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Development and implementation of large-scale quality control for the European bank for induced Pluripotent Stem Cells

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ABSTRACT

The European Bank for induced Pluripotent Stem Cells (EBiSC) has collected iPSC lines associated with genetic diseases and healthy controls from across Europe and made these available for research use to international academic and commercial users. Ensuring availability of consistently high quality iPSCs at scale and from various sources requires quality systems which are flexible yet robust, maximising the utilisation of available resources. Here, we outline the establishment and implementation of a quality control regime suitable for a large-scale operational setting. Strict release testing ensures the safety and integrity of distributed iPSC lines, whilst informational testing allows publication of full characterisation and assessment of iPSC lines. Quality control screening is underpinned by a 'fit-for-purpose' Quality Management System giving full traceability and supporting continuous scientific and process development. Evaluation and qualification of key assays and techniques ensures that assay sensitivities and limits of detection are acceptable. Use of rapid testing techniques in place of more 'traditional' assays allows EBiSC to respond quickly to user demand, generating fully qualified iPSC line banks in a labour-saving and cost-efficient manner.

1. Introduction

Since the discovery of reprogramming technology to generate induced pluripotent stem cells (iPSCs), the utility of disease specific iPSC lines has led to a dramatic increase in the numbers of lines being generated for disease modelling and drug discovery (Gunaseeli et al., 2010). However, the levels and effectiveness of quality control (QC) performed in different laboratories varies tremendously and the availability of ethically sourced, quality controlled, and well characterised iPSCs is a priority to ensure robust and high-quality research in this area (Stacey et al., 2013).

The EBiSC resource was initiated in 2014 to provide a central international supply of quality-controlled iPSC lines. EBiSC allows scientists to deposit iPSC lines generated within their distinct research projects into this central repository which are then distributed to researchers internationally strictly for research use (i.e. not for commercial or clinical applications). Hence EBiSC lines are collected from different research centres with varying approaches and capabilities in cell line generation, QC and characterisation and under widely variable levels of internal quality systems, from academic centres operating with minimal Quality Assurance (QA) systems to generation under formal Quality Management Systems (QMS with Good Laboratory Practice (GLP). Therefore, a robust and standardised QC system capable of ensuring the distribution of iPSC lines of different provenances whilst assuring end users of consistent high-quality cell supply, is critical. Here we report the key learning experiences resulting from establishing a QC regime suitable for rapid and efficient screening of large numbers of iPSC lines, following on from initial learnings previously reported

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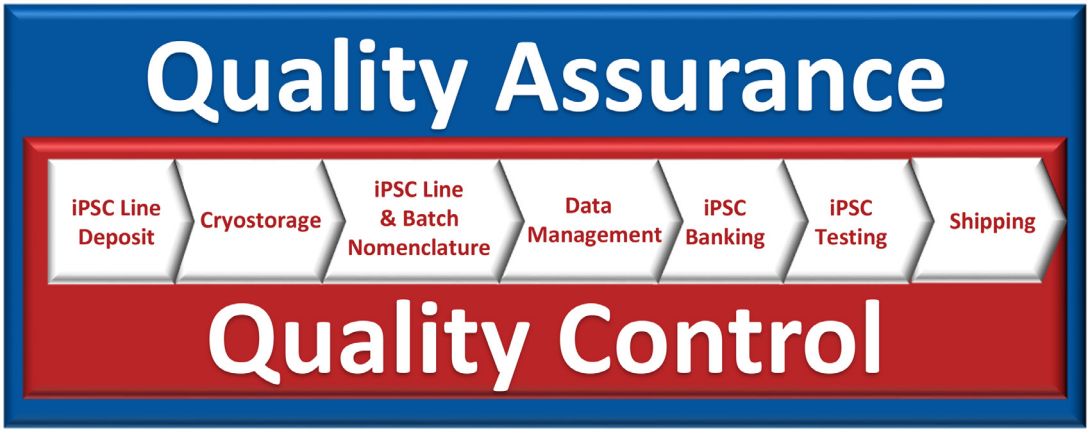

Fig. 1. A Robust Quality Management System supports deposit and distribution of high-quality iPSC lines. Key processes such as cell line shipment, assigning cell line names and batch identifiers, cell line processing and testing are underpinned by a Quality Assurance framework. The EBiSC QMS was designed to allow development of Quality Control systems which were flexible and suitable for a large scale and multi-centre setting, whilst still ensuring robust cell line characterisation and a high-quality end product.

(De Sousa et al., 2017a; 2017b). QC testing of individual cell lines is just one part of the QA and QMS which are required to efficiently deliver reliable supplies of iPSC lines to users of the EBiSC resource (Fig. 1). Encompassed in the QMS is control of the input materials (cells and raw materials), iPSC line nomenclature, quarantine, banking, cryostorage, QC, release, shipment and document and data management. At the outset of the EBiSC project, a key principle to adopt industry standards wherever applicable was agreed. The underpinning structure for the EBiSC QA production system was based on the DIN EN ISO9001:2015 standard for total Quality Management and a compliant Quality Manual was produced for EBiSC which covered relevant activities of each of the four operating centres responsible for delivery of the EBiSC resource (Roslin Cell Sciences, National Institute of Biological Standards and Control, European Collection of Authenticated Cell Cultures and Fraunhofer Institute for Biomedical Engineering).

During the establishment of EBiSC, a number of challenges and experiences led to refinement of the QC regime employed and, in this paper, we review the various developments and key learning points which led to the current EBiSC QC and characterisation regime and its ongoing programme of development under the second project phase, EBiSC2 (cells.EBiSC.org/).

2. Materials and methods

2.1. IPSC lines

All EBiSC lines are named using the hPSCreg nomenclature system (Kurtz et al., 2018). However here, iPSC lines have been coded, as for experimental purposes, not all assays were performed directly on EBiSC stocks. Additionally, non-EBiSC lines were also used for specific assays whereby cell line specific qualities were needed as positive or negative controls (for example, inclusion of non-EBiSC iPSC lines known to carry specific genomic abnormalities in Genome Status Analysis assessments). All iPSC lines used within this study are listed in Supplementary Figure 5.

2.2. Cell culture

All cell cultures were maintained at 37 °C, 5% CO_2. The iPSC lines were propagated on either Geltrex (Life Technologies) or Vitronectin (Life Technologies) coated 6 well plates using mTeSR1 medium (StemCell Technologies) or E8 (Life Technologies). Undifferentiated cells were passaged at 70% confluency every 4–5 days by manual passage or EDTA. Cell pellets for cell line identity, viral screening were collected when available.

2.3. Viral screen

Cell pellets or DNA were analysed by IDEXX Laboratories for presence of HIV1, HIV2, HBV or HCV nucleic acids.

2.4. Cell line identity

Cell pellets or DNA were analysed by Source Bioscience using Promega PowerPlex® 16 HS.

2.5. Mycoplasma qPCR

Cell culture supernatant was collected from confluent cultures and analysed using Venor®GeM qEP according to manufacturer's instructions.

2.6. Sterility

Spent media (at least 24 h old) collected from confluent cell cultures was inoculated into Fluid Thioglycollate Medium (FTM) and Tryptone Soya Broth (TSB) and incubated at 37 °C ± 2 °C and 22 °C ± 2 °C respectively for 14 days (World Health Organization., 2012).

2.7. Embryoid body differentiation

All samples were run in triplicate, with at least one passage between samples. All undifferentiated lines displayed typical morphology and stained positive (i.e. >70%) via flow cytometry for the stem cell associated markers SSEA4, and TRA-1–60 and negative for SSEA1 (i.e. < 10%). Briefly, media was removed and the cells were washed with PBS. TrypLE™ (Gibco) was added for 3 min and quenched by DMEM containing 20% FCS. The cells were centrifuged at 300 g for 3 min and resuspended in Apel media (Stem Cell Technologies) containing 10 μM Rock inhibitor at 30,000 cells per ml. 100 μl was then added to each well of a U-bottom 96 well plate and centrifuged at 300 g for 3 min. The plates were place in an incubator at 37 °C, 5% CO_2. EB's formed overnight and samples were collected at days 7 and 14. RNA was extracted using Maxwell RSC machine and kit, according to manufacturers instructions (Promega).

2.8. Genome health screening

Cells from the same bank were sent for each individual test. Bacs on Beads:1 million cells were sent to The Doctors Laboratory (TDL) who performed the assay according to their local procedures (García-

Table 1
EBiSC testing criteria for iPSC cell bank release.

Testing Category	*Assay*	*Method*	*Acceptance Criteria*[*]
Critical Release Testing	Mycoplasma	QPCR / Culture Method	Not Detected
	Viral Screening (HIV1, HIV2, HBV and HCV)	QPCR	Not Detected
	Growth of bacteria and fungi	Broth Inoculation	Not Detected
	Viability	Visual Assessment	Growth to confluence post-thaw
	Cell Line Identity	Microsatellite PCR	Must match identity profile of donor (where available) and/or comparator cell lines (where applicable)
	Morphology	Visual Assessment	Typical iPSC morphology with low-medium differentiation levels. Alert Limit: No / few typical iPSC colonies present
Informational Testing (cell line characterisation)	Expression of markers for hPSCs and self-renewal	Flow cytometry	SSEA4+ > 70% TRA–1–60+ > 70% POU5F1+ > 70% SSEA1+ < 10% Alert Limits: see[**].
	Karyology	G-Banding	Sex match to donor, 30 successful karyotypes recorded. >75% normal spreads classed as 'diploid karyotype'
	Pluripotency	Trilineage Differentiation	Up-regulation of markers for all germ layers. Alert Limit: Failure to show up-regulation for 1 or more germ layers.
	Vector Clearance	PCR / QPCR	Integrating vectors are silenced. Non-integrating vectors are not detected.
Additional characterisation for gene-edited lines.	Genetic Lesion	Sequencing or suitable alternative	Genotype of targeted locus confirmed, and sequence of clone confirmed as free of unrequired mutations or frameshift effects. Orientation of insertions confirmed.
	Vector Clearance (if vector used in gene-editing process)	PCR or alternative	Clearance / silencing of gene-editing vectors.

[*] N.B. all acceptance criteria based on molecular or antibody based assays are dependent on acceptable performance of the individual tests regarding control material results.

[**] Flow cytometry is an informational test and it is difficult to set generic pass-fail limits. However, a high value (>10%) for SSEA-1 or consistently low values for other markers may mean that recovery of a culture from the cell bank may be a cause for concern and further investigation.

Herrero et al., 2014). G-banding: A T25 flask containing cells at approximately 40% confluency sent to The Doctors Laboratory (TDL) who performed the assay according to their local procedures. SNP array: DNA was extracted from cell pellets at North East Thames Regional Genetics Laboratory and the cytoscan 750 K assay (Affymetrix) was performed as per manufacturer's protocol. The data was filtered to identify copy number variants over 3Mb in size.

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2.9. Flow cytometry

Prior to EB spontaneous differentiation, iPSC lines were assessed for the stem cell markers, SSEA-4, SSEA-3, SSEA-1 and TRA-1-60, using specific antibodies (all R&D Systems) on the BD Accuri flow cytometer according to manufacturer's instructions. For flow cytometry assessment during standard batch QC, iPSC lines were assessed for SSEA-4, SSEA-1, POU5F1 and TRA-1–60 (Cat 560477, Cat 560380 and Cat 553474 BD Biosciences) on a Guava® easyCyte instrument.

2.10. Confluency assessments

iPSC lines were grown on Vitronectin (Life Technologies) and mTeSR1 (StemCell Technologies) and placed in the IncuCyte® just after passage. Images were taken at multiple points for 5 days. The IncuCyte® System, a real-time quantitative live-cell imaging and analysis platform enables visualisation and quantification of cell behaviour over time by automatically gathering and analysing images.

2.11. Comparative real-time PCR

cDNA was produced using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions and analysed with gene-specific probes (Applied Biosystems) by standard methods and run on Quantstudio thermocycler (Life Technologies). For assessment of cell line pluripotency each differentiated sample was compared against its undifferentiated counterpart as a normalised control using the DDCt method, to give relative quantification (RQ) values using GAPDH and ACTB as reference genes.

2.12. Statistical analysis

P-values were determined using GraphPad Prism using a two tailed paired *t*-test, where $p > 0.05$ was considered significant.

2.13. Principal component analysis

Using a correlation matrix between samples, Principal Component Analysis was performed to investigate trends associated with particular cell lines. The number of principal components interpreted was chosen using a scree plot which shows how much variation is explained cumulatively by the principal components.

3. Establishing the EBiSC quality criteria and quality control regime

An important first step in evaluating cell lines for inclusion in EBiSC was a screening process to assess the core scientific properties, ethical provenance and ownership, and intellectual property landscape for each iPSC line. At the start of this process each line was registered in the hPSCreg database (www.hpscreg.eu/) providing a unique name for the cell line (Kurtz et al., 2018) and collecting a range of data points on donor consent, iPSC generation, gene-editing, quality control and characterisation (Seltmann et al., 2016). This includes critical data such as screening for human viral pathogens (e.g. HIV1, HIV2, HBV and HCV), and the clearance or silencing of reprogramming vectors in established iPSC lines. Use of hPSCreg eased the significant burden of data entry and management for hundreds of iPSC lines, with completion of mandatory data points required prior to cell line release. Establishment of the EBiSC Data Access Committee and subsequent deposit of

genomic datasets into the European Genome-Phenome Archive simplified both how EBiSC depositors can securely store and share genomic datasets and how external researchers can access these data to support their research goals. 'Historical' data on each iPSC line is supplemented with additional EBiSC generated characterisation and QC, performed during iPSC line expansion and after cryopreservation.

EBiSC quality criteria, applied to all cell banks prior to release and distribution, is identified in Table 1. All iPSC line batches released by EBiSC undergo 'Critical Release' testing to assure viability, cell line identity and lack of micro-organism growth, recognised by EBiSC as common failures and critical issues across the research community (De Sousa et al., 2017b). Additional cell line characterisation (such as flow cytometry for marker expression, assessment of iPSC morphology, genomic stability, and potential for trilineage differentiation) are performed and recorded as 'Informational Testing' i.e., for information only. These informational tests give an indication of how the line may perform in the hands of users and allow EBiSC to judge the quality of each line by assessing such informational QC data as a whole, rather than on a rigid strict pass/fail criterion on each assay individually. Through implementation of consistent QC on every batch, EBiSC can ensure that variability across subsequent batches is minimised, with in-assay controls ensuring consistent assay performance long term (see Section 5) and individuals at all central facilities being trained consistently within DIN EN ISO9001:2015 compliant QMSs. *QC of gene-edited iPSC lines*: Gene editing and in particular CRISPR-Cas9, provides tools which have transformed the way in which iPSCs can be used in disease research (Jehuda et al., 2015; Omole and Fakoya, 2018)). The introduction of gene-corrected and mutation-induced isogenic iPSC lines into the EBiSC catalogue prompted a review of the QC criteria, to ensure that sufficient data was available on these lines prior to them being made available to end users. After collaborative discussion with key experts on the challenge of delivering gene edited iPSC lines and key checks to be made in their development, additional characterisation was incorporated as a requirement for deposit of gene-edited iPSC lines including assessment of guide RNA quality, presence of original (wildtype) sequence, off-target effects (if known) and sequence analysis of the target pre and post gene-editing (Table 1).

4. Development of EBiSC QC procedures

4.1. Assuring identify, viability and purity of iPSC lines

4.1.1. Microbiological testing

Bacterial and fungal contamination: Antibiotic-free culture conditions were adopted by EBiSC consistent with good cell culture practice (Geraghty et al., 2014) and microbiological assessment was initially performed by visual assessment of media during routine culture. However, it was observed that the daily media change and possibly the serum-free hPSC media could inhibit the visible appearance of contaminants below levels that were visually detectable unless cells were left in the same media for at least 48 h. Hence, a test to screen for growth of micro-organisms (i.e. broth media inoculation) was introduced as standard for all cell line banks. As previously reported, 6.4% of cell lines deposited through the Hot Start program showed evidence of microbiological contamination (De Sousa et al., 2017b). Post Hot Start, EBiSC did not receive any contaminated cell line banks. It's feasible that this drop was partly contributed to by the availability of best practice workshops for EBiSC partners and the deposition of lines from other large scale iPSC projects such as HipSci (Kilpinen et al., 2017) and StemBANCC (Morrison et al., 2015) which also operated under agreed quality standards (Table 2).

High sensitivity Mycoplasma screening: A mycoplasma selective broth media inoculation is still frequently used as a 'gold-standard' Pharmacopeia reference test, but typically takes >28 days of inoculation before results are completed which is ill-suited to a rapid high scale setting. For the purposes of EBiSC, a high-sensitivity qPCR method was validated and a subset of lines tested using both methods to provide comparative data. Subsequently, cell banks released by EBiSC can be tested using a qPCR method that detects all mycoplasma species commonly arising in cell culture (Venor®GeM qEP). Surprisingly, given the reported rates of Mycoplasma contaminations (Nikfarjam and Farzaneh, 2012), cells from only 1 depositor were found to harbour Mycoplasma contamination during the project period. However, this one case resulted in 5 cell line banks having to be disposed of and re-banked. The central facility screened 467 lines between 2014 and 2017, and all tests proved negative. It is worth noting that all lines were required to have been tested prior to receipt at the central facility and this possibly had a major influence by early prevention of contaminated or suspect cultures being shipped to EBiSC and may not necessarily reflect the general incidence of mycoplasma in research laboratories.

Human Viral Pathogen screening: The European Federation of Pharmaceutical Industries and Associations (EFPIA) partners within EBiSC highlighted their requirement for human viral pathogen screening, recommending the inclusion of HIV1, HIV2, HBV and HCV as standard. At the initiation of testing in 2014, testing for HIV2 was the most difficult to establish in the absence of a nucleic acid-based test. However, it was considered acceptable if donors had been screened for serious blood borne viruses including HIV2, in replacement of screening the derived cell lines. Additionally, a suitable supplier was identified who showed capability for PCR screening for HIV2 viral nucleic acids. Att he time of writing, only two incidences of positivity for human viral pathogens were reported. In one instance, the donor was flagged as being HIV+, so a biosample was not collected. In the second instance, an iPSC line was discarded as it tested positive by PCR for HIV1. Interestingly, this line was initially reported as being reprogrammed by lentivirus, possibly causing a false positive due to the reprogramming vector using the HIV-1 viral backbone. However, this line was also implicated in a case of cell line cross-contamination so the root cause of the viral contamination could not be fully ascertained, and the cell line was withdrawn.

4.1.2. Cell line identity

In the Hot Start phase of EBiSC, 8 of 47 cell lines (17%) were shown to have incorrect cell line identity (De Sousa et al., 2017b). A number of root causes were identified for these issues, including poor data traceability, accidental mixing of cell lines, and labelling issues. Subsequent dissemination activities such as best practice discussions with iPSC generation centres, training workshops and availability of EBiSC cell culture manuals, in addition to collaboration with other quality strong iPSC projects highlighted this issue, appearing to contribute to a steep decline, with only 3 subsequent incidents of incorrect cell line identities. Considering the variance of sites for biosample sourcing and iPSC generation throughout the EBiSC project, the frequency of cell line identity issues is far below the estimated 10–20% of mis-identifications reported elsewhere for cell lines in general (Barallon et al., 2010; Bian et al., 2017; Cabrera et al., 2006).

4.1.3. Cell enumeration, viability and growth

An early challenge was to ensure that EBiSC stocks were of a consistent cell viability. A collaborative workshop established a standard operating procedure (SOP) for culture cryopreservation, recovery and assessment. This protocol established a 70% rate of recovery as an acceptable viability level and supplied users with representative images of iPSCs with optimal, acceptable and poor morphology for training use, in addition to a guide for scoring and recording iPSC morphology, adherence and performing viability cell counts prior to cryopreservation, available at cells.EBiSC.org/customer-information/.

The level of iPSC confluency during harvesting and passaging is critical for the consistency, viability and recovery rate of generated cell banks, hence, the consistency of confluency scoring was assessed across 9 EBiSC iPSC generation centres. Images of an iPSC line from low to high confluency were recorded on an IncuCyte™ and the confluency

Table 2
Summary of EBiSC critical release quality control testing (Satisfaction of critical EBiSC QC is required to allow release and distribution of EBiSC iPSC lines. Initial learnings in the early Hot Start project phase highlighted sterility and cell line identity as particular areas of concern and were the focus of process improvement efforts. Subsequent 'Additional' cell line depositions, including those performed in conjunction with HipSci, the Oxford Parkinson's Disease Centre, StemBANCC and cell line generation projects commissioned by EFPIA partners, showed improvements in the number of deposited cell lines carrying these issues, resulting in higher pass rates and efficiencies in cell line releases).

	Contamination Not Detected	Contamination Detected
Sterility	Hot Start: 92.9% (n = 52) Additional iPSCs: 100.0% (n = 352)	Hot Start: 7.1% (n = 4) Additional iPSCs: 0.0% (n = 0)
Mycoplasma	Mycoplasma Not Detected Hot Start: 100.0% (n = 58) Additional iPSCs: 98.4% (n = 315)	Mycoplasma Detected Hot Start: 0.0% (n = 0) Additional iPSCs: 1.6% (n = 5)
Viral Screening (HIV1, HIV2, HBV & HCV)	Viral Pathogen Not Detected Hot Start: 98.3% (n = 58) Additional iPSCs: 99.8% (n = 406)	Viral Pathogen Detected Hot Start: 1.7% (n = 1) Additional iPSCs: 0.2% (n = 1)
Cell Line Identity	Correct Hot Start: 86.4% (n = 51) Additional iPSCs: 99.2% (n = 396)	Incorrect Hot Start: 13.6% (n = 8) Additional iPSCs: 0.8% (n = 3)

level was calculated by the IncuCyte™ device (Fig. 3). The images were circulated to 9 EBiSC partner laboratories with instructions for >3 lab members to individually visually assess each image for% confluency and record the results. These estimates of confluency were subjected to statistical analysis and the approximate percentage variation from the electronically assessed confluency value are shown in Supplementary Figure 1. Overall, visual estimates of confluency were most accurate when the iPSC line was at 8%, 10% and 80% confluency with 55, 60 and 70% confluency showing most variability, in comparison to confluency values calculated by the IncuCyte™. In addition, estimates of confluency varied considerably between some workers even within the same laboratory. Overall these data focused EBiSC cell bank production on consistency of cell harvesting to assure reliable recovery of cultures in the hands of customers.

4.2. Informational QC testing for genomic health, phenotype and differentiation potential

4.2.1. Differentiation potential

A number of assays have been established to determine the pluripotent potential of human iPSC lines. Although the Teratoma assay has been viewed as a "gold" standard, concerns around reproducibility, cost and effort, plus an incompatibility with 3R principles, makes this impractical for a large-scale banking setting (Andrews et al., 2015; Buta et al., 2013). To establish a reliable pluripotency assay for EBiSC, an EB assay was developed by the UK Stem Cell Bank in which iPSC lines underwent spontaneous EB differentiation in a 96-well plate for 7 and14 days. Expression levels of genes from stem cell markers, and those representative of each of the three germ layers, were assessed by quantitative PCR. Twenty-nine iPSC lines (with the hESC line H9 used as a control) generated from multiple, different laboratories were assayed. All 30 hPSC lines screened, were capable of generating day 7 and day 14 trilineage EBs via the spin plate method (see Materials & Methods for details). Only expression changes greater than 3-fold were considered significant, and lines showing up-regulation in at least 2 markers for each germ layer, were considered to show pluripotent potential.

Self-renewal markers POU5F1, NANOG and SOX2 showed notably similar gene expression levels (SD 0.88, 1, and 0.94 respectively) in undifferentiated cultures of all lines. Some variation between the germ layer markers at Day 0 was present, with DCN, HAND1 and PITX1 having the biggest variations. (SD 3.7, 3, and 3.4 respectively), see Fig. 2A. Assayed iPSC lines were controlled against a CT standard range generated from Day 0 data, wherein tested lines had to result within range once normalised (Fig. 2A). Any cultures where CT values fell outside of these ranges were not used in EB assays. After 7 and 14 days of differentiation, stem cell markers (POU5F1, NANOG and SOX2) showed significant down regulation ($p < 0.0001$), indicating that the cells had lost their stem cell phenotype (Fig. 2B). Endoderm associated genes, FOXA2, CXCR4 and GATA6 had the most significant increases in gene expression at Day 7. Mesoderm associated genes all showed significant upregulation at D14 ($p < 0.0001$) with only PDGFRA not significantly upregulated at Day 7. All ectoderm associated genes except for Beta Tubulin showed significant upregulation in gene expression at both Day 7 ($p < 0.0001$) and Day 14 ($p < 0.0001$). Gene expression levels averaged for all lines tested are shown in Fig. 2B. It should be noted that failure to express such genes, which are important in human development, could mean that the respective cell lines may not give profiles for differentiation typical of in vivo development.

Analysis of the data indicated that a set of genes used for each germ layer were consistently and significantly expressed in each cell line (Endoderm; SOX17, CXCR4 and GATA6, Mesoderm; DCN, VIMENTIN, PECAM1, and Ectoderm; PAX6, NEUROD1 and HES5) at day 7. Accordingly, EBiSC adopted a streamlined EB assay analysing 9 genes at Day 7 EBs, thus realising significant benefits in cost reduction and improved testing turnaround.

Directed differentiation assays: Subsequent to the assay developmental phase, a number of samples failed the EB assay, requiring repeated rounds of differentiation to achieve satisfactory pass results for all germ layers, increasing QC costs and delaying release. In parallel, a commercial kit enabling pluripotency screening using 3 directed differentiation assays became available (STEMdiff™ Trilineage Differentiation Kit, Stem Cell Technologies, catalog #05230), presenting significant benefits in time and cost. The kit was trialled with 11 EBiSC lines, six of which were known to have passed the streamlined EB assay only after repeated analysis. The results (Supplementary Figure 4) showed that successful differentiation readouts in the assay were achieved for all 11 lines including those which had failed EB tests at the first analysis. In order to avoid the variability experienced with the streamlined EB assay this kit was adopted for all routine analysis due to its suitability for rapid testing.

4.2.2. IPSC morphology

As the number of unique iPSC lines cultured at the EBiSC central facilities increased, it became clear that whilst most cultures exhibited typical colony morphology, some variation was observed, as illustrated in Fig. 3. Two iPSC lines exemplified our general findings regarding lines exhibiting consistently atypical morphology. IPSC14 (derived from adult male, healthy control, fibroblasts) consistently showed a flattened morphology, growing more as a monolayer than distinct colonies. However, this line had typical marker expression and capacity for Trilineage differentiation, including terminal neuronal differentiation (Koch et al., 2011). Similarly, IPSC15 (derived from a young male affected by aplastic anaemia, fibroblasts) showed morphology with no

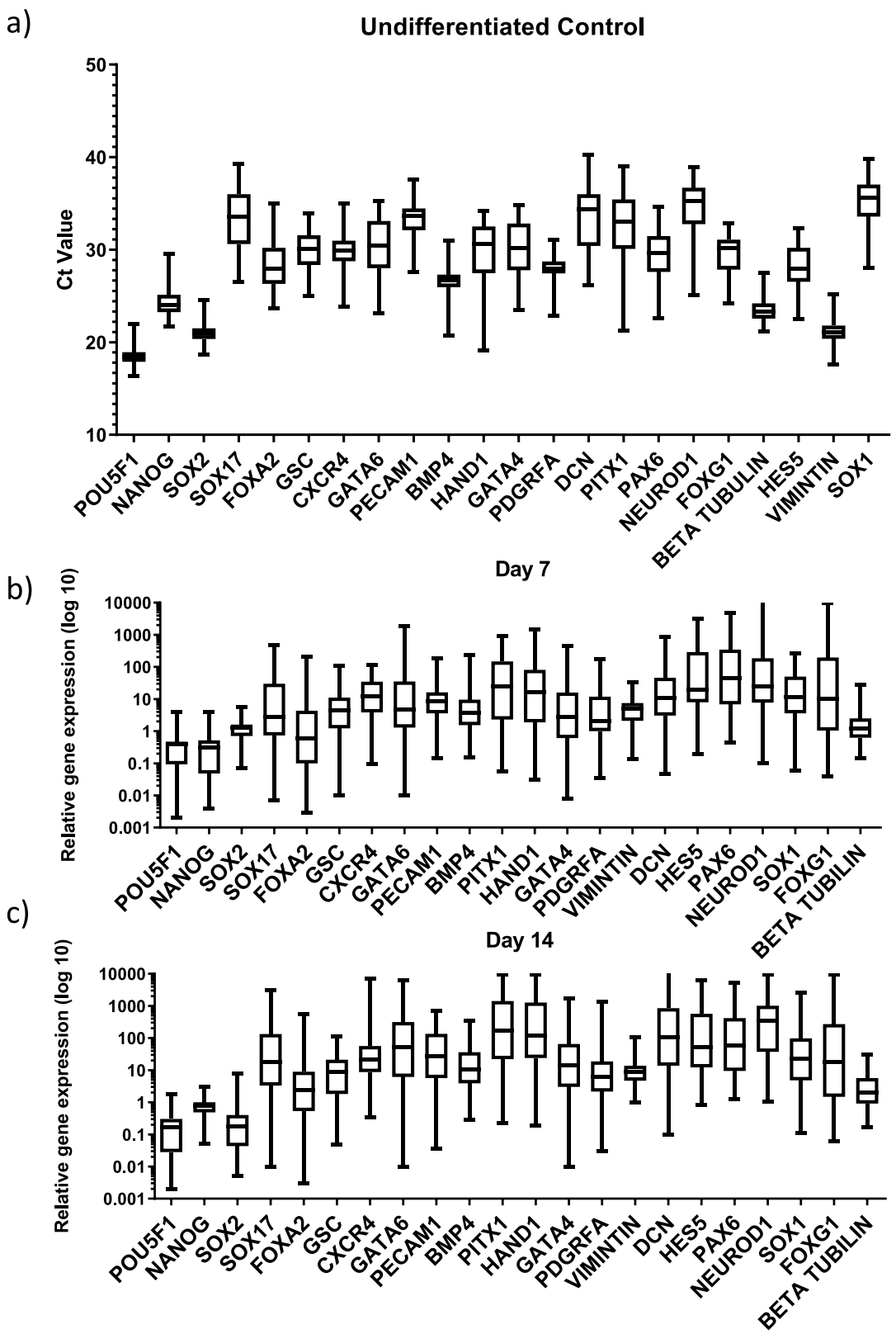

Fig. 2. Assessment of pluripotent potential. (a) Ranges of CT values for gene expression at Day 0 of differentiation (i.e. whilst undifferentiated) were used as internal control for the assay. Changes in gene expression at Day 7 (b) and Day 14 (c) after EB formation showed an expected increase in germ layer associated gene expression and decrease in expression of pluripotency regulators.

clearly defined colony borders but typical marker expression and acceptable differentiation potential. Additionally, regardless of atypical morphology, both lines have a pattern of Ct values from the pluripotency assay that falls inside the average standard variation seen in all other lines, indicating a consistent pluripotent identity as discussed in Section 4.2.1. This data demonstrated a good example of the use of "Informational QC testing" which allows characterisation data to be evaluated together, rather than on a test by test basis. In a number of

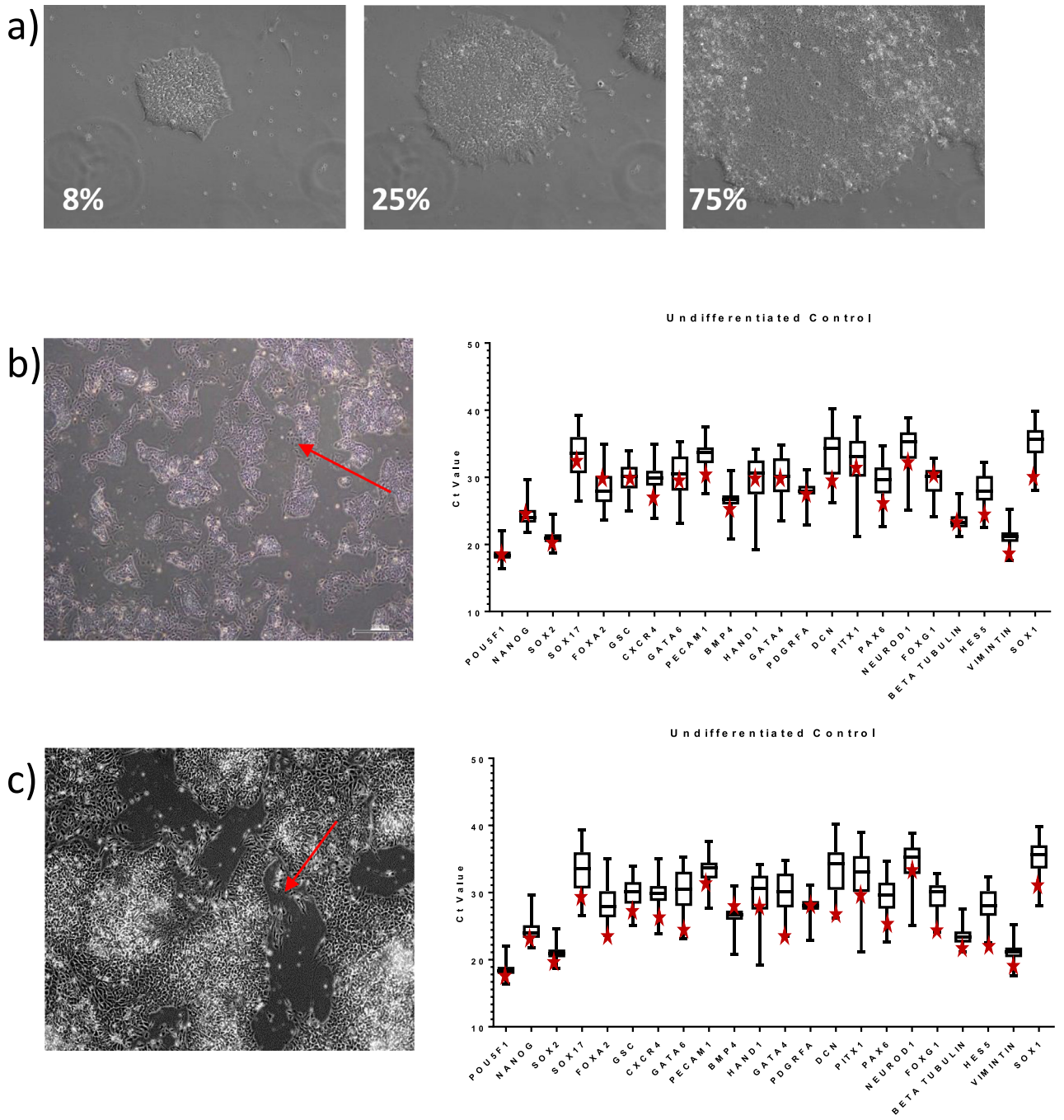

Fig. 3. Assessing typical and atypical iPSC morphology. A) Most EBiSC iPSC lines displayed typical iPSC morphology across low and high confluencies, as shown in exemplar images collected by the IncuCyte™ for confluency assessments. Contrastingly, two EBiSC iPSC lines displayed here consistently exhibited atypical colony morphology (B, IPSC14 and C, IPSC15) but otherwise showed gene expression identity (red stars) and phenotype consistent with a human pluripotent stem cell line.

other instances, iPSC lines which when first recovered by EBiSC scientists showed slow abnormal growth, could be improved through adaptation to alternative EBiSC culture conditions. Typically, such cultures could be recovered to normal morphology within 3–4 passages.

4.2.3. IPSC marker expression

Flow cytometry was used to assess the proportion of each iPSC line which expressed markers associated with pluripotency and self-renewal, including OCT4, SSEA-1, SSEA-4 and TRA-1–60. Of 90 EBiSC lines processed at the EBiSC Central facility, the majority of cultures analysed expressed high levels (>70%) of OCT4, SSEA-4 and TRA-1–60 and expected low levels of SSEA-1 (<10%), Fig. 4. Alert limits were used to flag iPSC lines for which results fell outside the expected ranges and informed decision making in conjunction with other QC parameters.

4.3. Genome status and genetic stability

4.3.1. Introduction to screening for genome stability

The assessment of a cell line's genomic stability is important. However, a method which is cost effective, rapid and robust is critical for large scale operations. Human PSCs are known to be susceptible to genetic drift over extended time in culture commonly leading to gains and deletions in chromosomes 12, 17, 20 and X (Amps et al., 2012; Mayshar et al., 2010; Weissbein et al., 2014; Werbowetski-Ogilvie et al., 2009). Although G-Banding has been considered the "gold" standard

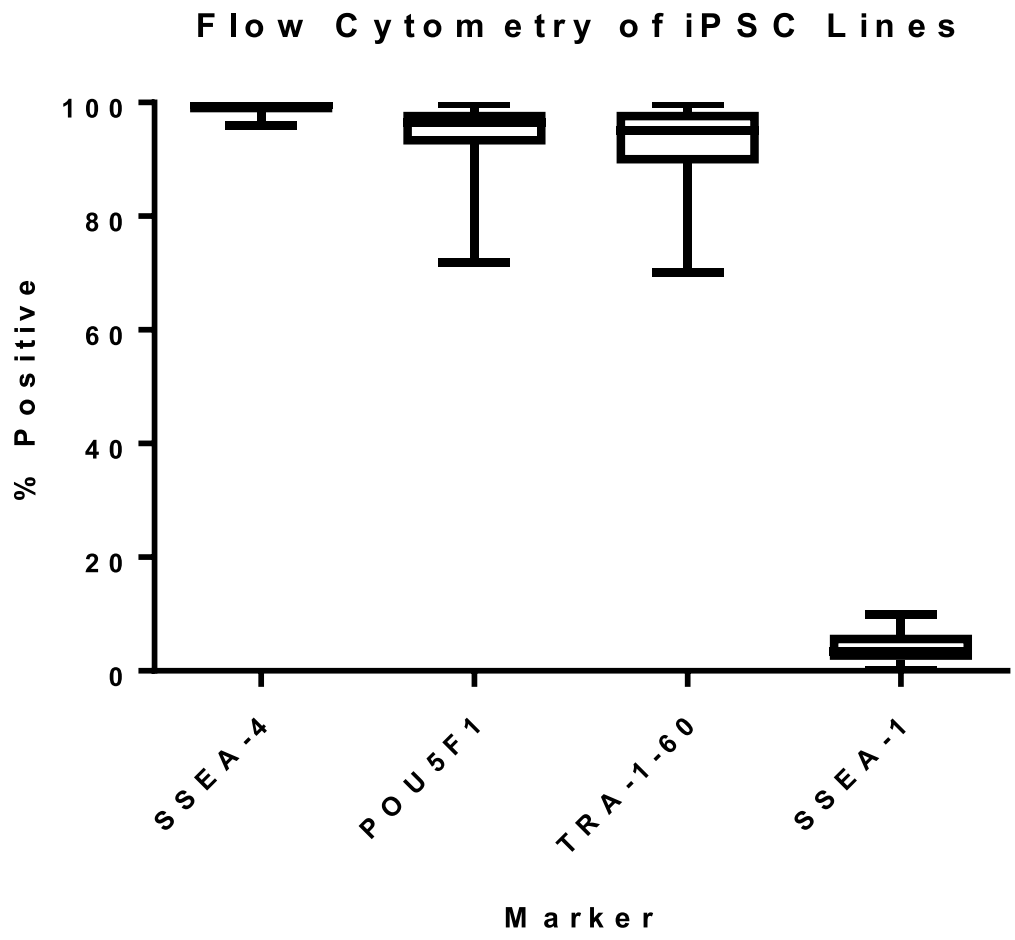

Fig. 4. Assessing pluripotent iPSC phenotype. A) The majority of EBiSC iPSC lines showed expected marker profiles for SSEA-1, SSEA-4, TRA-1–60 and POU5F1 using Flow cytometry.

Table 3
Comparison of methods for assessing genomic stability for routine cell bank screening.

	KaryoLite BoBs®	*G-Banding*	*SNP array*
Sample Requirements	>1 m cells	20–30 cells in metaphase	10^6cells
Cost	£~100	£~550	£~300
Sample Prep Time	0.5 h	5 h	0.5 h
Turnaround time	1 week	4–8 weeks	4 weeks
Resolution	Proximal and terminal regional of chromosome	5Mbp	<1Mbp
Sensitivity	20–30%	Dependant on operator	10–15%
Other Issues	Cannot detect inversions / translocations / balanced rearrangements.	Only 20–30 cells visualised.	Cannot detect inversions / balanced rearrangements. Mosaicism not seen. May pick up unexpected findings or variants that are of unknown significance.

method, this assay normally needs to be outsourced to specialist laboratories, can be extremely costly and commonly has slow turnaround time due to a lack of skilled service providers. In addition, this technique typically analyses a relatively small number of cells (normally 10 to 30). As newer technologies such as KaryoLite BoBs® and SNP arrays become more accessible, they have potential to ease the logistical and time burden of genome screening, in addition to offering higher resolution data for users. However, these methods have significant differences in sensitivity, resolution and each has limitations for routine QC in terms of cost and time, see Table 3.

4.3.2. A comparative study of different genome status analysis techniques

In order to establish a cost effective and efficient routine test to assess genome status, a comparative analysis of a range of assays (KaryoLite BoBs®, G-Banding and SNP analysis) was performed across 13 iPSC lines from multiple sources, including iPSC lines known to carry genomic abnormalities as positive controls, 3 hESC lines, a human dermal fibroblast line, see Table 4. Cell lines were banked and samples for G-Banding, SNP and KaryoLite BoBs® were collected in parallel for processing. Only 4 samples showed consistency of results across all 3 methods, with an additional 3 samples showing a predominantly

Table 4
Comparative analysis of hPSCs using different methods for detecting chromosomal abnormalities.

Cell Line	*Cell Line Type*	*KaryoLite BoBs®*	*G-Banding*	*SNP* array	*Consistency across Methods*
H9	hESC	No abnormality reported	No abnormality reported	No abnormality reported	3/3
IPSC1	iPSC	No abnormality reported	(28/30) Normal (1/30) Loss of Chromosome 20 (1/30) Loss of Chromosome 9	No abnormality reported	2/3
IPSC2	iPSC	No abnormality reported	(12/20) Normal (4/20) Loss of chromosome 20 short arm and gain of long arm. (2/20) loss of chromosome 16 short arm	No abnormality reported	2/3
IPSC3	iPSC	No abnormality reported	(25/30) Normal 2/30 Inversion of 13q. 1/30 Inversion of 8q. 1/30, translocation (p10:q10). 1/30 47 Gain of chromosome 5.	No abnormality reported	2/3
IPSC4	iPSC	No abnormality reported	(20/20) Translocation 14:22 (q24.3q11.2)	No abnormality reported	2/3
IPSC5	iPSC	No abnormality reported	No abnormality reported	Gain chromosome 3, Loss of chromosome 20	2/3
Hdf's	Human dermal Fibroblasts	Gain chromosome 5, Gain chromosome 10	(3/20) Chromosome 4 abnormal	Gain chromosome 5, Gain chromosome 10	0/3
IPSC6	iPSC	No abnormality reported	(20/20) Gain chromosome 20	No abnormality reported	2/3
IPSC7	iPSC	No abnormality reported	(20/20) Deletion chromosome 17(p13.3)	Gain chromosome 20 Gain chromosome 12	0/3
IPSC8	iPSC	Gain chromosome 20 Gain chromosome 12	(20/20) Gain chromosome 20 Gain chromosome 12	Gain chromosome 20 Gain chromosome 12	3/3
hESC1	hESC	No abnormality reported	(20/20) Gain chromosome 20 Gain chromosome 12	Gain chromosome 20 Gain chromosome 12	2/3
hESC2	hESC	Gain chromosome 20 Gain chromosome 12	(20/20) Gain chromosome 20 Gain chromosome 12	Gain chromosome 20 Gain chromosome 12	3/3
IPSC9	iPSC	No abnormality reported	(10/20) Deletion chromosome18(q21.3)	Deletion chromosome18(q21.3-q23)	2/3
IPSC10	iPSC	No abnormality reported	No abnormality reported	No abnormality reported	3/3
IPSC11	iPSC	No abnormality reported	No abnormality reported	No abnormality reported	3/3
IPSC12	iPSC	No abnormality reported	(20/20) Deletion chromosome 7 (q22.2q31.32)	Deletion chromosome7(q22.2q31.32)	2/3
IPSC13	iPSC	No abnormality reported	(4/20) Deletion chromosome18(q21.3)	No abnormality reported	2/3

consistent population across G-Banding and SNPs and. A number (4/13) of lines showed gains and/or deletions in genomic regions known to be susceptible in hPSCs (such as Chromosome 20 and 12) with G-Banding and SNPs (Amps et al., 2012; Mayshar et al., 2010; Weissbein et al., 2014; Werbowetski-Ogilvie et al., 2009), but were not observed with KaryoLite BoBs®. This may indicate that the Karyolite BoBs® assay is less sensitive in addition to its known inability to detect balanced chromosomal rearrangements. For these reasons, whilst KaryoLite BoBs® has distinct benefits in terms of cost, speed and ease, it was deemed inappropriate for the EBiSC QC setting. G-Banding gives detailed structural analysis on metaphase chromosomes in a small number of cells, however the assay also gives false positives in individual cells in terms of chromosomal gains, losses and re-arrangements which can be an artefact of the preparation process for karyotyping. The SNP array correctly detected most abnormalities present above the level of resolution but was unable to detect inversions and translocations in small populations of cells, which were detected by G-Banding. It is worth noting that the level of SNP resolution here was 300,000 base pairs, but this can be adjusted to suit particular needs. The preparation for SNP arrays is also significantly cheaper, quicker and simpler than for G-Banding, involving simple collection and snap freezing of a dry pellet suitable for high throughput processing, compared to dissociation, fixing and staining of cells required for G-Banding. For all three methods used, results must be qualified in terms of sensitivity and ability to detect particular kinds of genetic change. In terms of suitability for a large scale hPSC supply chain, SNP arrays give a rich data set for minimal cost and time to inform rapid cell line specific decision making, in addition to the possibilities for sharing genomic datasets with users through Data Access Committees. Where resources are available, this would optimally be combined with G-Banding to give a detailed insight into the chromosomal stability of essential iPSC lines, allowing them to be used for cost and time intensive procedures downstream, such as disease modelling and drug discovery.

Users should also consider the level of sensitivity required for their specific needs, the genomic region affected by any mutations or re-arrangements detected, and the possible impact which this may or may not have on the intended use of the cells, although there is often no clear correlation between a specific genetic change and a biological effect (Allison et al., 2018). The genomic stability of a PSC may vary due to many factors and genetic change at some level is an inevitable consequence of culturing cells in vitro for which the consequences are typically unclear. Currently, the most a stem cell resource can do is to attempt to limit such changes by minimising passage number and selecting optimised cell culture media (Stacey et al., 2019). Rejecting cell banks based on observation of a minor genetic change is not currently recommended by EBiSC, but genetic stability data may become a more informative tool in cell culture collection management as more data is collated linking to adverse consequences for cell function (Allison et al., 2018).

5. Developing suitable quality control regimes

All assays should go through robust qualification prior to use, including, where appropriate, validation of the limit of sensitivity, robust and appropriate positive and negative assay controls and qualification with multiple test samples, including those whose performance may be less than ideal. As a case example, early qualification of a Mycoplasma qPCR assay within EBiSC ensured that the selected assay performed robustly at low detection limits across a range of differing media types. Due to this assay validation and the incorporation of Limit of Detection controls, a loss of assay sensitivity after 2 years of use was rapidly detected during routine screening and could be immediately addressed

with no impact on end users. Hence, assay qualification and use of appropriate controls is critical to understand assay limitations. The level of qualification should be context dependant, for example, as the cell lines supplied by EBiSC are prohibited from use in humans, safety screening under the requirements of GMP is not appropriate for these cell lines. Relevant QMSs should include SOPs, document control and management of quality incidents to reflect the required context in an appropriate manner. Risk management is valuable in facilitating decisions on user safety and impact of changes in processing, equipment and raw materials. However, these approaches should not be over engineered and should be designed to meet the specific needs of the respective operational setting, which for some resource centres may include meeting regulatory standards for clinical or diagnostic applications (Andrews et al., 2015; Detela and Lodge, 2019). A robust banking regime should be at the core of the QMS with appropriate quality assessment steps at critical timepoints (for example, morphology assessments prior to cryopreservation). Processes detailing batch IDs, cell nomenclature and labelling should be agreed and tested with multiple scenarios prior to initiating banking. Inclusion of Master cell banks (for long term storage) and Working banks (for general use and distribution) is recommended (Stacey et al., 2013). It is inevitable that once quality-controlled vials of cells are recovered in user labs, some cases of cell line switching, microbial contamination, phenotypic drift and genetic shift may occur which will not be obvious to users just by microscopic observation. EBiSC therefore developed user guidance on how routine iPSC line QC should be performed by users as part of local good practice. This includes guidance on testing of cell identity, mycoplasma contamination status and genomic state and is available at cells.EBiSC.org/customer-information/qc/.

6. Conclusions and future of quality control

Our experience developing and establishing a QC regime for EBiSC has emphasised the need for flexibility and robustness in iPSC qualification. Clearly, if critical release criteria (e.g. viability, sterility, mycoplasma, STR) are not adhered to, the resource becomes unreliable, can present risk to end users and is unlikely to sustain itself in the long term. However, for other testing, maintaining a flexible approach and making judgement calls to balance different demands on resources for QC is important to assure the resource continues to be available and retains its value in a manner transparent to the end user. Through application of Informational testing and review of cell line characterisation as a whole, the entirety of a cell line's performance can be reviewed and assessed, giving the 'full picture' of how the line may perform in the hands of an end user. Consistent use of the same QC across multiple batches also allows EBiSC QA procedures to track performance and assess the impact of culture, cryopreservation and recovery, highlighting significant differences between cell stocks and flagging any progressive or abrupt change. It is also acknowledged that future media developments may improve stability of hPSC cultures and reduce variability.

Repeating cell line characterisation already performed by depositors is not always essential but can be introduced as part of exploratory activities if issues arise during downstream use. For example, EBiSC does not as standard, screen for residual vector persistence in iPSC lines if already performed by the depositor prior to sharing with EBiSC. Nevertheless, the emergence of vector-expressing clones previously present but below detectable levels, could potentially impact on differentiation potential of the iPSCs. Significant effects on early stages of differentiation should be detected in the pluripotency assays applied and can thus be investigated.

Assays such as screening for microbiological growth and G-Banding are reliant on historical methods which are well tested but can be expensive and time consuming. Assay development and transition to rapid testing methods such as the Mycoplasma qPCR screen adopted here have the potential to drastically reduce both the cost and time required to fully characterise an iPSC line. Investment of the time and effort for appropriate validation can help the resource centre to understand assay limitations and thus apply it and interpret results appropriately. Improved access to high throughput genotyping methods such as SNP arrays are clearly an important facet of biobank development with the potential to both reduce costs and time and enrich subsequent datasets available to users. EBiSC already shares whole genome sequencing datasets through a system of managed access by application to the EBiSC Data Access Committee so SNP datasets could soon also be included.

Current monolayer iPSC culture systems are labour intensive and require costly materials which are a major cause of the high cost of pluripotent stem cell facilities. Upscaling iPSC banking processes through use of bioreactors and high-throughput 2D technologies will reduce costs and increase efficiencies. Establishment of an associated QC regime must ensure that the assays used, the required sample types and the associated sampling time points are suitable for the method of upscaling selected. Due to the significant skills levels and reagent cost it is likely that in the future there will be a demand for differentiated cell preparations rather than the original pluripotent stem cell lines themselves. A second project phase, EBiSC2, launched in early 2019 and plans to meet that demand with a substantial investment in development of automation and preservation technologies. Thus, generation of protocols for bulk production of differentiated cell populations such as neural and cardiac progenitors and delivery of differentiated cells by semi-automated systems in a ready to use format will support users in a cost-effective manner. Novel and robust QC regimes will allow in-progress monitoring during differentiation and ensure consistency and reliability of differentiated cell types. Thus, large iPSC resources need not only to be responsive to developing optimal culture media and surface treatments, but also have to be innovative in responding to user demand.

CRediT authorship contribution statement

Orla O'Shea: Methodology, Validation, Investigation, Supervision, Formal analysis, Writing - original draft, Writing - review & editing. **Rachel Steeg:** Writing - original draft, Writing - review & editing, Supervision, Project administration, Investigation. **Charlotte Chapman:** Methodology, Validation, Investigation, Formal analysis. **Peter Mackintosh:** Methodology, Validation, Investigation. **Glyn N Stacey:** Writing - original draft, Writing - review & editing, Supervision, Conceptualization, Project administration.

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Supplementary materials

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