Stem Cell Reports
Forum
ISSCR
OPEN ACCESS

# A call for consensus guidelines on monitoring the integrity of nuclear and mitochondrial genomes in human pluripotent stem cells

Andrea Rossi,[1,*] Selene Lickfett,[2,3] Soraia Martins,[1] and Alessandro Prigione[2,*]
[1]Genome Engineering and Model Development Lab (GEMD), IUF-Leibniz Research Institute for Environmental Medicine, Düsseldorf 40225, Germany
[2]Department of General Pediatrics, Neonatology, and Pediatric Cardiology, Medical Faculty, Heinrich Heine University, Düsseldorf Germany
[3]Department of Anatomy, Medical Faculty, Heinrich Heine University, Düsseldorf, Germany
*Correspondence: andrea.rossi@IUF-Duesseldorf.de (A.R.), alessandro.prigione@hhu.de (A.P.)
https://doi.org/10.1016/j.stemcr.2022.01.019

Despite the widespread use of human pluripotent stem cells (hPSCs), there is no general consensus with respect to monitoring the integrity of nuclear and mitochondrial genome of hPSCs. We argue that clear guidelines should be in place, and we have identified frequently asked questions that can be used to develop a practical guide for hPSCs users.

Human pluripotent stem cells (hPSCs) are becoming an integrative part of several disciplines beyond classic stem cell research. Core facilities for the generation and handling of hPSCs are being routinely established at research institutes worldwide. Nonetheless, there is a lack of consensus regarding how and when to monitor the integrity of the nuclear and mitochondrial genome of hPSCs. Loss of nuclear or mitochondrial genome integrity can cause downstream changes in cellular phenotype, as variations in common genetic traits affect the gene expression profile and may even lead to detrimental consequences on cell viability or increase in tumorigenesis (Halliwell et al., 2020; Wei et al., 2021). However, how and when to monitor the genomic integrity of hPSCs is currently left to the individual scientists to decide. This lack of consensus hinders data reproducibility and transfer of material, as different standards are applied in different laboratories, ultimately undermining clinical translational efforts. We believe that the international stem cell community should put forward clear guidelines on genome monitoring of hPSCs. We have identified key frequently asked questions on this topic. We propose that these questions could represent the basis on which to develop practical-oriented guidelines for scientists wishing to apply hPSCs to their research questions.

## How can I monitor the genome integrity of hPSC lines?

Several techniques are available to analyze hPSC genome integrity, although with different resolution, sensitivity, sample multiplexing, and costs (McIntire et al., 2020) (Table 1). While time-consuming, G-banding is widely used for karyotyping because it offers a spatial resolution to detect chromosomal alterations, it can visualize individual cells and individual chromosomes, and it is cost-effective. G-banding requires an actively growing source of cells and the analysis of at least 20 metaphase nuclei. The major pitfall of G-banding is its limited resolution of ~5–10 Mb, which prevents the detection of small abnormalities. Chromosomal microarray (CMA) platforms, such as comparative genomic hybridization (CGH) or single-nucleotide polymorphism (SNP) arrays, are high-resolution alternatives to G-banding. CMA can detect copy-number variants (CNVs) and genomic differences as small as 0.5 Mb, and can also be used for high-throughput karyotyping. However, CMA cannot detect balanced chromosomal rearrangements such as translocations with no change in copy number. Whole genome sequencing (WGS) using short reads can overcome the shortcomings of conventional cytogenetic methods and CMA, as it allows the detection of all types of mutations, including chromosomal rearrangements and single-nucleotide changes. WGS is still associated with high costs, however. Oxford Nanopore Technology (ONT) is a lower-cost alternative that allows the real-time sequencing of ultra-long reads, thereby enabling the detection of CNVs in hPSCs. Nevertheless, ONT raw reads may be associated with high error rates dominated by false deletions, and common protocols are not yet widely available.

## How often should I monitor the genome integrity of hPSC lines?

It has been estimated that hPSCs may have a mutation rate of $\sim 1 \times 10^{-9}$ mutations per base pair per cell division (Kuijk et al., 2020). Some of the commonly acquired mutations may confer a selective growth advantage. During cell culture expansion, a genetically abnormal clone may thus quickly overtake the whole culture. Specific culture conditions such as low oxygen conditions could help minimize the occurrence of these adverse events (Thompson et al., 2020). Routine genome monitoring should therefore be implemented as a standard operating procedure (SOP) in stem cell laboratories (Table 1). However, the precise frequency of genome monitoring of hPSCs has not been clearly defined by the stem cell community. Moreover, for mutant or patient-derived hPSCs that may be, for instance, more susceptible to

**Table 1. Monitoring the genome integrity of human pluripotent stem cells: from critical questions to implementable strategies**

| Questions | Approaches | Implementations |
|---|---|---|
| How can I assess the genome integrity of hPSC lines? | • G-banding (from at least 20 metaphases)<br>• Chromosomal microarrays (CMAs)<br>• Whole-genome sequencing (WGS)<br>• Oxford Nanopore Technology (ONT) | • Journals should require authors to show proof of genome integrity for hPSC lines<br>• Standard operating procedures (SOP) for genome integrity data analysis could be shared across laboratories<br>• Uniform guidelines for stem cell banks, repositories, and journal resource articles<br>• Funding agencies should be made aware of the importance of monitoring genome integrity in hPSCs, so that they can specifically ask scientists to implement these analyses<br>• Research institutes could require and support routine genome integrity analyses<br>• Discussion and regular updating of the guidelines during international stem cell conferences |
| How can I perform routine monitoring of genome integrity of hPSC lines? | • G-banding<br>• CMA<br>• WGS<br>• ONT<br>• fluorescence *in situ* hybridization (FISH)<br>• qPCR<br>• digital PCR | |
| How should I check the genome integrity of hPSC lines after genome editing? | Inspect the presence of off-targets by:<br>• WGS<br>• targeted PCR followed by high-resolution melting (HRM) analysis or PCR and sequencing | |
| How could I determine the integrity of mitochondrial genome of hPSCs? | • mtDNA PCR amplification or mtDNA enrichment, followed by sequencing<br>• analysis of mtDNA sequences from WGS | |

oxidative damage, it may be advisable to lower the passage limit for the next genome integrity assessment. There is also no consensus regarding the method of choice for constant monitoring of the integrity of the nuclear genome of cultured hPSCs. To decrease the costs of routine genome monitoring of several hPSC lines, one could first focus on excluding the presence of genetic aberrations that are known to occur with higher frequency in cultured hPSCs (Kuijk et al., 2020). The identification of recurrent abnormalities can be performed using fluorescence *in situ* hybridization (FISH) or quantitative polymerase chain reaction (qPCR) (McIntire et al., 2020). In addition, digital PCR has been suggested as a cost-effective and sensitive approach for the routine genome monitoring of hPSCs (Assou et al., 2020).

## How should I check the genome integrity of hPSC lines after genome editing?

The ability to genetically modify hPSCs to obtain isogenic cell lines allows an assessment of molecular and cellular phenotypes that result from a certain mutation rather than the cellular genetic background. Isogenic hPSC lines can be generated by repairing a certain mutation in patient-derived cells, or by introducing it in cells derived from healthy individuals. Which of these two strategies is preferable may depend on the underlying scientific questions. Despite continuous advances in editing technologies, genome integrity defects may occur during this process due to potential off-target mutations generated by such techniques. Therefore, the genomic characterization of edited hPSCs remains crucial (Halliwell et al., 2020). While it is essential to perform karyotyping of genome-edited hPSCs, it is not trivial to decide how many edited clones need to be monitored. It is reassuring that if the bulk of cells that went through genome engineering does not display chromosomal abnormalities, then the single clones that have been genotyped will likely be karyotypically normal (Chen and Pruett-Miller, 2018). In this case, karyotyping of many clones may be avoided, reducing costs and unnecessary work. For clustered regularly interspaced short palindromic repeats (CRISPR)-based genome editing, there is a growing number of tools available that can predict potential off-target effects. In addition, different assays can be performed to fully assess the presence of off-target events. WGS is a powerful tool to detect genome-wide

off-target mutations. A lower-cost alternative is represented by target PCR of the predicted off-target loci followed by high-resolution melting (HRM) analysis and sequencing (Ramachandran et al., 2021). Furthermore, the use of different clones generated from different guide RNAs may help in addressing some of the off-target concerns.

## Should I also monitor the integrity of the mitochondrial genome of hPSC lines?

Next to the nuclear DNA, every cell also contains multiple copies of mitochondrial DNA (mtDNA), which is often overlooked in genome integrity testing of hPSCs. Human cells can harbor a mixed population of mtDNA copies, both wild-type mtDNA and mutated mtDNA. A high percentage of mtDNA copies carrying specific mutations cause severe multi-system mitochondrial disorders, and also contribute to common age-related disorders such as Parkinson's disease. Changes in the mtDNA mutation level can occur upon reprogramming somatic cells into hPSCs, leading to an altered respiratory capacity of the differentiated cells (Prigione et al., 2011; Zambelli et al., 2018). Although mtDNA variants may remain relatively stable during differentiation, changes in their amount can occur at high frequency during the derivation of hPSCs. A study in 146 hPSC lines derived from healthy individuals observed that 76.6% of the lines displayed altered mtDNA mutation levels when compared to their parental somatic cells, with a mutation rate of $\sim 8.62 \times 10^{-5}$/bp (Wei et al., 2021). Similar changes have been observed in hPSCs obtained from patients carrying pathogenetic mtDNA mutations (Palombo et al., 2021). Monitoring mtDNA integrity should therefore be included in the characterization of newly generated hPSC lines (Table 1). The quantification of mtDNA changes can be performed by PCR, followed by Sanger sequencing, next-generation sequencing (NGS), or Nanopore sequencing. Primers for amplifying mtDNA molecules into two segments need to be located outside the regions involved in the generation of breakpoints underlying mtDNA deletions (Palombo et al., 2021). Alternatively, high-depth mtDNA sequences can be extracted from WGS (Wei et al., 2021). In every case, specific software and expertise may be required to analyze mtDNA, given its multi-copy nature that can contain both wild-type and mutated DNA molecules.

## Are there ethical aspects related to monitoring the genome integrity of hPSC lines?

Working with hPSCs can have important ethical implications to consider, even when hPSCs are generated from non-embryonic material by reprogramming somatic cells. Ethical regulations can vary among different countries, but genetic information is usually particularly sensitive. For example, in accordance with the European Union (EU) General Data Protection Regulation, information on genome integrity and confidential documents, such as donor consent and donor information forms, cannot be made public. These measures may result in restrictions in analyzing and reporting genomic changes in individual hPSC lines or in sharing those lines with other laboratories. Scientists working with hPSCs should be aware of these important ethical aspects, and the potential limits in sharing data and material. To enable the transparency and reproducibility of hPSC work, there are registries that catalog published hPSCs. One example of a freely accessible global registry is the Human Pluripotent Stem Cell Registry (hPSCreg) (https://hpscreg.eu/). However, the information on genome integrity within such registries is usually not provided by all users in the same manner, given the lack of standardized guidelines with respect to reporting the genome integrity of hPSCs. The stem cell community would therefore benefit greatly from having clear guidelines for genome integrity monitoring, as all published hPSC lines registered in available repositories could list the information on potential genomic abnormalities in a standardized and comparable manner.

## How can we ensure the implementation of nuclear and mitochondrial genome monitoring in hPSC research?

While there is a significant consensus on the importance of genome integrity for hPSCs, we lack clear guidelines for implementation strategies. This is particularly important for translational research, as even clinical grade hPSCs may harbor genetic defects (Thompson et al., 2020). The International Stem Cell Initiative highlighted the need for a widespread agreement on the risk assessment of genetic changes in hPSCs (Andrews et al., 2017). They suggested establishing an international advisory group that could evaluate the genetic risks and ultimately propose safety assessment approaches. In addition to this, other implementation strategies may be pursued by different stakeholders (Table 1). Scientific journals and hPSC repositories could ask authors to provide proof of the genome integrity of hPSCs in a manner that is transparent and easily comparable across laboratories. Standardized SOP for genome integrity data analysis (e.g. pipelines for CMA analysis) may be made available and shared among core facilities and individual scientists. Funding agencies and research institutes could require and support scientists in performing routine genome integrity analysis. Finally, the International Society for Stem Cell Research (ISSCR) and other stem cell research networks may provide a hub for discussing and improving the monitoring guidelines in an open and inclusive manner.

Ultimately, it is in the interest of the whole community that hPSC studies are reproducible and sharable, and that the generated findings can have a meaningful impact on future clinical applications of stem cell research.

## ACKNOWLEDGMENTS

A.P. acknowledges support from the Deutsche Forschungsgemeinschaft (DFG) (#PR1527/5-1), the German Federal Ministry of Education and Research (BMBF) (#01GM2002A), the United Mitochondrial Disease Foundation (UMDF), and the University Hospital Düsseldorf (Forschungskommission UKD). A.R. acknowledges support from the Leibniz Association.

## AUTHOR CONTRIBUTIONS

Concept and supervision, A.R. and A.P. Writing – original draft, A.R., S.L., and A.P. Writing – review & editing, A.P. Comments and final acceptance, A.R., S.L., S.M., and A.P. Table preparation, S.M.

## CONFLICTS OF INTEREST

The authors declare no competing interests.

## REFERENCES

Andrews, P.W., Ben-David, U., Benvenisty, N., Coffey, P., Eggan, K., Knowles, B.B., Nagy, A., Pera, M., Reubinoff, B., Rugg-Gunn, P.J., et al. (2017). Assessing the safety of human pluripotent stem cells and their derivatives for clinical applications. Stem Cell Rep. *9*, 1–4.

Assou, S., Girault, N., Plinet, M., Bouckenheimer, J., Sansac, C., Combe, M., Mianne, J., Bourguignon, C., Fieldes, M., Ahmed, E., et al. (2020). Recurrent genetic abnormalities in human pluripotent stem cells: definition and routine detection in culture supernatant by targeted droplet digital PCR. Stem Cell Rep. *14*, 1–8.

Chen, Y.H., and Pruett-Miller, S.M. (2018). Improving single-cell cloning workflow for gene editing in human pluripotent stem cells. Stem Cell Res. *31*, 186–192.

Halliwell, J., Barbaric, I., and Andrews, P.W. (2020). Acquired genetic changes in human pluripotent stem cells: origins and consequences. Nat. Rev. Mol. Cell Biol. *21*, 715–728.

Kuijk, E., Jager, M., van der Roest, B., Locati, M.D., Van Hoeck, A., Korzelius, J., Janssen, R., Besselink, N., Boymans, S., van Boxtel, R., et al. (2020). The mutational impact of culturing human pluripotent and adult stem cells. Nat. Commun. *11*, 2493.

McIntire, E., Taapken, S., Leonhard, K., and Larson, A.L. (2020). Genomic stability testing of pluripotent stem cells. Curr. Protoc. Stem Cell Biol. *52*, e107.

Palombo, F., Peron, C., Caporali, L., Iannielli, A., Maresca, A., Di Meo, I., Fiorini, C., Segnali, A., Sciacca, F.L., Rizzo, A., et al. (2021). The relevance of mitochondrial DNA variants fluctuation during reprogramming and neuronal differentiation of human iPSCs. Stem Cell Reports *16*, 1953–1967.

Prigione, A., Lichtner, B., Kuhl, H., Struys, E.A., Wamelink, M., Lehrach, H., Ralser, M., Timmermann, B., and Adjaye, J. (2011). Human induced pluripotent stem cells harbor homoplasmic and heteroplasmic mitochondrial DNA mutations while maintaining human embryonic stem cell-like metabolic reprogramming. Stem Cells *29*, 1338–1348.

Ramachandran, H., Martins, S., Kontarakis, Z., Krutmann, J., and Rossi, A. (2021). Fast but not furious: a streamlined selection method for genome-edited cells. Life Sci. Alliance *4*, e202101051.

Thompson, O., von Meyenn, F., Hewitt, Z., Alexander, J., Wood, A., Weightman, R., Gregory, S., Krueger, F., Andrews, S., Barbaric, I., et al. (2020). Low rates of mutation in clinical grade human pluripotent stem cells under different culture conditions. Nat. Commun. *11*, 1528.

Wei, W., Gaffney, D.J., and Chinnery, P.F. (2021). Cell reprogramming shapes the mitochondrial DNA landscape. Nat. Commun. *12*, 5241.

Zambelli, F., Mertens, J., Dziedzicka, D., Sterckx, J., Markouli, C., Keller, A., Tropel, P., Jung, L., Viville, S., Van de Velde, H., et al. (2018). Random mutagenesis, clonal events, and embryonic or somatic origin determine the mtDNA variant type and load in human pluripotent stem cells. Stem Cell Reports *11*, 102–114.