

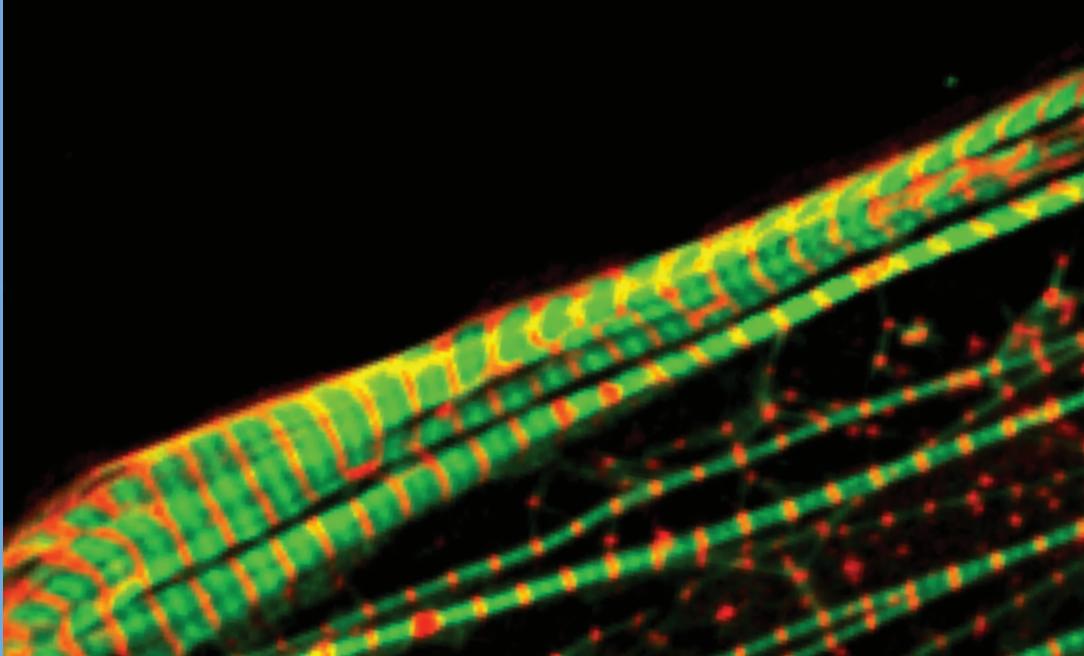
Disease model generation: 5 steps to model cardiac diseases

Follow this proven guide to facilitate generation
of iPSC-derived disease models

Introduction

A variety of somatic cells can be reprogrammed into human induced pluripotent stem cells (hiPSCs) that can be further differentiated into disease-relevant cell types, such as neurons and cardiomyocytes. This technology has now enabled scientists to use patient-derived cells for *in vitro* modeling of disease, applicable to biological studies and drug discovery. The availability of genome editing tools, such as the CRISPR-Cas9 and TALEN™ systems, further enhance disease modeling studies by allowing scientists to introduce or correct disease-related genetic changes, such as single-nucleotide polymorphisms (SNPs), to study their contribution to the disease phenotypes.

The ideal disease model consists of an isogenic pair (i.e., the control and mutated hiPSC lines) that was generated through genome editing and fully characterized before differentiation of the hiPSC lines into relevant cell types. The differentiated cells can then be used for the visualization of disease phenotypes *in vitro*. This guide highlights the 5 key steps required to build a cardiac disease model in hiPSCs.



1



Edit—design and order genome editing tools

2



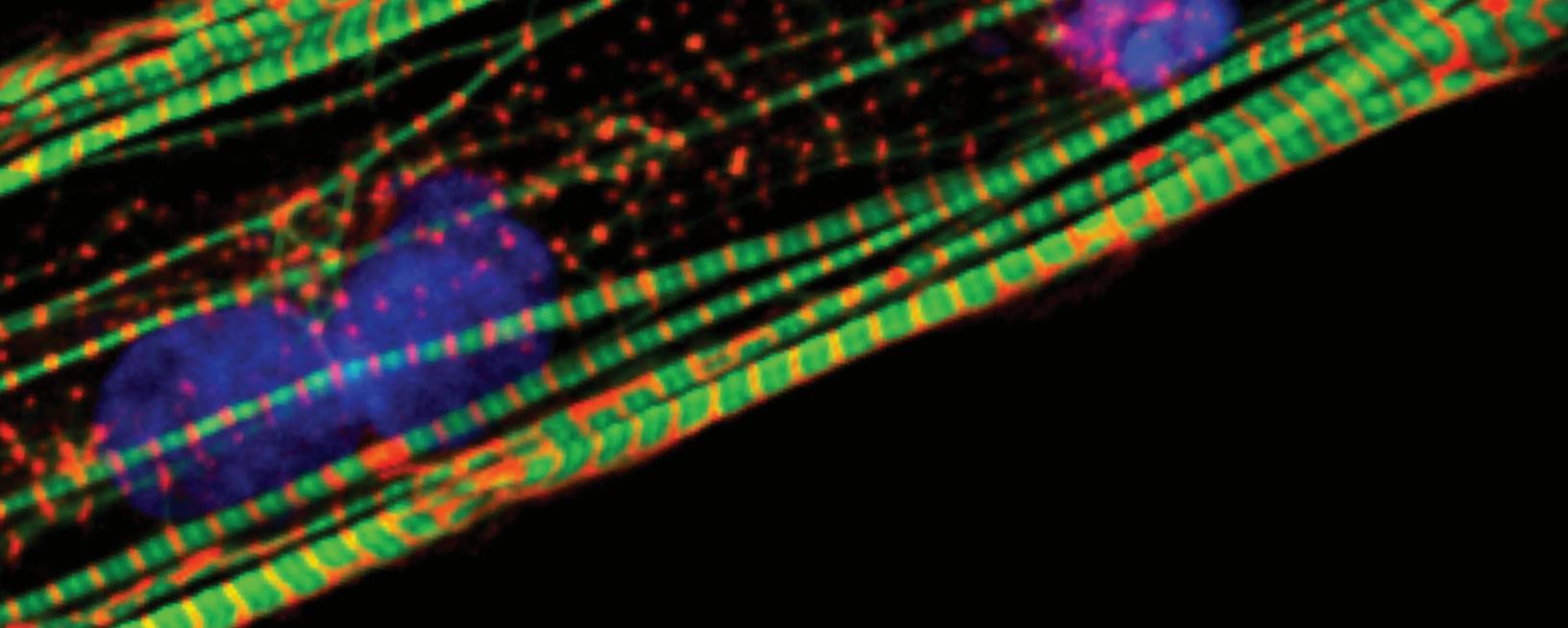
Clone—isolate and expand clones from a pool of genome-edited hiPSCs

Key reagents and tools:

- Invitrogen™ Pluripotent Stem Cell (PSC) Gene Editing Demo Kit
- Invitrogen™ TrueCut™ Cas9 Protein v2 and TrueGuide™ gRNA
- Invitrogen™ GeneArt™ Precision gRNA Synthesis Kit
- Invitrogen™ GeneArt™ PerfectMatch™ TALs
- Invitrogen™ Neon™ Transfection System
- Gibco™ StemFlex™ Medium
- Gibco™ RevitaCell™ Supplement
- Gibco™ rhLaminin-521
- Invitrogen™ GeneArt™ Genomic Cleavage Detection Kit
- Ion GeneStudio™ S5 System and Ion AmpliSeq™ primers

Key reagents and tools:

- StemFlex Medium
- RevitaCell Supplement
- rhLaminin-521
- Invitrogen™ TRA-1-60 kit for live-cell imaging
- Invitrogen™ Propidium Iodide



3



Characterize—check pluripotency and genome stability after editing

Key reagents and tools:

- PluriTest™ services and tools
- Applied Biosystems™ KaryoStat™ services and tools
- Applied Biosystems™ TaqMan® hPSC Scorecard services and tools
- Ion GeneStudio S5 System and Ion AmpliSeq primers
- Invitrogen™ Attune™ NxT Flow Cytometer
- Thermo Scientific™ CellInsight™ CX5 and CX7 High-Content Analysis (HCA) Platforms
- Invitrogen™ EVOS™ Imaging Systems
- Invitrogen™ Pluripotent Stem Cell 4-Marker Immunocytochemistry Kit

4



Differentiate—differentiate hiPSCs into cardiomyocytes

Key reagents and tools:

- Gibco™ PSC Cardiomyocyte Differentiation Kit
- Invitrogen™ Human Cardiomyocyte Immunocytochemistry Kit

5



Measure—perform disease-relevant assays in hiPSC-derived cardiomyocytes

Key reagents and tools:

- Invitrogen™ Fluo-4 fluorescent indicators
- Invitrogen™ FluoVolt™ Membrane Potential Kit
- Applied Biosystems™ TaqMan® qPCR probes and reagents
- CellInsight CX5 and CX7 HCA Platforms
- Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader

Step 1: Edit

Design and order genome editing tools

To improve the genome editing workflow in hiPSCs, editing efficiency should be maximized to facilitate clonal isolation downstream. Aside from locus-specific differences, genome editing efficiency in hiPSCs mainly depends on the editing tools used and the method implemented to deliver those tools. Genome editing can be achieved using TALEN technology or the CRISPR-Cas9 system, with the latter being easier to implement (Figure 1). With both tools, DNA breaks are introduced that may be randomly repaired by nonhomologous end joining (NHEJ), yielding a mixture of insertions and deletions (indels), or may be selectively repaired through homology-directed repair (HDR). NHEJ-generated indels may be used to create gene knockout cell lines, while HDR may be used to change single bases or introduce small or large tags (e.g., 6xHis or GFP), resulting in mutated or tagged cell lines.

Tips

- SNPs can be introduced using a 100 nt single-stranded DNA (ssDNA) oligo as a donor, which works well with CRISPR-Cas9 and TALEN-based genome editing.
- Protocols that use the Neon Transfection System may need to be optimized for genome editing efficiency and cell survival for your own hiPSC line, but in general program 7 provides good delivery of CRISPR editing tools and cell survival with maintenance of high editing efficiency.
- A complete protocol for delivery of Cas9 protein, gRNA, and oligos into hiPSCs can be found at thermofisher.com/diseasemodeling.

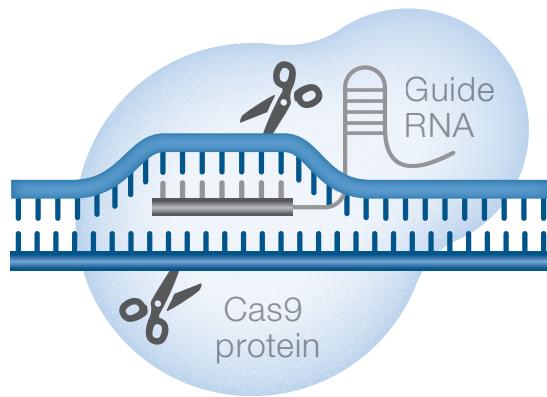
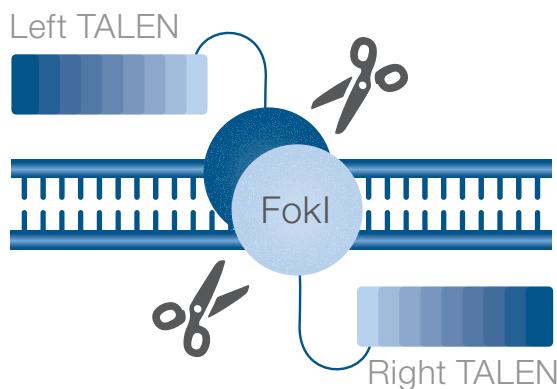


Figure 1. TALEN and CRISPR-Cas9 genome editing tools. Each editing tool binds and cleaves double-stranded DNA at specifically determined loci in the genome. Specificity is determined by the TALEN or guide RNA (gRNA) sequence, which can be changed to target the desired genomic DNA sequence. While the CRISPR-Cas9 system is easy to use due to simple design and ordering of gRNAs, sequence constraints may limit the loci that can be targeted. PerfectMatch TALs can target any sequence in the human genome and may have fewer off-target effects due to the longer DNA-binding site.

Product highlights

- Pluripotent Stem Cell (PSC) Gene Editing Demo Kit: This kit offers the critical reagents for successfully generating and detecting knockouts and point mutations in your cell line of interest. It allows optimization of conditions to yield efficient genome editing in hiPSCs, with reduced hands-on time and greater success. Learn more at thermofisher.com/pscgeneditingkit.
- Cas9 iPSCs and TrueCut Cas9 Protein v2: CRISPR-Cas9 editing in hiPSCs can be achieved by transfecting gRNA into a stable, Cas9-expressing hiPSC line such as Invitrogen™ Cas9 hiPSCs. Alternatively, a highly efficient Cas9 nuclease such as TrueCut Cas9 Protein v2 can be delivered into cells along with gRNA (Figure 3). These methods provide superior editing efficiencies compared to plasmid- or RNA-based methods. Explore our CRISPR products and services at thermofisher.com/crispr.

- GeneArt Precision gRNA Synthesis Kit: Make your own *in vitro*-transcribed (IVT) gRNAs that are compatible with the available editing and delivery tools. To obtain gRNA sequences, use our Invitrogen™ GeneArt™ CRISPR Search and Design Tool at thermofisher.com/crisprdesign.
- PerfectMatch TALs: PerfectMatch TALs provide locus-specific localization of the FokI nuclease. Unlike other TAL effector proteins, PerfectMatch TALs are engineered to remove the 5' base constraint and thus can be designed to target any desired sequence in the genome. Explore our TALEN products and services at thermofisher.com/tals.
- Custom genome editing services: Rely on our trusted products and expertise to have genome editing done for you. To send us a project inquiry, go to thermofisher.com/cellmodels.

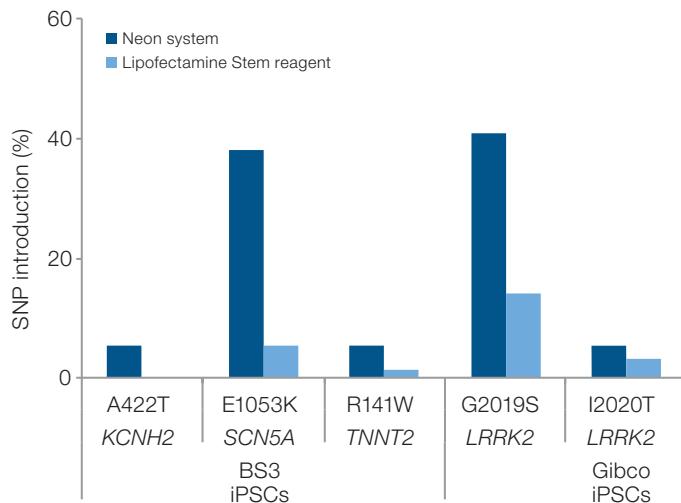


Figure 2. A comparison between delivery of editing tools using the Neon Transfection System and Invitrogen™ Lipofectamine™ Stem Transfection Reagent. Gibco™ Episomal and BS3 iPSC lines were edited at the indicated genomic loci using TrueCut Cas9 Protein v2, IVT gRNAs, and single-stranded oligo donors.

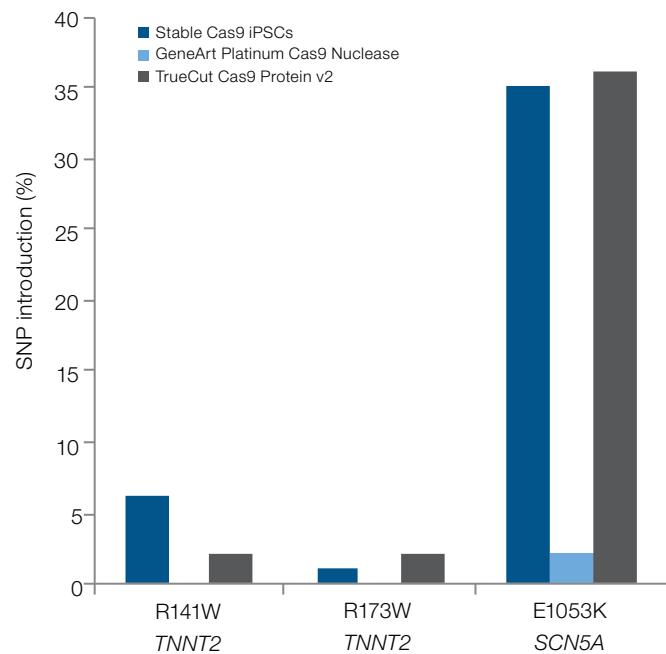


Figure 3. TrueCut Cas9 Protein v2 can induce SNP formation at frequencies similar to stable Cas9 iPSCs. HDR-mediated genome editing with gRNA and single-stranded oligo donor was performed using stable Cas9 hiPSCs or Cas9 protein variants, using the Neon device for delivery of the tools.

Step 2: Clone

Isolate and expand clones from a pool of genome-edited hiPSCs

After genome editing is achieved, the next step involves isolation of clonal hiPSC populations. Manual isolation of clones emerging after sparse plating of singularized cells is tedious and often requires multiple rounds of subcloning to isolate a fully clonal population. Single-cell isolation through cell sorting-based methods is a viable alternative, which had been challenging with hiPSC lines until recently. With the availability of state-of-the-art hiPSC culture reagents that are optimized for single-cell dissociation and survival of hiPSCs, we can now reliably isolate single-cell clones using cell sorting, with clonal recovery rates of 20–60% depending on the hiPSC line. The right extracellular matrix, growth medium, and survival supplements are key to a high recovery rate. In addition, stringent settings for cell sorting are required to reliably isolate single, viable hiPSCs and can be achieved through gating on cells that are negative for propidium iodide (PI) and positive for the pluripotency marker TRA-1-60 (Figure 4A). About 2 weeks after sorting, single-cell clones can then be easily visualized and further expanded for genetic analysis to identify clones with the edit of interest (Figure 4B). A protocol for clonal hiPSC isolation and expansion is available at thermofisher.com/diseasemodeling.

Tips

- Reagents developed for single-cell recovery dramatically improve cell survival of hiPSCs after sorting.
- Medium should not be changed for 3 days after single-cell sorting, to avoid aspiration of the single cells that are not fully attached.
- When using StemFlex Medium, medium changes every 3 days are sufficient for expansion of clones.
- Single-cell sorting into 96-well plates allows automation of downstream clone processing.

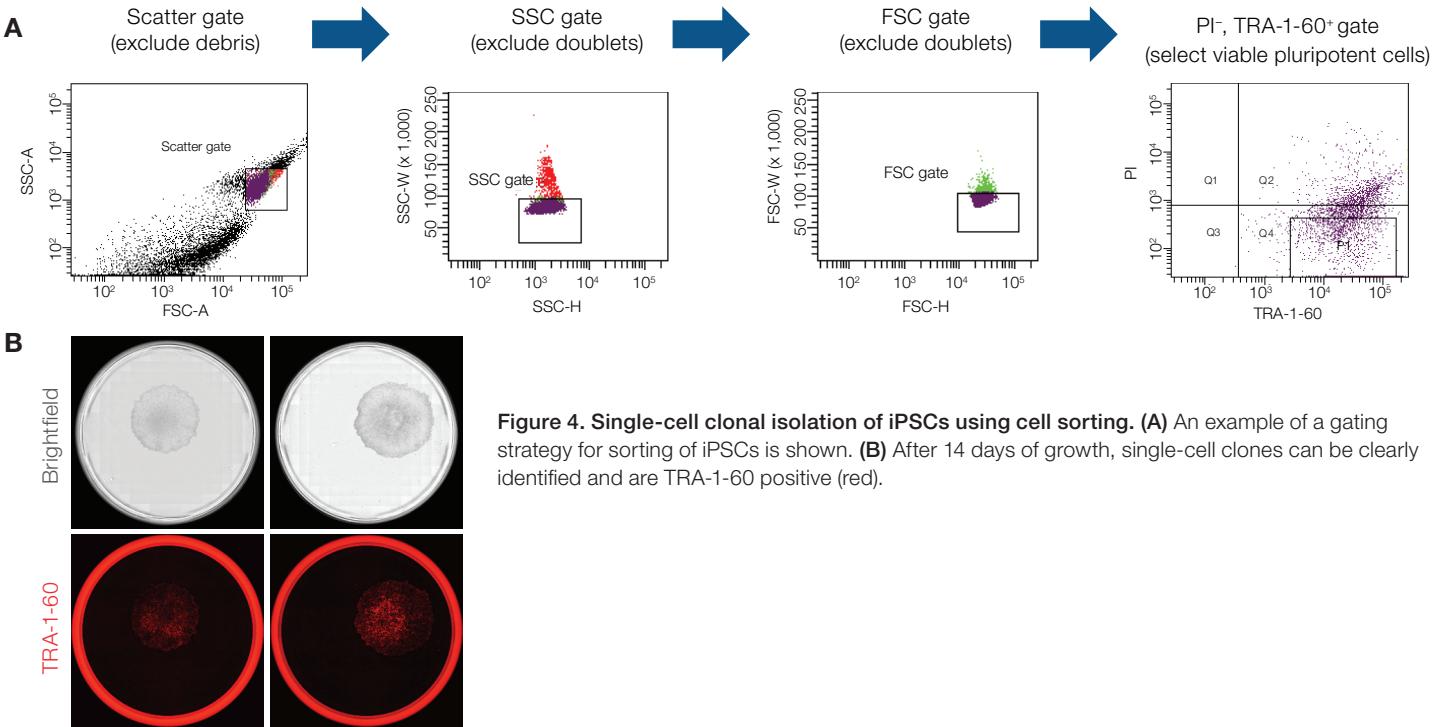


Figure 4. Single-cell clonal isolation of iPSCs using cell sorting. (A) An example of a gating strategy for sorting of iPSCs is shown. (B) After 14 days of growth, single-cell clones can be clearly identified and are TRA-1-60 positive (red).

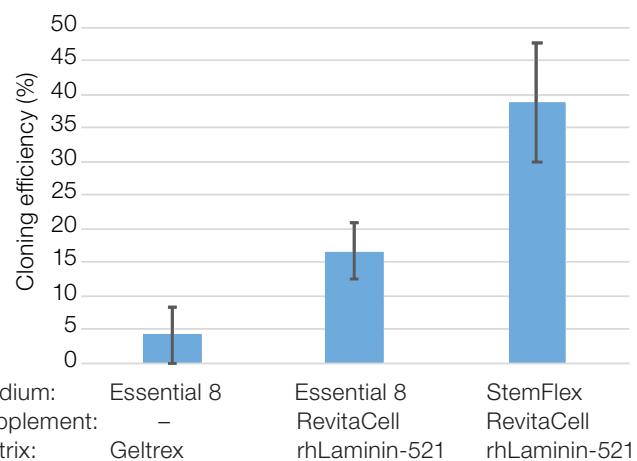


Figure 5. StemFlex Medium enhances cloning efficiency after sorting.
StemFlex Medium, RevitaCell Supplement, and rhLaminin-521 are optimized for challenging applications such as single-cell passaging and clonal isolation.

Product highlights

- StemFlex Medium: Single-cell sorting of hiPSCs requires a robust medium that allows culture of single hiPSCs and extended culture of hiPSCs without the need for daily medium changes (Figures 5 and 6). Go to thermofisher.com/stemflex for protocols and additional application data.

- rhLaminin-521: Attachment of single hiPSCs is the key for effective clonal recovery after sorting. Our rhLaminin-521 provides superior single-cell attachment and survival of hiPSCs compared to other matrices such as vitronectin.
- RevitaCell Supplement: Cell survival cocktails greatly enhance the recovery of hiPSCs after singulation. In addition, the presence of such cocktails in the medium after sorting provides an additional boost to clonal recovery.

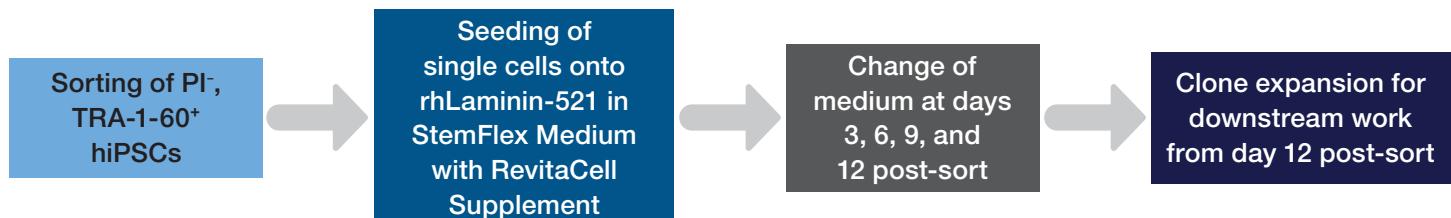


Figure 6. Workflow for isolation and recovery of single-cell hiPSC clones using cell sorting.

Step 3: Characterize

Check pluripotency and genomic stability after editing

Genome editing and clonal isolation of hiPSCs can be very stressful to the cells, and while in general no adverse effects are observed, it is important to characterize the hiPSCs before and after genome editing and clonal isolation experiments. Several options are available to easily assess the pluripotency status of hiPSCs at the protein or mRNA level, including systems that correlate your hiPSC line with a large reference set of commonly used PSC lines (Figure 7). Genomic stability is generally measured using traditional G-band karyotyping or through more objective digital karyotyping methods where DNA probes across the genome are used to map chromosomal gains and losses. To explore these options and more, go to thermofisher.com/stemcellanalysis.

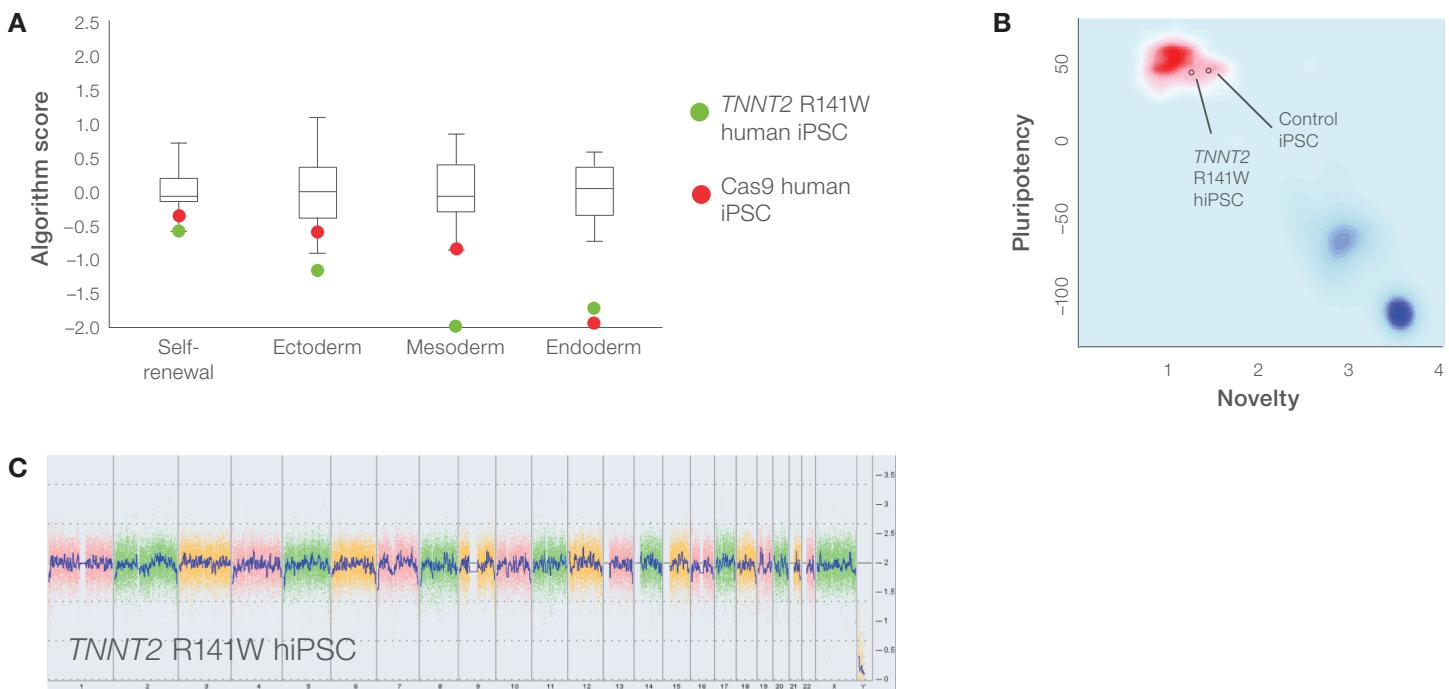
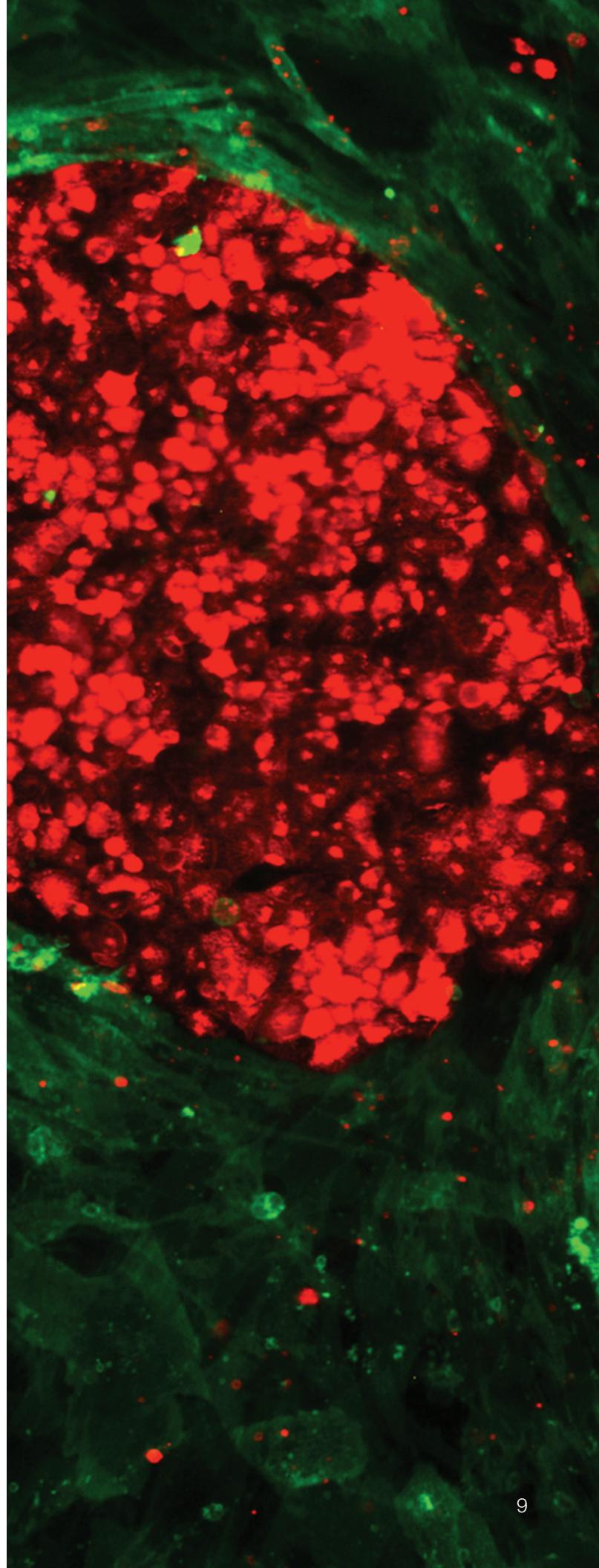


Figure 7. Characterization of a cell model of cardiac disease: dilated cardiomyopathy (DCM). A DCM-associated mutation of the cardiac muscle troponin T gene (*TNNT2* R141W) was introduced by gene editing of Cas9 hiPSCs. A combination of approaches showed that pluripotency and karyotype were unaffected by genome editing. **(A)** The TaqMan hPSC Scorecard Panel compares the gene expression profile of the sample to that of a reference set (colored dots and gray box plots, respectively). This assay uses over 90 genes and a static database of 13 PSCs for the comparison. **(B)** The PluriTest Assay uses microarray data to confirm pluripotency marker expression via a pluripotency score (reflecting degree of pluripotency) and novelty score (reflecting degree of differentiation). This assay uses more than 36,000 transcripts and a fluid reference set of over 450 cell and tissue types for the comparison. **(C)** The KaryoStat Assay offers whole-genome coverage for accurate detection of copy number changes and genomic aberrations.

Product highlights

- TaqMan hPSC Scorecard Panel: Teratoma formation is one of the methods used to demonstrate true pluripotency of an hiPSC line, but it is a lengthy process that requires the use of animals. Using mRNA isolated from hiPSCs and embryoid bodies (an unbiased way to generate all three germ layers from hiPSCs) in gene expression analysis, pluripotency can be confirmed through the presence of pluripotency genes in the hiPSCs and germ layer genes in the embryoid bodies [1,2].
- PluriTest Assay: RT-qPCR methods allow pluripotency assessment through mRNA expression analysis of a selected panel of well-established genes. However, unbiased methodologies based on mRNA from the entire genome allow a more accurate comparison against the mRNA profile of a collection of well-characterized hiPSCs [2,3]. The PluriTest Assay is a bioinformatics-based assay that compares the transcriptional profile obtained by microarray analysis of a sample to an extensive reference set of hESC lines, iPSC lines, somatic cells, and tissues.
- KaryoStat Assay: Genomic stability of hiPSCs should be checked regularly since hiPSCs are known to have an unstable genome. Traditionally, the karyotypes of hiPSCs have been analyzed using G-band-based karyotyping to reveal chromosomal aberrations. The KaryoStat Assay takes an alternative, molecular approach where the genomic DNA of the hiPSC line is surveyed on a microarray containing DNA probes across the genome. Probe intensities are then used to obtain a detailed view of the copy number and aberrations in DNA regions across the genome.



Step 4: Differentiate

Differentiate hiPSCs into cardiomyocytes

To visualize disease-relevant phenotypes, hiPSCs must be differentiated into the cell type where the disease manifests itself. Differentiation typically occurs in multiple stages that can take up to 2 months, depending on the cell type. Functionally relevant cardiomyocytes, expressing most of the functional proteins such as TNNT2, NKX2.5, MYH6, and ACTN1 and capable of contracting in culture, can be obtained in as little as 2 weeks. To monitor efficiency of the differentiation process, it is important to check the cells during differentiation using immunocytochemistry or qPCR for markers that are specific to the fate of interest (Figure 8).

Tips

- The PSC Cardiomyocyte Differentiation Kit simplifies generation of cardiomyocytes from PSCs.
- Formed cardiomyocytes can be easily identified through their spontaneous contraction, typically observed 10–14 days after the start of differentiation.
- Cardiomyocytes can be generated in bulk from PSCs and replated for high-throughput downstream assays.

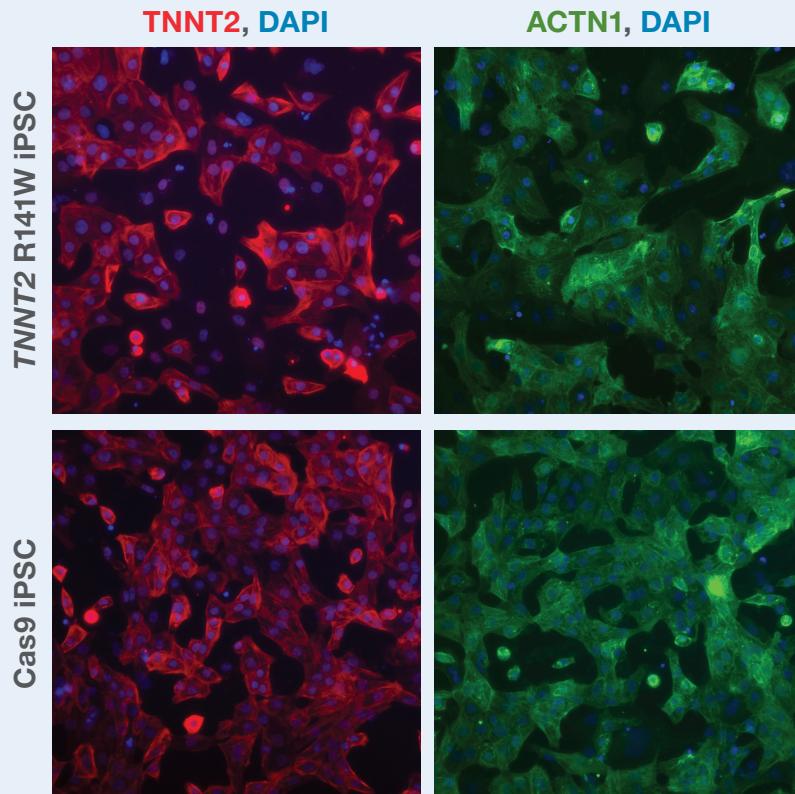
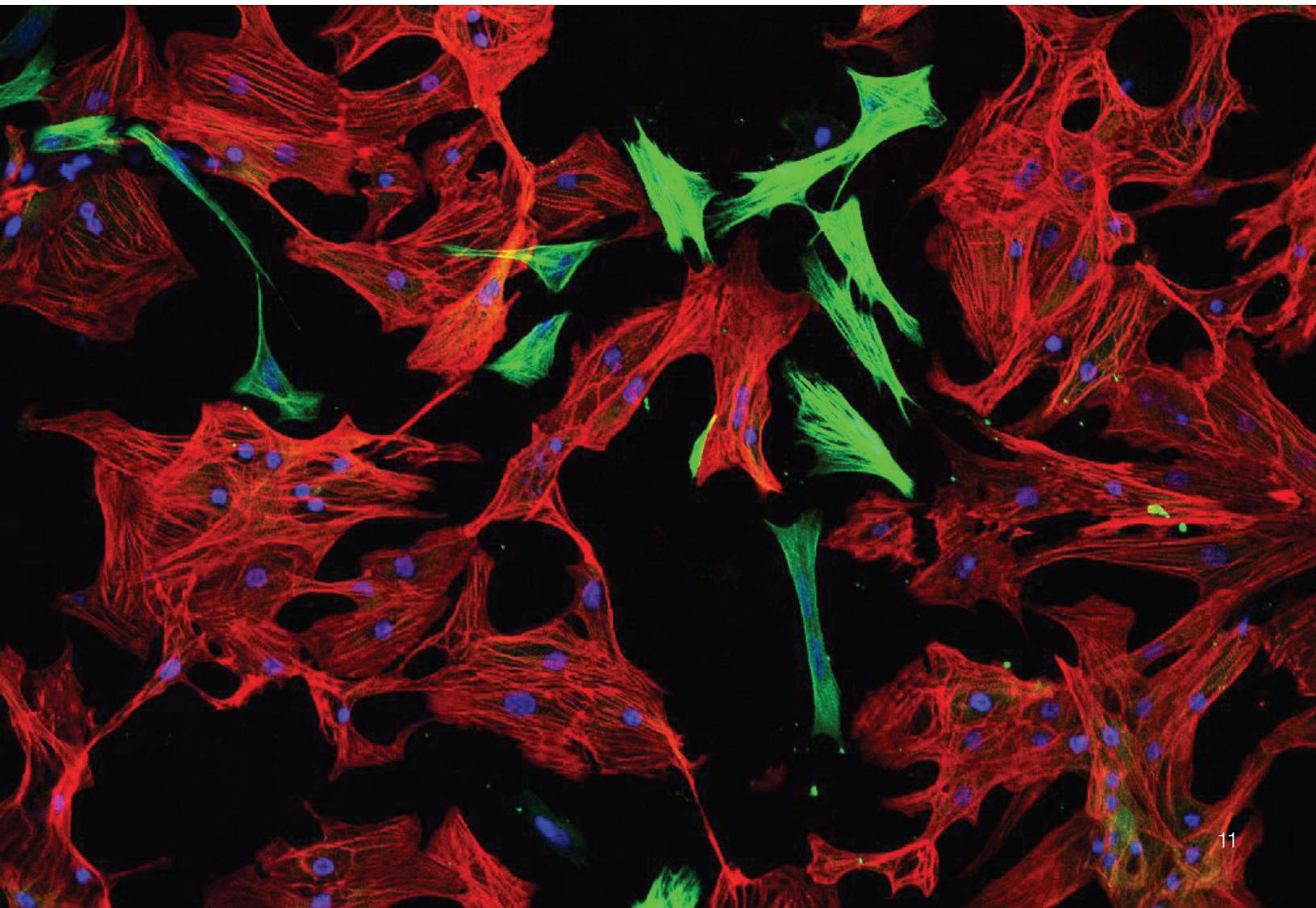


Figure 8. Cardiomyocytes derived from iPSCs with the TNNT2 R141W mutation. Cells were stained for TNNT2 and ACTN1, two structural proteins that are essential parts of the contractile apparatus in cardiomyocytes.

Product highlights

- PSC Cardiomyocyte Differentiation Kit: Differentiation into specialized cells such as cardiomyocytes can be challenging due to complicated and lengthy processes. Kits that simplify hiPSC differentiation in the lab are now available, relying on a small number of media that are used to specify progenitors and ultimately the mature cell type. Protocols and data for this kit are available at thermofisher.com/cardiacdiff.
- Human Cardiomyocyte Immunocytochemistry Kit: Throughout the differentiation process, efficiency during the different stages can be evaluated through protein expression analysis of fate-specific markers such as NKX2.5 and TNNT2.



Step 5: Measure

Perform disease-relevant assays in hiPSC-derived cardiomyocytes

Disease-relevant assays should always be performed on cells derived from an edited hiPSC line and its matching isogenic control. A wide variety of Invitrogen™ assays can be used, including endpoint assays to measure mitochondrial function (MitoTracker™ probes), proliferation (CyQUANT™ Direct kits), and apoptosis (CellEvent™ assays), or dynamic functional assays to measure kinetic changes in calcium flux (Fluo-4 assay), potassium flux (FluxOR™ assay), or membrane voltage (FluoVolt assay). In DCM, cardiac function is affected, and the heart may stop contracting during periods of stress or overexertion [4]. *In vitro* analysis of a disease model of the *TNNT2* R141W mutation indicates that functional phenotypes are indeed observed, reminiscent of the actual disease (Figures 9 and 10).

Tips

- Cell behavior and health can be measured over time using an HCA instrument that has an onstage incubator, such as the CellInsight CX7 HCA Platform.
- Hypertrophy of cardiomyocytes can be easily analyzed on HCA instruments by measuring cell size.
- Cardiomyocyte function can be visualized using Fluo-4 (calcium) or FluoVolt (membrane potential) fluorescent probes, for toxicity and disease model studies.
- To facilitate large-scale screening exercises, it may be more convenient to generate the cardiomyocytes in bulk, and replate for downstream assays.

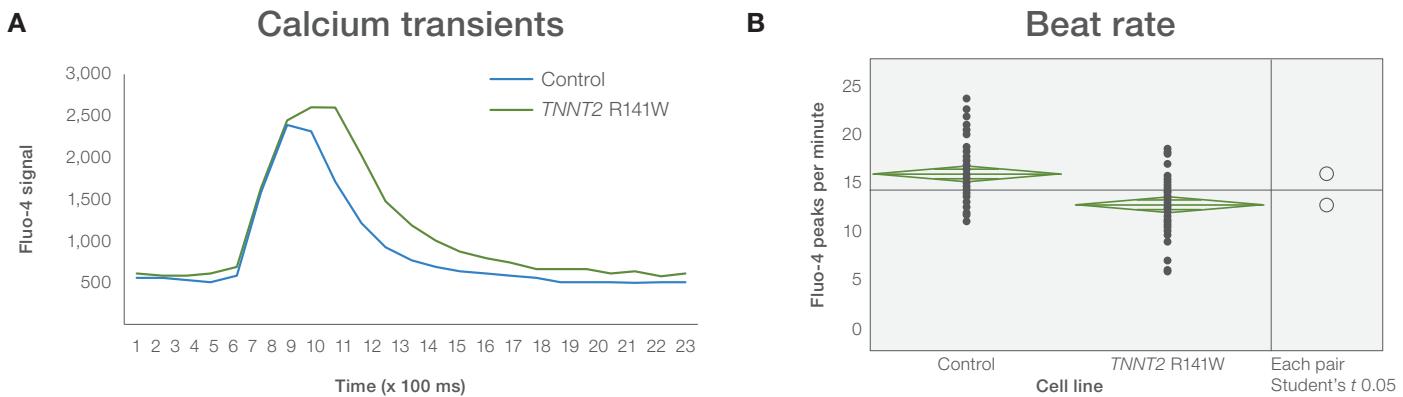


Figure 9. The DCM-associated mutation *TNNT2* R141W affects cardiomyocyte function *in vitro*. (A) Representative Fluo-4 traces indicating calcium handling in control and *TNNT2* R141W edited cardiomyocytes. A slight delay in the calcium level decay time can be observed. (B) The beat rate in DCM cardiomyocytes is slightly reduced, most likely caused by the slower calcium release as observed in panel A.

Product highlights

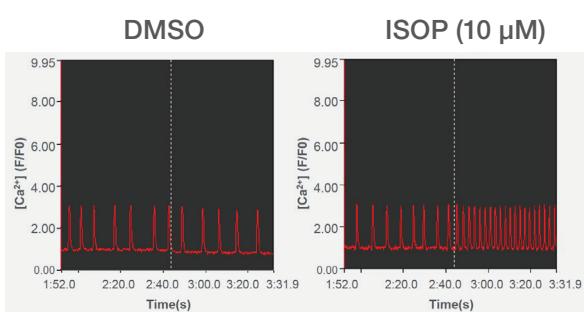
- FluoVolt Membrane Potential Kit: Cardiovascular diseases are often characterized by abnormal cardiac action potentials. The FluoVolt voltage-sensitive fluorescent indicator is a fast-response probe that can be used simultaneously with ion-sensitive fluorescent probes to confirm changes in voltage-gated ion channels in heart failure. Find out more about this kit at thermofisher.com/fluovoltbp70.



- CellInsight HCA platforms with onstage incubator: To observe and measure biological activity and changes over time, assays for disease models require precise control of temperature, humidity, and CO₂ levels. CellInsight HCA systems support long-term imaging studies, the data from which are the basis for quantitative analysis studies, which implement image analysis software to generate meaningful data. Find out more about live-cell HCA at thermofisher.com/hca.

A

Control



B

TNNT2 R141W

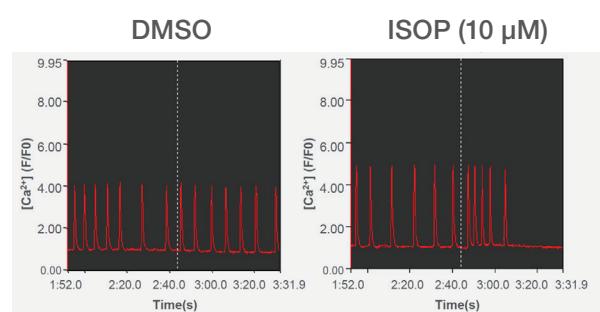


Figure 10. Cardiomyocytes carrying a DCM mutation adversely respond to adrenergic stimulation. (A) In normal, healthy cardiomyocytes, the beta-adrenergic stimulator isoproterenol (ISOP) increases beat rate, while the vehicle DMSO does not (white dashed line indicates compound addition). (B) DCM cardiomyocytes initially respond when treated with ISOP, but quickly lose the ability to contract spontaneously. Assays were performed on a Hamamatsu™ FDSS™ 6000 system.

Notes

Notes

References

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Find out more at thermofisher.com/cardiomodel

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