Culturing Human Pluripotent Stem Cells: Influence of Diverse Culture Conditions on Genomic Stability

Marianne P. Henry [1,2*], Jennifer Man[1], Craig Nowell[1], J. Ross Hawkins[2], Jennifer Boyle[2] and Joanna M. Bridger [1*]

[1] Laboratory of Nuclear and Genomic Health, Division of Biosciences, Centre of Genome Engineering and Maintenance, Department of Life Sciences, College of Health, Medicine and Life Sciences, Brunel University London, London UB8 3PH, UK

[2] Advanced Therapies Division, National Institute for Biological Standards and Control (NIBSC), Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK

* Correspondence: marianne.henry17@gmail.com; joanna.bridger@brunel.ac.uk

Abstract: Maintaining and analyzing the genomic health of stem cells is a priority, asstem cell therapies are expected to become commonplace clinical treatments for many illnesses and ailments, including natural ageing. Allogeneic stem cell therapies employed will require expansion in culture and then subsequent analysis of their genome complement and behavior. But to determine if they are safe to use, even with a certain level of genomic abnormalities or dis-organization, *in vivo* data is required from such samples. Any health issues that arise for the organism would need to be . In this pilot study, we have grown human pluripotent cells on four commercially available matrices for stem cells in combination with three commercially available stem cell growth media. The study has focused on using ddPCR™ to detect changes in copy number of a gene on chromosome X, *AMELX,* and a gene on chromosome 4, *ALB,* as well as the positioning of these two gene loci in interphase nuclei. The data demonstrate that the growth media can influence the level of aneuploidy and that major differences in gene loci positioning can occur when cells are grown in different media and matrix combinations.

Keywords: human pluripotent stem cells; genomic instability; cell culture conditions, interphase, aneuploidy, gene positioning

Abbreviations:
BAC – Bacterial artificial chromosome
DAPI - 4′,6-diamidino-2-phenylindole
dPCR – digital polymerase chain reaction
ddPCR – droplet digital polymerase chain reaction
ESC – embryonic stem cells
FISH – fluorescence in situ hybridization
HDF – human dermal fibroblasts
hESC – human embryonic stem cells
hPSC – human pluripotent stem cells
RIN – RNA integrity number
UKSCB – UK stem cell bank

Introduction

The isolation and successful cultivation of human pluripotent stem cells (hPSCs) in 1998 marked a seminal breakthrough in regenerative medicine [Thomson et al., 1998, Shamblott et al., 1998]. Subsequent research has elucidated essential growth factors, such as fibroblast growth factor (FGF) and leukemia inhibitory factor (LIF), along with optimized culture conditions, including substrates and extracellular matrix components, crucial for maintaining hPSC pluripotency [Williams et al., 1988, Smith et al., 1988, Braam et al., 2008, Rodin et al., 2010]. Simultaneously, investigations have underscored the importance of genomic stability within prolonged hPSC culture, addressing

concerns regarding the accumulation of genetic aberrations [Taapken et al., 2011, Henry et al., 2019, Hussein et al., 2011]. These dual imperatives, sustaining pluripotency and minimizing genomic instability, constitute the cornerstone of advancements in human pluripotent stem cell culture, promising transformative possibilities in regenerative medicine and tissue engineering. Notably, the stringent regulatory requirements necessitating Good Manufacturing Practice (GMP)-compliant cell culture components have constrained the use of many products, thereby presenting limitations and challenges in achieving optimal culture conditions.

With emerging practices for the upscaling of hPSCs, novel methods are required to monitor and standardize the use and production of cellular materials intended for therapeutic use [O'Shea et al., 2020, Sullivan et al., 2018, Yuan et al., 2015]. Despite the increasing availability of novel techniques to increase the yield and/or quality of cells, the quality control for these cells remains unchanged; karyology, flow cytometry, differentiation potential and gene expression studies typically make up the characterization of hPSCs. More sensitive and high-throughput methods need to be developed to understand how the novel techniques may affect the potential end-products. For example, despite the advancement in the large-scale production of human pluripotent stem cells, the low resolution and insensitive karyotyping method, G-banding, still remains the gold standard for karyology. Similarly, the use of methods, such as qPCR for observing gene expression remains a widely used method for stem cell pluripotency characterization, despite the need for standard curves for relative quantification. Digital PCR (dPCR) is a method utilizing partitioning for the absolute quantification of target molecules, this improving the overall sensitivity of the assay [Hindson et al., 2011, Quan et al., 2018]. The development and use of novel analytical tools are essential for the characterization of human pluripotent stem cells (hPSCs) while also being practical for widespread use across different laboratories. Additionally, high-throughput methods are necessary to accurately screen hPSCs, determining both their pluripotency and genomic stability.

Chromosomes are organized into distinct compartments called chromosome territories [Cremer and Cremer 2001], a structural organisation conserved across many species [Marshall et al., 1996, Bridger and Bickmore 1998, Parada and Misteli 2002, Tanabe et al., 2002, Foster et al., 2010] and is influenced by gene density and guanine-cytosine (GC) content [Cremer and Cremer 2001, Dietzel et al., 1998, Visser et al., 1999, Bridger et al., 2000, Federico et al., 2006, Meaburn et al., 2007a], transcription activity [Volpi et al., 2000, Mahy et al., 2002a, Mahy et al., 2002b], cellular differentiation [Parada et al., 2004, Wiblin et al., 2005, Meshorer and Misteli 2006, Szczerbal et al., 2009] and can undergo alterations during infection [Knight et al., 2011, Arican-Goktas et al., 2014], upon serum removal [Mehta et al., 2010] and in early stages of disease states [Cremer et al., 2003, Meaburn et al., 2007b Meaburn and Misteli 2008, Meaburn et al., 2009, Leshner et al., 2016, Meaburn et al., 2016, Mehta et al., 2011]. Given the latter observation, exploring impact of various cell culture system compositions on gene positioning in stem cells bears the potential to unveil significant changes in the growth of human pluripotent stem cells, notably concerning the emergence of genomic instability, such as aneuploidy formation.

In this study, we have concentrated on employing two methodologies to assess human pluripotent stem cell cultures. Droplet Digital PCR™ (ddPCR™) was used to quantify potential aneuploidies arising in the hPSC population, and fluorescence *in-situ* hybridization (FISH) was used to reveal the specific radial nuclear location of genes within the cells as they progressed through the culture timeline.

2. Materials and Methods

Cell Culture

Human embryonic stem cell line, H9 (WiCell), was expanded from a single vial, originating from passage 29 of a master cell bank. Initial culture establishment involved five passages on inactivated human dermal fibroblasts (HDFs). Subsequently, the cell line was maintained through 12 different combinations of media and matrices, as per manufacturer guidelines (summarized in Table 1). Samples were systematically collected at each passage for flow cytometry, G-banding, embryoid

body (EB) formation, and qPCR analyses. Enzymatic passaging was conducted using TrypLE Express Enzyme™ (ThermoFisher Scientific), and cells were grown in standard 6-well plates in a 5% CO2 atmosphere at 37^{o}C. Culture medium was replenished every 2-3 days, and cells were passaged weekly at a 1:6 ratio, ensuring controlled and reproducible culture conditions for subsequent analyses. Quality control (QC) of the starting material before the beginning of this study was performed by the UK Stem Cell Bank (UKSCB) to ensure that the cells were karyotypically normal (G-banding of 20 metaphase spreads) and pluripotent (cell surface marker expression via flow cytometry and gene expression via qPCR). Ethical approval was granted for both addresses. Lymphoblastoid cells were cultured in RPMI-1640 medium (Sigma, UK) supplemented with 10% (v/v) Foetal Calf Serum (FCS; Sigma) and 5% (v/v) L-glutamine (Sigma) in a 5% CO2 atmosphere at 37^{o}C. Medium was changed approximately every four days and the cells were split once a week.

Table 1. List of media and matrices used in the study

Media	**Code**	**Matrix**	**Code**
Essential 8 (ThermoFisher Scientific)	A	Matrigel (Corning).	1
NutriStem XF/FF (Sartorius)	B	Laminin 5-1-1 (BioLamina)	2
mTesR2 (STEMCELL Technologies)	C	Laminin 5-2-1 (BioLamina)	3
		Vitronectin XF (STEMCELL Technologies)	4

Nucleic Acid Extraction

For DNA extraction, the GenElute DNA Extraction Kit (Merck) was utilized according to the manufacturer's instructions, and DNA quantification was performed using the Qubit 2.0 Fluorometer (ThermoFisher Scientific). Sample purity was assessed using the Nanodrop ND-1000 (ThermoFisher Scientific), while DNA integrity was determined through analysis on a TapeStation Bioanalyzer (Agilent Technologies) or agarose gel electrophoresis.

RNA extraction was conducted using the Maxwell® RSC simplyRNA Cells Kit in conjunction with the Maxwell® RSC Instrument (Promega), following the manufacturer's protocols. Quantification and assessment of RNA quality were achieved using the QIAxpert instrument (Qiagen). To ensure high-quality RNA, a minimum RNA Integrity Number (RIN) value of 8 was verified by resolution on the TapeStation Bioanalyzer.

Droplet Digital PCR™

The QX200 ddPCR™ system with the AutoDG system (BioRad) was employed for ddPCR™ experiments in strict accordance with the manufacturer's instructions. Genomic stability monitoring was performed using DNA extracted from each passage of the various cell lines. All ddPCR™ data were extrapolated from the Quantasoft software Version 1.7.4.0917 (BioRad); the thresholds for the ddPCR™ plots were set manually.

Two wild-type female lymphoblastoid cell lines, FCWES01 and FCWES02, with normal karyotypes were used as controls for the normalization in the aneuploidy quantification (see Meaburn et al., 2005). For the statistical analysis of significance from the diploid control cell lines, a one sample t-test was used; *p* values and symbols for the statistical significance are shown in Table 2.

Table 2. *P* Values and Symbols for Statistical Significance

P value	Result	Symbol
< 0.001	Extremely significant	***
0.001 to 0.01	Very significant	**
0.01 to 0.05	Significant	*
≥ 0.05	Not significant	ns

All primers were HPLC-purified (Integrated Device Technology), while TaqMan® MGB (minor groove binder) probes, equipped with a 5' reporter and a 3' non-fluorescent quencher (NFQ), were procured from ThermoFisher Scientific. The ddPCR™ Supermix for Probes or ddPCR™ Supermix for Probes (no UTPs) served as the master mix (BioRad). For our duplex assay, *AMELX* and *AMELY* genes [George et al., 2013] were chosen, targeting chromosomes X and Y, respectively. Additionally, *ALB* was selected as the reference gene due to its location on a stable chromosome (Chromosome 4) in hESCs. It is worth noting that aberrations have been reported in all chromosomes of hPSCs, except for chromosome 4 [Amps et al., 2011]. Notably, the region of the gene subjected to PCR amplification was not identified as a "micro-instability" according to the Supplementary Data from the International Stem Cell Initiative in 2011, where this information was obtained. The primer concentration was set at 900µM, probes at 250µM, and the master mix at 2X, with each reaction containing 40ng of DNA. The sequences of the primers and probes are provided in Table 3.

Table 3. ddPCR™ primer and probe sequences.

	Sequence
AMELX Forward Primer	CCCTGGGCTCTGTAAAGAATAGTG
AMELX Reverse Primer	CAGGCTTGAGGCCAACCAT
AMELX Probe	ATCCCAGATGTTTCTCAA
ALB Forward Primer	GCTGTCATCTCTTGTGGGCTGT
ALB Reverse Primer	AAACTCATGGGAGCTGCTGGTT
ALB Probe	CCTGTCATGCCCACACAAATCTCTCC

Prior to their utilization, all primers and probes for individual genes, as well as combinations of genes in duplex reactions, underwent rigorous testing on a temperature gradient to ascertain a distinct demarcation between positive and negative signals. A temperature of 59^{o}C was identified as the optimal threshold for DNA annealing and elongation in all duplex reactions. The ddPCR™ thermocycling conditions, as detailed in Table 4, were subsequently employed for our experiments.

Table 4. ddPCR™ Thermocycling Conditions

	Temperature (^{o}C)	Time (Minutes)	No. of Cycles
Step 1	95	10	1
Step 2	95	0.2	39
Step 3	59	1	
Step 4	98	10	1
Step 5	12	Hold	

The H9 cells underwent evaluation using ddPCR™ to detect changes in the *AMELX* to *ALB* ratio, serving as a marker for X chromosome aneuploidy. To facilitate data analysis, the samples were categorized into three groups based on their respective culture media: mTeSR2, NutriStem XF/FF, and Essential 8

Fluorescence In-Situ Hybridisation

Cell samples were treated with hypotonic solution (0.075M KCl) for 15 minutes at room temperature, followed by fixation in ice-cold methanol and acetic acid (3:1 vol/vol), repeated 5-7 times. Cells were aged on SuperFrost slides (ThermoFisher Scientific), dehydrated with ethanol, and subjected to probe or DNA paint application. After a 2-day incubation, slides were washed, blocked,

and incubated with labeled probes. Following probe hybridization, slides underwent stringent washes, were counterstained with DAPI, and covered with 22 x 40 coverslips.
For gene-specific FISH, Bacterial Artificial Chromosomes (BACs obtained from ThermoFisher Scientific, Clone IDs RP11.580P21 and RP11.121K9 for *ALB* and *AMELX*) were grown on LB agar with chloramphenicol (Merck), and isolated using an alkaline lysis method. The BACs were tagged with fluorescent labels (nick translation with biotin or digoxygenin), and then used as probes after precipitation and denaturation. Hybridization was performed with a hybridization buffer/mix, and slides were washed, counterstained, and mounted with coverslips [Godwin et al., 2021].

Image Capture and Analysis
Nuclei were observed at 100x magnification using immersion oil on either the Olympus BX41 or Leica DM 4000 immunofluorescence microscope. Manual settings were used for fixed time exposure. Image capture was facilitated by "SmartCapture 3.0" for the Olympus microscope (Digital Scientific) and "LAS AF" for the Leica microscope (Leica Geosystems), with approximately one hundred images captured per slide.
For quantifying the spatial distribution of gene loci within interphase nuclei, a bespoke positioning script (see Clements et al., 2016) was utilized. Nuclei were segmented into five concentric shells of equal area radiating from the nuclear periphery (Shell 1) to the nuclear interior (Shell 5). The software assessed signal intensities for both DAPI (DNA) and the target chromosome or gene within each shell; for normalization of the data the intensity of the gene loci signals was divided by the intensity of the DAPI signal. A minimum of 50 nuclei were analyzed per samples; a Kruskal-Wallis test in combination with Dunn's multiple comparisons test was then performed to compare each of the samples' shells' intensities to the lymphoblastoid cells; *p* value threshold set are shown in Table 2.

Results
ddPCR™ for assessing AMELX copy number alternations in pluripotent H9 cells exposed to different media matrix combinations

Understanding genomic instability, as well as the karyotype and genome status of cultured and expanded stem cells is crucial for their potential use in clinical therapies. These analyses include assessing the levels of potential aneuploidies present in samples. Thus, initially this investigation centered on assessing the *AMELX* to *ALB* ratio within each sample collected (Figure 1A). This is because the sex chromosomes X and Y are frequently found to be aneuploid in stem cells, however *ALB* representing chromosome 4 is the most stable. When H9 cells were cultured in Medium A, no significant differences in the number of *AMELX* copies were observed between the test samples and the diploid female control. Additionally, no discernible variations were noted across the different timepoints or when different matrices were utilized in conjunction with Medium A.

Concerning H9 cell cultures with Medium B, significant differences were evident between the control and the Medium B cultured samples across the multiple matrices and timepoints (Figure 1B). The only combination that did not demonstrate a statistically significant difference from the control was Medium B-Matrix 3 (at time point a). However, in subsequent passages (b and c), this combination depicted a decrease in *AMELX* copy numbers to 82.7% and 78.77%, respectively (calculated from the *AMELX* to *ALB* ratio). Similar reductions in *AMELX* copy numbers, approximately 60% and 90%, were observed in the Matrix 1 and 2 combinations, respectively. Conversely, the Matrix 4 combination exhibited an increase in *AMELX* of approximately 5% across a single passage.

In the case of H9 cell cultures with Medium C, significant differences were noted in seven out of nine combinations when compared to the control samples (Figure 1C). Notably, the sole exceptions, Medium B-Matrix 1 (at time point b) and Matrix 3 (at time point c), did not display statistical

significance in comparison to the control. Interestingly, in contrast to the Medium B samples, a gradual increase in *AMELX* copy numbers was observed across passages in all matrix combinations. However, this increase in *AMELX* copy numbers ranged from approximately 1% to 5%.

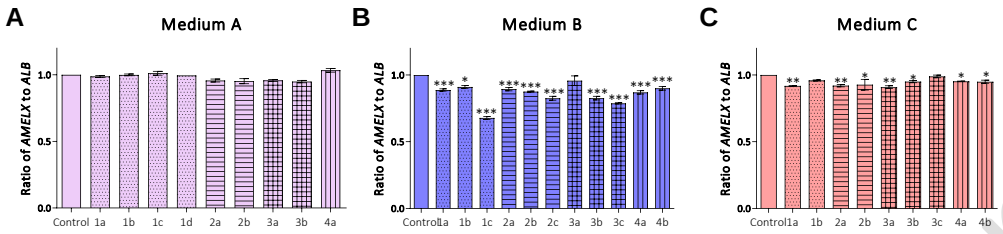
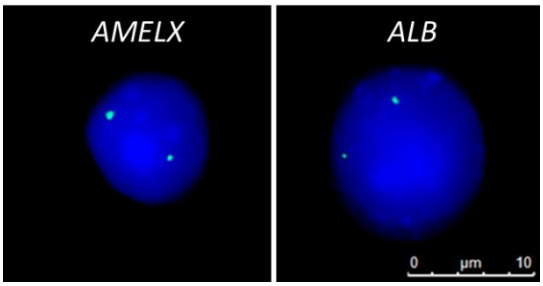


Figure 1. Relative *AMELX* Copy Number Measurements via ddPCR™ in H9 Cells Grown in Different Media-Matrix Combinations
ddPCR™ results for the ratio of *AMELX* to *ALB* are shown for each sample. H9 cells were grown in different media (A-C) and matrix (1-4) combinations, with multiple timepoints collected (a-d). A t-test was performed for each sample collected against the control diploid samples. Both control and the samples were analyzed in triplicate; error bars represent the standard error of the mean.

Exploring gene locus radial positioning in H9 human pluripotent stem cells under different combinations of media and matrices using fluorescence in situ hybridization and image analysis

The spatial location of a gene within interphase nuclei has been shown to be correlated with regulation of its expression in a plethora of studies. To map *ALB* and *AMELX* in H9 cells, we performed 2-dimensional fluorescence *in-situ* hybridization (FISH) for the *ALB* gene located on chromosome 4 and the *AMELX* gene found on the X chromosome (Figure 2). For the *ALB* gene, an examination of nine distinct media-matrix combinations revealed a notable variance in its positioning within interphase nuclei (Figure 3). A significant deviation was identified primarily within the nuclear interior in contrast to the lymphoblastoid samples where the *ALB* gene predominantly resided in the second shell of the nuclei at the nuclear periphery. This shift towards the nuclear interior was observed in five out of the nine test combinations. Among these, the most substantial relocation of the *ALB* gene to the nuclear interior occurred in the Medium B-Matrix 3 combination. Interestingly, this relocation did not exhibit a consistent pattern associated with specific media or matrices in the other experimental conditions. Conversely, the Medium C-Matrix 2 combination showcased a gene positioning most akin to the control cells, lacking significant differences across all five nuclear shells.

Figure 2. *AMELX* and *ALB* Nuclear Positioning
Gene loci positioning in diploid female lymphoblastoid cells. Cells were subjected to fluorescence in-situ hybridization displaying *AMELX* and *ALB* gene loci in green and nuclear DNA stained with DAPI in blue. Scale bar is 10 µm.



Similarly, the positioning of the *AMELX* gene in the lymphoblastoid samples reflected a peripheral localization within the nuclei. Only three out of the nine media-matrix combinations demonstrated a significant deviation from the lymphoblastoid samples (Figure 4). Despite a lower statistical deviation in the positioning of the chromosome X gene (*AMELX*), distinct patterns emerged within the media-matrix combinations, unlike the observations with *ALB* positioning. Notably, a consistent positioning of the gene in shell 1 in the peripheral region was evident across all cells cultured on Matrix 2, irrespective of the media used. Furthermore, three out of the four combinations cultured with Medium C displayed a similar positioning of *AMELX* at the periphery, apart from when grown on Matrix 3, where the loci were positioned internally. Conversely, with Medium B, all combinations exhibited some form of bimodal distribution in the gene's positioning, meaning that both gene loci were in different nuclear locations in a population of cells or one gene at the periphery and the other in the interior in all the cells.

Figure 3. *ALB* Gene Loci Nuclear Positioning in Different Media-Matrix Combinations

Cell line H9 was cultured under different combinations of media (A-C) and matrix (1-4) combinations. On average, positioning is shown in 80 nuclei for each sample combination using a bespoke erosion analysis script. A Kruskal-Wallis test, in conjunction with Dunn's multiple comparisons test, was performed on the intensity of each of the samples' shells compared to the lymphoblastoid cells. Significant differences are indicated by asterisks (*) and the standard error of mean is depicted as the error bars.

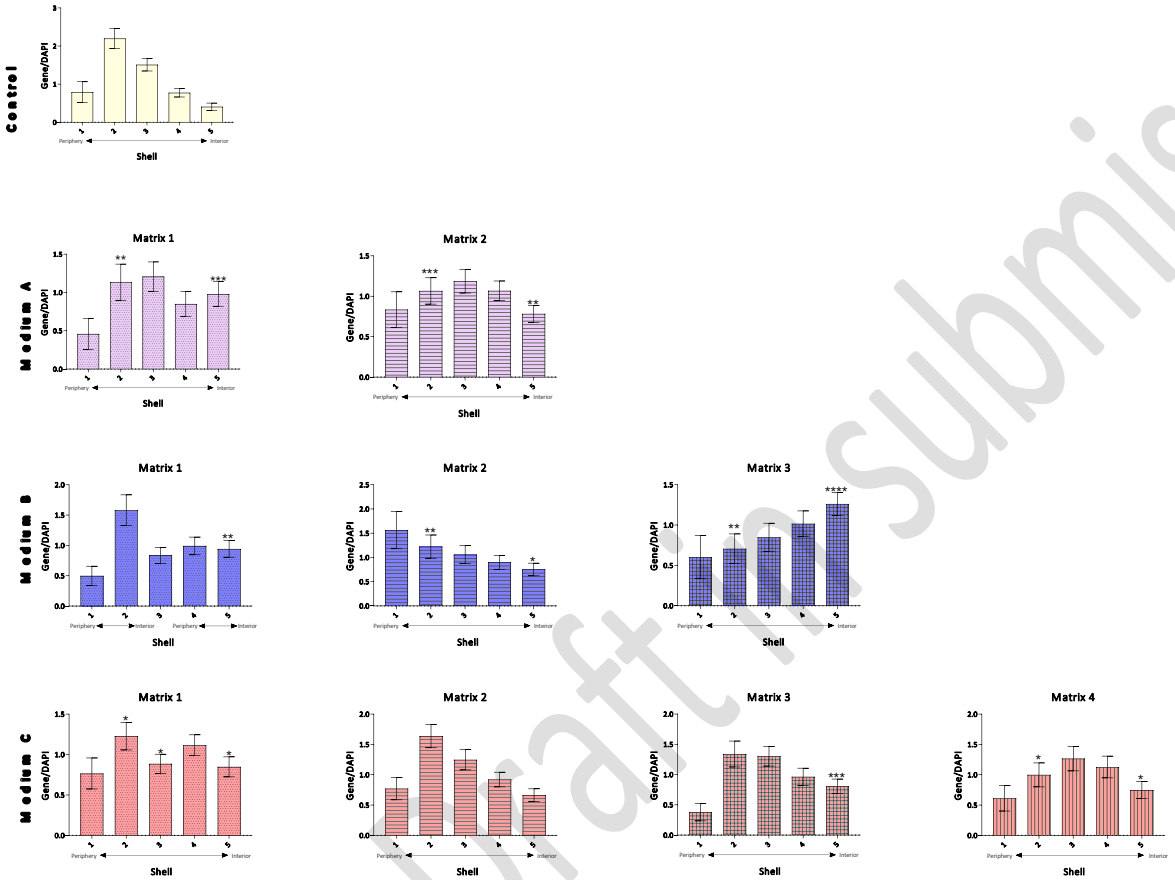
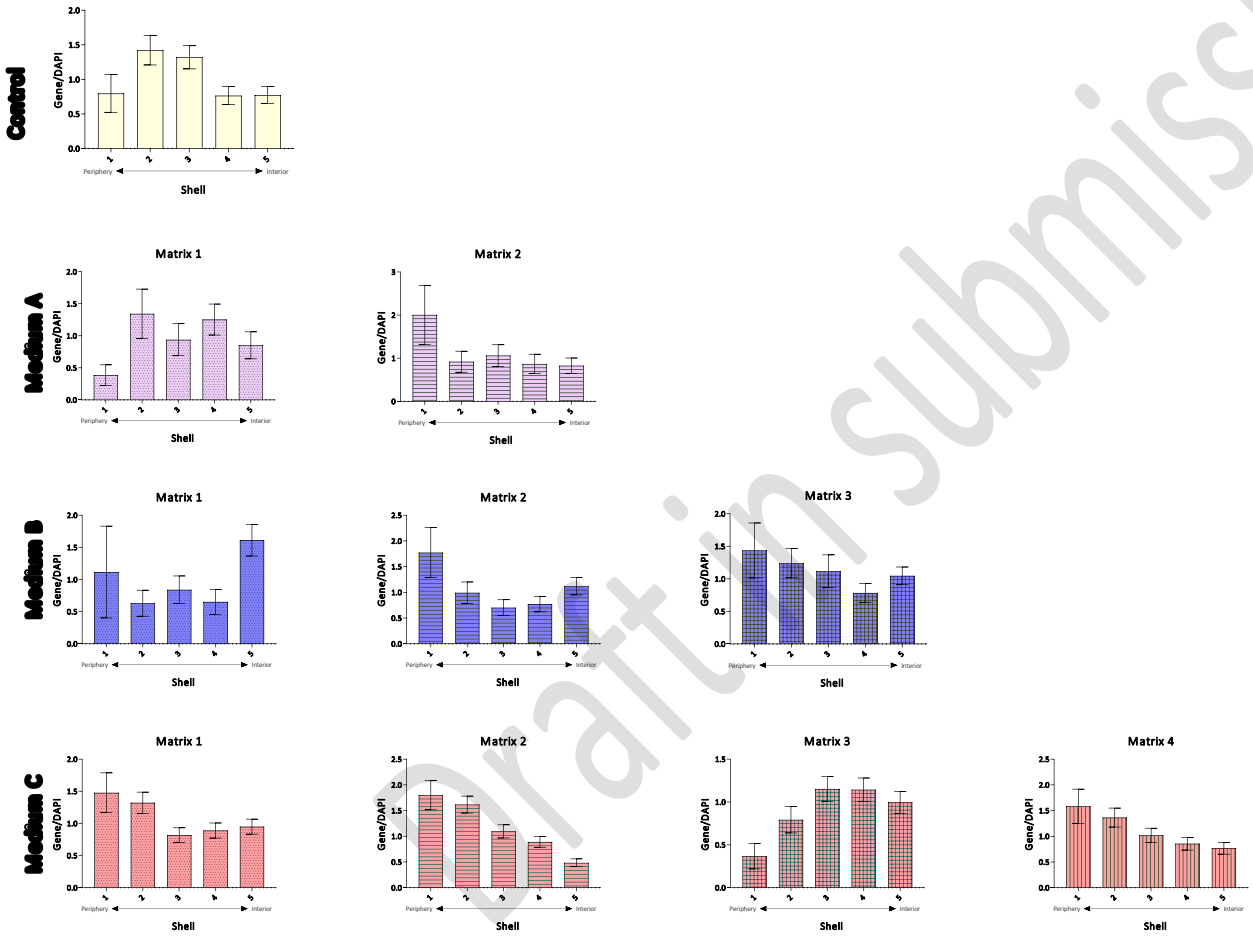


Figure 4. *AMELX* Gene Loci Nuclear Positioning in Different Media-Matrix Combinations

Cell line H9 was cultured under different combinations of media (A-C) and matrix (1-4) combinations. On average, positioning is shown in 80 nuclei for each sample combination using the positioning script. A Kruskal-Wallis test, in conjunction with Dunn's multiple comparisons test, was performed on the intensity of each of the samples' shells compared to the lymphoblastoid cells. Significant differences are indicated by asterisks (*) and the standard error of mean is depicted as the error bars.



4. Discussion

Ensuring the optimal culturing conditions for human embryonic stem cells to preserve genome integrity is crucial for their potential application in clinical stem cell therapies. Indeed, stem cells with unstable genomes, genomic aberrations and aneuploidies have the propensity to be deleterious to the long-term health of a patient by either causing stem cell-derived tumorgenicity or by promoting apoptosis or senescence in themselves and/or to surrounding endogenous cells. Human pluripotent stem cells have been widely reported to accumulate gains and/or losses of whole chromosomes [Amps et al., 2011, Draper et al., 2004, Baker et al., 2007, Avery et al., 2013]. Suggestions have been made that poor cell culture techniques may be to blame [Kim et al., 2012], and more recently, the quality of the starting material has been suggested to be dictated by the individual cell's size, morphology and the medium [Stacey et al., 2017]. In this study, we focused on the effects of the combinations of different media and matrices on aneuploidy and loci positioning of a gene found on the X chromosome and a control gene on chromosome 4 in H9 cells.

The results obtained from the ddPCR™ indicate the presence of X chromosome instability in the H9 cells, as evidenced by the *AMELX* copy number changes. In our model, this instability suggests a potential loss of chromosome X in up to 35% of the cells, contingent upon culture conditions, including the type of media and/or matrix utilized. However, the precise impact of genomic instability in human pluripotent stem cells remains unknown, particularly when contemplating the utilization of highly sensitive assays like the one employed in this study. Although the accumulation of recurrent aneuploidies in culture can lead to cells with growth advantage in culture [Amps et al., 2011, Avery et al., 2013, Peterson and Loring 2014], small copy number variations have not been reported to affect the differentiation ability of hPSCs [Tosca et al., 2015]. Potential theories behind the reason for the accumulation of aneuploidies has been reviewed in detail in previous studies [Henry et al., 2019], however the impact *in-vitro* is not well understood. Certainly, extra chromosomes may be excluded, and cells with insufficient chromosomes may be eliminated. Reports indicate that cell division in early embryos may not be tightly regulated, often resulting in recoverable aneuploidy, which could serve a functional purpose [Zhang et al., 2018, Momcilovic et al., 2010, Desmarais et al., 2012, Milagre et al., 2023]. Furthermore, tumor development from non-host origin has been reported after the injection of karyotypically normal neural stem cells in a patient [Amariglio et al., 2009], however details of this case had not been disclosed with the suggestion of the lack of characterization of the transplanted cells being the reason for this tumour development [Baker et al., 2009]. On the other hand, Fazeli *et al*., had found altered patterns of differentiation in karyotypically abnormal cells, suggesting that although small copy number variations may not affect the differentiation of hPSCs, larger abnormalities may significantly disrupt both gene expression and differentiation of these cells.

Despite the genomic instability revealed by ddPCR™, the G-banding analysis of the cells did not identify any significant number of chromosomal gains and/or losses in the samples collected. This is noteworthy given the well-documented high instability of human pluripotent stem cells [Milagre et al., 2023], which often exhibit recurrent aneuploidies, including those involving chromosome X [Amps et al., 2011, Baker et al., 2007, Brimble et al., 2001, Maitra et al., 2005, Harrison et al., 2007]. Nevertheless, karyotyping via G-banding is known to have limitations in the sensitivity and consistency of aneuploidy detection [Henry et al., 2019] with frequent failures in observing genomic instability in chromosomal regions less than 10 Mbs [Miller et al., 2010]. While ddPCR™ may not be able to detect balanced translocations, its utility lies in the ability to design assays to screen known areas of instability in smaller regions of the genome, thus offering a more comprehensive understanding of cells cultured *in-vitro*. Incorporating such analysis into routine quality control, alongside G-banding, would enhance the assessment of sample quality generated by laboratories.

The organization of the genome and the positioning of genes within the radial structure of interphase nuclei are closely linked to gene regulation. Specific genes tend to be consistently located in the same spatial compartments and anchored to specific structures within the nucleoskeleton, such as lamina-associated domains (LADs), nucleolar-associated domains (NADs), and well-organized topologically associated domains (TADs) associated with the nuclear lamina [Bridger et al., 2022]. Alterations in the nuclear positioning could affect replication timing in stem cells [Marchal et al., 2019] and accessibility of chromatin for transcription before and after differentiation in H9 cells [Melendez-Ramirez et al., 2021]. The positioning of genes by 2D-FISH has been demonstrated to be a reproducible,

fast, reliable, as well as a relatively low-cost method to analyze spatial gene positioning. We have shown that genes from chromosome X and 4 i.e. *AMELX* and *ALB*, respectively, are located towards the nuclear periphery in the diploid lymphoblastoid cells, as has been found in many other human cell lines, including young proliferating dermal fibroblasts from which the hPSC were made from although not the same cell strain. What is strikingly evident from the analysis of the H9 stem cells is the noticeable differences in gene loci positioning across the various cell culture conditions. This variability poses challenges in determining the optimal growth environment in terms of genome organization. Furthermore, while it may appear crucial for genomes to be stable, diploid, and organized according to our standards, it's conceivable that these factors may not significantly impact the cells once they are within the body. As stem cells differentiate into specific cell types, genomic regulation and organization may undergo substantial changes, rendering initial observations less consequential.

Based on our FISH data, the most atypical positioning of *AMELX* and *ALB* was observed in Media A and B compared to the control. Regarding *ALB* positioning, across various combinations of media and matrices, a consistent pattern of peripheral or intermediate positioning was observed for the gene. However, Medium B and Matrix 2 exhibited a notably different internal positioning of the gene. *ALB* gene expression can vary according to the growth factors that supplement the medium [Shirahashi et al., 2004, Davidson et al., 2014]. This may offer a possible explanation for the observed differences in the positioning of *ALB* across the different growth conditions, despite the stability of the gene copy number.

In contrast, the positioning of *AMELX* exhibited significantly more varied results across the various test conditions compared to *ALB*. This observation suggests that in the context of a normal diploid genome, it is plausible for two chromosomes or gene copies to be present, with one chromosome positioned in the periphery while the other is localized more centrally within the nuclei. From the ddPCR™ findings, it's clear that under Media B and C conditions, the samples showed a decrease in *AMELX* copy number by approximately 15-20%. With the results from the gene loci positioning being the most varied in *AMELX*, one potential explanation for this observation is that with the loss of chromosome X in a subset of the cell population, the nuclei with monosomy X tend to assume a more central positioning, while those with diploid X chromosomes retain their peripheral positioning. Indeed, alterations in centromere and chromosome positioning have been noted in human ESCs following aneuploidy [Wiblin et al., 2005, Shete et al., 2014]. Additionally, one study has shown that the X chromosome tends to be more centrally localized in early human embryos, indicating that embryonic cells may exhibit greater flexibility in their chromosome positioning [Finch et al., 2008]. These findings suggest that chromosomal positioning may not pose significant concerns until the differentiation process occurs.
Chromosome rearrangement detected by FISH can be tissue-specific in diseases and aid in diagnostics and prognosis [Leshner et al., 2016, Meaburn et al., 2016]. Whilst in the context of cell therapy manufacturing, it is essential to possess analytical tools that are capable of sensitively and reliably evaluating the genomic health of human pluripotent stem cell cultures, further research into the impact of stem cell aneuploidies is required. Whilst no single standard method of stem cell culture is established, multiple initiatives have been set up to discuss the different conditions globally [Allegrucci and Young 2007]. Currently, one of the biggest challenges facing the scientific community is understanding the crucial impact of pluripotent stem cell aneuploidies and other suboptimal characteristics. Finally, the development of novel methods that can ascertain the low-level presence of aneuploidies is necessary for improving release assays, thus ensuring the safety of potential cell therapy products.

This study provides insight into how aneuploidy in human pluripotent stem cells could be measured sensitively and accurately giving a good indication of copy number, whilst specific genes can be easily localized using 2-D FISH effectively. The deleterious impact of aneuploidy in assisted reproductive technology (ART) embryos is well-documented, however whether such cells would be deemed acceptable in a clinical stem cell application remains uncertain. Furthermore, genome organization may or not be a concern when deciding upon cells for a clinical treatment, but it is clear from these studies that different media and matrix combinations have an impact on both aneuploidy and gene positioning. For this study, it seems that Medium A is beneficial for the maintenance of genomic stability, whereas Medium C is preferable for consistent gene positioning. Further research is required to better understand the impact of low-level aneuploidies and smaller regions of genomic instabilities on the therapeutic potential of human pluripotent stem cells. Certainly, this is not the first study to question genome

functionality in growth conditions for stem cells, however achieving certainty for *in-vivo* treatments necessitates conducting numerous additional studies and implementing sensitive and robust screening assays before product release and subsequent use.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the UK Stem Cell Bank (UKSCB) Steering Committee and Brunel University London (Ethical approval no: UREC2016-12).

Conflicts of Interest: The authors declare no conflicts of interest.

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