Running title: Protein and mRNA quantification in small hiPSC-CMs samples

Protein and mRNA quantification in small samples of human induced pluripotent stem-cell-derived cardiomyocytes in 96-well microplates

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Summary/Abstract

We describe a method for protein quantification and for mRNA quantification in small sample quantities of human induced pluripotent stem-cell-derived-cardiomyocytes (hiPSC-CMs). Demonstrated here is how the capillary-based protein detection system Wes™ by ProteinSimple and the *Power* SYBR™ Green Cells-to-CT™ Kit by Invitrogen can be applied to individual samples in a 96-well microplate format and thus made compatible with high-throughput (HT) cardiomyocyte assays. As an example of the usage, we illustrate that Cx43 protein and GJA1 mRNA levels in hiPSC-CMs are enhanced when the optogenetic actuator, channelrodopsin-2 (ChR2), is genetically expressed in them. Instructions are presented for cell culture and lysate preparations from hiPSC-CMs, along with optimized parameter settings and experimental protocol steps. Strategies to optimize primary antibody concentrations as well as ways for signal normalization are discussed, i.e. antibody multiplexing and total protein assay. The sensitivity of both the Wes and Cells-to-CT kit enables protein and mRNA quantification in a HT format, which is important when dealing with precious small samples. In addition to being able to handle small cardiomyocyte samples, these streamlined and semi-automated processes enable quick mechanistic analysis.

Key Words

High-throughput, hiPSC-CMs, Wes™ ProteinSimple, Cells-to-CT™, Cx43, ChR2, optogenetic

1. Introduction

Human stem-cell-derived cardiomyocytes are an important driver in personalized medicine through the development of patient-specific high-throughput assays*(1-4)*. These assays yield a range of functional outputs from viability to metabolic function to arrhythmia predictions using all-optical or other technologies*(5-10)*. It is essential to be able to perform quick analysis of protein and gene expression with minimal cell material, compatible with the HT format of these functional assays.

Western Blots (WB) have been widely used for protein quantification since their development in the 1970s *(11-13)*. In traditional WB, protein samples are denatured and separated based on molecular weights by SDS-PAGE (sodium dodecyl sulfate, SDS – polyacrylamide gel electrophoresis, PAGE). The separated protein components are transferred onto either nitrocellulose membrane or PVDF (polyvinylidene fluoride) for target protein immunoprobing and chemiluminescent quantification. While this classic WB method is widely used, it is difficult to detect targets in total protein amounts less than 8μg per sample. Thus, it cannot be used to detect protein expression levels in small cell collections completed in 96-well format.

Improvements of the technique have been made to increase the detection sensitivity, throughput and reproducibility. Utilizing sequential lateral flow, automation of the immunoprobing steps is commercially available from iBind (Thermo Fisher). A bead-based microarray assay immobilizes separated protein components onto hundreds of microspheres and achieves high-throughput by analyzing bead collections *(14)*. Microfluidics-based immunoassays have integrated protein separation and detection in microchips *(15-17)* which has allowed for the reduction of starting material to a single cell level *(18)*. Capillary electrophoresis-based approaches have also been

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pursued, with dispersion or ligand binding to generate signals along a narrow capillary at nano-liter volumes for high sensitivity quantification with also very small starting material *(19,20)*.

Based on capillary electrophoresis, the Wes™ ProteinSimple platform for protein quantification was commercialized and has demonstrated high sensitivity, wide dynamic range and good reproducibility *(21-23)*. Since deployment, it has been widely used in cancer *(24,25)* and neuroscience *(26,27)*, with some recent applications to cardiac research *(28-34)*. The key advantages of this system over standard WB include the small starting material needed (as low as 0.8µg per sample), the level of automation and high throughput (runs up to 24 samples concurrently) and faster turnover (3-5 hours assay time), all of which make it ideal for applications requiring analysis of many protein samples limited by size. Due to high-cost considerations and the need for high-throughput mechanism insights into drug screening applications with human cells, such a system for protein quantification is particularly valuable for studies with human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). Indeed, the Wes has been already applied in several cases recently, including signaling pathway inquiries with hiPSC-CMs *(35)*, cardiac pharmacology and toxicology *(36)*, hiPSC differentiation *(37,38)*, gene therapy *(39)* and quantifying ion channel expression to assess maturity*(34)*.

It is often useful and/or more straightforward to perform gene expression analysis as a surrogate or as a complement to protein quantification. The basis of the current "gold standard" in quantifying gene expression is the polymerase-chain-reaction (PCR) method developed in the 1980s by Mullis *(40,41)*. A variety of newer techniques have emerged in this area, including the quantitative reverse-transcription qPCR, application of microarrays and RNAseq, extending recently to single-cell and spatial transcriptomics*(42)*.

Critical developments in the 1990s included the use of fluorescent labeling for kinetic (real-time) qPCR to quantify mRNA *(43,44)*. This allows for very sensitive detection of gene expression in small samples. Rigorous quantification of mRNA based on a critical time or threshold cycle (Ct) from the amplification curves was developed *(45-47)*. To further streamline the process and eliminate the RNA isolation step, the direct "Cells-to-CT" method *(48)* was made commercially available. In this method, the cell lysate is directly incorporated in the qPCR workflow, which makes the process faster and enables further reduction of sample size needed to run the reactions. This technique has been applied to human iPSC-CMs and human cardiovascular progenitor cells in a limited number of studies *(34,49-51)*.

Here, we provide a detailed protocol of protein detection using the Wes and mRNA quantification using the Cells-to-CT kit and qPCR in hiPSC-CMs samples in a 96-well format, overview in **Figure 1**. Frequently used system settings, sample preparation, antibody linear dynamic range test, antibody multiplexing dilutions, and recommended qPCR settings are summarized in tables for reference. Optimization of antibody multiplexing is illustrated using several pairs of proteins in hiPSC-CMs – with options to extend the method to other protein targets.

Using this method, we demonstrate the potential influence of ChR2 on connexin43 (Cx43) in hiPSC-CMs. ChR2 is introduced into hiPSC-CMs by adenoviral infection and comparison was done with respect to both non-infected samples and an Ad-eYFP control. In the presence of ChR2, there is an increase of GJA1 at the transcriptional level and enhanced expression of the Cx43 protein in hiPSC-CMs.

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2. Materials and Reagents

2.1. hiPSC-CMs cell culture

1. Human induced pluripotent stem cell-derived cardiomyocytes iCell Cardiomyocytes[2] CMC-100-012-001, derived from a female donor, purchased from Fujifilm Cellular Dynamics International.
2. MyCell hiPSC-CM-1X 01395, derived from a male donor, purchased from Fujifilm Cellular Dynamics International.
3. Cell plating medium and maintenance medium are provided by the manufacturer (Fujifilm Cellular Dynamics International).
4. Fibronectin (Corning)
5. 1X phosphate buffered saline (PBS)
6. 96-well glass bottom plate (Cellvis)
7. (optional to illustrate usage by adenoviral infection) Ad-CMV-hChR2 (H134R)-eYFP (Vector Biosystems Inc.)
8. (optional to illustrate usage by adenoviral infection) Ad-CMV-eYFP as a control (Vector Biosystems Inc.)

2.2. Cell lysis and protein denaturing for WB

1. 1X PBS
2. Qproteome Mammalian Protein Prep Kit (Qiagen)
3. Sterile 1.5 mL microcentrifuge tubes
4. Plate shaker, **Figure 2a**

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5. Refrigerated high-speed Eppendorf centrifuge (Millipore-Sigma), **Figure 2b**

6. Heat block with inserts for microcentrifuge tubes, **Figure 2c**

7. Microcentrifuge/spinner, **Figure 2d**

8. PlateFuge plate centrifuge, **Figure 2e**

2.3. Immunoprobing

1. Kit for running an assay by Protein Simple, containing:

   a. Jess/Wes 25-capillary cartridge, **Figure 2f.**

   b. Jess/Wes separation module (12-230kDa) with a pre-filled microplate with split running buffer, **Figure 2g.**

   c. Wash buffer and 10X sample buffer.

   d. EZ standard pack with lyophilized material in three tubes, as follows: biotinylated ladder, fluorescent standard 5X master mix and dithiothreitol (DTT).

2. Wes system (Protein Simple), **Figure 2h.**

3. Primary antibodies tested in this study:

   a. Cx43 (ab11370), Abcam

   b. Kir2.1 (ab65796), Abcam

   c. alpha-tubulin (ab7291), Abcam

   d. GAPDH (ab181602), Abcam

   e. LDH (H-10) (sc-133123), Santa Cruz.

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4. Secondary antibodies were anti-mouse and anti-rabbit detection modules (Protein Simple), which contained luminol, peroxide, milk-free antibody diluent and streptavidin-HRP in addition to anti-mouse/ anti-rabbit secondary HRP antibody.

5. PlateFuge plate centrifuge, **Figure 2e.**

6. Pipettes and tips.

2.4. qPCR with the Cells-to-CT™ kit

1. Power SYBR Green Cells-to-CT™ kit (Thermo Fisher)

2. 1X TE Buffer, pH 8.0

3. MicroAmp™ Optical 96-well Reaction Plate (Thermo Fisher)

4. MicroAmp™ Optical Adhesive Film Kit (Thermo Fisher)

5. QuantStudio™ 3 Real-Time PCR instrument (Thermo Fisher)

6. Thermal cycler (Eppendorf)

7. QuantStudio™ Design & Analysis Software (Thermo Fisher) was used for to conduct differential expression analysis

8. Nuclease-free microcentrifuge tubes and pipette tips

3. Methods

The detailed workflow for protein quantification is illustrated in **Figure 2**, with relevant equipment shown.

3.1. Culturing hiPSC-CMs in a 96-well plate (50,000 cells per well)

Thawing and plating of hiPSC-CMs is done following manufacturer's instructions, adapted by user and summarized as follows:

1. Thaw the commercial plating and maintenance medium at 4°C overnight; equilibrate to room temperature upon usage.

2. Coat wells (in the 96-well plate) with 100 µl of 50 µg/mL fibronectin diluted in 1X sterile PBS in 37°C cell culture incubator for at least 2 hours. Remove fibronectin solution right before cell plating.

3. Immediately transfer hiPSC-CMs cryovial from liquid nitrogen to 37°C water bath. Hold the cryovial using floating tube rack and immerse the cryovial in 37°C water bath for 3 min without submerging the cap (see note 1).

4. Move the cryovial into the biosafety cabinet after sterilizing with 70% ethanol.

5. Transfer 1 mL of cell suspension from the cryovial into a 50 mL conical tube. Use 1 mL cell plating medium to rinse the remaining cells in cryovial and add dropwise into the cell suspension at a rate of 1 drop per 5 seconds and swirl between drops.

6. Gently drop additional 8 mL cell plating medium into the 50 mL conical tube for a total of 10 ml solution, slowly swirl the tube in the process.

7. Gently plate 100 µL cell suspension into each 96-well for a plating density of 50,000 cells per well. Slowly swirl the conical tube while plating as cells will settle over time.

8. Place the plate into 37°C cell culture incubator after plating, exchange plating media to maintenance medium 4 hours after the thaw.

9. Gently replace maintenance medium every 2-3 days. Tilt the plate 45 degree and add in medium along the edges. Avoid dropping culture medium directly onto the cell layer (see note 2).

3.2. Adenovirus infection – optional to illustrate usage

1. Start adenovirus infection 5 days after cell plating.

2. Thaw adenovirus from -80°C to 4°C on ice. Predilute the virus in sterile 1x PBS.

3. Conduct infection in maintenance media at multiplicity of infection (MOI 50). For calculations, follow the detailed published protocols *(52,53)*.

4. After 2 hours of incubation at (37 °C, 5% CO_2), exchange viral medium with normal maintenance medium.

5. Collect cell lysates 48 hours after infection.

3.3. Protein collection from a 96-well microplate

1. Prepare QProteome Mammalian Protein lysis buffer and store on ice. Conduct all subsequent steps on ice.

2. Place the 96-well plate on ice. Aspirate cell culture medium and wash with cold 1x PBS. Completely aspirate the 1X PBS and add 10 µl protein lysis buffer in each well.

3. After adding the lysis buffer, incubate on ice with shake for 5min.

4. Scrape each well and collect complete lysate into epitubes (see note 3).

5. Centrifuge all samples at 2000 rpm for 30 min at 4°C and separate the supernatant (lysate) from the pellet.

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6. Transfer the samples to -80°C freezer for long term storage. Upon usage, thaw samples on ice.

3.4. Protein quantification using Wes™

An overview of the process for protein quantification using the Wes is illustrated in **Figure 2**. In the Wes workflow, the cell lysates and reagents, including the primary antibodies and secondary horseradish peroxidase (HRP) antibodies, are loaded into small compartments in a special assay microplate (25 samples). The microplate is placed onto the system and interfaces a cassette with 25 thin capillaries. Size-based separation of proteins occurs as the proteins migrate in the separation matrix within the capillaries and are immobilized to the walls using proprietary photo-activated chemistry. Within 3-5 hours, the protein components in each sample are separated based on molecular weight, immobilized in the capillaries and detected using chemiluminescence. System readout is in the form of digitized "electropherograms", which can be converted to virtual or "pseudo" blot lanes automatically. Target peaks are quantified as area under the curve.

3.4.1 Experiment planning and setting the Compass software

It is crucial to carefully plan the sample layout and to enter all system settings in the software before running an assay.

1. Each 10 µL cell lysate, obtained from a 96-well plate sample, allows for several Wes protein quantification runs, depending on protein target expression levels. For lower-expressing proteins, e.g. Kir2.1 in hiPSC-CMs, undiluted (or "neat") samples are preferred and cell lysates from one 96-well may be enough for up to two runs. For abundantly expressed proteins like LDH, Cx43 and GAPDH, samples can be diluted up to four times (see note 4).

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2. Based on the primary antibody's dilution factor and testing well number, one can calculate the primary antibody volume needed. Prepare for one more well to account for pipette errors.

3. Create a new file in the Compass software based on the type of assay. Choose 'Wes size' for typical immunoassay, including antibody multiplexing, or choose 'Wes total protein' if testing samples include total protein detection. There are some differences between the two programs, e.g. the total protein assay has 30 min of biotin labelling time instead of antibody diluent time in the immunoassay. Frequently used system settings are summarized in Table 1.

4. Enter the sample layout and primary antibodies attributes in the software. Adjust the running parameters. Save the file and print out the layout for referencing on the experimental day.

Table 1. Frequently used system setting

Parameters	Wes size run	Total protein run
Separation matrix load time	200 sec	200 sec
Stacking matrix load time	15 sec	18 sec
Sample load time	9 sec	9 sec
Separation time	31 min	31 min
Separation voltage	375 volts	375 volts
Standards exposure	4 sec	4 sec
EE immobilization time	200 sec	200 sec
Antibody diluent time	30 min	/

Biotin labeling time	/	30min
Primary antibody time	30 min	30 min
Secondary antibody time	30 min	30 min

3.4.2 Sample and primary antibody preparation for protein quantification

1. Thaw samples from -80°C freezer on ice.

2. Preset temperature of the heat block to 75°C.

3. Start the Wes and the Compass software. Initiate the instrument self-test and save the result. If failure occurs – try again and report the problem if it persists.

4. Open an EZ standard pack and prepare the reagents following the manufacturer's instructions.

5. Dilute the 10x sample buffer to 0.1x buffer to dilute the cell lysates as needed. For analysis of low-abundance proteins, the cell lysates should be used "neat" (undiluted).

6. Mix cell lysates with the 5X fluorescent master mix 1:4 (lysate: fluorescent mix).

7. Denature all samples at 75°C for 10 min (or at 90°C for 5 min) on heat block. Vortex and spin the tubes before and after the heating to ensure that the cell lysate and the fluorescent master mix are well homogenized.

8. Place the denatured samples back on ice and spin them in a centrifuge for 5 min at 2500 rpm at room temperature before sample loading.

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9. Dilute the primary antibodies with Antibody Diluent2, based on intended final concentration. Working concentrations of each antibody will need optimization as necessary concentrations are antibody dependent. Store in the pre-labeled microcentrifuge tubes.

3.4.3 Microplate loading and start of a Wes run

For reagent loading into the microplates, please see **Figure 3** and details below.

1. Take the sealed microplate from the Jess/Wes separation module. To minimize the microwell exposure, peel the Wes microplate foil row by row when pipetting.

2. Loading row A: pipet 5 µL biotinylated ladder in the first well of row A. Pipet 3 µL of each prepared protein sample into the remaining 24 wells of the microplate. (see note 5)

3. For the biotinylated ladder column (default is the first column), pipet 10 µL of Antibody Diluent2 in row B & C, 10 µL of Streptavidin-HRP in row D.

4. Loading rows B, C and D with antibody diluent2, primary and secondary antibodies:

4a. For running an immunoassay, for each of the 24 sample wells, pipette 10 µL of Antibody Diluent2 in row B, 10 µL primary antibody in Row C and 10 µL of the corresponding secondary antibody in row D.

4b. For running a total protein assay, for each of the 24 sample wells, pipette 10 µL of Biotinylation reagent in row B, 10 µL of Antibody Diluent2 in row C and 10 µL of total protein SA-HRP in row D.

5. Loading row E: Mix 200 µL of Luminol with 200 µL of Peroxide and fill row E with 15 µL of Luminol-Peroxide mixture in each well.

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6. Cover the microplate with its lid and centrifuge at 2500 rpm for 5 min (using the PlateFuge) at room temperature to eliminate all bubbles; use an unopened microplate for balance.

7. Fill the first three rows of the bigger compartments on the microplate with 500 µL (each) of Wash Buffer.

8. Peel off the assay plate bottom foil. Carefully pop all bubbles in the Separation Matrix wells using 5 µL pipette tips.

9. Open the Wes's door and insert a newly-opened capillary cartridge into cartridge holder. The interior light will change from orange to blue.

10. Place the assay plate on the plate holder and make sure the plate is attached with the edge of the tray.

11. Start the Wes run and assign the file save directory. Watch for 10 minutes to make sure the sample has loaded normally (fluorescent dots travel down evenly).

12. The machine will indicate the end-time point for the run (typically takes around 3 h), **Figure 4**, inset. After the test, take out and discard the microplate and capillary cartridge.

3.4.4 Data analysis of Wes data

1. Check the fluorescent standards assignment in the biotinylated ladder and each sample by clicking on Standard → Single View in the Compass Analysis Graph pane. Standard peaks for 12-230 kDa kit are 1, 29, 230 kDa. 1kDa standard is the highest peak. The position of the 29 kDa and 230 kDa peaks should be similar for the ladder and the samples. If needed, correct peak position by right-clicking on peak and selecting "Not a Standard" or "Force Standard".

2. If the 1 kDa peaks vary a lot among samples and standard, exclude that peak to increase the accuracy of the protein size assignment. To exclude the 1 kDa standard peak for protein

size fit, click Analysis in Edit tab. In the pop out window, under Standard tab, unclick the box for 1MW (kDa) fit. Click Apply to save the adjusted setting.

3. Confirm biotinylated ladder peaks assignment by clicking on Samples → Single View of 1st capillary in Compass Analysis Graph pane. Peaks in 12-230 kDa kit are 12, 40, 66, 116, 180, 230 kDa.

4. Make sure the Sample Baseline Corrected and Fit Baseline Corrected are checked in the View Menu pull down options.

5. Name the detected peaks by going to Analysis in the Edit tab, click on the Peak Names tab in pop out window. Add names in the Analysis Groups and assign MWs (kDa), Color and Range (%) for each peak. 10% is commonly used for Range (%), decrease the percentage if two detected peaks have similar molecular weights. Apply setting to desired groups. Click on Apply after the peak assignment.

6. To confirm the peaks assignment, check the fitted peaks in Graph Options (right top corner of the Analysis window). Under Graph pane, assigned peaks are colored as assigned. Check the peaks recognition in each sample and adjust analysis setting if needed.

7. Sample information is listed at the bottom of the Analysis view in Peaks tab, and it includes sample name, primary antibody, capillary number, peak number, peak name, peak position, molecular weight (MW(kDa)), peak height, peak area, percentage area (% peak area/ area under the curve), peak width, signal noise ratio (S/N), averaged baseline.

8. For peaks analysis, go to the Capillaries tab at the bottom of Analysis view. The peaks areas are listed with peak names and capillary number. Choose the Area display, copy the sheet by clicking Ctrl + A and Ctrl + C. Paste the data into an Excel file for analysis.

9. Virtual blotting results ("lanes") available in the Compass Lane pane. Add sample, primary antibody, secondary antibody, their attributes and capillary No. information from Gels Options. Adjust display contrast with the Slider if needed. Copy or screenshot the Lane view.

10. The Compass software can be downloaded for free for further examination and plotting of the data offline.

3.5 Primary antibody dilution optimization

With consistent plating and culturing conditions, the hiPSC-CMs samples collected from 96-wells yield similar protein concentration, therefore the protein amount in each Wes sample loading will be similar. When using an antibody for the first time, optimal primary antibody dilution should be tested. The primary antibody needs to be at a saturating concentration to make sure the HRP signal change is proportional to protein expression difference. Over-saturation will increase the signal background and cause non-specific detection.

If a target protein has been quantified in traditional WB, a good starting point for the referencing primary antibody dilution in Wes will be to use 20 times higher antibody concentration than in traditional WB. If a target protein could be clearly identified at the expected molecular weight with referencing primary antibody dilution and system peak height/ baseline ratio $\geqslant 3$, further tests of signal linear response under this dilution could be conducted. Otherwise, increase or decrease the primary antibody dilution according to the signal.

Proper controls should be included to distinguish signal from noise and to validate detection specificity, which includes:

- Neat sample with only Antibody Diluent2 in the microwell of 'primary antibody'
- 0.1x sample buffer in the microwell of 'sample' with testing antibody

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- Positive and negative controls, i.e. samples confirmed to have abundant & no target protein expression

For quantifying a protein expression level, confirmation is needed that the measurements are in the linear dynamic range. Prepare four sample attributes using one sample and varying dilutions: neat, 0.75, 0.5 and 0.25 (actual sample attributes are 0.8, 0.6, 0.4 and 0.2, because samples need to be mixed with the Fluorescent Master Mix, as described earlier).

A sample collected from a single 96-well is enough for one linearity test. Repeat the following steps with multiple samples in the same test or in multiple tests to confirm the linearity.

1. For sample preparation, label four microcentrifuge tubes as 'neat', '0.75', '0.5' and '0.25'. Mix cell lysis, 0.1x sample buffer and Fluorescent Master Mix as described in Table 2.

Table 2. Sample preparation for antibody linear dynamic range test

Microcentrifuge sample	Cell lysis	0.1x Sample buffer	Fluorescent Master Mix
Neat	4 µL	0 µL	1 µL
0.75	3 µL	1 µL	1 µL
0.5	2 µL	2 µL	1 µL
0.25	1 µL	3 µL	1 µL

2. Other steps are the same as previously described for running an immunoassay by Wes.

3. After the immunoassay, in the Compass Analysis Graph pane, select one sample and click the View Selected on top left corner. Check the All exposures in the Graph options pull down menu. 1s exposure usually has the highest peak value. If the antibody is not over-saturated, the 1 s, 2 s,

and 4 s peaks should be close to each other. Consider decreasing the antibody dilution if the peaks from different exposure times are far apart.

4. Export the peak areas as previously described (see note 6). Plot the signal over sample attributes as a dot plot.

5. Add linear regression trendline to the dot plot. Acquire R-squared value of the linear regression. R-squared value above 0.9 suggests the tested antibody dilution has linear response in the range of detection.

The optimized primary antibody dilution is acquired when the high R-squared value of linearity test is consistent, and no over-saturation is observed.

3.6 Normalized protein expression by antibody multiplexing and total protein linear response test

Signal normalization can be achieved by antibody multiplexing or total protein quantification. Both methods have merits. Antibody multiplexing with a loading control protein requires less material, two or more tests could be conducted from a 96-well hiPSC-CMs collection. Total protein quantification could be used when no proper loading control is available.

For antibody multiplexing, the primary antibody of a target protein and the loading control need to be mixed in a proper ratio. Tested target protein & loading control combinations are summarized in Table 3; also see note 7. Example results are shown in **Figure 5** for antibody multiplexing using Cx43 and GAPDH (loading control), including the electropherogram with peaks (with areas quantified on the bottom) and the Compass-generated lane view.

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Table 3. Wes antibody multiplexing dilution examples for use in hiPSC-CMs

Target description	**Target protein (antibody)**	**Loading control**	**Dilution factor**
LDH-A: lactose dehydrogenase-A, linked to metabolic state	LDH (H-10) (Santa Cruz sc-133123)	Alpha tubulin (Abcam ab7291)	1:300 LDH 1:50 Alpha tubulin
Cx43 – Connexin43, gap junctional protein found in ventricular heart tissue	Cx43 (Abcam ab11370)	GAPDH (Abcam ab181602)	1:25 Cx43 1:2000 GAPDH
Kir2.1 – ion channel (inward rectifier) responsible for the resting membrane potential	Kir2.1 (Abcam ab65796) (Alomone APC-159)	GAPDH (Abcam ab181602)	1:10 Kir2.1 1: 4000 GAPDH

For simplicity, start making new antibody combinations by choosing two primary antibodies from the same host species. First, optimize the primary antibody dilution of each target. Make sure the multiplexed antibodies have clean baseline and are sufficiently different in molecular weight. Second, vary the antibody dilution to make the detected signals appear at a comparable level. In most cases, the expression levels of two targets are distinct and can differ by order of magnitude. Increase the antibody concentration of the less abundant target protein to balance the signal level. Or further dilute the abundantly-expressed protein antibody. Rerun the signal linear response test to make sure the diluted concentration is saturated.

If varying the antibody dilutions can't adjust the signal to a comparable level, alternative solutions are provided by Protein Simple:

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- Choose different species for the abundantly-expressed protein. Also mix secondary antibodies and ensure they reach saturation. The company provides 20x secondary antibody formulation to ensure saturation of the secondary antibodies.
- Apply an HRP-conjugated secondary with lower HRP load. For example, Simple Western charge secondary antibodies (Anti-Rabbit HRP PN 040-656, Anti-Mouse HRP PN 040-655).
- Mix unlabeled secondary with the HRP-conjugated secondary. Be aware of the stability of secondary antibody mixtures.
- Use HRP-conjugated primary antibody as direct detection. It limits the amplification effect induced by indirect detection, but it is less specific comparing with indirect detection.

Follow Protein Simple's instruction on how to apply these methods.

For signal normalization by total protein assay, total protein linear detection range needs to be identified and included in each immunoassay. Use neat, 0.75, 0.5 and 0.25 sample attributes as four detection points, conduct total protein assay as previously described. Plot the total protein signal over sample attributes and do the linear regression to determine the total protein detection range. If titrated samples show good linearity (R-squared value > 0.9), the 4-times diluted sample could be used for antibody optimization and total protein assay could be used for signal normalization. If including 0.25 sample attribute influences the signal linearity, choose four attributes above 0.25 and rerun the linear detection range tests until finalizing the detection limit.

3.7 mRNA quantification in hiPSC-CMs using Cells-to-CT™ kit and qPCR

1. qPCR analysis is run using the Power SYBR-Green Cells-to-CT™ kit (Thermo Fisher) and following the manufacturer's instructions

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2. Prepare DNase1 and Lysis solution 1:100 and use 50 uL of it for each well.

3. Aspirate the maintenance medium from the hiPSC-CMs. Wash cells with cold 1X PBS.

4. After aspirating the PBS, add 50 uL of DNase1 and Lysis Solution to each well. Pipet up and down with the pipette set to 30 uL (so to not introduce bubbles) 5 times.

5. Incubate plate at room temperature (19-25°C) for 5 min.

6. Add 5 uL of stop solution to each well, mix 5 times, and incubate at room temperature for an additional 2 minutes.

7. Store samples at -80°C as needed or proceed to the next step.

8. Program thermal cycler as indicated in Table 4.

Table 4. Thermal cycler settings for Reverse Transcriptase

	Stage	**Reps**	**Temp**	**Time**
Reverse Transcription	1	1	37°C	60 min
RT inactivation	2	1	95°C	5 min
Hold	3	1	4°C	indefinite

9. Prepare a Reverse Transcriptase Master mix of 25 uL RT buffer, 2.5 uL Reverse Transcriptase, and 12.5 uL nuclease-free water per lysate.

10. Add 10 uL of lysate per 40 uL Reverse Transcriptase Master Mix and mix.

11. Run thermal cycler as indicated.

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12. After reverse transcriptase, store samples at -20°C or proceed to the next step.

13. Program the real-time PCR instrument as indicated in Table 5. Reps (cycles) determines the level of amplification.

Table 5. Real-Time PCR Cycling Parameters

	Stage	**Reps (Cycles)**	**Temp**	**Time**
Enzyme Activation (hold)	1	1	95°C	10 min
PCR (cycle)	2	40	95°C 60°C	15 sec 1 min
Dissociation Curve	3	(use default settings)		

14. Prepare PCR cocktail as indicated in Table 6. To detect GJA1 mRNA, use Fw_GGTGGTACTCAACAGCCTTATT and Rv_ACCAACATGCACCTCTCTTATC primers and to detect GAPDH, use Fw_GGTGTGAACCATGAGAAGTATGA and Rv_GAGTCCTTCCACGATACCAAAG primers respectively.

Table 6. PCR Cocktail Conditions

Component	20uL PCRs (Each reaction)
Power SYBR Green PCR Master Mix	10 uL
Forward and Reverse PCR Primers	250 nM final concentration of each PCR primer
Nuclease Free Water	variable
cDNA	4 uL

15. Dilute forward and reverse primers in nuclease free water as needed. Add PCR cocktail and cDNA to each well of the MicroAmp™ Optical 96-well Reaction plate. Scale volumes as needed to account for 3 technical replicates and the various targets. Per one 96-well of lysate collected, about 12 different gene targets can be tested.

16. Seal MicroAmp™ Optical 96-well Reaction plate with MicroAmp™ Optical Adhesive Film and seal all edges with plastic MicroAmp® Adhesive Film Applicator to form a tight seal between the plate and adhesive film.

17. Run plate on QuantStudio™ 3 Real-Time PCR instrument under the parameters listed on Table 5.

18. Use QuantStudio™ Design & Analysis Software to extract Ct values and normalize Cx43 gene expression quantification to housekeeping gene GAPDH using standard ΔΔCt method*(45-47)*.

4. Example application

We illustrate the described methods for quantification of protein and mRNA within small cell samples (50,000 cells) in a 96-well microplate format by examining the effects of optogenetic transduction (ChR2) on the transcript and protein levels of gap junctional protein Cx43.

Connexin 43 is the main gap junctional protein in ventricular cardiomyocytes with a critical role in their structural and functional maturity*(2,54)*. A previous optogenetics study, using HEK293T cells (with minimal Cx43 expression) suggested possible augmentation of Cx43 expression in those cells upon genetic expression of Channelrodopsin-2 (ChR2), a light-gated ion channel*(55)*.

Using the methods described here, we probe for effects of ChR2 on Cx43/GJA1 in human iPSC-CMs from individual wells in a 96-well microplate.

With optimized Cx43 & GAPDH multiplexing in the Wes assay, the Cx43 protein expression levels are compared in non-infected, Ad-CMV-eYFP infected and Ad-CMV-hChR2 (H134R)-eYFP infected hiPSC-CMs using two different hiPSC-CM lines, iCell Cardiomyocytes[2] CMC-100-012-001 (female differentiated) and MyCell hiPSC-CM-1X 01395 (male differentiated). In addition, GJA1 normalized to GAPDH mRNA levels were quantified in the respective conditions using the Cells-to-Ct kit and qPCR.

The results suggest highest Cx43 protein expression levels (after normalization by GAPDH) in ChR2 transduced hiPSC-CMs when compared to Ad-CMV-eYFP infected and non-infected hiPSC-CM cell lines (**Figure 6a-b**). At the transcriptional level, elevated GJA1 is also seen in ChR2-treated hiPSC-CMs compared to the eYFP-expressing cells but not compared to the untreated (**Figure 6c-d**). The results indicate that the presence of ChR2 may enhance Cx43 expression in hiPSC-CMs, though no statistical significance was reached at the transcription level. Although the mechanism is not clear and warrants further investigation, the findings suggest that an optogenetic perturbation may have an overall positive effect on hiPSC-CMs coupling.

Notes

1. Timing the water bath time is critical for cell viability when handling the iPSC-CMs.

2. Keeping the integrity of the cell monolayer is important for a successful protein collection. Proper dilution of fibronectin, sufficient fibronectin coating time, gentle operation during hiPS-CMs thaw and culture could help improve cell attachment and viability.

3. In the cell lysing process, timing is important - try to operate as quickly as possible to avoid sample degradation. In, addition, all operations should be conducted on ice.

4. To minimize the difference in migration speed due to protein concentration, it is preferable to use samples with the same dilution factor in a particular run.

5. Minimize bubble formation by pointing pipette tip to the bottom of each well while pipetting and do it gently.

6. Default exposure is “High Dynamic Range 4.0”; different exposure times yield similar results.

7. Testing is required for each new antibody being considered. Antibodies working in standard WB may not work with Wes, even for the same samples. Different lot numbers of the same product number antibody also can produce variable results and need to be re-tested. ProteinSimple maintains a database of Wes-tested antibodies and target proteins in different cell types that can be useful.

Author contributions

WZL, JLH and EE conceived the project. WZL designed and performed the Wes system validation and optimization, antibody multiplexing optimization, total protein linear response test and wrote the first draft. JLH and WZL designed and conducted proof of concept experiments, including qPCR and Wes quantification of Cx43 in non-infected, Ad-eYFP infected and Ad-CMV-hChR2 (H134R)-EYFP infected hiPSC-CMs. WZL, JLH and EE edited the draft.

Acknowledgements

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Figures

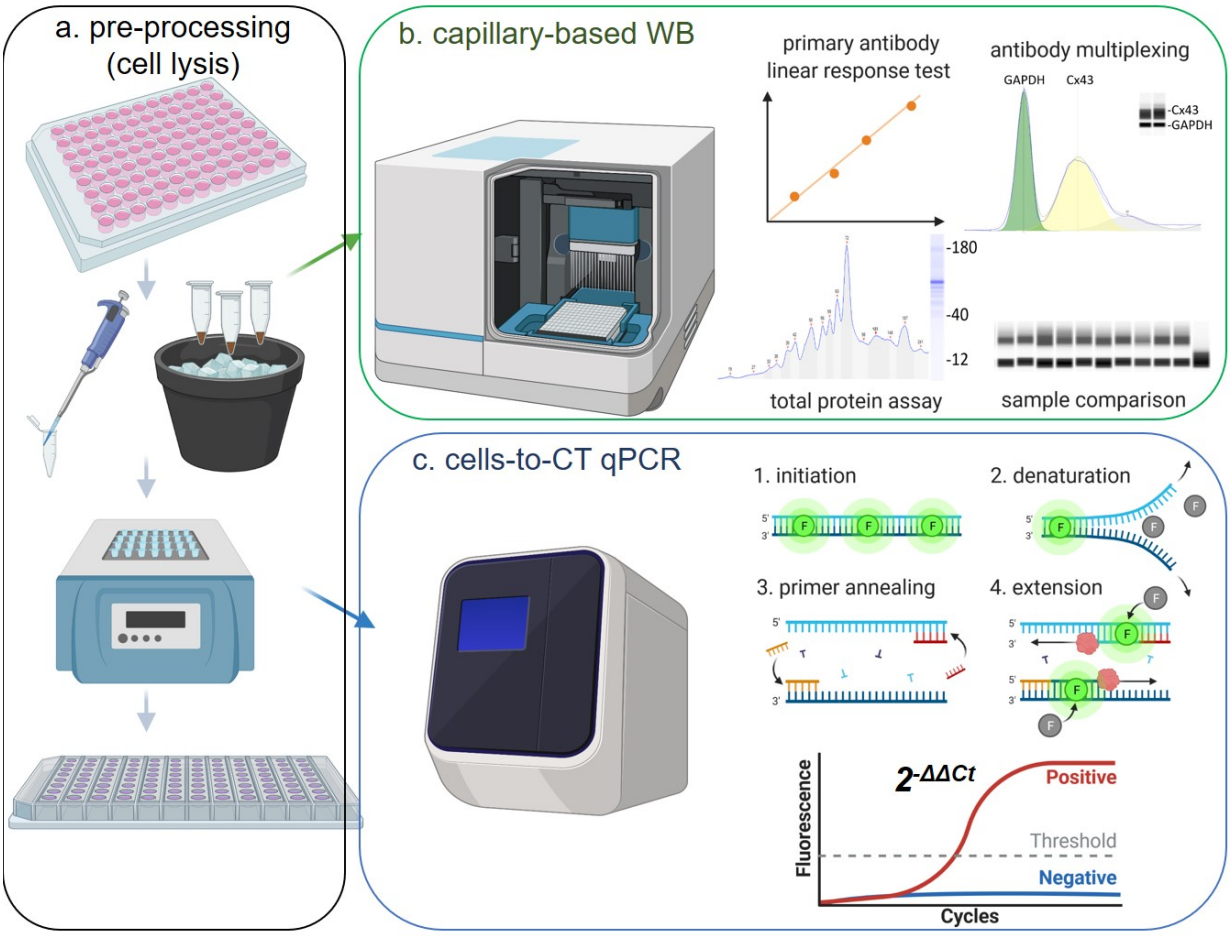

Figure 1. Small hiPSC-CM sample protein and mRNA quantification overview

(a) hiPSC-CMs lysates collected from each of the 96-wells are used directly for protein or mRNA quantification. For Wes quantification, the cell lysate is mixed with reaction reagent and denatured at 75°C for 10 min. (b) Twenty-four samples can be loaded in each Wes microplate and quantified concurrently. After 3-5 hours of Wes run, quantification results including target protein linear response, antibody multiplexing or total protein assays can be analyzed in the Compass software, and sample comparison can be displayed using pseudo-gel-bands. (c) For qPCR quantification,

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mRNA in the whole cell lysates from individual wells can be quantified with the Cells-to-CT qPCR approach, involving the steps shown; quantification is by the ΔΔCt method. Figure created with Biorender.

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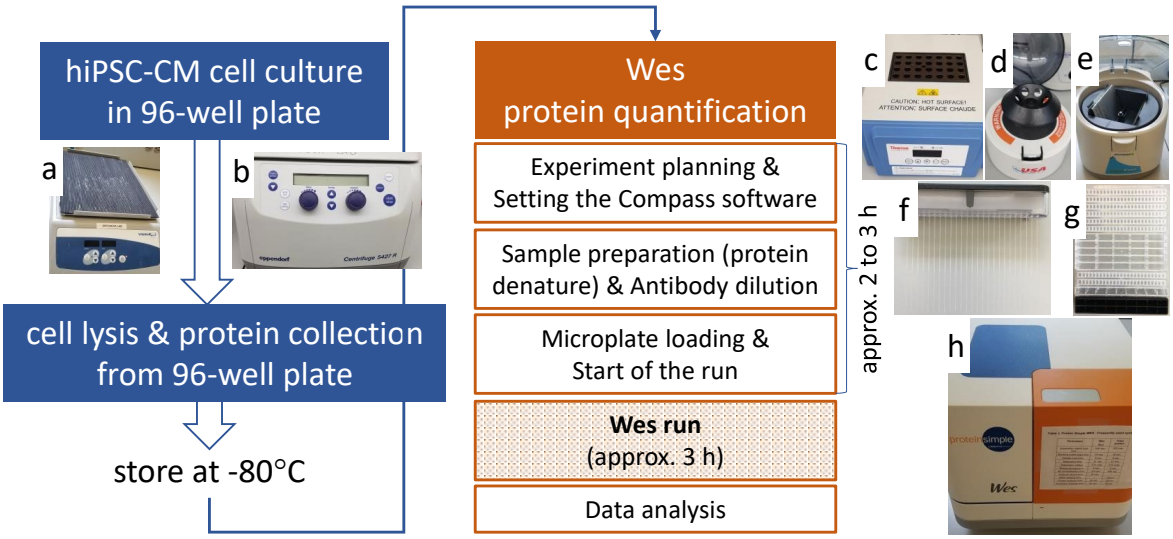

Figure 2. Detailed workflow and equipment needed for Wes protein quantification

Wes protein analysis workflow can be separated to three main parts: protein collection, experiment preparation and data analysis. Plate shaker (a), a box of ice and refrigerated high-speed centrifuge (b) are needed in protein collection. In experiment preparation, the heating block (c), microtube centrifuge (d) and microplate centrifuge (e) are needed. Wes capillary set (f) and the prepared microplate (g) are inserted in the Wes machine (h) for the run.

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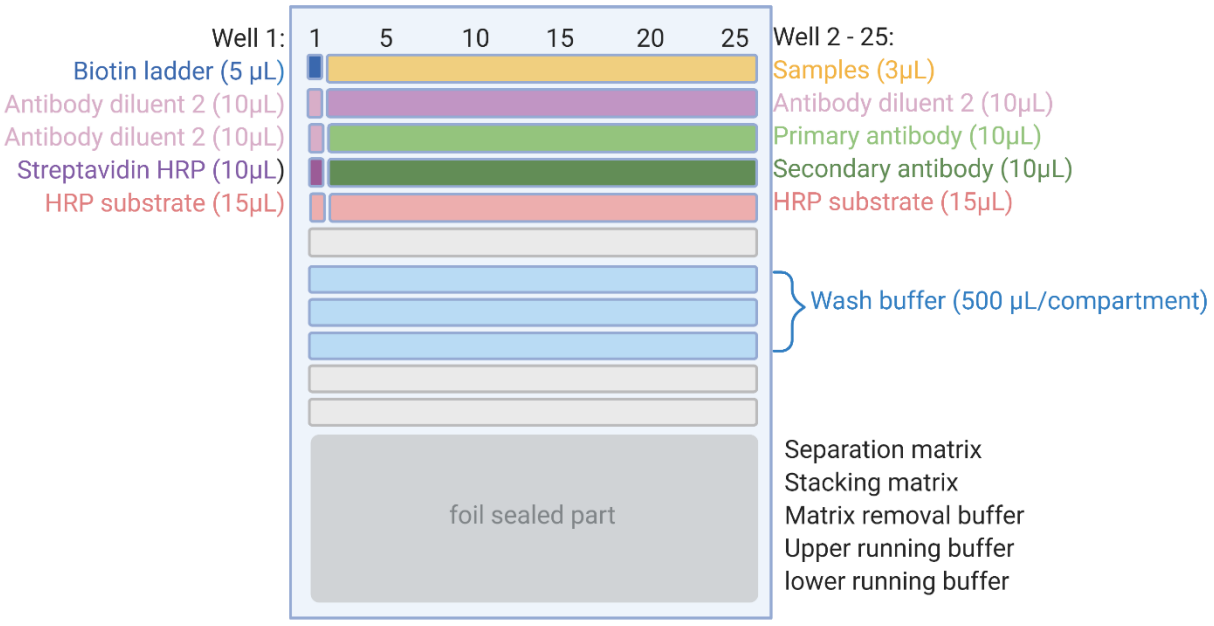

Figure 3. Microplate loading with reagents

The layout of a Wes microplate includes 6 rows of wells (25 wells per row), 5 rows of wash buffer troughs and bottom manufactured part. The first 5 rows of oval wells and the first 3 rows of wash buffer troughs are usually loaded as indicated in the figure. Figure created with Biorender.

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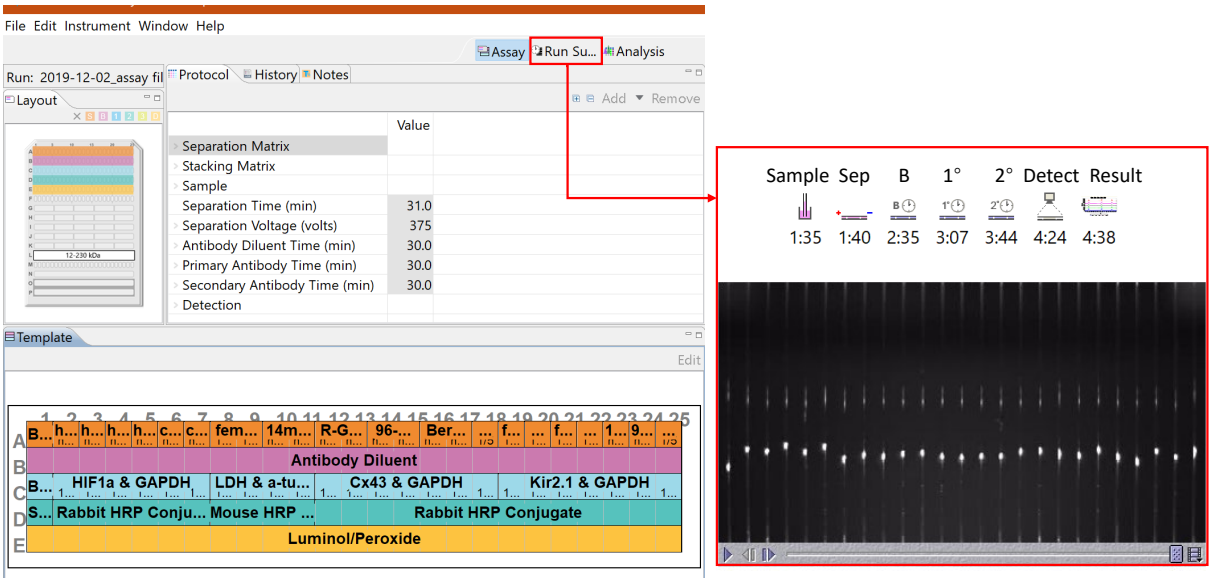

Figure 4. Compass - data acquisition and analysis software

After creating a new run, the experimental settings, including testing protocol, sample layout and antibody dilutions are saved as Assay in Compass Data File (.cbz). Once a new run is started, the experimental timeline can be seen in the Run summary. The progression of the sample loading can be monitored in real-time in the Run summary, which is stored as a video after the experiment. Quantification result and signal analysis are available in Analysis.

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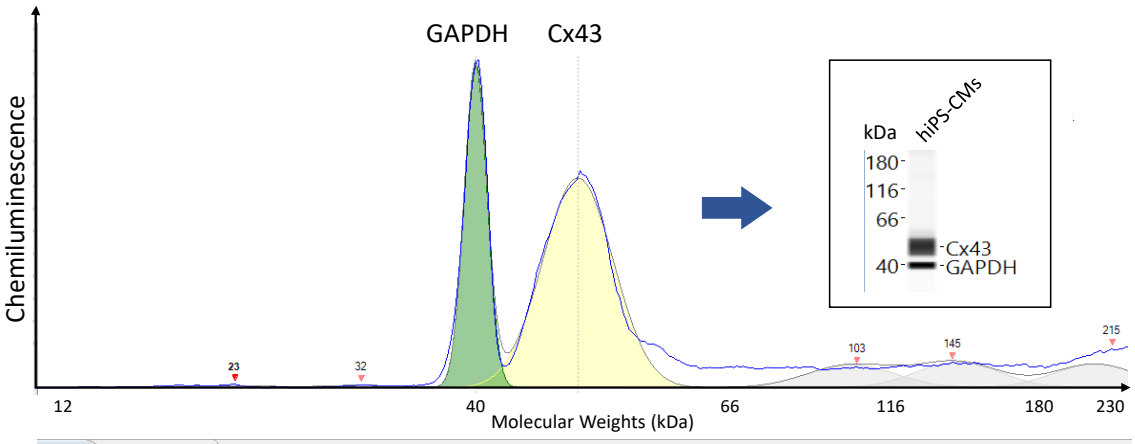

Peaks | Capillaries

Sample	Primary	Cap	Peak	Name	Position	MW (kDa)	Height	Area	% Area	Width	S/N	Baseline
hiPS-CMs	Cx43 & GAPDH	12	3	GAPDH	378	40	724551.5	6653170	33.1	8.6	10000.9	1000.8
hiPS-CMs	Cx43 & GAPDH	12	4	Cx43	415	50	468053.1	13448959	66.9	27.0	2214.7	1104.9

Figure 5. Example result of Cx43 & GAPDH multiplexing in human iPSC-CMs

Electropherogram and its conversion to a virtual lane view result of Cx43 & GAPDH multiplexing, using a 96-well collected human iPSC-CMs sample. Information including peak position, molecular weights, height, area, percentage area, width, signal noise ratio and baseline are quantified in the Compass software.

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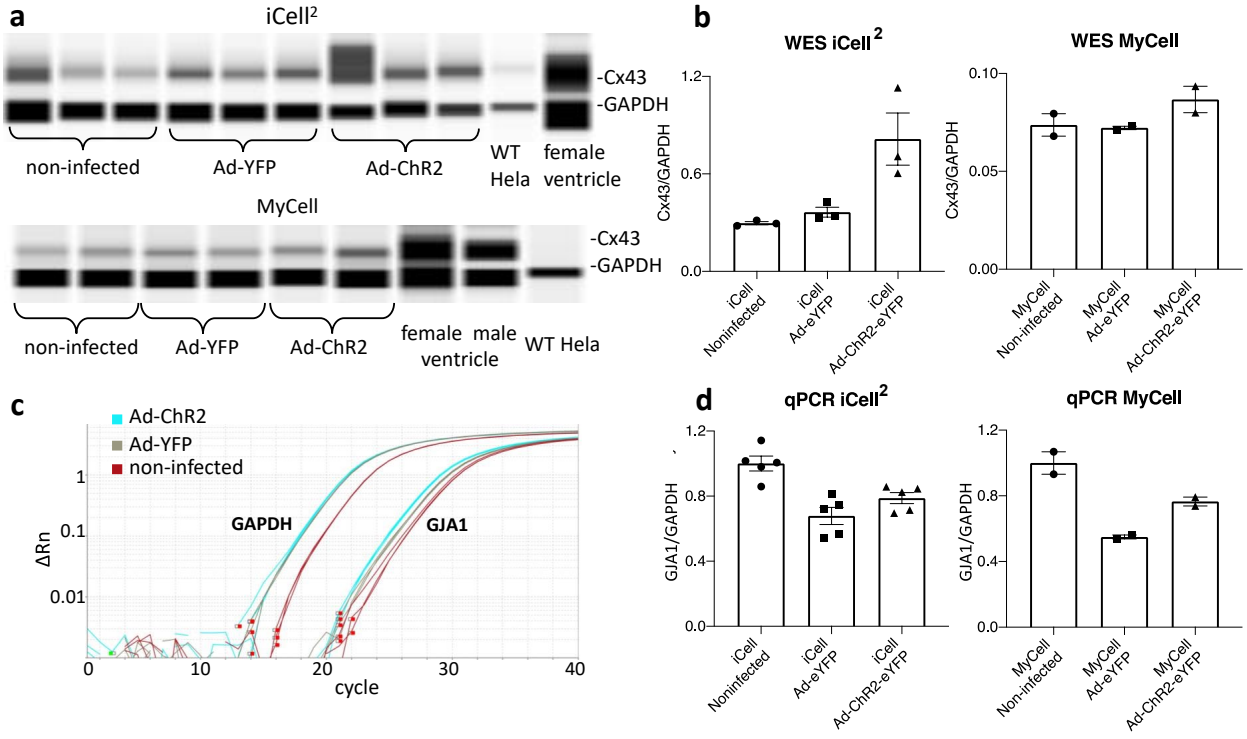

Figure 6. Quantification of protein (Cx43) and mRNA (GJA1) after ChR2 expression in female iCell² and male MyCell hiPSC-CMs

(a-b) Protein quantification (Cx43 & GAPDH) in iCell² (a) and MyCell iPSC-CMs from individual 96-wells (control non-infected samples, Ad-eYFP expressing cardiomyocytes and Ad-ChR2-eYFP-expressing cardiomyocytes); human ventricular tissues as positive control and wild type HeLa as negative control. Panel (a) shows the pseudo-bands automatically generated in the Compass software. Panel (b) displays the normalized Cx43/GAPDH values automatically calculated from areas under the curve in the electropherograms. Data are presented as n = 3 technical replicates in iCell² and n = 2 biological replicates in MyCell. (c-d) mRNA quantification (GJA1 & GAPDH) in iCell² (a) and MyCell iPSC-CMs from individual 96-wells. Panel (c) shows example PCR amplification curves from one run with iCell² samples. Panel (d) shows the

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normalized values GJA1/GAPDH calculated using the ΔΔCt method. Data are presented as n = 5 biological replicates with n = 3 technical replicates for iCell^2 and n = 2 biological replicates with n = 3 technical replicates for MyCell. All biological replicates displayed along with the mean.