Highly sensitive and quantitative detection of remnant Oct3/4$^+$ pluripotent cells in differentiated cell preparations for stem cell–based therapies

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Introduction

One of the major challenges of the cell manufacturing process for cellular therapies is the exact analysis of the cell product's identity after differentiation. Detection of non-differentiated cells that might contaminate the cell product is especially important due to the teratogenic potential of pluripotent cells.
We developed a flow cytometry–based assay for highly sensitive and quantitative detection of remnant Oct3/4$^+$ pluripotent cells in a population of differentiated cells. Flow cytometry enables a standardized and fast readout of protein levels on single cells and the absolute number of cells that are positive for a given marker. In our assay, cells were labeled with the Oct3/4-specific recombinant REAfinity™ Antibody clone REA622 conjugated to APC, measured using the MACSQuant® Analyzer 10, and analyzed by MACSQuantify™ Software. To define the assay's sensitivity, the limit of blank (LoB), limit of detection (LoD), and limit of quantification (LoQ) were determined. The assay was then used to analyze human pluripotent stem cell (hPSC)–derived mesencephalic dopaminergic (mesDA) progenitors, which are a promising cell source for treatment of Parkinson's disease. Apparent percentages of Oct3/4$^+$ cells among differentiated mesDA cells were below the detection limit.
In summary, we developed a quantitative assay that shows a high sensitivity with an LoB of 0.002%, an LoD of 0.008%, and an LoQ of 0.01% for detection of remnant pluripotent cells, with defined settings for flow cytometric acquisition and data analysis.

Results

1 Principle of the assay for highly sensitive detection of Oct3/4$^+$ pluripotent cells

We defined the flow cytometry assay's sensitivity by determination of the limit of blank (LoB), limit of detection (LoD), and limit of quantification (LoQ) for cells labeled with the Oct3/4-specific antibody clone REA622.
While the LoB defines the highest apparent analyte concentration that can be detected in a sample that contains no analyte, the LoD describes the lowest analyte concentration that can be reliably distinguished from the LoB and is likely to yield a result that exceeds the LoB. Furthermore, the LoQ is the lowest concentration that can be reliably quantified and at which pre-defined goals for accuracy are fulfilled.[1]
For the assay, cells were fixed using the FoxP3 Staining Buffer Set. Subsequently, samples of 1×10^6 cells (volume: 500 µL) were labeled with the Oct3/4-specific antibody conjugated to APC. Flow cytometry was performed using the MACSQuant Analyzer 10, which enables automated measurement of the accurate cell count. The complete volume of each sample was taken up and measured with a high flow rate. To minimize the risk of carryover between different samples, an extended washing procedure was performed after each measurement. Analysis was carried out using the MACSQuantify Software. First, debris was excluded in the forward scatter versus side scatter plot (fig. 1A). Subsequently, the gate for detection of Oct3/4$^+$ cells was defined using a sample of Oct3/4-stained pluripotent cells (fig. 1B). Then the accurate numbers of Oct3/4-APC$^+$ events and total events were determined to calculate the accurate percentage of Oct3/4$^+$ cells (fig. 1C).

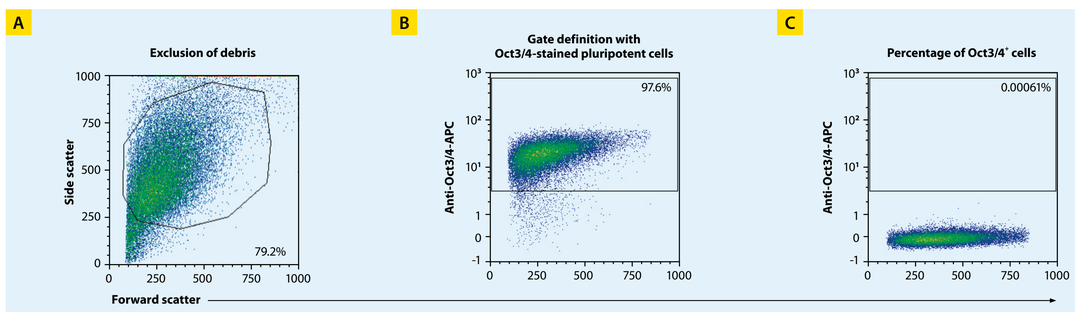
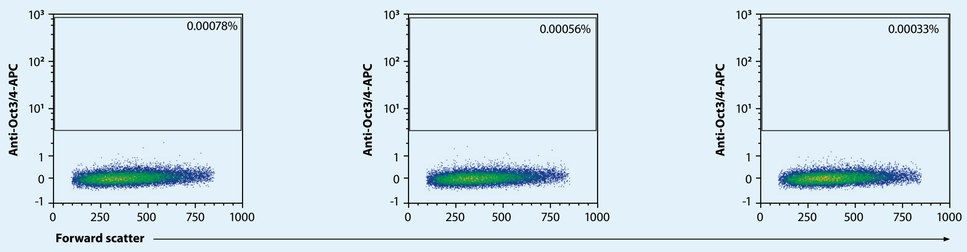


Figure 1

2 Limit of blank, limit of detection, and limit of quantification for detection of Oct3/4$^+$ pluripotent cells

The LoB was determined by measuring 20 replicates of unlabeled cells. Samples showed a very low background in the APC channel, with a percentage of 0.00076±0.00039 positive events. According to these data, the LoB was 0.002%. This means that a sample containing no Oct3/4$^+$ cells displays a background of 0.002% positive events (fig. 2). Assuming a Gaussian distribution of the signals from blank samples, 99.9% of the observed values were below the LoB, and only 0.1% of the samples exceeded the LoB and thus produced a false-positive signal (type I error).

Limit of blank (LoB): defines the highest apparent analyte concentration that can be detected in a sample that contains no analyte.



Percentage of Oct3/4$^+$ cells = 0.00076%±0.00039% (n = 20)

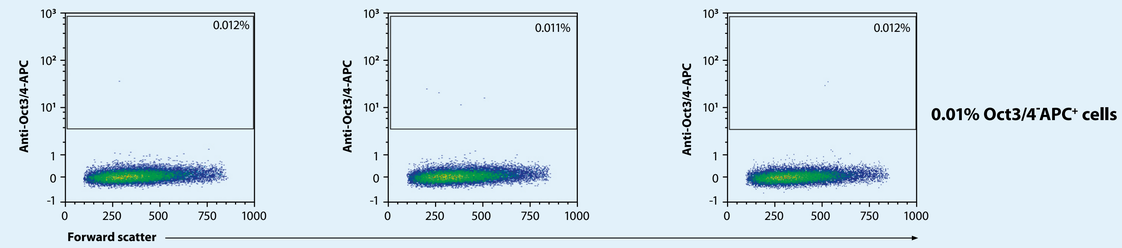
$$LoB = Mean_{blank} + 3.09 \times SD_{blank}$$
$$= 0.00076\% + 3.09 \times 0.00039\%$$
$$= 0.002\%$$

Error probability: 0.1%, assuming a Gaussian distribution of the signals

Figure 2

One approach to define the LoD is to analyze samples that contain low but defined percentages of positive cells and compare the signals from these samples to a blank. In this way, it is possible to determine which minimum percentage of Oct3/4$^+$ cells is required to distinguish the presence from the absence of positive events. To this end, 0.01% Oct3/4$^+$ pluripotent, stained cells were spiked into a suspension of unlabeled cells. Using the flow cytometry assay, the exact percentage of Oct3/4$^+$ cells was determined to be 0.010% ± 0.002% (n = 20), leading to an LoD of 0.008% (fig. 3).
This means that, by this assay, a percentage of ≥0.008% Oct3/4-positive events can be distinguished from the LoB and only 0.1% of the low-concentration samples will produce a false-negative signal below the LoB (type II error).

Limit of detection (LoD): defines the lowest analyte concentration that can be reliably distinguished from the Limit of Blank (LoB).



Percentage of Oct3/4$^+$ cells = 0.010%±0.002% (n = 20)

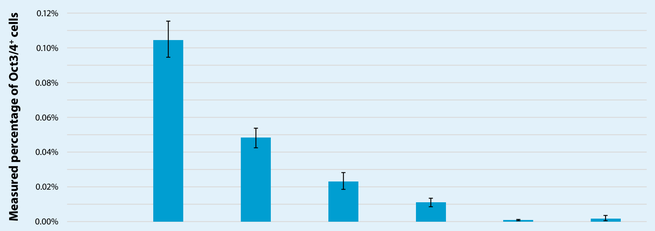
$$LoD = LoB + 3.09 \times (SD_{low\ concentration})$$
$$= LoB + 3.09 \times (SD_{0.01\%\ Oct3/4})$$
$$= 0.002\% + 3.09 \times 0.002\%$$
$$= 0.008\%$$

Error probability: 0.1%, assuming a Gaussian distribution of the signals

Figure 3

While the LoD provides only information about whether a sample contains positive cells or not, the LoQ defines the lowest concentration that can be reliably determined quantitatively, and at which some pre-defined goals for accuracy are met (coefficient of variation (CV) ≤ 20%). For definition of the LoQ, 0.01%, 0.025%, 0.05%, and 0.1% of Oct3/4$^+$ pluripotent, stained cells were spiked into a suspension of unlabeled cells (n = 20) and the exact percentage of Oct3/4$^+$ cells was determined.

Percentages of positive cells detected in the spiked samples nicely matched the theoretical values (fig. 4) and the coefficient of variation (CV) was determined to be 20%, 20%, 10%, and 10% for 0.01%, 0.025%, 0.05%, and 0.1% Oct3/4$^+$ cells, respectively. As the CV was ≤20% in case of the 0.01% sample, the assay resulted in an LoQ of 0.01%.



Sample	0.10% Oct3/4$^+$ (n = 20)	0.05% Oct3/4$^+$ (n = 20)	0.025% Oct3/4$^+$ (n = 20)	0.01% Oct3/4$^+$ (n = 20)	hESC unstained (n = 20)	mesDA stained (n = 20)
Measured percentage of Oct3/4$^+$ cells	0.104% (±0.01) CV = 10%	0.048% (±0.005) CV = 10%	0.023% (±0.005) CV = 20%	0.010% (±0.002) CV = 20%	0.00076% (±0.00039)	0.00188% (±0.00148)

Figure 4

3 Flow cytometric assay for quality control of hPSC-derived mesencephalic dopaminergic progenitors

The assay was applied to analyze hPSC-derived mesDA progenitors. In addition to Oct3/4, positive and negative mesDA progenitor markers were included in the assay for identification of the cellular phenotype.
hPSC-derived mesDA progenitors were generated using a protocol for fully adherent cultivation (fig. 5A).[2] Neural conversion and regionalization were induced by i) inhibition of glycogen synthase kinase 3 (GSK3) with CHIR99021 for rostro-caudal patterning and ii) activation of sonic hedgehog (SHH) for ventralization. In addition to the Oct3/4-specific antibody clone REA622, we defined a set of antibodies specific for mesencephalic floor plate markers (FoxA2, Otx2, IAP) as well as forebrain, hindbrain, and lateral midbrain markers (Pax-6, Sox1, Nkx2.1, Nkx6.1), enabling a clear characterization of the cellular phenotype. Differentiated cells showed a high frequency of Otx2$^+$FoxA2$^+$IAP$^+$ cells after 16 days of differentiation and lacked expression of Sox1, Pax-6, and Nkx6.1. We found that Nkx2.1 was expressed on the majority of mesDA progenitors at the protein level, while it was absent at mRNA level. Some proliferative potential was indicated by expression of Ki-67, and only 0.00109% Oct3/4$^+$ pluripotent cells were detected (fig. 5B). In total, 20 samples of mesDA progenitors were analyzed and showed an apparent percentage of 0.00188%±0.00148% Oct3/4-positive events, which was below the detection limit and in the range of the LoB (fig. 4, 5).

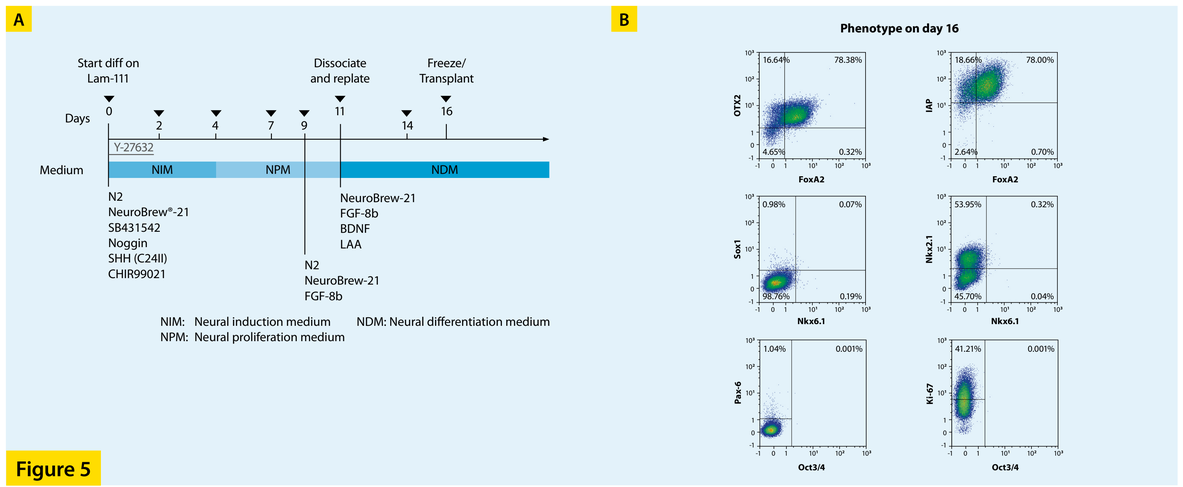


Figure 5

Summary and outlook

- We present a highly sensitive and quantitative assay for detection of potentially contaminating pluripotent cells in populations of differentiated cells, with defined settings for flow cytometric acquisition and data analysis.
- The assay is characterized by a limit of blank of 0.002%, a limit of detection of 0.008%, and a limit of quantification of 0.01% for detection of Oct3/4$^+$ pluripotent cells.
- hPSC–derived mesDA progenitor cells showed an apparent percentage of 0.00188%±0.00148% Oct3/4$^+$ cells, which is below the detection limit.

References
1. Armbruster, D.A. and Pry, T. (2008) Clin. Biochem. Rev. 29 (suppl 1): S49–S52.
2. Kirkeby, A. *et al.* (2017) Nat. Protoc. 12: 1962–1979.

Funding source
Research activities were supported by Neurostemcellrepair (European Community's Seventh Framework Programme grant agreement).

NeuroStemcellRepair
European Stem Cell Consortium for Neural Cell Replacement Reprogramming and Functional Brain Repair

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