact nuclei of HeLa cells.
Figure 3A shows typical peak patterns obtained using nuclei aliquots digested with increasing amounts of DNaseI. Several peaks show significantly decreased peak height with increased DNaseI concentration, while others remain virtually unchanged. Nine of 11 probes targeting previously described DNaseI hypersensitive sites in HeLa cells show a clear decrease in normalized peak heights (defined as < 75% of the equivalent peak in undigested DNA), whereas all nine probes targeting non-sensitive loci show no significant decrease (figure 3B). These results were highly reproducible, with the technical and biological replicates giving an $r^2 > 0.9$.

We also tested the same probe mix on DNA directly isolated from HeLa cells. As can be seen in Figure 3C, there are no significant changes in normalized peak heights with increasing DNasel digestion. This was expected as these DNA samples should have no chromatin structure, and all cuts by DNasel should therefore occur in a random fashion.

Comparison of results using other cell lines
We then analysed the same 20 loci for DNaseI hypersensitivity in other cell lines. For this comparison we used HEK293, C28 and S97 cells. While the results for C28 cells were very similar to those of HeLa cells (data not shown), we could identify differences in sensitivity for several probes in HEK293 and S97 cells. As shown in Figure 4, some probes identifying hypersensitive sites in HeLa cells stay unchanged in the other cell lines (probes S9 in HEK293 cells, S6 in S97 cells) and two of the probes not showing the expected sensitivity in HeLa cells (S3 and S4) do show sensitivity in HEK293 cells.

Determining the minimum cell number
We also tried to determine the minimum cell number that could be used with this approach. As we were able to obtain robust results and sufficient DNA for several replicates from aliquots containing nuclei from about 2.5 × 10^5 cells, we estimated the lower limit of required cells to be around 5 × 10^4 (theoretically ~300 ng DNA). To confirm this number, we used aliquots of 5 × 10^4 HeLa cells and treated them as described above and compared the results with those obtained from experiments using 2.5 × 10^5 cells per aliquot. Figure 5 shows the comparison of results from 5 × 10^4 and 2.5 × 10^5 cells ($r^2 = 0.97$).

Discussion
We describe here a simple technique that allows the rapid analysis of many DNaseI hypersensitive sites using little starting material. For a proof of principle we chose loci that had been analysed by both array analysis and deep sequencing as part of the ENCODE project [21].

We used 20 probes in one reaction (11 sensitive, 9 non-sensitive in HeLa cells according to ENCODE data). Nine of 11 of the probes targeted to hypersensitive sites showed the expected decrease in peak height for HeLa cells. The fact that two sensitive sites did not show the expected drop in peak heights is probably due to different growth conditions, differences in passage numbers or to our batch of HeLa cells being different from the one used in the ENCODE study rather than to limitations of our method. This is supported by the high correlation observed for both technical and biological replicates ($r^2 > 0.9$) and the fact that those two probes showed sensitivity in HEK293 cells.

We also examined three other cell lines, in order to examine the cell line specificity of our experimental approach. We could show clear differences in hypersensitivity for some loci compared to HeLa cells. Again, the conclusion that these are cell type-specific differences is supported by the concordance of replicate experiments ($r^2 > 0.9$).

To determine suitable DNaseI digestion conditions for our method, a range of different DNaseI concentrations and incubation conditions were tested. Incubating the digestion reactions with the indicated DNaseI amounts at room temperature for 20 minutes gave more reproducible
Figure 3
MLPA on DNA from HeLa cells. A) Representative MLPA peak patterns obtained from nuclei isolated from HeLa cells and subsequent treatment with 0, 0.5 and 2 units of DNaseI. Red peaks represent size standards, blue peaks the signals from the probes after PCR amplification. Black numbers show probes designed to bind to non-sensitive regions, blue numbers to sensitive regions for HeLa cells as published by the ENCODE consortium. B) Analysis of experiment shown in 3A. Data were obtained from two independent experiments and two technical replicates (n = 4). Results shown as mean ± SD. C) Analysis of data derived from DNaseI treatment of naked DNA isolated from HeLa cells. In this case DNaseI digestion was with either 0.25 units or 1 unit DNaseI for 1 minute on ice, as this DNA was far more susceptible to degradation than DNA in intact nuclei. Probes are grouped into sensitive and non-sensitive, and then ordered according to their length. N: non-sensitive, S: sensitive for HeLa cells as published by the ENCODE consortium.
results than, for example, using less DNaseI and incubating at 37°C or incubations on ice (data not shown). By analysing the digested DNA on an agarose gel it was possible to identify the most appropriate conditions. Most of our experiments were carried out using nuclei from 2.5 × 10^5 cells per DNaseI digestion aliquot, providing sufficient DNA for several technical replicates. However, in the case of HeLa cells this number could be reduced to only 5 × 10^4 cells, still providing highly reproducible results as shown by an r^2 of 0.97 when comparing the results of the experiments with both cell numbers. Furthermore, we found that two different DNaseI concentrations and an undigested control are sufficient to analyse hypersensitive sites, which reduces the amount of total starting material required to about 1.5 × 10^5 cells.

Although we have used 20 probes in this study, there is the potential to significantly increase the degree of multiplexing. Reducing the interprobe spacing to 3 bp would allow up to 40 loci to be examined. Indeed, by designing probes that can be labelled with different fluorophores [22,23] it would be possible to analyse > 100 loci in a single reaction.

**Conclusion**

We describe here a rapid and accurate method for assaying DNaseI hypersensitive sites. In contrast to genome-wide approaches such as deep sequencing or microarray analysis, we consider the primary strength of this approach to be when < 100 genomic loci are being analysed. As all loci are analysed in a single reaction, relatively little starting material is required. We have shown that < 10^5 cells per DNaseI treatment can be used, which will allow the study of e.g. embryonic organ development without extensive pooling. The protocol is straightforward and results can be easily obtained from many samples within 48 hours. Finally, using this method makes it possible to also detect heterozygous and homozygous deletions and duplications of the examined regions, which is an artifact that is known to occur in cultured cells.

**Methods**

**Cell culture**

HEK293 (human embryonic kidney cells), C28 (chondro-geic cells), HeLa (cervical cancer cells) and S97 (dermal fibroblasts) were grown in DMEM containing 10% FBS and supplemented with L-glutamine in T-25 flasks and incubated at 37°C containing 5% CO2 in a humidified atmosphere.

**Cell harvest and isolation of nuclei**

After reaching 95-100% of confluence, cells were washed with PBS and harvested using 1 ml of 0.025% Trypsin-EDTA. After 5-10 min incubation at 37°C, cells were washed with 2 ml of 10% FBS containing DMEM and carefully resuspended in a 1.5 ml Eppendorf tube in cold PBS and placed on ice. To limit changes in chromatin structure during treatment, nuclei isolation was performed using 2.5 × 10^5 to 10^6 cells as soon as possible.

For isolation of nuclei, the NE-PER Kit (Thermo Scientific) was used with the following modifications. To prevent nuclei from excessive clumping and releasing DNA, twice the recommended volumes of the solutions were used and resuspension of cells/nuclei was carried out by carefully pipetting up and down rather than vortexing. Centrifugation steps were carried out at 250 × g at 4°C.

**DNaseI treatment of nuclei**

Isolated nuclei were washed in 500 μl cold DNaseI buffer containing 2% glycerol and carefully resuspended in 75 μl DNaseI buffer containing 2% glycerol. 25 μl aliquots of the nuclei suspension were added to 2 ml Eppendorf tubes containing one control without DNaseI and increasing amounts (0.5 - 2 units) of DNaseI (Promega) in 50 μl DNaseI buffer containing 2% glycerol. The solutions were mixed by carefully flicking the tubes and incubated for 20 min at room temperature (23°C).

**Isolation and purification of DNaseI treated genomic DNA**

DNaseI treated nuclei were lysed by adding 250 μl of nuclei lysis buffer (200 mM NaCl, 150 mM Tris HCl pH 8,
10 mM EDTA pH 8, 0.2\% SDS) containing 50 μg of Proteinase K and incubated for 45 min at 55°C. 20 μg of RNaseA (Sigma) was added and incubated for 30 min at 37°C. The DNaseI treated DNA was isolated and purified using the HighPure PCR Purification Kit (Roche) according to the manufacturer’s instructions. DNA was eluted with 50 μl elution buffer provided with the kit. Purified DNA was checked for quality and degree of DNaseI digestion by agarose gel electrophoresis. DNA concentrations were measured using the NanoDrop.

**MLPA and fragment analysis**

The MLPA probe mix was created by combining each MLPA oligonucleotide in table 1 to a final concentration of 4 nM. The extension MLPA was performed essentially as described [20]. 100-200 ng of DNaseI-treated DNA of each sample was denatured at 98°C for 5 min, and allowed to cool to room temperature. 1.5 μl MLPA buffer (MRC-Holland, the Netherlands) and 1.5 μl MLPA probe mix were added to each sample, denatured at 95°C for 1 min then hybridized overnight at 60°C. The following morning a ligation and elongation step was carried out at 54°C by adding 3 μl ligase buffer A, 3 μl ligase buffer B, 1 μl Ligase-65 (all MRC Holland, the Netherlands), 2 mM dNTPs, 1 U Stoffel Taq polymerase (Applied Biosystems) and H\_2O to a final volume of 40 μl. After 15-20 min the ligase was inactivated by heating to 95°C for 5 min. The PCR reaction consisted of 5 μl of ligated mix added to 2 μl SALSA PCR buffer, 1 μl SALSA enzyme buffer, 1 μl SALSA PCR primer, 0.25 μl SALSA polymerase (all MRC-Holland, the Netherlands) and 15.75 μl H\_2O. The PCR reaction started with a 1 min denaturation step at 95°C, followed by 35-38 cycles of amplification (each cycle consisting of 95°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec) and concluded with 20 min at 72°C.

Fragment analysis was performed on an ABI3130 capillary sequencer. Peak data was extracted using GeneMarker software (Soft Genetics) and exported to Excel (Microsoft) for further analysis.

**MLPA data analysis**

Basic data analysis was performed as described [24]. Peak data for the naked DNA experiment was normalised to the average of all probes in the examined samples, with all other reactions being normalized to all non-sensitive probes. To assist in visualisation of the results, normalized ratios of all untreated samples were set to 1.

![Figure 5](image_url)

**Comparison of results obtained using different cell numbers.** Correlation of results obtained from experiments with 5 × 10^4 (n = 1) and 2.5 × 10^5 (n = 4) HeLa cells per DNase treatment aliquot.
Table 1: Probe sets used in this study.

| Name | Probe1     | Probe2     | Genomic location   | Product size | Gap size |
|------|------------|------------|--------------------|--------------|----------|
| N1   | 1AAGCACAGATCGACGACAAGC AACCTTG | GAGCGGCTCTTTCTCTCCTCCTTCG | chr2:234,440,746-234,440,797 | 94           | -        |
| N2   | 1GAGCCAGCCTTTAGAACGAGGCAACGC GCGCTGTCG | CCAGGAATGCAGAAGGCTTGGCAG AAGG2 | chrX:153,258,524-153,258,587 | 106          | 9        |
| N3   | 1GACCTTTACCTTTGTAAGGACA GCTCGTCG | CATGGGTGCCGAGGTGGAATGT ATC2 | chr2:231,073,083-231,073,161 | 121          | 25       |
| N4   | 1CCCACTGTAGCTACAGTAGAAGT CTCTCTGCTC | GCTGTTCCTCGGTCCTATCACCT CGTAAGG2 | chr2:234,444,778-234,444,867 | 132          | 26       |
| N5   | 1CAATTAGTGCTCAGTGATCCTG TTPGACG | GACAGAATCGAGAAGCAAATACCG TTCTCATTCTATGAGGC | chr5:131,655,909-131,656,020 | 154          | 44       |
| N6   | 1GACAGAAGAGATCAAGCTTCATGGTGGTGCAGGCTTTGCTCATGTG | CTCTCAGGCTCCTTTCGAGTCATG AATCAC2 | chr2:231,377,023-231,377,144 | 164          | 64       |
| N7   | 1GCTGTTCAGCTTGAGTTGGAAGGCAAGA TTCTGAGATC | CTCCAGAACACCGTTAGGCAAAGAG AAGATT2 | chr2:231,306,976-231,306,976 | 170          | 70       |
| N8   | 1CTAAGTGGGACTCGCTCTCTG GTGATTGC | GACGTTCATCGCCGCTGATTCTC GGAAGTGTTTATC2 | chr5:131,660,201-131,660,338 | 180          | 74       |
| N9   | 1GGAAGAGGACGAGGAAAGGGA ATCTGCTC | GCTTTCTCTCGCTTCGCTGAAGCCATG ACGG2 | chr7:27,143,394-27,143,543 | 192          | 94       |
| S1   | CTGTTGGGTAGGGGATGCTCGGCA GAGGCAGG | GAGGTCCGAAAGCCGAATACAGC TGATC2 | chr19:59,386,440-59,386,478 | 96           | -        |
| S2   | 1GCTTTAAGAGAATTAGGGAACACGAGGCTTGCTCAGCG | GGGGCAGCAGTCAGGCGAGATGAGT GCCATG | chr19:59,396,645-59,396,701 | 99           | -        |
| S3   | 1GCTGGAGCTGAGATGCTGCTCGGCTTGAG | GCTGTTTCTCTATGCTGAGCTGAGCTG AGTTGCAG | chr19:59,386,011-59,386,072 | 104          | -        |
| S4   | 1GTACCCCGACTCATCTGTTGACGACG | CCTTTACCAGGAAAACGGACATAC TCTGCTC2 | chr2:231,378,757-231,378,822 | 106          | 14       |
| S5   | 1GGCCACAAACTCAAATAGGAGA CTTGG | CGTTTTCTCTTATGGGCTAGAGCGG AAGCGC2 | chr2:234,427,935-234,428,007 | 115          | 17       |
| S6   | 1ATGGGCTCGACCTCTGTGCTGCG TGCTG | CCTGATGACCTCTAGGACAGGAAATACG TGTTGCAC2 | chr7:27,149,886-27,149,967 | 124          | 25       |
| S7   | 1AACCTAGTCTCCCATGATTAGGACCGA GCGCTG | CGACCGCAAGCGCCTTCTTCTGCA TCTG2 | chr19:59,396,374-59,396,459 | 128          | 35       |
| S8   | 1GTGGGAGGACCTTGTTACTGCTG GCAG | CATCAGGAGGTATACGCGTAC GACA2 | chr5:131,655,970-131,655,967 | 140          | 46       |
| S9   | 1CCGAGAGTGGGAGAGCTACTCTG TGGAGG | CCTTTAATTAAGGCTGAGGAGCA CCTAGG2 | chrX:153,252,787-153,252,889 | 145          | 46       |
| S10  | 1TCTCGACGATGATCGAGCTTGGT GGGCCTG | CGAATTCAGCCTGCTGAGACGCTG TGGG2 | chr5:131,657,322-131,657,462 | 183          | 86       |
Table 1: Probe sets used in this study. (Continued)

| S11 | 1GCTTTGGATCGCTCTCGGT | CGTCTGGCAGCCTAACCGGAAGGCG | chr21:33,066,198-33,066,362 | 207 |
|-----|-----------------------|-----------------------------|-------------------------------|-----|
|     | GGTTCCCTAGGCTGGA      | GGTTCCCTAGGCTGGA             |                               |     |
|     | GTAA2                 | TCTAGATTGGATCTTGCGC          |                               |     |

N(n): non-sensitive, S(n): sensitive probes for HeLa cells according to data published by the ENCODE consortium. The genomic coordinates are from the human genome assembly of June 2006 (hg18).

Authors' contributions

TO and SJW conceived the experimental design. TO, SE, WNL, AHS, and SJW participated in acquisition, analysis, and interpretation of the data. TO and SJW drafted the manuscript. TO, SE, WNL, AHS, and SJW participated in critical revision and approved the final manuscript.

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