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Research article

TRACKING CHROMATIN STATES USING CONTROLLED DNase I TREATMENT AND REAL-TIME PCR

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Abstract: A novel approach to DNase I-sensitivity analysis was applied to examining genes of the spermatogenic pathway, reflective of the substantial morphological and genomic changes that occur during this program of differentiation. A new real-time PCR-based strategy that considers the nuances of response to nuclease treatment was used to assess the nuclease susceptibility through differentiation. Data analysis was automated with the K-Lab PCR algorithm, facilitating the rapid analysis of multiple samples while eliminating the subjectivity usually associated with C_t analyses. The utility of this assay and analytical paradigm as applied to nuclease-sensitivity mapping is presented.

Key words: DNase I-sensitivity, Differentiation, Spermatogenesis, Method

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Abbreviations used: Actb – β-actin; BSA – bovine serum albumin; C_t – cycle crossover threshold; DNase I – deoxyribonuclease I; EDTA – ethylenediamine tetraacetic acid; E_{max} – maximum efficiency of PCR reaction; FSB – frozen storage buffer; Hbb – β-globin, HEPES – 4-(2-hydroxyethyl)-1- piperazineethanesulfonic; NP40 – Nonidet-P40; PBS – phosphate buffered saline; PCR – polymerase chain reaction; Prm – protamine; Tnp2 – transition protein 2; RPMI-1640 Medium - Liquid Media; T_n – concentration of template at cycle n

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INTRODUCTION

The preferential digestion of potentiated-open or 'active' chromatin by nucleases [1] has proven to be a powerful technique to identify gene regulatory elements. These have included regulatory regions of acute sensitivity [2] located sometimes at a distance from a gene's promoter. From its humble beginnings of mapping the actively transcribed globin genes in erythrocytes [1] by Cot analysis and regulatory regions of the *Drosophila* heat shock genes [3, 4], using Southern hybridization-based techniques [5] the technique of using nucleases to examine chromatin organization continues to evolve [6-8]. Large-scale strategies are now emerging that include the use of the polymerase chain reaction to map chromatin domains [9] and genome-wide microarray-based analyses [6, 8].

The protocol described below represents the next phase in this evolution to analyze the relative DNase I-sensitivity of genomic sequences in cells of the spermatogenic lineage. To dissect this dynamic mechanism, various cell types along the pathway were surveyed. The method described below evaluates its utility as a function of the response of the different cell types.

MATERIALS AND METHODS

Reagents were purchased from Amresco Inc. (Solon, OH). All buffers and solutions were freshly-prepared excluding Frozen Storage Buffer, FSB [9], composed of 50 mM HEPES buffer pH, 7.5, containing 10 mM sodium chloride, 5 mM magnesium acetate, 25% glycerol. FSB was prepared, and then stored as single use aliquots at -20°C. Single somatic cell suspensions were obtained by mincing freshly dissected whole organs in 10 ml of ice-cold RPMI 1640 (Mediatech, Inc., Hernden, VA) supplemented with 20 mM L-glutamine (Invitrogen Corporation, Carlsbad, CA), 100 U/ml penicillin G, 100 µg/ml streptomycin and 250 ng/ml Amphotericin B (Invitrogen). In brief, the tissue was mechanically disaggregated, with five strokes in a Potter-Elvehjem homogenizer. The resulting suspension was then treated with 0.5mg/ml trypsin (1:300; Invitrogen), 85 U/ml collagenase-type 1 (Worthington Biochemical Corporation, Lakewood, NJ), and 50 U/ml DNase I, at 33°C for 15 min with gentle agitation. The digestion was terminated with the addition of 0.1 mM phenylmethylsulfonyl fluoride and BSA to a final concentration of 0.5%. The resulting suspension was then filtered through a 500 micron pore size 250 micron and finally, 80 micron nylon mesh (Sefar Filtration, Kansas City, MO) to remove clumps of cells. Cells were then centrifuged at 600 x *g* at 4°C for 10 min. The cell pellets were washed twice with ice cold PBS, then filtered through an 80 µm nylon mesh, cell concentration determined then divided into aliquots of approximately 2 x 10^7 cells. Adult liver and spleen single-cell suspensions were then cryogenically preserved in FSB in nitrogen vapor, then stored in liquid nitrogen.

Unit gravity sedimentation is routinely used to isolate cells from the spermatogenic lineage [10-12]. Sexually mature mice yield enriched populations

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of later-stage spermatocytes, round and elongating spermatids [12]. Maturing spermatozoa can be isolated from the epididymides of adult mice. Earlier cell types can be isolated from pre-pubertal mice [10]. Cells recovered, were then cryogenically preserved in FSB and then stored in liquid nitrogen.

Optimization: determining [DNaseI]$_{50}$

Tracking changes in chromatin organization through spermatogenesis requires one to examine several cell types that could each potentially respond differently to nuclease treatment. For example, different cell types could, by virtue of different nuclear envelope constituents [13-15] and amounts of DNA, respond in a unique manner when treated with a nuclease. Accordingly, this protocol employs an initial survey step to determine optimal digestion thereby developing a digestion profile unique to that cell type. The resulting digestion profile can be modeled using an exponential decay function (Fig. 1A) and in turn, used to calculate the [DNaseI]$_{50}$, the concentration of DNase I that will digest 50% of the input DNA template. To determine how discrete regions respond to DNase I digestion, cells are treated with two concentrations of nuclease; the [DNaseI]$_{50}$ and a minimal concentration (0.5 U/million cells). DNA is purified then used for parallel real-time PCR. This method has worked well using fresh or frozen cells.
Aliquots of 1-3 x 10^7 cells frozen in FSB were rapidly hand thawed. Cells were washed in 1 vol. of cold PBS by repeated inversion then pelleted by centrifugation at ~400 x *g*, at 4°C for 5 min. The supernatant was removed and cells were resuspended in 3 ml of cold PBS. To 900 µl of suspended cells, the optimization aliquot, 2.7 ml of ice cold PBS and 400 µl of a 10 x DNase I solution composed of a 500 mM HEPES buffer pH, 7.5, containing 100 mM sodium chloride, 100 mM calcium chloride, 50 mM magnesium acetate, 10 mM dithiothreitol, were added. The remaining cells and the experimental aliquot were reserved on ice. To the optimization aliquot, Nonidet P-40 was then added drop-wise to a final concentration of 0.2% and then mixed by inversion. Nuclei were permeabilized by incubation on ice for 5 min then pelleted as before. After centrifugation nuclei were resuspended in 1.3 ml of 1 x DNase I buffer and concentration determined using a haemocytometer prior to partitioning into six-200 µl aliquots of 0.5-1.5 x 10^7 cells/ml. Nuclei were then incubated for 10 min, at 15°C. The amount of DNase I added was standardized to the number of cells in each aliquot (Fig. 1A). Each 200 µl aliquot was treated with increasing amounts of DNase I for 5 min, at 15°C. DNase I digestion was terminated following the addition of three volumes of binding solution, composed of a 10 mM Tris-HCl buffer pH, 8.0, containing 6 M Guanidine-HCl, 1mM EDTA pH, 8.0. The resulting suspension was incubated at room temperature for 5 min.
DNA isolation was accomplished using Quantum Prep, a silicaceous earth-based matrix, (Bio-Rad, Hercules, CA). The Quantum Prep matrix was added to the terminated reaction and the DNA was isolated according to the manufacturer's protocol. A 1.5-fold excess of matrix was added assuming that 10^6 diploid

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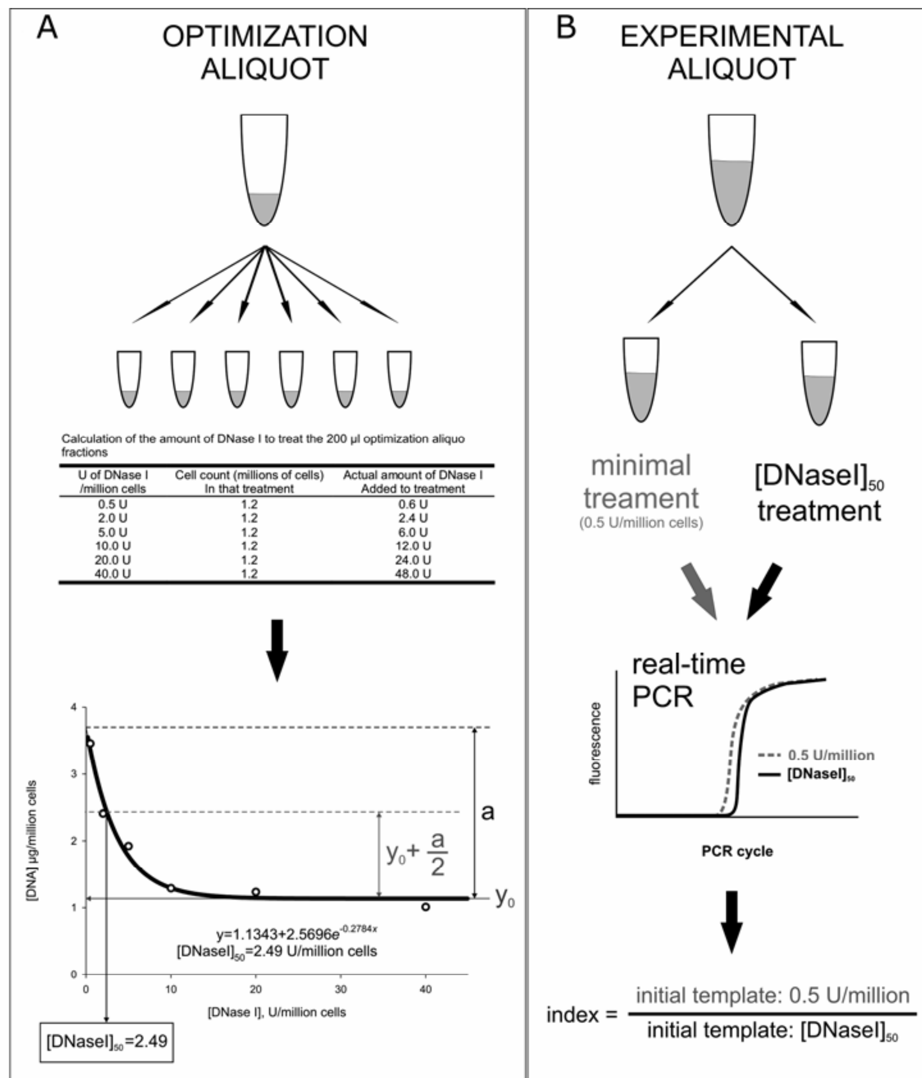

Fig. 1. Optimization and experimental steps to using controlled DNase I digestion to survey chromatin structure. A - Optimization aliquot of cells is divided into six equal fractions, treated with increasing concentrations of DNase I, at 15°C for 5 min. The digestion is modeled by the exponential relationship, $y = y_0 + ae^{-bx}$, where y_0 is the lower asymptote and a represents the rise from the asymptote when $x = 0$. The concentration of DNase I that will digest 50% of the input template, the $[DNaseI]_{50}$, can thus be determined. B - The remaining experimental aliquot is partitioned in half and treated at two DNase I concentrations, a minimal treatment (0.5 U/million cells) and the $[DNaseI]_{50}$ concentration. The resulting DNAs are used for parallel real-time PCR. Indices of sensitivity are determined as ratios of initial template calculated for the minimally-treated sample compared to the initial template calculations for the $[DNaseI]_{50}$-treated sample.

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mammalian cells contain ~7 µg of DNA. The DNA was bound to the matrix at room temperature by inversion then pelleted in a microcentrifuge. The matrix containing DNA was washed twice with 1 ml of wash solution, of 20 mM Tris-HCl buffer pH, 7.5, containing 2 mM EDTA pH, 7.5, and 100 mM NaCl, 50% ethanol. Subsequent to the second wash, the pellet was air-dried for 5 min. DNA was then eluted in 1 matrix-volume of TE buffer pH, 8.0, by suspension then incubation at 72°C for 5 min, prior to centrifugation to separate the released DNA. This was repeated, eluates combined then DNA yield determined per aliquot [16].

The relationship between DNA yield and DNase I concentration resolved as an exponential decay curve (Fig. 1A). The $[DNaseI]_{50}$, the point at which 50% of the DNA is digested, was calculated by solving the decay relationship, $y = y_0 + ae^{-bx}$, for x when $y = (y_0 + a/2)$. This concentration of DNase I was then used to treat half of the remaining experimental aliquot cells (Fig. 1B). The $[DNaseI]_{50}$ ranged from 1.99 ± 0.3 U/million cells for round spermatids to 14.6 ± 0.9 U/million cells for splenocytes. The other half of the experimental aliquot was treated with a minimal amount of DNase I (0.5 U/million cells; Fig. 1B).

DNase I-sensitivity: treating the experimental aliquot of cells

To the remaining Experimental Aliquot of cells, 5.1 ml of ice cold PBS and 800 µl of 10 x DNase I buffer were added, nuclei permeabilized with NP40 then recovered as described (Optimization). The permeabilized nuclei were resuspended in 1 ml of 1 x DNase I buffer and then divided into two equal 500 µl aliquots. The nuclei were incubated at 15°C for 10 min, then one aliquot was treated at $[DNaseI]_{50}$, and the other aliquot subjected to minimal DNase I treatment at 0.5 U/million cells for 5 min at 15°C. The reactions were terminated and the DNA isolated.

Real-time polymerase chain reaction

The minimally- and $[DNaseI]_{50}$-treated pools of Experimental Aliquot DNA were used in parallel real-time SYBR-green PCR assays. Custom oligonucleotide primers were designed using Oligo 6.0 Primer (Molecular Biology Insights, Cascade, CO). Primers varied from 18-25 bp in length, with a G + C-content of approximately 50%, possessing annealing temperatures that ranged from 55-60°C targeted to amplicons of 100-500 bp in length. The regions interrogated included the mouse protamine gene cluster, *Prm1*→*Prm2*→*Tnp2*, as well as mouse β-Actin, *Actb* and mouse β-globin *Hbb* (Tab. 1). Each 20 µl PCR contained 2-5 ng of DNA template as summarized in Tab. 2. Real-time quantitative PCR was carried out for 55 cycles using the Chromo4 real time PCR detection system (Bio-Rad). An index of DNase I sensitivity was calculated as the initial template from minimally treated nuclei divided by the initial template from $[DNaseI]_{50}$-treated nuclei (Fig. 1B). To enable comparison, indices of sensitivity were also calculated using $2^{\Delta Ct}$, where the ΔC_t was difference in cycle threshold between minimally- and $[DNaseI]_{50}$-treated nuclei.

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Tab. 1. Oligonucleotide primer pairs used for PCR.

Region surveyed	Oligonucleotide sequence	Amplicon size	T_{READ} [°C]*
Prm1	fwd 5' TCCTGGTCCTCTTTGACTTCATAAT 3' rev 5' ATCTGCTCCTGCTTTTGCTG 3'	211 bp	85
Prm2	fwd 5' GAGGCCATCTCACATTCAATA 3' rev 5' GCTCCTCATTCGGTAGCG 3'	323 bp	85
Tnp2	fwd 5' GTGTCCATTTGATCCCAA 3' rev 5' AAAGCTGCTGACTCAAGACTA 3'	142 bp	80
Actb	fwd 5' ATGTCACGCACGATTTCCC 3' rev 5' AACACCCCAGCCATGTACG 3'	254 bp	87
Hbb	fwd 5' CAACCAGCAGCCTAAAAAG 3' rev 5' ACTCACAACCCCAGAAACAG 3'	243 bp	84

Prm1, *Prm2* and *Tnp2* survey the three genes of the mouse protamine domain, β-Actin (*Actb*) and β-Globin (*Hbb*). * Read temperature determined as previously described [17].

Tab. 2. Conditions and reagents used for polymerase chain reaction.

PCR reagents	Volume per reaction [µl]	Final concentra-tion	Cycling conditions
HotStarTaq (Qiagen)	0.2	1.0 U	1. 95°C hot start, 15 min
10 x PCR Buffer (Qiagen)	2.0	1.0 x	2. 94°C denature, 30 s
SYBR Green (Invitrogen)	2.0	0.25 x	3. 56°C anneal, 30 s
dNTPs (10 mM ea.; Invitrogen)	0.4	0.2 mM	4. 72°C extension, 30 s
Oligonucleotide primers	1.0	0.5 µM, each	5. T_{READ}, 10 s
Nuclease-free water	12.4		6. fluorescence read
			7. go to step 2 (55 cycles total)
Minimally digested or $[DNaseI]_{50}$-digested DNA (diluted 3-fold in water)	2.0		8. melting curve
			9. 4°C hold
Total volume	20		

KLab-PCR: analysis of Real-time PCR data

Quantitative approaches to real time PCR typically utilize standards containing a known amount of input DNA. Often a PCR fragment cloned into a vector is used as a means to quantify how much product is generated at a given cycle threshold (C_t). The assumptions and related degrees of error associated with this approach are compounded when differences in amplification efficiency brought about by different template sequences [18] and DNA isolation methods [19] are considered. The differences in amplification efficiency inherent with different primer sets and targets suggested that we revisit C_t analysis.

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It can be assumed that PCR amplification becomes hindered by resource depletion, degradation of polymerase activity and the accumulation of competitive elements. Modeled in equation (1), the reaction efficiency E_{max} is systematically reduced by G such that it rapidly departs from exponential amplification and ultimately plateaus. Thus each element that hinders the reaction contributes to G relative to T_n, the total template produced during the previous amplification cycle.

$$T_{n+1} = T_n\left(E_{\max} - G(T_n)\right) \quad (1)$$

Given an estimate of E_{max}, G is:

$$G\left(T_n\right) = E_{\max} - \frac{T_{n+1}}{T_n}$$

Ideally, each PCR yields a doubling of product at each cycle, and thus E_{max} will be 2 and $T_n + 1/T_n$ will remain 2 at every cycle, hence G will be constant and zero. However, G cumulatively approaches 1 with each cycle, during which time the reaction deviates from its exponential rate and is eventually quenched. From the moment that competing factors make G detectable, log-linear modeling [17, 20] of real-time PCR analysis is compromised. For any given reaction E_{max} is ~1.7 and thus G is greater than 0 even during the initial cycles when the fluorescence signal is rising above background. Hence a log linear model is limited by the background signal. KLab-PCR models G as a power series. E_{max} is modeled using an initial curve fitting procedure in which a simple non degenerate G is used. The initial estimate of G is then refined by employing higher order series elements to model the PCR amplification. An estimate of initial fluorescence, T_0, is then calculated, corresponding to the amount of initial DNA template.

A test data set was created to assess the veracity of the KLab-PCR analysis method. The *Prm2* amplicon was interrogated by real-time PCR (Tabs. 1 and 2), using a concentration range of 5 pg to 3 ng of mouse genomic DNA. The reaction data were analyzed using KLab-PCR and C_t. C_t analysis was carried out using the Opticon Monitor Real-time detection software (v.3.1; Bio-Rad). The baseline was calculated from the first fifteen cycles, and the threshold of detection was set to twice the standard deviation over the same cycle range. To directly compare the results obtained by C_t analysis to KLab-PCR, the values obtained from C_t analysis were transformed. This assumed that the gain in fluorescence (F) varied directly with initial template (T_0) as a function of the product generated at threshold C_t, assuming a doubling at each cycle; $F = T_0 \cdot 2^{Ct}$. One can then calculate the amount of initial template when F equals the threshold. A dose response of the mouse *Prm2* promoter was generated by both methods of analysis; producing a linear relationship over three orders of magnitude (Fig. 2). A five-fold series should produce a linear relationship with a slope of five. The KLab-PCR method yielded a slope of 5.3 whereas the C_t approach yielded a slope of 10.5; an overestimation by a factor of two. This over-estimation is likely to reflect the inability of C_t to consider differences in

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amplification efficiency (E) as a doubling (E_{max} = 2) between cycles is assumed. Given that the observed E_{max} is ~1.7, fold-change calculated as $2^{\Delta Ct}$ would yield a consistent over-estimate.

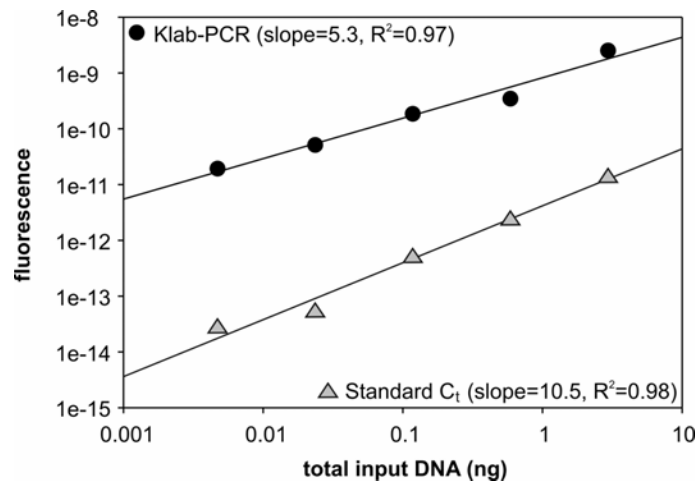

Fig. 2. Comparison of real-time PCR analysis methods. A region in the *Prm2* promoter was interrogated by real-time PCR using serially diluted mouse genomic DNA. Fluorescence data was analyzed by the KLab-PCR algorithm as well as standard C_t approaches.

RESULTS AND DISCUSSION

To test this modified protocol, three regions within the mouse protamine domain as well as a constitutively sensitive region, *Actb*, and constitutively insensitive region, *Hbb,* were interrogated by real-time PCR in round spermatids, the cell type in which the cluster is expressed and splenocytes, a somatic cell lineage in which the protamine cluster is not expressed. Each cell type demonstrated a varying sensitivity to DNase I digestion. Indices of sensitivity were computed using both KLab-PCR and standard C_t approaches. As shown in Tab. 3, the mouse protamine cluster is approximately 4-5 fold more sensitive in round spermatids than in somatic cells, as previously reported [21]. *Actb*, a constitutively expressed housekeeping gene was DNase I-sensitive, but in both germ cells and somatic tissue. As expected, since *Hbb* expression is restricted to the erythroid lineage this region remained DNase I-insensitive in all cell types investigated. The control regions were digested to a similar extent in both cell types even though the amount of DNase I used for spleen cells was greater than round spermatids (Tab. 3). This likely reflects differences in permeability since the lamin content of nuclei of spermatogenic lineage cells is stage-dependent [13-15]. Thus, the $[DNaseI]_{50}$ method compensated for the differential susceptibility to nuclease treatment observed in the round spermatid and spleen cell response.

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Tab. 3. The mouse protamine cluster is in a DNase I-sensitive conformation in the round spermatids, but remains insensitive in somatic tissues.

$[DNaseI]_{50}$	Round spermatids 1.99 ± 0.3 U/million cells		Splenocytes 14.6 ± 0.9 U/million cells	
	Indices of DNase I sensitivity			
	C_t	KLab-PCR	C_t	KLab-PCR
Prm1	4.12 ± 1.47	4.06 ± 0.84	1.16 ± 0.53	1.21 ± 0.09
Prm2	5.54 ± 1.90	4.41 ± 0.58	1.36 ± 0.12	0.94 ± 0.17
Tnp2	4.13 ± 1.02	3.77 ± 1.24	1.69 ± 0.77	1.49 ± 0.04
Actb	3.25 ± 0.96	4.83 ± 0.96	6.37 ± 1.44	4.04 ± 0.27
Hbb	0.81 ± 0.08	0.81 ± 0.08	1.20 ± 0.12	1.01 ± 0.17

Values are mean indices of sensitivity to DNase I ± 1 standard deviation of the mean ($n = 4$ for round spermatids and $n = 2$ for splenocytes). KLab-PCR values were calculated using initial template estimates derived from a novel real-time PCR analysis algorithm; whereas C_t values were computed using standard C_t approaches.

This assay can be readily scaled enabling one to scan large chromosomal regions to define chromatin domains like the multi-genic protamine domain [22]. As the depth of coverage increases, i.e., the number of regions interrogated per unit length, DNase I- hypersensitive sites are resolved. These often exhibit lower initial template values. This reflects their susceptibility to cleavage as they are frequently devoid of nucleosomes and other chromatin proteins. They are often orders of magnitude more sensitive than insensitive regions (index ≈ 1).

The quantitative nature of real-time PCR renders it a powerful tool. This can be compromised by analyses that introduce subjectivity and error. Automation of the KLab-PCR algorithm provides a means of rapid analysis for multiple samples without the inherent subjectivity of the threshold selection while considering the kinetics of the PCR. The KLab-PCR algorithm quickly identifies reactions that failed to amplify as expected, or that display markedly different kinetics from other replicates. This feature is useful for high-throughput analysis.

The strategy described above scales well. For example, as the depth of coverage of a given region increases the likelihood of identifying DNase I-hypersensitive sites often associated with trans-acting factors and promoter associated regions increases [6, 22]. Identifying the extent of the region of chromatin that adopts a sensitive conformation provides key regulatory information facilitating the identification of chromatin domains and regulatory sites of interaction.

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