Increased Single-Cell Cloning Efficiency of Human Pluripotent Stem Cells Using CloneR™

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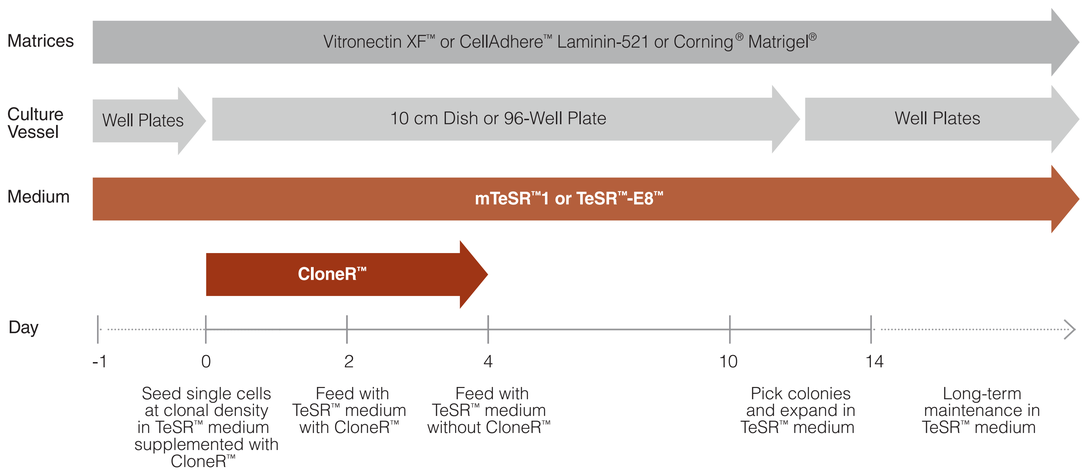
Introduction

Recent advances in gene-editing techniques, such as CRISPR, have led to more accessible and cost-effective methods to generate variant human pluripotent stem cell (hPSC) lines for a wide range of research areas. A major hurdle for gene-editing in hPSCs is the extremely low cloning efficiency of these cells (< 5%), making the generation of clonal hPSC lines an inefficient process. To address this hurdle, we have developed a novel hPSC cloning supplement, CloneR™. CloneR™ significantly increases the cloning efficiency of both human embryonic stem (hES) and induced pluripotent stem (hiPS) cells to 15 - 40% without the need for single-cell adaptation. The supplement is compatible with both mTeSR™1 and TeSR™-E8™ on a range of different matrices. CloneR™ supports hPSCs seeded both at clonal density (< 25 cells/cm^2) and single-cell deposition (1 cell/well). This novel supplement will facilitate gene editing in hPSCs through the rapid and highly efficient generation of clonal cell lines.

Methods

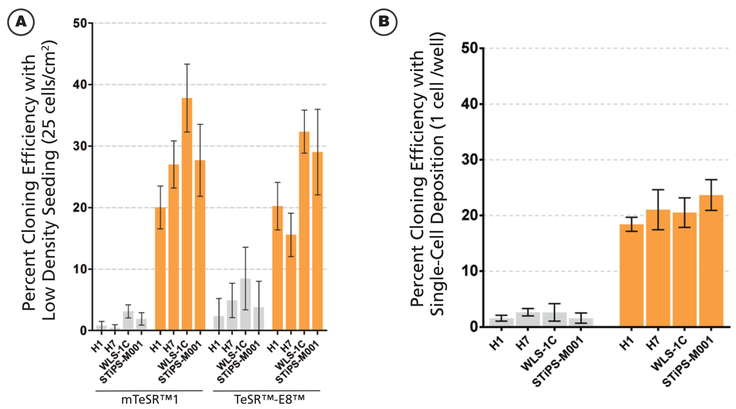
Figure 1. hPSC Single-Cell Cloning Workflow with CloneR™



On day 0, gene-edited or unmodified hPSCs are seeded as single cells at clonal density (e.g. 25 cells/cm^2) or sorted by FACS at 1 cell per well in 96-well plates in TeSR™ (mTeSR™1 or TeSR™-E8™) medium supplemented with CloneR™. On day 2, the cells are fed with TeSR™ medium containing CloneR™ supplement. From day 4, cells are maintained in TeSR™ medium without CloneR™. Colonies are ready to be picked between days 10 - 14. Clonal cell lines can be maintained long-term in TeSR™ medium.

Results

Figure 2. CloneR™ Increases the Cloning Efficiency of hPSCs Using Different Cell Seeding Protocols without Single-Cell Adaptation



TeSR™ medium supplemented with CloneR™ (orange) increases hPSC cloning efficiency compared with cells plated in TeSR™-containing ROCK inhibitor (10 µM Y-27632; gray). hPSCs were seeded **(A)** at clonal density (25 cells/cm^2) in mTeSR™1 or TeSR™-E8™ and **(B)** by single-cell deposition (seeded at 1 cell/well) in mTeSR™1. CloneR™ increases cloning efficiency in both hES (H1, H7) and iPS (WLS-1C, STiPS-M001) cell lines.

Figure 3. CloneR™ Increases the Cloning Efficiency of hPSCs at Low Densities Minimizing the Risk of Selecting Mosaic Colonies

10 µM Y-27632
0.6% Cloning Efficiency
(95 colonies)

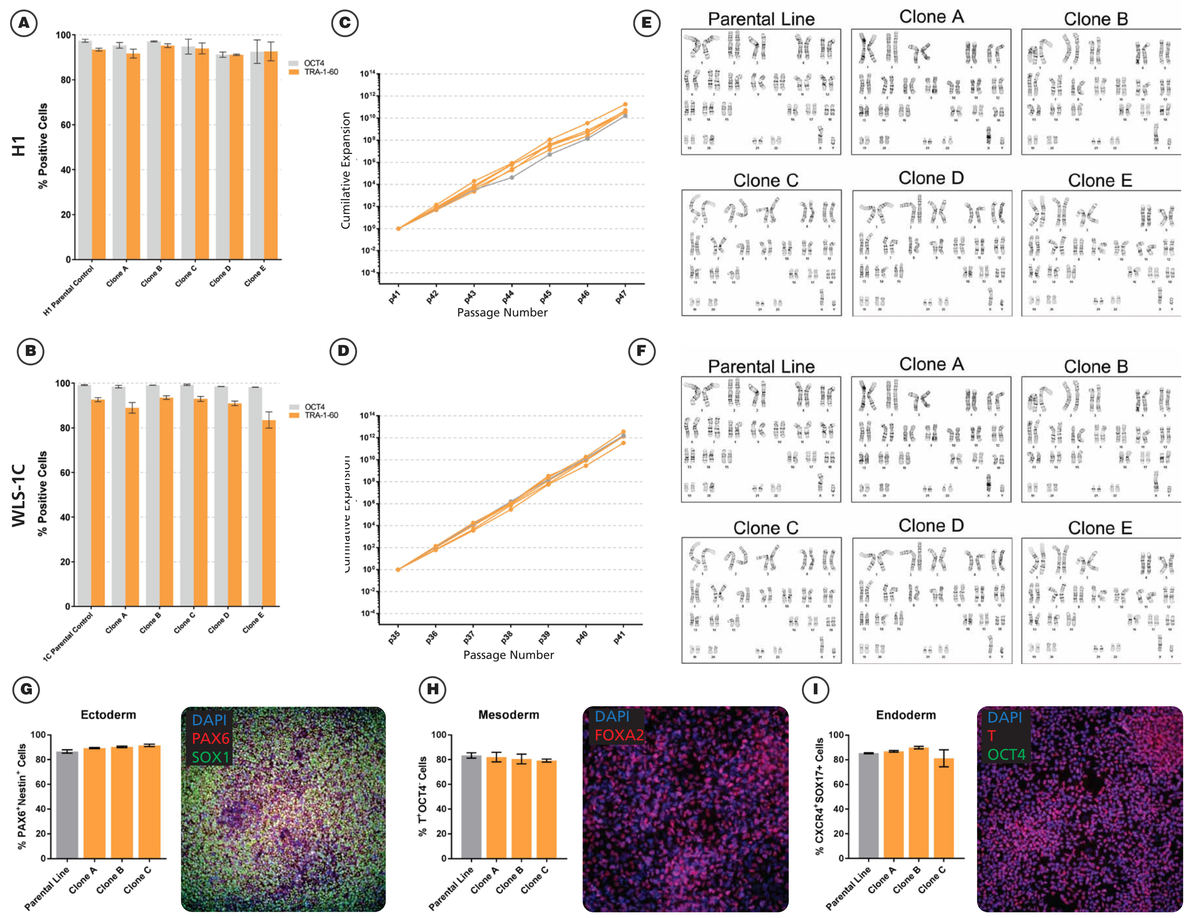
CloneR™
16% Cloning Efficiency
(2398 colonies)

10µM Y-27632
1% Cloning Efficiency
(3 colonies)

CloneR™
27% Cloning Efficiency
(82 colonies)

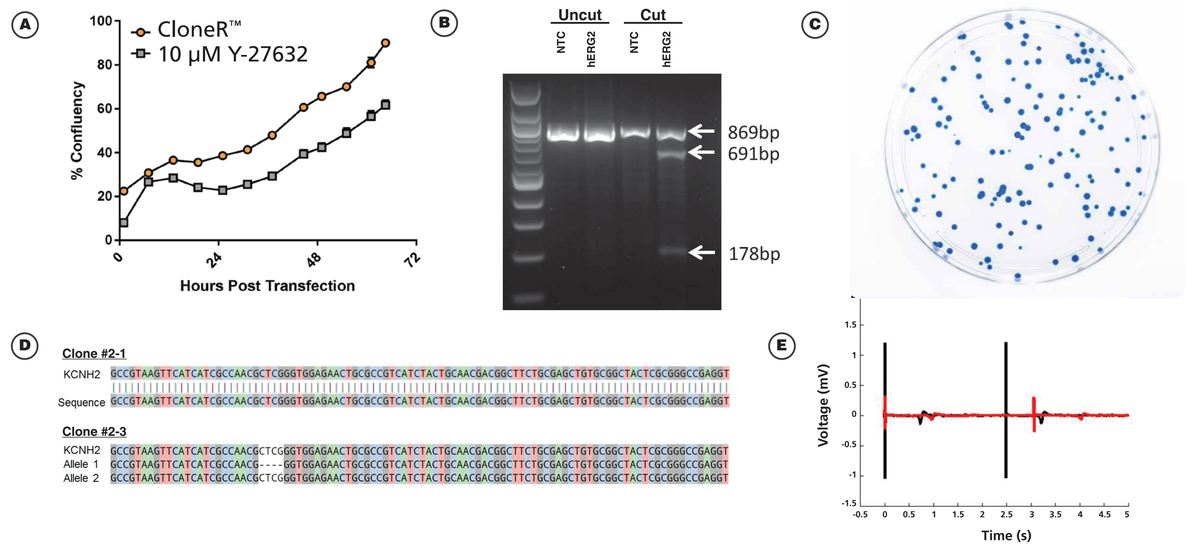
In accordance with current methods for generating clonal hPSC lines, H1 hES cells were plated at 15,000 cells per 10 cm dish either in mTeSR™1 supplemented with **(A)** ROCK inhibitor or **(B)** CloneR™. The superior performance of CloneR™ demonstrates that colonies generated at this density are likely not clonal but rather represent mosaic colonies. This is further illustrated by seeding the H1 hES cell line at 300 cells per 10 cm dish either in mTeSR™1 supplemented with **(C)** ROCK inhibitor or **(D)** CloneR™. Seeding cells at very low densities in medium supplemented with CloneR™ minimizes the risk of cells migrating and mixing to form mosaic colonies (colonies were fixed and stained with alkaline phosphatase at day 7).

Figure 4. Clonal hPSC Lines Generated Using CloneR™ Exhibit Pluripotency Characteristics Similar to Their Respective Parental Line, Indicating CloneR™ Does not Select Abnormal Cells



Clonal hPSC lines derived using CloneR™ express undifferentiated markers OCT4 and TRA-1-60 equivalent to their respective parental line (**A:** H1 hES cells, **B:** WLS-1C iPS cells). CloneR™ does not select for clones with increased growth rates (**C**: H1 hES cells, **D**: WLS-1C iPS cells). All clones derived from karyotypically normal parental lines using CloneR™ were karyotypically normal following five passages after cloning (**E**: H1 hES cells, **F**: WLS-1C iPS cells) (20 out of 20 cells analyzed). Clonal hPSC lines were capable of trilineage differentiation, three H1 hES clones shown following differentiation towards **(G)** ectoderm using the STEMdiff™ SMADi Neural Induction Kit, **(H)** mesoderm using STEMdiff™ Mesoderm Induction Medium and **(I)** endoderm using the STEMdiff™ Definitive Endoderm Kit.

Figure 5. CloneR™ Facilitates Gene-Editing of hERG2 to Model Long QT Syndrome In Vitro



STiPS-M001 hiPS cells were seeded at 50,000 cells/cm^2 in mTeSR™1 supplemented with CloneR™ following electroporation with Cas9/hERG2 RNP complex. **(A)** Cells recovered to confluency over 72 hours post-seeding with ~40% editing efficiency **(B)** determined using the T7 endonuclease assay. **(C)** Cells were harvested at 72 hours and plated at 400 cells per 10 cm dish (7 cells/cm^2) and cultured for 12 days diplaying 35% cloning efficiency (colonies were fixed and stained with alkaline phosphatase). **(D)** Sequencing confirmed mono-allelic frameshift deletion compared to unedited parental line. **(E)** Following differentiation to cardiomyocytes, edited cells display decreased spike amplitude and longer beat frequency duration (red line) compared to un-edited parental line (black line).

Summary

- CloneR™ is a specialized supplement developed to significantly increase single-cell survival of hPSCs
- hPSC lines derived using CloneR™ display equivalent marker expression, growth rates and pluripotent differentiation to their parental line
- Clones derived in CloneR™ from karyotypically normal populations maintain their genomic integrity
- Using CloneR™ in combination with TeSR™ media can advance gene-editing studies by facilitating the rapid and reproducible generation and establishment of new and clonal hPSC lines