NOVEMBER 2024

# GENETIC INTEGRITY WHITE PAPER

ENSURING **GENETIC INTEGRITY** IN **HUMAN PLURIPOTENT STEM CELLS** - CHALLENGES AND SOLUTIONS -

stemgenomics
cell integrity

# Content


**Short summary:**

Human pluripotent stem cells (hPSCs) are invaluable tools for regenerative medicine, disease modeling, and drug discovery. However, maintaining their genomic stability during in vitro culture remains a significant challenge. This white paper investigates key factors that contribute to genetic instability in hPSCs, including (i) reprogramming methods, (ii) prolonged culture conditions, which include environmental factors (e.g. oxygen levels, cell density, temperature), and (iii) gene-editing technologies such as CRISPR-Cas9. By addressing these factors and providing analytical characterization strategies, this work provides best practices to ensure the genomic integrity of hPSCs, ensuring their reliability for clinical and research applications.


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# List of abbreviations

| | |
|---|---|
| hPSCs | human Pluripotent Stem Cells |
| ESCs | Embryonic Stem Cells |
| iPSCs | induced Pluripotent Stem Cells |
| CNVs | Copy Number Variations |
| SNVs | Single Nucleotide Variations |
| ROS | Reactive Oxygen Species |
| HIF | Hypoxia-Inducible Factor |
| CRISPR | Clustered Regularly Interspaced Short Palindromic Repeats |
| Cas9 | CRISPR-associated protein 9 |
| LOH | Loss of Heterozygosity |
| DSBs | Double-Strand Breaks |
| gRNA | guide RNA |
| RNPs | Ribonucleoproteins |
| MDM4 | Mouse Double Minute 4 |
| TP53 | Tumor Protein p53 |
| MEFs | Mouse Embryonic Fibroblasts |
| FISH | Fluorescence In Situ Hybridization |
| WGS | Whole-Genome Sequencing |
| CGH | Comparative Genomic Hybridization |
| aCGH | Array-based Comparative Genomic Hybridization |
| SNPs | Single Nucleotide Polymorphisms |
| ddPCR | Droplet Digital PCR |

# I. Introduction

## Introduction: genetic abnormalities in human pluripotent stem cells during culture

Human pluripotent stem cells (hPSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are essential tools in regenerative medicine, disease modeling, and drug discovery. However, maintaining their genomic integrity during in vitro culture remains a major challenge. Over time, hPSCs tend to acquire genetic abnormalities, which can compromise their therapeutic potential and research applications (Assou *et al.,* 2018, 2020; Vales & Barbaric, 2024).

One of the earliest studies of hPSCs showed that genetic variations could be acquired during in vitro culture, particularly recurrent chromosomal aberrations such as gains of chromosomes 12 and 17. These chromosomal alterations confer a selective growth advantage, allowing the expansion of genetically altered cell populations that eventually come to dominate the culture (Draper *et al.,* 2004). More recent research has identified a broader range of genetic alterations, including copy number variations (CNVs) and single nucleotide variations (SNVs). Specific amplifications on chromosomes 1, 12, 17, and 20 are frequently observed, with gains in the 20q11.21 region being particularly common in hPSC lines (Figure 1) (Maitra *et al.,* 2005; Amps *et al.,* 2011). These recurrent mutations confer increased cell survival, decreased apoptosis, and enhanced proliferation, allowing genetically unstable cells to outcompete normal cells in culture (Avery *et al.,* 2013). These defects can occur as early as the 5th passage in culture and at regular intervals, requiring appropriate genomic stability testing throughout the culture workflow. Regular genomic stability assessment every 5 to 10 passages is therefore recommended in PSC cultures to detect the emergence of genetic abnormalities that may accumulate due to selective pressures in vitro. This routine testing helps maintain the genomic integrity of PSCs, which is essential for both research and clinical applications (McIntire *et al.,* 2020; Pamies *et al.,* 2017; Assou *et al.,* 2020).

Mitotic errors have played a critical role in driving genetic changes in hPSCs. Unlike somatic cells, hPSCs exhibit a higher frequency of chromosome segregation errors during mitosis, often leading to improper spindle-kinetochore attachments and lagging chromosomes during mitosis (Zhang *et al.,* 2019). In addition, the unique cell cycle dynamics of hPSCs, particularly their shortened G1 phase, contribute to replication stress. This shortened phase limits the time available for cells to prepare for DNA replication, thereby increasing the risk of DNA damage during S phase (Ahuja *et al.,* 2016). Moreover, the hyper-transcriptive nature of hPSCs exacerbates this replication

stress, as the high levels of gene expression place additional demands on the DNA replication machinery. This pressure can lead to further disruptions in genomic integrity, ultimately contributing to genomic instability within hPSC populations (Bowry *et al.,* 2021). As a result, these factors combine to create an environment in which genetic abnormalities are more likely to occur, posing significant challenges for the therapeutic application of hPSCs.

Although hPSCs have the ability to eliminate damaged cells through apoptosis, certain mutations can arise and confer resistance to this programmed cell death, particularly mutations affecting genes such as BCL2L1 and TP53. These mutations can trigger the proliferation of these resistant cells, which in turn leads to the clonal expansion of genetically abnormal cells, posing significant risks for clinical use (Merkle *et al.*, 2017; Avery *et al*., 2013). Therefore, these mutations raise serious concerns about the potential for tumorigenesis when these genetically modified cells are used in therapy and require close monitoring (Ben-David *et al*., 2014). With these challenges in mind, this white paper provides a detailed analysis of the various factors that contribute to genetic instability in hPSCs during in vitro culture. It examines both internal and external influences, including reprogramming methods, prolonged culture conditions, environmental factors (e.g. oxygen levels, cell density, temperature), and gene-editing technologies. The goal is to provide strategies to mitigate these risks and thereby ensure the safe and effective use of hPSCs in both research and clinical settings.

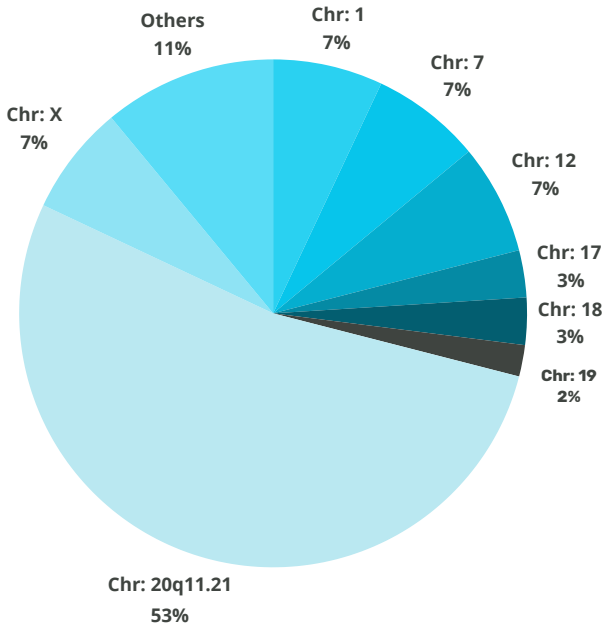



*Figure 1: Localization of the most frequent genomic abnormalities observed in Stem Genomics testing using digital PCR*

This figure illustrates the distribution of genetic abnormalities in human pluripotent stem cells (hPSCs) across different chromosomes as detected by digital droplet PCR. The most affected region was 20q11.21, followed by chromosomes 12, 7, 1 and X, which showed similar patterns. The graph underscores the importance of regular genomic monitoring to maintain the integrity of hPSCs for both research and clinical applications. The data, derived from 2,124 abnormal samples, identified 2,374 chromosomal abnormalities, as some samples had multiple alterations. This proprietary data was provided by Stem Genomics.

# II. Risk factors and genetic instability

**This chapter examines three major areas that contribute to these risks: the use of integrative reprogramming methods, prolonged culture conditions, and gene editing technologies such as CRISPR-Cas9.**

## 1) Impact of integrative reprogramming methods on genetic stability

Integrative methods, such as retroviral and lentiviral vectors, were initially used to reprogram somatic cells into iPSCs. While effective, for generating iPSCs, these techniques also pose significant risks to the genetic stability of the resulting cells. The integration of viral vectors into the genome can lead to insertional mutagenesis, where random insertions disrupt critical genes. This disruption can activate oncogenes or inactivate tumor suppressor genes, leading to tumor formation. For instance, the oncogene c-Myc often utilized in the reprogramming process, is associated with an elevated risk of tumorigenesis if it remains active post-reprogramming (Hu, 2014; Takahashi & Yamanaka, 2006). Another concern is the residual expression of reprogramming factors. Ideally, these genes would be silenced after the successful reprogramming of somatic cells, however, in some cases, they continue to be expressed, which can contribute to genomic instability and promote tumorigenesis.

## Specific recommendations

### Adopt non-integrative methods:

The main recommendation is to avoid integrative reprogramming methods. Several non-integrative methods have been developed. These include:

- **Episomal Plasmids:** derived from Epstein-Barr Virus (EBV), episomal plasmids replicate without integrating into the host genome. While this method reduces the risk of insertional mutagenesis, episomal vectors are often silenced by the host cell, reducing reprogramming efficiency over time (Yu *et al.,* 2011).

- **Sendai Viral Vectors:** Sendai viral vectors (SeVV) are non-integrative RNA-based vectors that replicate exclusively in the cytoplasm. This method is efficient and avoids the risk of permanent genomic alterations. However, residual viral RNA can persist, requiring careful monitoring (Fusaki *et al.,* 2009).

- **Synthetic mRNA:** synthetic mRNA avoids the risk of genomic integration entirely by providing a transient source of reprogramming factors. However, this method requires repeated transfections and careful optimization to mitigate immune responses in the host cell (Warren *et al.,* 2010).

## Specific recommendations

**o Ensure complete silencing of reprogramming factors:**

if you are using an iPSC line that has been reprogrammed using integrative vectors, in addition to a thorough check of genomic instability, check your cell line to be sure that the reprogramming factors are fully silenced.

Persistent c-Myc expression, in particular, increases the likelihood of uncontrolled cell growth (Okita *et al.,* 2007). Moreover, integrative methods can lead to the emergence of Copy Number Variations (CNVs) like deletions and duplications. Early-passage iPSCs often show deletions in tumor suppressor genes and duplications in oncogenic regions, such as chromosome 12, which contains the pluripotency gene NANOG (Laurent *et al.,* 2011; Hussein *et al.,* 2011). These genetic changes undermine the stability of iPSCs, particularly when cultured over extended periods. Thus, understanding and addressing these challenges is crucial for the safe application of iPSCs in therapeutic contexts.

## 2) Prolonged culture conditions

### a) Feeder types and adhesion substrates for stem cells

The choice of feeder cells or adhesion substrates is critical to maintaining the genetic integrity of hPSCs. Traditional feeder layers, such as mouse embryonic fibroblasts (MEFs), provide essential growth factors, extracellular matrix components, and cues that support the pluripotency and self-renewal of hPSCs. MEFs have been widely used in hPSC culture systems due to their ability to secrete critical factors such as basic fibroblast growth factor (bFGF), which plays a key role in maintaining stem cells in an undifferentiated state. However, despite these advantages, the use of MEFs presents several challenges that can compromise genetic integrity. First, there is a risk of cross-species contamination, where mouse-derived proteins or genetic material could be inadvertently introduced into hPSC cultures, potentially affecting their genomic stability. In addition, variations in the quality of MEF batches can lead to inconsistent hPSC support, increasing the likelihood of genetic abnormalities. Over time, this variability can contribute to the accumulation of genetic mutations or epigenetic changes in hPSCs, which may compromise their utility for clinical applications. As a result, there has been a growing shift toward the use of defined, xeno-free culture systems that eliminate animal-derived components. Synthetic substrates and human-derived feeder cells provide a more controlled environment, reducing the risks associated with traditional MEF-based systems and better supporting the genetic integrity of hPSCs in long-term culture.

#### i) MEF-based feeder systems

The use of feeder cells, such as MEFs, can introduce variability due to differences in feeder cell quality between batches. This variability can lead to inconsistent growth and differentiation responses in hPSC cultures. Such inconsistency can create selective pressures that favor the expansion of genetically unstable subclones, potentially compromising the overall quality of the culture. In addition, there is a significant risk of cross-species contamination, particularly with mouse-derived pathogens or cellular components, which can compromise the purity and genetic stability of hPSC lines. Prolonged exposure to non-human feeder cells can also lead to inadvertent incorporation of foreign DNA into the hPSC genome, further compromising their genetic integrity (Ludwig *et al.,* 2006). In addition, long-term culture on MEF feeders has been associated with the development of chromosomal abnormalities in hPSCs, such as CNVs and aneuploidies. These genetic alterations can accumulate over time due to the selective pressure exerted by feeder cells, leading to clonal expansion of genetically abnormal cells. Such genetic instability poses a significant threat to the

utility of hPSCs in research and clinical applications, as it may compromise their differentiation potential and increase the risk of tumorigenesis (Al Delbany, 2024). Therefore, the choice of culture systems must be carefully considered in order to maintain the genetic stability of hPSCs over time.

## Specific recommendations

to limit the impact of MEFs system on stem cell genetic integrity:

**o Switch to human-derived feeders:** reduce cross-species contamination by using human feeder cells instead of mouse embryonic fibroblasts (MEFs) (Ledwig, 2006).

**o Ensure quality control:** minimize batch-to-batch variability in feeder layers to prevent selective pressures favoring genetically unstable cells (Loring & Rao, 2006).

### ii) Feeder-free systems

Feeder-free culture systems, such as those using Matrigel or recombinant vitronectin, have been developed to eliminate the variability and contamination risks associated with traditional feeder-layer MEFs. These systems provide a more defined environment and reduce the risks associated with cross-species contamination. However, despite these advantages, feeder-free systems have been associated with an increased risk of genomic instability. Specifically, cells grown under feeder-free conditions show a higher incidence of genetic aberrations, particularly gain of chromosome 1q. This chromosomal gain is mediated by the overexpression of MDM4, which confers a selective growth advantage to the cells, promoting faster proliferation and reduced apoptosis. As a result, these genetically altered cells can outcompete normal cells in culture, leading to clonal expansion of unstable cell populations. Over time, the accumulation of such genetic alterations becomes a critical concern, especially in the context of long-term culture. The presence of chromosomal abnormalities such as 1q gains could undermine the therapeutic potential of hPSCs, as these cells may not perform reliably in clinical applications and may even increase the risk of tumorigenesis. Therefore, while feeder-free systems help mitigate some of the challenges of traditional feeder-based methods, they introduce new risks that must be carefully monitored, particularly when considering the use of hPSCs for regenerative medicine and other therapies (Narva *et al.,* 2010; Stavish *et al.,* 2024).
A key finding by Barbaric *et al.* (2024) demonstrated that feeder-free systems, particularly when combined with specific culture media such as E8 or NutriStem, promote the selective expansion of cells with MDM4-mediated gains of chromosome 1q. This chromosomal gain not only increases cell proliferation but also decreases apoptosis, allowing genetically abnormal cells to dominate the culture. Overexpression of MDM4 also interferes with the regulation of the tumor suppressor gene TP53, resulting in reduced apoptosis in response to

genomic damage. This selective advantage for cells with genetic abnormalities underscores the risks associated with feeder-free culture systems, particularly when these cells are being considered for clinical therapies (Stavish *et al.,* 2024). In addition, feeder-free systems have been shown to induce higher levels of genomic damage compared to feeder-based systems. Increased markers of DNA damage, such as gH2AX, a key indicator of double-strand DNA breaks, are more commonly observed in cells grown under feeder-free conditions.
This increased genomic damage creates an environment that favors the survival and proliferation of cells with genetic alterations, such as the gain of chromosome 1q. Cells with these genetic alterations have enhanced survival mechanisms that allow them to survive and proliferate despite ongoing DNA damage. This highlights the importance of carefully monitoring and optimizing culture conditions to minimize genomic damage and maintain the genetic integrity of stem cell populations (Stavish *et al.,* 2024).

## Specific recommendations

to mitigate the impact of feeder-free system on stem cell genetic integrity according to Stavich *et al.*, 2024 study:

**o Use defined media:** implement serum-free, defined media like E8 to avoid contamination and variability.

**o Control gene expression:** tighten regulation of genes like MDM4 to avoid chromosomal gains that promote abnormal cell expansion.

**iii) Adhesion substrates**

Adhesion substrates also play an important role in maintaining stem cell behavior and genomic stability. The physical properties of these substrates, such as their stiffness and elasticity, can significantly affect essential cell processes, including differentiation potential, proliferation rates, and overall stem cell maintenance. Rigid substrates can alter focal adhesion points, affecting cell signalling pathways and contributing to genetic instability. The altered signalling can promote abnormal cell growth patterns, contributing to a higher risk of accumulating genetic mutations over time.
In addition, synthetic materials designed to mimic the extracellular matrix (ECM) can also influence cell behavior in feeder-free systems. Although these materials are designed to provide a supportive environment for stem cells, they can inadvertently influence the rate of cell differentiation and proliferation. When cells are exposed to suboptimal substrate conditions, the stress on cellular processes can accelerate the accumulation of genetic abnormalities. This is of particular concern in long-term cultures, where even small variations in substrate mechanical properties can lead to the expansion of genetically unstable cell populations. In feeder-free systems, where the absence of feeder cells removes a layer of biological support, the mechanical properties of the substrate become even more critical. If the substrate is too rigid or poorly mimics the natural ECM, it can exacerbate genetic instability and accelerate the accumulation of mutations

that can compromise the therapeutic potential of the cells. This highlights the importance of optimizing substrate conditions to maintain the delicate balance required for genomic stability in hPSCs (Azarin & Palecek, 2010).

## Specific recommendations

to mitigate the impact of adhesion substrates on stem cell genetic integrity according to Azarin & Palecek, 2010 study:

- **Optimize substrate rigidity:** use softer or tunable substrates that better mimic the natural extracellular matrix (ECM) to maintain appropriate cell signaling and reduce stress on focal adhesion points, which can minimize genetic instability.
- **Utilize defined synthetic substrates:** adopt chemically defined synthetic substrates to ensure reproducibility and eliminate variability that may arise from biological materials like Matrigel.
- **Functionalize substrates with ECM components**: integrate ECM proteins such as fibronectin, collagen, or laminin into synthetic substrates to support proper stem cell adhesion and reduce the risk of genomic alterations.

### b) Passaging and dissociation methods

**Enzymatic dissociation**

Enzymatic passaging, particularly with the use of Accutase, has been associated with a higher accumulation of genetic aberrations compared to mechanical passaging techniques. Studies have shown that enzymatic dissociation can lead to chromosomal duplications and deletions, with duplications occurring more frequently. For example, recurrent duplications in chromosome 12 and the 20q11 region have been observed in multiple stem cell cultures, which is of concern because these genetic alterations may confer a selective growth advantage to the cells, potentially leading to the expansion of genetically abnormal populations (Garitaonandia *et al.,* 2015).

However, not all dissociation methods appear to have the same effect on genomic stability. Other studies have reported different results when using alternative enzymes. For example, Tosca *et al.* (2015), using collagenase-IV, and Beers *et al.* (2012), using EDTA as a dissociation agent, found no significant effect on the genetic stability of hPSCs, even after long-term culture. These results suggest that the type of enzyme used for passaging plays a critical role in determining the extent of genomic alterations. The contrasting results indicate that some enzymatic methods, such as Accutase, may induce higher levels of genomic stress and instability, possibly due to more severe or frequent disruption of cell-cell and cell-substrate interactions during passaging. This underscores the importance of carefully selecting dissociation methods to minimize the risk of genetic abnormalities in hPSC cultures, particularly for regenerative medicine applications where genomic integrity is paramount. Optimizing dissociation protocols to reduce the risk of chromosomal aberrations could improve the safety and reliability of hPSCs in both research and clinical settings.

## Specific recommendations

to avoid the negative impact of enzymatic digestion on genomic integrity of hPSCs based on Tosca *et al.* (2015) study:

**o Mechanical Dissociation:** mechanical dissociation methods, such as manual cutting or scraping, involve separating cells into clumps rather than dissociating them into single cells. Although these methods are labor-intensive and less scalable, they result in less DNA damage and fewer mutations compared to enzymatic approaches. Mechanical dissociation has been shown to preserve the pluripotency and genetic stability of hPSCs during long-term culture, making it a more reliable method for maintaining stem cell lines intended for clinical applications.

**o Consider Combination Techniques:** if enzymatic methods are necessary, consider combining them with mechanical dissociation to reduce the overall impact on genomic stability. This hybrid approach may offer a balance between scalability and maintaining genetic integrity.

### C) Environmental factors

Several environmental factors can affect the genetic stability of hPSCs in prolonged culture:

- Cell density and physical culture conditions: cell density plays a crucial role in maintaining the genetic stability of hPSCs during prolonged culture periods. Both high and low cell densities pose risks to genomic integrity, requiring careful monitoring and adjustment of culture conditions to preserve cellular integrity.

- **High-density cultures:** in high-density cultures, cells are closely packed together, which can create an environment conducive to increased genetic instability. At high densities, cells experience nutrient competition and limited access to oxygen, which can lead to hypoxic conditions. These factors contribute to oxidative stress, a known cause of DNA damage, and can lead to the accumulation of genetic changes such as CNVs and chromosomal abnormalities. Over time, continued exposure to cellular stress from high-density conditions exacerbates the risk of accumulating these genetic abnormalities and further compromises the genomic integrity of the cell population (Cui *et al.,* 20-24; Jacobs *et al.,* 2016);

- **Low-density cultures:** conversely, low density cultures, while avoiding nutrient deprivation, may lack the paracrine signals essential for maintaining pluripotency. This can lead to spontaneous differentiation and genetic drift, compromising the genomic stability of hPSCs. Therefore, balancing cell density is critical to maintain both growth and genetic integrity (Ie *et al.*, 2018).

## Specific recommendations

to reduce the impact of cell density on the genetic integrity of hPSCs based on Dubose *et al.* 2022 and Cui *et al.,* 2024 studies:

**o Optimal cell seeding density:** avoid high cell density to prevent metabolic stress and DNA damage; maintain appropriate seeding levels for genomic integrity.

**o Frequent medium changes:** regularly replace medium, especially at high cell densities, to remove byproducts like lactate and maintain stable growth conditions.

**o Use of 3D culture systems:** transition to some 3D culture systems such as alginate encapsulation to prevent overcrowding, promote natural growth, and reduce stress from high-density 2D cultures (Cohen *et al.,* 2023). Engineering 3D micro-compartments for highly efficient and scale-independent expansion of hPSCs in bioreactors.

**o Monitor culture conditions:** use real-time monitoring of pH, oxygen, and lactate levels to detect stress early and adjust conditions as needed.

**o Optimize growth medium:** use optimized media to improve cell health and minimize genetic instability.

This can lead to spontaneous differentiation and genetic drift, compromising the genomic stability of hPSCs. Therefore, balancing cell density is critical to maintain both growth and genetic integrity (le *et al.,* 2018).

### i) Oxygen levels

Oxygen levels critically affect the genetic stability of hPSCs during prolonged culture. Studies suggest that low oxygen tension (hypoxia) can significantly improve the maintenance of pluripotency and reduce oxidative stress, a major cause of DNA damage in hPSCs (Nit *et al.,* 2021). Under normoxic conditions (standard atmospheric oxygen levels), reactive oxygen species (ROS) accumulate and can cause double-strand breaks and mutations that accumulate over time, especially in long-term cultures.

- **Impact of hypoxia:**

Under hypoxic conditions, the reduced availability of oxygen helps to create an environment that reduces the production of reactive oxygen species (ROS). ROS are highly reactive molecules that can cause significant cellular damage, including double-strand breaks in DNA and various mutations. By limiting ROS formation, hypoxia reduces the risk of genetic changes that can accumulate over time, especially during long-term culture.

- **Challenges of normoxia:**

Conversely, when hPSCs are cultured under normoxic conditions, the higher oxygen concentration can lead to increased generation of ROS. As these reactive molecules accumulate, they can have a number of deleterious effects on cellular components, leading to oxidative stress. This stress can exacerbate DNA damage, resulting in mutations that can compromise the genetic integrity of cells. Such damage is of particular concern in long-term cultures, where the risk of accumulating genetic abnormalities increases significantly.

## Specific recommendations

to manage oxygen levels to reduce the impact on the genetic integrity of hPSCs based on Nit *et al.,* 2021 study:

**o Maintain hypoxic conditions** (3-5% O2): culturing hPSCs in 3-5% oxygen reduces reactive oxygen species (ROS), which supports pluripotency and preserves genetic stability during long-term culture, while avoiding normoxic (21% O2) conditions that increase ROS and cause DNA damage.

**o Use HIF regulation:** hypoxia activates HIF1α and HIF2α, reducing oxidative stress and maintaining pluripotency, helping prevent DNA damage.

**ii) Temperature:**

Temperature and physical culture conditions are both critical to maintaining the genetic stability of hPSCs. The optimal culture temperature is 37°C, and deviations from this temperature can lead to cellular stress and the production of reactive oxygen species (ROS), which cause DNA damage and genetic mutations. Maintaining a consistent temperature is essential to prevent oxidative stress and ensure pluripotency and genomic stability of hPSCs (DuBose *et al.,* 2022; Cui *et al.,* 2024). In addition to temperature, the physical culture environment also affects genetic integrity. 3D culture systems more closely mimic in vivo conditions, providing better nutrient and oxygen distribution while reducing mechanical stress. This helps minimize chromosomal aberrations and increases genomic stability compared to traditional 2D cultures (Cui *et al.,* 2024). Both stable temperature control and optimized culture conditions are essential for maintaining the long-term genetic health of hPSCs.

## Specific recommendations

to manage temperature impact on the genetic integrity of hPSCs according to DuBose *et al.,* 2022; Cui *et al.,* 2024 studies:

**o Maintain optimal culture temperature (37°C):** keep the temperature at 37°C to prevent ROS production and DNA damage in hPSCs. Use incubators with tight regulation to maintain genomic stability.

**o Monitor for temperature fluctuations:** use continuous monitoring systems to quickly detect and fix temperature changes, minimizing oxidative stress and protecting pluripotency.

### iii) pH and Ionic balance

- **Importance of pH Regulation:** maintaining the optimal pH range of 7.2-7.4 is essential for hPSC culture. Deviations can trigger reactive oxygen species (ROS) pathways, which cause DNA damage, mutations, and chromosomal aberrations. Without consistent pH control, prolonged culture increases the risk of genomic instability (Jacobs *et al.,* 2016; Wilmes *et al.* 2017).
- **Culture Medium Optimization:** optimizing culture media to maintain pH and ionic balance is essential. Buffered media systems and controlled electrolyte levels help stabilize pH and support genetic stability in long-term hPSC cultures, reducing the risk of mutations (Jacobs *et al.,* 2016; Liu *et al.,* 2018).
- **Ionic Balance and Homeostasis:** ionic balance, particularly in sodium (Na+), potassium (K+), calcium (Ca2+), and magnesium (Mg2+), is crucial for cellular homeostasis and DNA repair. Calcium, in particular, plays a role in DNA repair mechanisms, and imbalances can impair these processes, leading to oxidative stress and increased genetic instability over time (Souza *et al.* 2023).

### iv) Serum batch variability in prolonged culture

Serum has been essential in the early history of hPSC culture to provide growth factors and nutrients. However, differences between serum batches can introduce variability in culture conditions, leading to altered gene expression, changes in cell behavior, and most critically, genomic instability (Baker *et al.,* 2007; Loring & Rao, 2006). Variability in serum composition can create inconsistent selective pressures on cells, promoting the emergence of subclonal populations with distinct genetic mutations. This increases the risk of genetic instability, including the occurrence of CNVs and chromosomal abnormalities, which can compromise the

## Specific recommendations

to manage pH and ionic balance impact on the genetic integrity of hPSCs:

- **Maintain optimal pH (7.2-7.4):** regulate pH within the 7.2-7.4 range to prevent ROS-related DNA damage. Use buffered media and pH monitoring systems.
- **Use buffered media:** implement buffered culture media to ensure pH stability and prevent genetic instability during long-term culture.
- **Ensure ionic balance:** control levels of Na+, K+, Ca2+, and Mg2+ in the medium. Calcium is key for DNA repair, and imbalances can lead to oxidative stress and mutations.
- **Monitor pH and electrolytes:** use real-time monitoring to detect fluctuations in pH and ionic balance, ensuring stable culture conditions.
- **Optimize culture media:** tailor media formulations to meet the metabolic needs of hPSCs, maintaining pH and electrolyte levels that support genomic stability.

long-term integrity and viability of cells (Loring & Rao, 2006). Maintaining consistent serum quality or using defined serum-free media is key to reducing these risks in hPSC culture. To mitigate these risks, it is essential to maintain consistent serum quality, but the best strategy is to use defined serum-free media in hPSC culture. This approach can help ensure a stable environment, promoting the genetic integrity and functionality of the stem cells over extended culture periods.

## Specific recommendations

- **Use defined media:** switch to serum-free, defined media like E8 or mTeSR1 to avoid variability in serum quality.
- **Test serum lots:** perform quality control tests on new serum batches to ensure they don't affect growth or genomic stability.
- **Batch freezing:** create large stocks of cells cultured under the same serum batch to ensure consistency over time.
- **Regular genomic checks:** conduct routine karyotyping or genomic integrity tests to catch early signs of instability.

## d) Microbial and mycoplasma contamination

### i) Mycoplasma contamination in hPSC cultures

Mycoplasma contamination is a major concern for hPSCs in long-term culture. These bacteria, which lack a cell wall, are common contaminants in cell culture and can grow silently without obvious signs such as medium turbidity. Globally, studies suggest that mycoplasma infections affect between 15% and 35% of cell cultures, with some laboratories reporting alarmingly high contamination rates of 65% to 80% (Uphoff & Drexler, 2014). This high prevalence of contamination can have significant consequences, including altered cell behavior, changes in gene expression, and compromised experimental results. Mycoplasmas can interfere with several cellular processes, including cell growth, pluripotency, and genetic stability.

They alter cell membrane composition, enzyme activities, and metabolic pathways, leading to chromosomal aberrations and genomic instability. This can severely compromise the quality of hPSCs, making them unsuitable for therapeutic applications. Regular testing using PCR-based or traditional culture methods is recommended to detect and prevent the spread of contamination.

### ii) Effects on genetic integrity

Infected hPSCs show alterations in growth rate, pluripotency, and viability. Mycoplasma contamination can induce DNA damage and disrupt proper cell division, leading to chromosomal abnormalities. In mouse

models, mycoplasma contamination reduced growth rates and impaired pluripotency of stem cells (Borchsenius *et al.,* 2020). The same is true for hPSCs, where the long-term integrity of their genome is compromised, leading to potential risks when these cells are used in clinical settings.

## Specific recommendations

- **Regular screening:** perform routine PCR-based mycoplasma tests to catch contamination early.
- **Antibiotic-free methods:** use 0.1 µm filters for media, as they are effective in blocking mycoplasma, which typically range in size from 0.2 to 0.3 µm, and ensure cell lines are authenticated to prevent contamination.
- **Frequent media changes:** regularly change media to lower contamination risks.
- **Use antibiotics:** apply plasmocin as a curative action to eliminate mycoplasma contamination effectively.

## 3) Impact of gene editing on hPSC genetic stability

Gene editing technologies have revolutionized molecular biology, with CRISPR-Cas9 emerging as the most versatile and widely used system. Its ability to induce double-strand breaks (DSBs) at precise locations allows for targeted gene modifications, including disruptions, insertions, and deletions. This technology has become invaluable for both basic research and therapeutic applications. One of the most promising applications of CRISPR-Cas9 is the manipulation of hPSCs (Zhu *et al.,* 2022). While the potential of CRISPR-Cas9 in hPSCs is significant, it is not without risk. The introduction of DSBs can lead to unintended genetic consequences, both on-target and off-target, including chromosomal rearrangements and other forms of genetic instability. These results raise concerns about the safety and reliability of CRISPR-Cas9, particularly in clinical applications where maintaining genetic integrity is critical. This section examines the impact of CRISPR-Cas9 on the genetic stability of hPSCs, exploring the implications of chromosomal abnormalities and the broader risks associated with this transformative technology.

### a) Chromosomal instability and off-target effects in CRISPR-Cas9 edited hPSCs

CRISPR-Cas9 technology poses significant risks to the genetic stability of hPSCs, particularly due to chromosomal instability and off-target effects. A major concern is the potential for CRISPR-Cas9 to induce chromosomal rearrangements, as has been observed in cancer cell lines where it has exacerbated alterations such as aneuploidies, deletions, and translocations. For example, in COLO320 cells, the MLH1 locus on chromosome 3 was translocated to another chromosome, illustrating the ability of CRISPR-Cas9 to cause significant chromosomal disruptions (Rayner *et al.,* 2019). While these findings were observed in cancer cells, they highlight the risk of similar disruptions in hPSCs, where such changes could compromise both cell safety and functionality. Furthermore, CRISPR-Cas9 has been shown to induce megabase-scale chromosomal truncations following a single double-strand break, raising further concerns about its potential to cause unintended large deletions (Cullot *et al.*, 2019). In addition to the risk of chromosomal instability, CRISPR-Cas9 has also been associated with off-target effects, where unintended regions of the genome are inadvertently edited. In the context of hiPSCs, off-target modifications can lead to large-scale genetic changes, including deletions, duplications, and translocations, all of which threaten the genetic stability of the cells. Research focused on correcting mutations in critical genes, such as HBB (associated with beta-thalassemia) and DMD (associated with Duchenne muscular dystrophy), underscores the need for thorough screening of CRISPR-edited cells. This screening is essential to ensure the accuracy and reliability of CRISPR applications, especially for therapeutic applications (Frangoul *et al.,* 2020; De Masi

*et al.,* 2020). Given these risks, it is imperative to implement rigorous validation processes to protect the integrity of hPSCs and minimize the potential for adverse outcomes in clinical settings.

### b) Loss of heterozygosity

Loss of heterozygosity (LOH) refers to the genetic phenomenon in which one allele of a gene is lost, resulting in the absence of a parent's genetic contribution in certain regions of the genome. LOH is commonly associated with deletions, recombination errors, or gene conversions, and is known to play a significant role in several genetic disorders, including cancer (Smith *et al.,* 2012). This mechanism is of particular concern in the context of CRISPR-Cas9, where unintended genetic consequences can occur.

### c) Chromothripsis as a consequence of CRISPR-Cas9 editing

Chromothripsis, a phenomenon in which a chromosome breaks and reassembles in a chaotic manner, has also been observed as a consequence of CRISPR-Cas9-induced DSBs. This catastrophic event leads to complex chromosomal rearrangements, including CNVs and LOH (Leibowitz *et al.,* 2021). A study investigating the relationship between CRISPR-Cas9 and chromothripsis found that DSBs can lead to the formation of micronuclei and chromosome bridges, both of which are prone to chromothripsis (Smith *et al.,* 2020).

The extreme genetic alterations resulting from chromothripsis pose significant risks, particularly in therapeutic contexts where genomic integrity is essential. Such alterations can disrupt gene function, promote tumorigenesis, or lead to unpredictable cellular behavior, raising significant safety concerns for the use of CRISPR-Cas9 in clinical applications. Given these potential consequences, it is critical to employ robust monitoring and validation strategies when using CRISPR technology in research and therapy to mitigate the risk of such catastrophic genetic events.

## Specific recommendations

- **Use high-fidelity Cas9 variants:** employ high-fidelity Cas9 variants, which reduce off-target effects and unintended DNA damage.
- **Implement short-exposure Cas9 Ribonucleoproteins (RNPs):** use Cas9 RNPs to limit exposure and reduce the likelihood of large-scale chromosomal rearrangements.
- **Optimize guide RNA (gRNA) design:** carefully design gRNAs with algorithms to minimize mismatches and avoid off-target effects, crucial for maintaining genomic integrity.
- **Employ dual gRNA and nickase strategy:** use dual gRNAs combined with Cas9 nickase to increase specificity and minimize double-strand breaks (DSBs), which are linked to chromosomal instability and off-target effects.

# III. Testing methods and recommended strategies

To ensure the stability and safety of hPSCs, several genomic testing methods have been developed to detect chromosomal abnormalities and genetic instability. Technologies such as digital droplet PCR (ddPCR) effectively screen for recurrent genetic abnormalities and offer a targeted approach with an effective resolution (200 bp), rapid results (1-3 days), lower costs and in-process control compatibility. Karyotyping remains the gold standard for identifying large chromosomal changes, although it may miss smaller ones, provides results in 2-3 weeks, and requires a cytogenetics specialist for data interpretation. Fluorescence in situ hybridization (FISH) complements karyotyping by allowing visual detection of large rearrangements and off-target effects, with a similar turnaround time of 1 week. Whole-genome sequencing (WGS) provides a comprehensive view of large and small mutations at single-base resolution, but requires bioinformatics expertise and takes up to 4-5 weeks. Comparative genomic hybridization (CGH) and its array-based counterpart (aCGH) are used to detect copy number variations (CNVs), unbalanced translocations, and aneuploidies, and offer a middle ground in terms of resolution (50 kb) and turnaround time (2-3 weeks). In addition, single nucleotide polymorphism (SNP) genotyping identifies smaller genetic changes that may occur during culture, while exome sequencing targets specific coding regions to detect mutations. Together, these methods provide an integrated approach to monitoring the genetic integrity of hPSCs, enabling both targeted and exhaustive detection of genomic changes that may affect the safety and stability of these cells in research and clinical applications (Table 1).

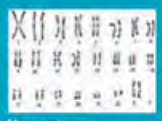
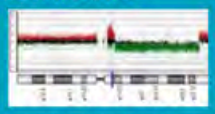
| Technology | Resolution | Detection | Mosaicism | Min. samples No. | Result | Timing | Price |
|---|---|---|---|---|---|---|---|
|  Karyotype | Poor 5-10 Mb | All variants > 5-10 Mb | Good 10% | From 1 sample | Cytogenetics specialist required | Average 2-3 weeks | Average $$$ |
|  NGS: WGS, WES | Excellent 1 bp | Exhaustive | Excellent 1% | From 1 sample | bioinformatician required | Poor 4-5 weeks | High $$$$ |
|  Arrays: aCGH, SNP | Average 50 kb | Aneuploidy, CNVs Unbalanced translocations | Average 20% | 8 or 24 samples | bioinformatician required | Average 2-3 weeks | Average $$ |
|  digital PCR | Good 200 bp | Targeted CNVs, aneuploidies | Average 20% | From 1 sample | Easy | Excellent 1-3 days | Excellent $ |
|  FISH | Average 100-300kb | Targeted regions | Good 10% | From 1 sample | Cytogenetics specialist required | Good 1 week | Average $$ |

*Table 1: Comparative Genomic Testing Technologies*

A combination of these methods as well as regular testing is recommended to pick up genomic abnormalities in hPSC as early as possible in a workflow. The schematic figure below shows at a glance the key stages during which Stem Genomics recommends testing human hPSCs in culture for genetic variants. Beyond avoiding the deleterious effects these defects can have on the final product, the aim is also to avoid the unnecessary waste of resources and time on potentially abnormal cell lines (figure 2).

**Key stages when genetic testing is recommended:**

- **Acquisition of a new line:** before starting any work, it is critical to ensure the genomic stability of the initial material. Most purchased lines will come with some kind of genomic stability tests, but sometimes small size abnormalities not detectable by G-Banding karyotype can emerge. A good method at this stage can be coupling G-Banding with digital PCR, or running NGS testing, if budget allows.

- **Reprogramming and gene editing:** these procedures could favor the generation and selection of genomic aberrations in PSCs. A quick screening of clones and colonies is recommended. Digital PCR's quick turnaround, sensitivity and affordable cost makes it an ideal technology at this stage.

- **In-process control during cell amplification & maintenance:** this constitutes another stressing factor likely to generate genetic defects in hPSCs. Considering the speed at which a recurrent abnormality can take over the culture, it is advisable to test PSCs

at least every 5-10 passages. This is realistically only feasible using digital PCR, as its multiplexing capabilities allow a high scope of detection with a fast turnaround and affordable cost.

- **Pre-banking characterization:** genomic stability is one of the critical features required for the complete characterization of a cell line before banking. For this stage, the method selection can be a combination of G-Banding and digital PCR or NGS-based assays.

- **Differentiation monitoring:** it is recommended to continue to check for genomic abnormalities until cells have achieved full differentiation, and to do so when changing media. Once again, the speed of digital PCR makes it ideal for that stage.

- **End of process:** a final check will be requested before publication, but also before moving to the clinical stage. If an NGS test has been performed at the start of the process, it is good practice to perform it again at the end of the process and compare the results. An alternative would be a G-Banding karyotype coupled with digital PCR, as it offers the usual structural rearrangement analysis combined with digital PCR's resolution, which will pick up sub-karyotyping abnormalities such as 20q. This constitutes a suitable testing strategy at the end of an RUO process.

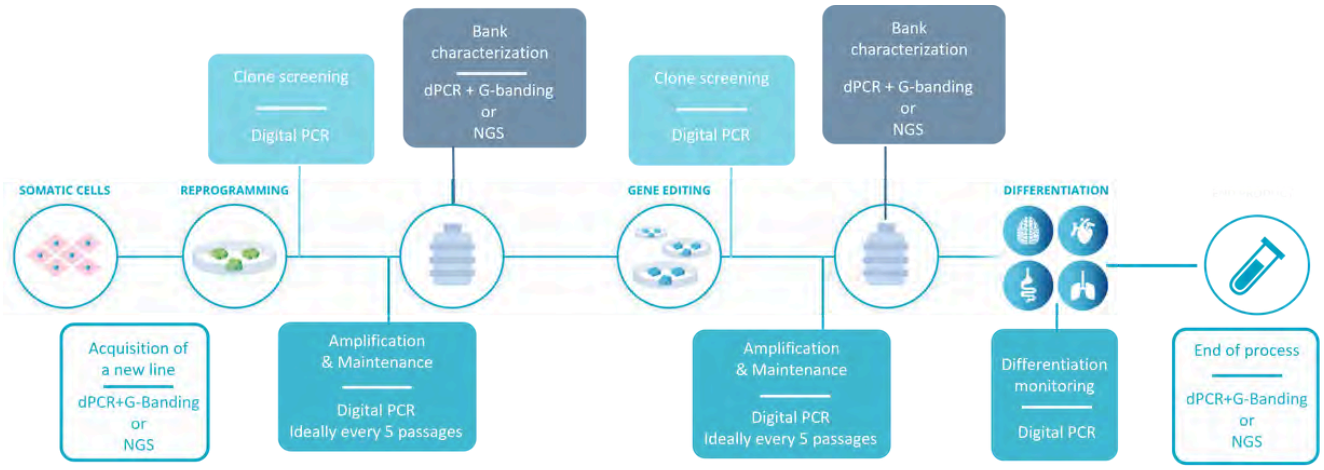



*Figure 2. Recommended quality testing workflow for genomic stability. Source Stem Genomics*

# IV. Conclusion: Best Practice

Ensuring the genomic integrity of hPSCs is a critical aspect for their use in both research and therapeutic applications. To minimize the risk of genetic abnormalities in hPSCs, it is essential to implement:

### 1-Standardized cell culture conditions

The adoption of standardized cell culture practices is critical. The use of a quality management system (QMS), such as ISO 9001:2015, is highly recommended to ensure consistency and reproducibility of cell culture conditions (Molina-Ruiz *et al.*, 2022).

### 2-Genetic integrity testing

The importance of regularly assessing the genetic integrity of stem cells is emphasized in all of the publications cited in this white paper. Routine genomic monitoring is consistently recommended to detect chromosomal abnormalities, copy number variations, and other mutations that may occur during long-term culture. In addition to the stressors mentioned in this paper, such as reprogramming methods, prolonged culture conditions including environmental factors (e.g. oxygen levels, cell density, temperature), and gene editing technologies using CRISPR-Cas9, it is also critical to remain vigilant during key stages of the culture process. Key stages include the acquisition of a new cell line, in-process controls during cell amplification and maintenance, pre-banking characterization, the differentiation phase, and the end of the process. Regular assessments during these phases ensure the stability and safety of stem cells for both research and clinical applications, preserving their functionality and therapeutic potential.


### Acknowledgments

I would like to express my sincere gratitude to Dr. Said Assou and Prof. John De Vos for their invaluable feedback and corrections to this document. I would also like to thank Juline Vincent for her diligent work on data collection.


**Thank you for reading.**

## About Stem Genomics

Stem Genomics is a biotech company specialized in the design of innovative testing technologies for stem cells. A spin-out of the Institute for Regenerative Medicine & Biotherapy (IRMB), Stem Genomics has emerged from a prestigious scientific environment. It was co-founded by Prof. John de Vos and Dr. Said Assou, both experts in stem cell genomic integrity.

Founded in 2018, Stem Genomics works with +150 clients worldwide, from academic institutions to biotech companies and core facilities. The company services North American clients from their laboratory based in the Research Triangle in Durham, North Carolina, and their European and other international clients from their headquarters in Montpellier, France.
Stem Genomics aims to provide a one-stop-shop solution for the quality control needs of stem cell scientists. This includes various genomic stability tests (digital PCR, G-Banding, and NGS-based assays), identity (STR) and sterility testing. Visit www.stemgenomics.com to learn more.

## About the author

Dr Reda Zenagui obtained his PhD in Molecular Genetics from the University of Montpellier, He has been a key contributor to the Montpellier Hospital for over a decade. He specialized in cutting-edge next-generation sequencing (NGS) techniques and bioinformatics analysis for targeted genetic studies. His expertise in variant interpretation ensured accurate patient diagnosis. In his current role as R&D Director at Stem Genomics, he continues his contribution to the genomic field by identifying molecular and chromosomal abnormalities, drawing on his extensive experience in testing the genomic integrity of stem cells.

**Stem Genomics France,** Cap Sigma, 1682 rue de la Valsière, 34 790 Grabels.
**Stem Genomics US,** 5501 Fortunes Ridge Drive, Suite 0, Durham, NC 27 713.
www.stemgenomics.com - contact@stemgenomics.com

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