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## Introduction

Human pluripotent stem cell (PSC)–derived cardiomyocytes (CMs) are of high interest for use in drug testing, heart disease modeling, and therapeutic applications. Therefore, standardized protocols for the efficient generation of CMs are needed. Even though several protocols for cardiac differentiation have been published, the majority of them have to be adjusted for each stem cell clone, e.g., by titration of small molecule and cytokine concentrations, in order

to obtain the optimal differentiation efficiency and cell yield. Moreover, lot-to-lot variations of media components also influence the outcome of differentiation. These protocol optimizations are costly and time consuming. In order to circumvent these limitations, we developed a cardiac differentiation medium, the StemMACS™ CardioDiff Kit, enabling robust and standardized cardiac differentiation, without the need for media adjustments.

## Results

### 1 Cardiac differentiation of PSCs

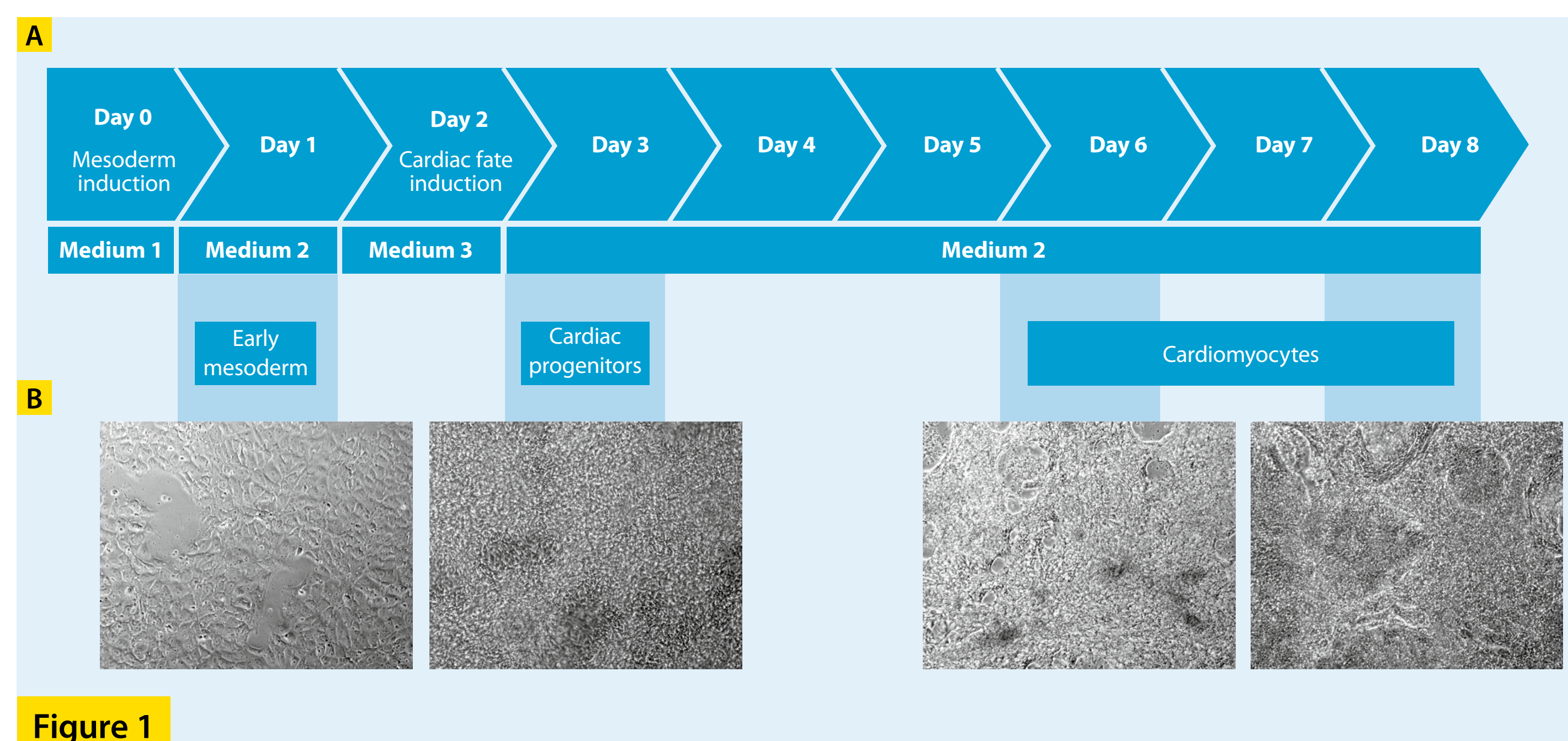


Figure 1

Cardiac differentiation of PSCs was induced stepwise by using three different media (fig. 1). First, PSCs were seeded as single cells in mesoderm induction medium on plates coated with Matrigel®. Cardiac fate was induced on day 2 of differentiation. Depending on the time point of differentiation, morphological changes could be observed. On day 1, cell clusters became visible, which disappeared on day 3 of differentiation. From

days 4 to 6, cardiac clusters started to form (fig. 1). First contracting cells were observed on days 6–8 of differentiation. This new workflow protocol allows for robust, highly efficient, and scalable generation of CMs within less than 10 days of differentiation, thereby solving several technical issues related to the generation of PSC-derived CMs.

### 2 StemMACS™ CardioDiff Kit enables standardized CM differentiation

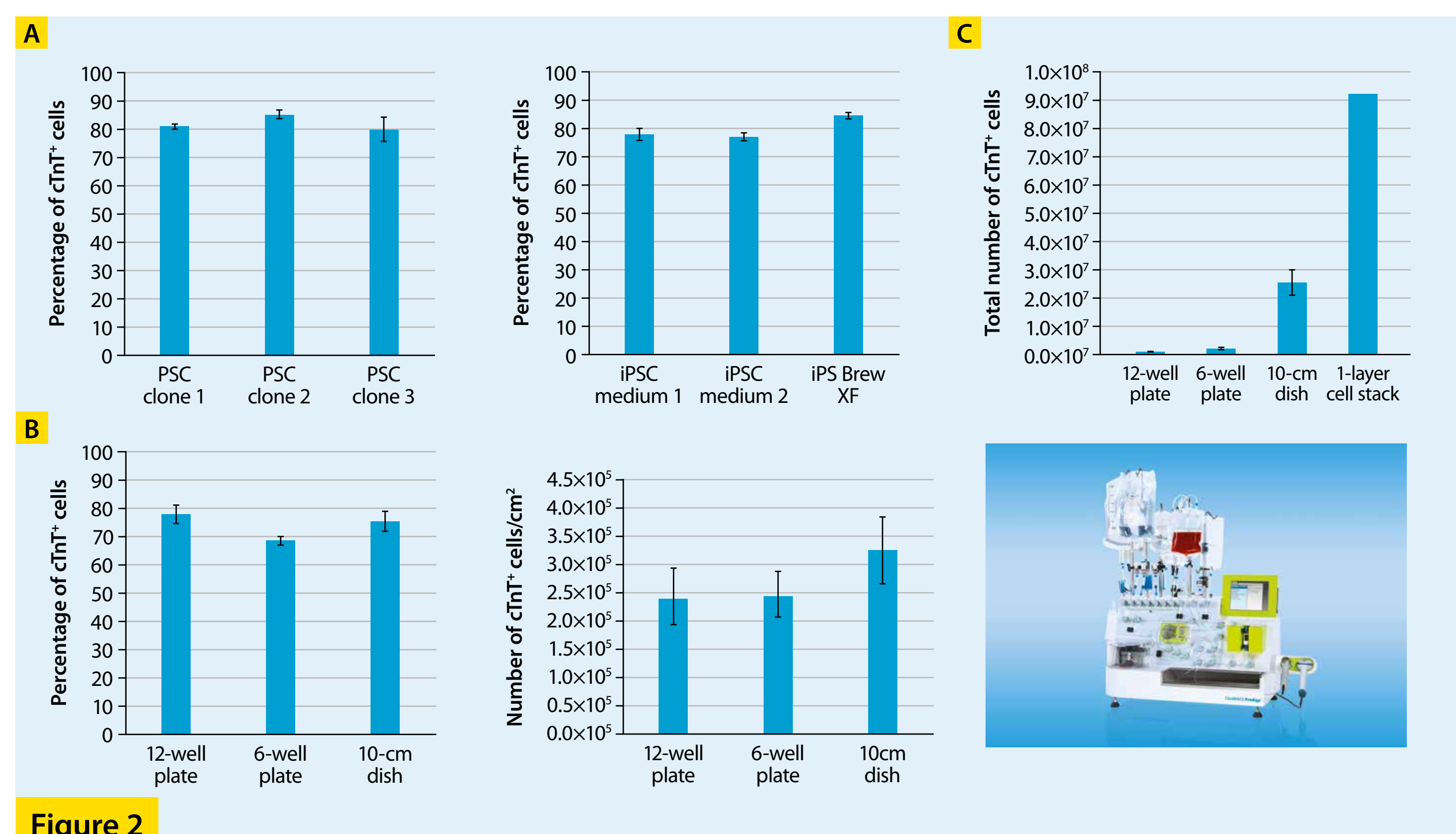


Figure 2

Application of the protocol to different PSC clones yielded differentiation efficiencies of up to 90% as shown by flow cytometry analysis of cardiac troponin T (cTnT, fig. 2A). Moreover, the StemMACS™ CardioDiff Kit supported cardiac differentiation of PSCs that were cultured in StemMACS iPS-Brew XF, human or two other commercially available stem cell media (fig. 2A). Further experiments have shown the scalability of the protocol to different multiwell plate formats or 10-cm dishes, yielding similar differentiation efficiencies and numbers of CMs/cm<sup>2</sup> (fig. 2B). This is the basis for standard-

ized, automated generation and large-scale differentiation of PSC-derived CMs in the functionally closed system of the CliniMACS Prodigy®. In a next step, our workflow protocol for CM generation will be transferred to this GMP-compliant cell manufacturing platform, enabling the scale-up of CM manufacturing. First data indicated that 9×10<sup>7</sup> CMs could be generated in a single automated production run of the CliniMACS Prodigy connected to a one-layer cell stack system (fig. 2C). All graphs: n ≥ 3; means±SD; except data for one-layer cell stack (fig. 2C): n = 1.

### 3 Analysis of cardiac differentiation

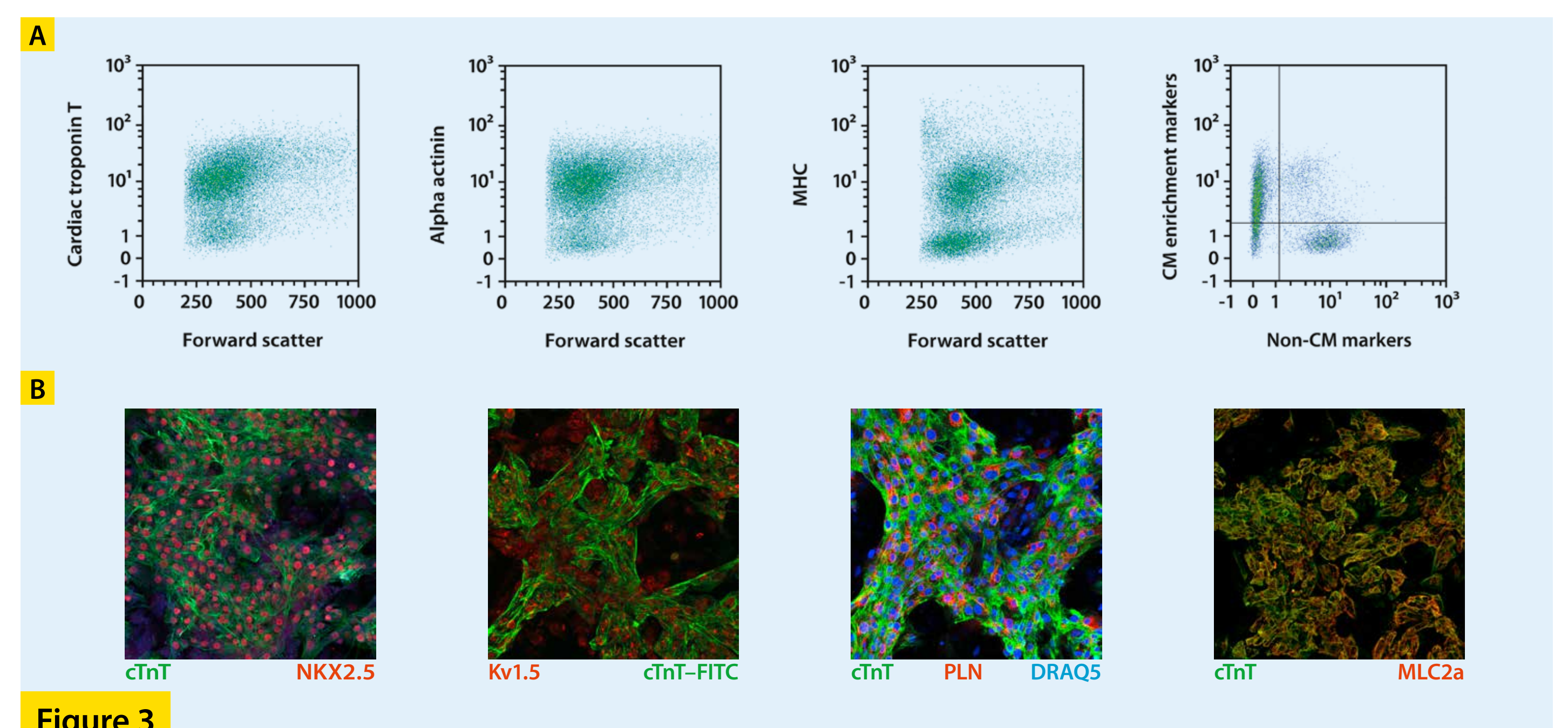


Figure 3

Final characterization by flow cytometry of CMs generated by the StemMACS CardioDiff Kit showed the expression of typical CM markers such as cTnT, alpha actinin, and MHC. Moreover, costaining of depletion and enrichment markers revealed compatibility with the PSC-derived Cardiomyocyte Isolation Kit, human, thus enabling further purification of CMs (fig. 3A).

Immunofluorescence staining for Kv1.5 and phospholamban (PLN) showed the presence of CMs with electrical properties and Ca<sup>2+</sup> handling machinery, which enables further characterization through functional studies based on the patch-clamp technique or the analysis of Ca<sup>2+</sup> transients (fig. 3B).

### 4 Differentiation of hPSCs into atrial- or ventricular-like CMs

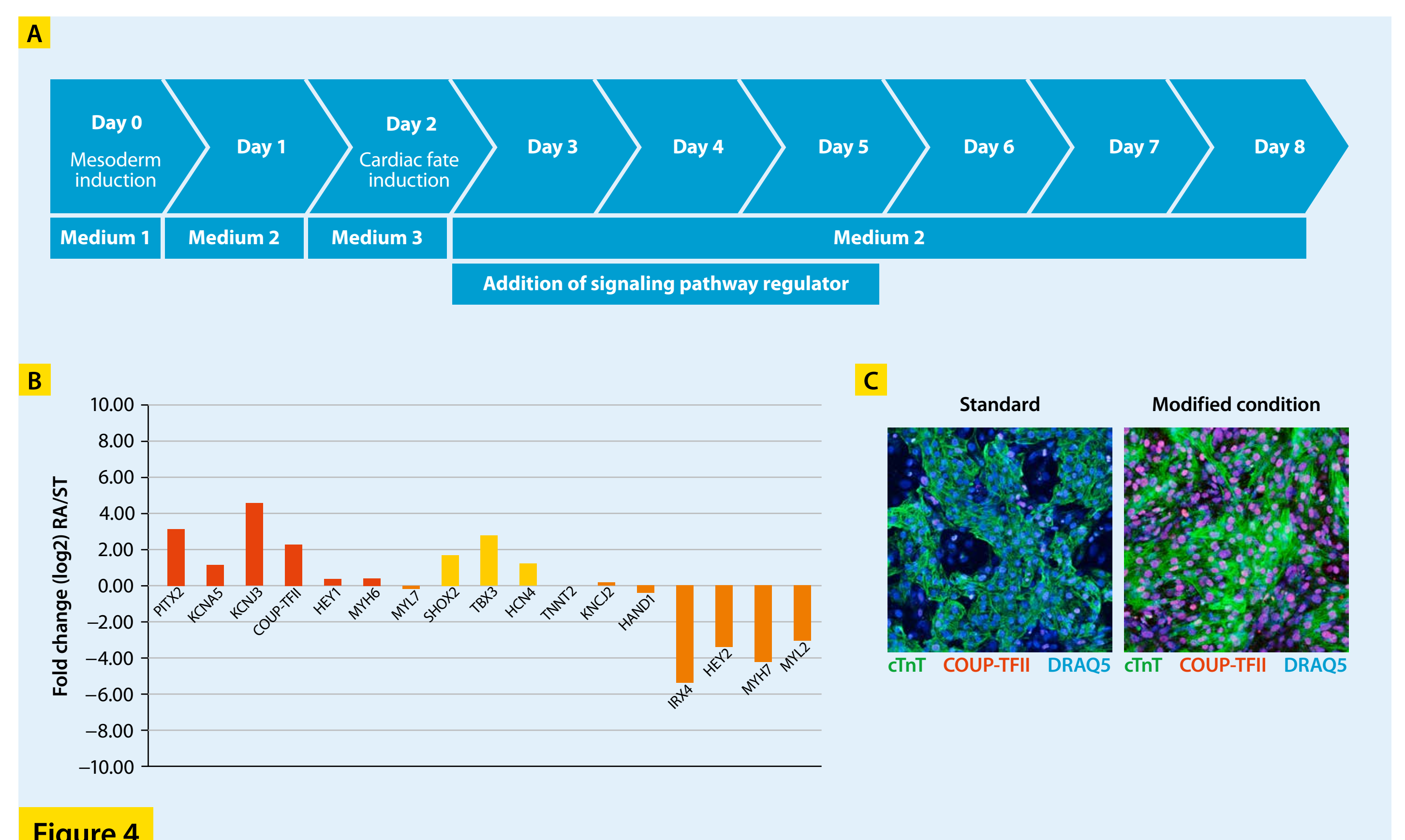


Figure 4

Expression analysis of CM subtype markers, e.g., IRX4 and MYL2, revealed a ventricular-like CM subtype. Interestingly, addition of a signaling pathway modulator to the StemMACS CardioDiff Kit at the stage of cardiac mesoderm specification enabled induction of an atrial-like cell fate (fig. 4A). Gene expression analysis by qRT-PCR showed the up-regulation of known atrial markers, such as COUP-TFII, PITX2 (transcription factors), KCNJ3, KCNA5 (ion channels), and down-regula-

tion of ventricular markers such as MYL2, IRX4, HEY2 (fig. 4B). This was in line with immunofluorescence data showing high expression levels of COUP-TFII after atrial fate induction. In contrast, COUP-TFII was not detected in CMs generated using the standard protocol (fig. 4C). In conclusion, the StemMACS Cardio Diff Kit generates CMs biased towards a ventricular-like fate, while the addition of signaling pathway modulators can lead to an atrial-like fate switch.

## Conclusion

The StemMACS CardioDiff Kit perfectly integrates into our complete workflow covering controlled cardiac differentiation as well as CM harvesting, purification, storage, and analysis. Transferring this complete workflow and the reagents to the automated cell processing platform of the CliniMACS Prodigy will pave the way for standardized, large-scale manufacturing of PSC-derived CMs.

- The StemMACS CardioDiff Kit enables short, robust, and scalable cardiac differentiation that can be applied to different stem cell clones.
- Generated CMs show typical cardiac marker expression.
- The StemMACS CardioDiff Kit generates CMs biased towards a ventricular-like fate, while the addition of signaling pathway modulators can lead to an atrial-like fate switch.

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