WHITE PAPER

Quality control for human cell lines and other human samples manipulated *ex vivo*

Here we discuss:

- The importance of quality control for isolated human cells and tissues
- The tools available for genotypic authentication, molecular karyotyping, pluripotency analysis, and determining the oncogenic potential of isolated human cells and tissues
- Quality control guidelines for induced pluripotent cell research

Introduction

Our understanding of how cells work would not be possible without the isolation and manipulation of cells removed from living organisms. This type of work is known as *ex vivo* ("out of the living") experimentation. Using *ex vivo* cells and tissues to study normal human biology, disease, and development enables investigators to perform tests and measurements that would not be possible or ethical using live subjects.

There are several ways cells can be used *ex vivo* (Figure 1). Among the most common and productive model systems for understanding human cells are immortalized cells grown in culture [1]. While immortalized cells have been useful for basic research, advances in the last decade have given scientists unprecedented power to use *ex vivo* cells for cell-based therapeutic research. For example, T cells can be isolated and manipulated to express novel chimeric antigen receptors [2]. These are known as CAR T cells, and they can target cancer-specific antigens for cancer immunotherapy. Researchers have identified a set of genes that, when expressed in isolated differentiated cells, can reprogram the cells to become undifferentiated stem cells [3]. These so-called induced pluripotent stem cells (iPSCs) can be reprogrammed to become a cell type that is unrelated to the starting isolate. Studies of iPSCs have great potential to provide insight into differentiation mechanisms and allow pathways to therapies for certain pathologies.

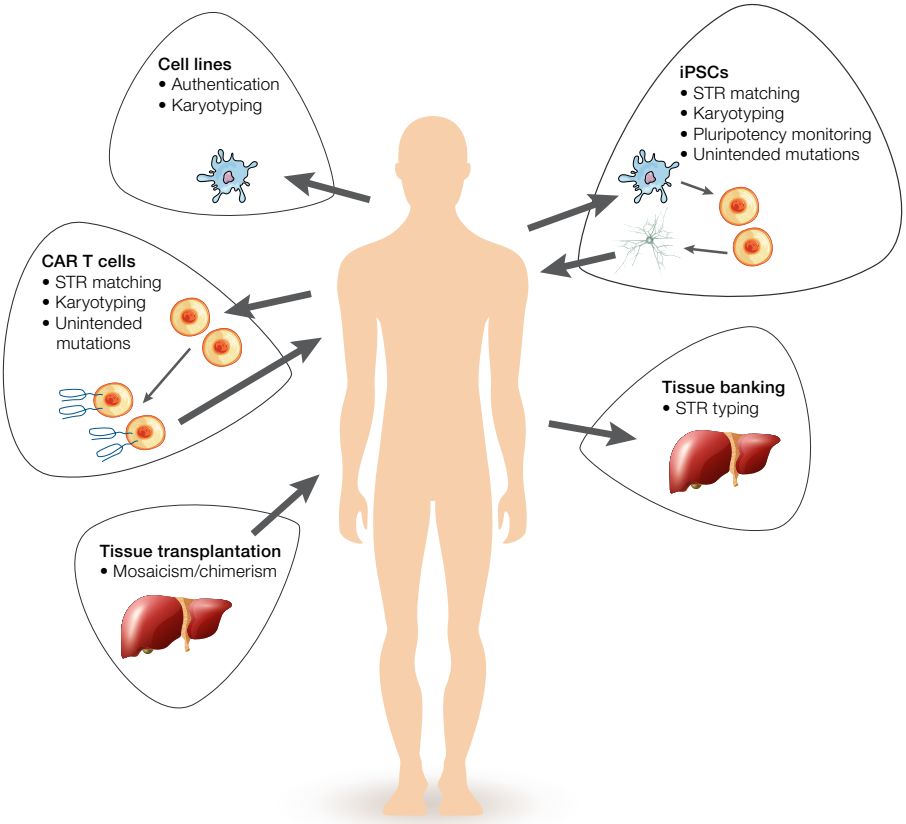

Figure 1. General categories of human *ex vivo* cells and samples. The Applied Biosystems™ portfolio includes tools and techniques for each of these strategies to help ensure quality and verify the identity of *ex vivo* cells and tissues.

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Whole tissues or biopsies from donors are also valuable sources of biological information. Clinical researchers, biobanks, and other repositories can provide raw materials for studies. They often offer matched samples of different tissue types from the same individual, such as lung and colon, or breast tumor and normal adjacent tissue. There has also been remarkable progress in growing tissues, or organoids, in culture from isolated cells [4].

Although these experimental *ex vivo* cell and tissue systems are integral to disease research, they are not without problems. For example, cells grown in culture can be misidentified or contaminated with unrelated or undesired cells. Cells cultured *in vitro* ("in glass") can exhibit genomic rearrangements and other mutations that can limit their usefulness as disease models or cell-based therapeutics. iPSCs may not be truly pluripotent after induction, or they may not completely lose pluripotency once they differentiate. Biobanks and cell and tissue repositories must be able to ensure that matched samples are truly from the same individual. Although it is not strictly an *ex vivo* experimental model, tissue transplantation involves the excision of cells or tissue from one individual and transplantation into another. Transplant researchers must be able to determine the degree to which transplanted tissue has been incorporated in the host.

There is thus a growing need to have quality control (QC) strategies in place for *ex vivo* cells and tissues. Thermo Fisher Scientific has a unique portfolio, under the Applied Biosystems brand, for human cell QC with PCR, capillary electrophoresis (CE), microarray, and sequencing applications. Here we describe how different tools can be used to prevent problems associated with *ex vivo* cell and tissue research.

Short tandem repeat (STR) profiling and authenticating isolated human cells

One of the greatest challenges with cells cultured *in vitro* is the difficulty of characterizing them based on morphology alone. It is thus necessary to authenticate cells (i.e., determine that they have the expected provenance and identity) by molecular analysis. The most widely used method for human cell authentication involves STR analysis. STRs are microsatellite sequences in the human genome, and the number of repeating units of a given microsatellite varies widely from person to person. For example, individuals can have between six and fifteen repeats of the sequence AGAT at the *CSF1PO* locus. A very specific molecular fingerprint can be generated for any human sample with a combination of several microsatellite loci (Figure 2). These molecular fingerprints are used extensively for forensics and other human identification (HID) strategies.

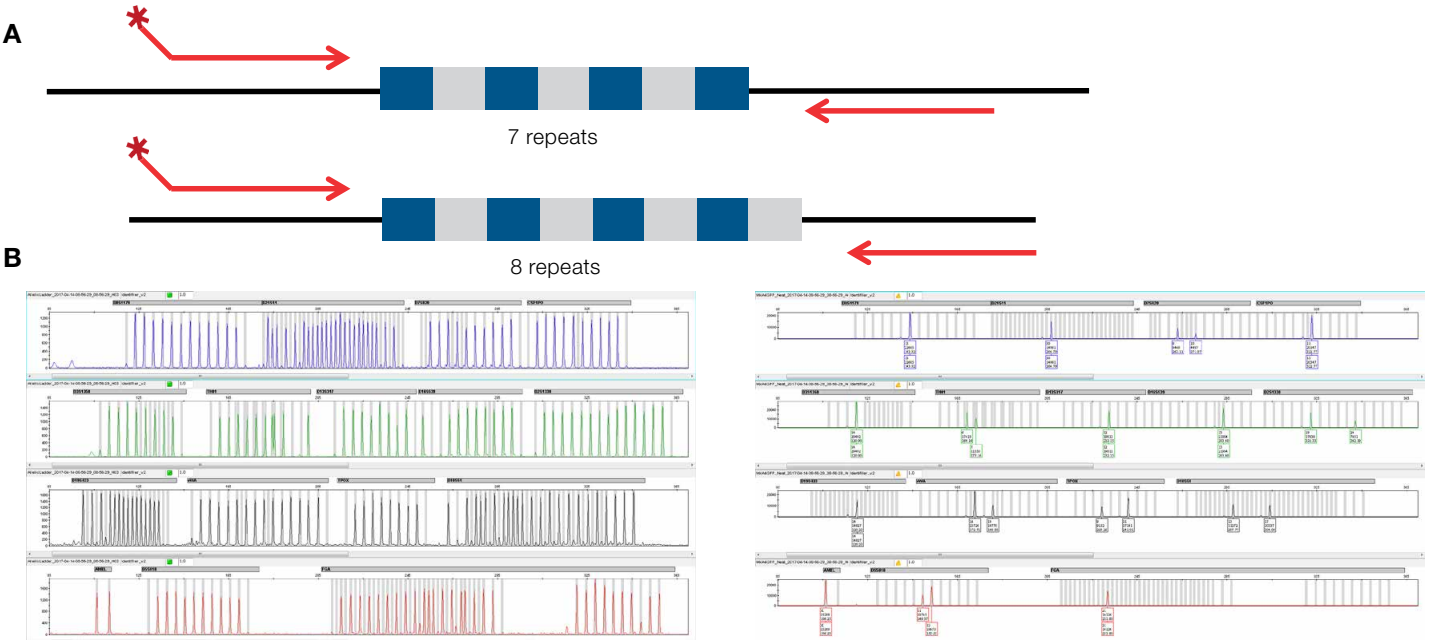

Figure 2. Molecular fingerprinting to confirm the identity of derived cells. (A) STR genotyping is used to define a set of highly polymorphic alleles associated with an individual that can be used to authenticate human samples. Multiplex PCR for STR analysis using the Applied Biosystems™ AmpFLSTR™ IdentiFiler™ Plus PCR Amplification Kit generates a library of amplicons with different lengths. The amplicons can then be analyzed by performing CE fragment analysis. **(B)** Applied Biosystems™ Gene Mapper™ Software facilitates analysis by providing an allelic ladder (left) that can be used to define alleles in purified genomic DNA (right). Samples such as starting and manipulated cells can be assumed to originate from the same source if they contain the same set of alleles. The boxes below the peaks show the allele number, peak height, and the size of the fragment in base pairs.

The Applied Biosystems brand has leading global recognition for kits for forensic and HID investigators. The Applied Biosystems™ IdentiFiler™ Kit includes 15 different informative microsatellite loci and one sex-linked identifier, and the Applied Biosystems™ GlobalFiler™ Kit has twenty-two autosomal and two sex-linked markers. Although the high specificity and accuracy of these kits make them ideal for forensic use, the same STR typing principle can be applied to authenticate any human-derived biological sample. STR typing is also useful for authenticating immortalized human cell lines.

Although cell lines derived from tissues have been enormously valuable for understanding basic cell biology and disease, there is growing recognition that the cell lines may not always be what investigators think they are. This is often due to storage mishaps, culturing errors, or contamination. There are numerous examples of publications that are based on large amounts of data obtained from misidentified cells [5–9]. It has been shown that the MDA-MB-435 cell line, which is used extensively for breast cancer research, is identical to a melanoma cell line derived from a male donor [6]. To reduce the number of publications based on data acquired with misidentified cell lines, many journals and funding organizations now require authors to have a quality assurance plan for cell line authentication throughout the course of their work [10,11]. Several agencies have published recommendations for cell line authentication [12,13], and various companies and research organizations provide searchable databases for cross-checking STR profiles against known cell lines [14,15]. A screenshot of an ATCC database query is shown in Figure 3.

STR genotyping can be useful for ensuring that dissected tissues from the same individual match for downstream molecular analyses. For example, Liu et al. [16] were interested in identifying common molecular markers in gastrointestinal tract adenomas in the esophagus, stomach, colon, and rectum. They collected tumor tissue, matched normal tissue from different individuals, and compared the mutational profiles of the adenocarcinomas. Because they needed to compare tumor tissue to normal tissue from the same individuals before looking for commonalities between the tumors, they needed to verify their sample sources. To do this, they generated STR profiles for both the tumor and normal samples to ensure that the dissociated tissue genotypes matched those of the donors. This enabled them to identify differences between the DNA methylation patterns in the tissues. An STR profile can unambiguously confirm that two tissue samples come from the same individual. For this reason, biobanks usually generate STR profiles for incoming tissues to ensure matching identities for storage and shipping purposes [17]. STR profiling is also an important step in iPSC quality control. In addition to authenticating a pluripotent stem cell line by STR profiling before initiating an experimental program, it is important to confirm that the STR profiles of undifferentiated and differentiated cells from the same donor match expectations (Table 1). In their investigation of direct RNA transfer to generate iPSCs from fibroblasts, Kogut et al. used IdentiFiler STR kits to ensure that the identities of the original fibroblasts and resulting iPSCs matched [18]. Ali et al. conducted a transcriptomic analysis of iPSCs derived from psoriasis patients and performed IdentiFiler STR typing to confirm that cell lines derived from the same subject were identical [19].

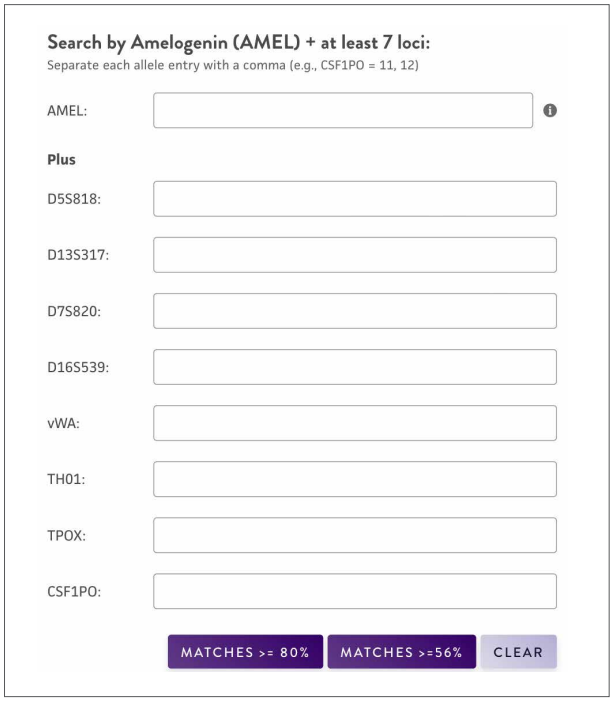

Figure 3. Comparing a STR profile to known cell line profiles in a database provides authentication confidence. A screenshot of an ATCC database query is shown above. Other organizations have similar database query options. For more information about this query form, see the ATCC webpage.

Table 1. Confirming and matching donor T cells and derived CAR T cells with an IdentiFiler™ PCR amplification kit [20].

	Donor 1		Donor 6		Donor 7	
	T cells	CAR T	T cells	CAR T	T cells	CAR T
AMEL	X, Y	X, Y	X, Y	X, Y	X, X	X, X
CSF1PO	10, 11	10, 11	11, 12	11, 12	10, 12	10, 12
D13S317	8, 14	8, 14	10, 12	10, 12	12, 13	12, 13
D16S539	11, 12	11, 12	10, 12	10, 12	13, 13	13, 13
D18S51	14, 17	14, 17	12, 14	12, 14	12, 16	12, 16
D19S433	13, 16.2	13, 16.2	13, 13	13, 13	13, 13	13, 13
D21S11	26, 30	26, 30	29, 32.2	29, 32.2	29, 31	29, 31
D2S1338	18, 20	18, 20	17, 19	17, 19	20, 24	20, 24
D3S1358	15, 16	15, 16	15, 17	15, 17	16, 19	16, 19
D5S818	11, 11	11, 11	12, 12	12, 12	11, 14	11, 14
D7S820	10, 11	10, 11	10, 11	10, 11	10, 10	10, 10
D8S1179	10, 11	10, 11	13, 15	13, 15	12, 13	12, 13
FGA	21, 22	21, 22	25, 25	25, 25	20, 22	20, 22
TH01	8, 9.3	8, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3
TPOX	8, 11	8, 11	9, 9	9, 9	9, 11	9, 11
vWA	15, 17	15, 17	18, 18	18, 18	17, 18	17, 18

Directors of stem cell core laboratories also employ STR profiling for fingerprinting and to ensure a match between original donor cells and the resulting iPSCs [20]. There are currently more than 20 preclinical research trials that involve stem cells [21]. As the number of personalized iPSC treatment options increases, quality control measures to ensure matching identities of donor cells and treated cells will become even more important.

Transplant research is another field for which STR typing is useful. Because STR typing provides a very specific fingerprint, it can be used to track the outcome of a transfer. This type of analysis is known as mosaic or mixed sample analysis, and understanding the degree to which a donor sample has been incorporated by a recipient is crucial. In a recent study, small molecules that influenced graft-versus-host disease (GVHD) were analyzed with hematopoietic cells [22]. They used an IdentiFiler kit to analyze chimerism and tracked host-versus-donor genotypes in lymphocytes 28, 56, 90, 180, and 365 days post-transplant. Additional reports on applying STR profiling to analyze chimerism in GVHD have been recently published [23–25]. Findings from their own studies and work of others prompted Carnevale-Schianca et al. [22] to posit that research on hematological malignancies and monitoring CAR T cells will continue to benefit from chimerism analysis.

Establishing karyotypes for manipulated cells

Long-term culture of human stem cells has shown that pluripotent stem cells can accumulate culture-driven mutations, and some data suggest that iPSCs may be less genetically stable than other PSC populations (26–28). Therefore, attention must be given to the genomic integrity of any cells grown *ex vivo*. Genomic integrity can be evaluated by Giemsa staining, which reveals distinct banding patterns (G-banding). However, the difficulty of the protocol, limited resolution, and the need for a skilled cytogenetic analyst to interpret and report the results limit the practical utility of this technique for research purposes. Molecular karyotyping with high-density microarrays is increasingly being adopted for *ex vivo* cell research and method development. Applied Biosystems™ KaryoStat™ and KaryoStat™ HD kits enable accurate genotyping for sample identification and provide whole genome coverage to accurately identify chromosomal abnormalities in human cells. Applied Biosystems™ Chromosome Analysis Suite (ChAS) software is the industry standard for analysis of karyotyping data. The software makes it easy to observe changes in the chromosomal complement of analyzed cells [34]. Disease models that make use of continuously cultured cell lines are subject to complications caused by chromosomal instability in the cultured cells. The chromosomal complements of many of these lines have changed extensively, so the cells are not expected to have normal karyotypes. However, best practices dictate establishing a baseline for a cell line karyotype and periodically checking it during a long-term study. This can confirm that the genome has remained relatively stable, if not completely normal, over the course of the study.

Because iPSCs have potential for use in novel therapies, the stability of iPSC karyotypes is of particular interest. This has been recognized by researchers in the field who are making karyotyping an integral part of their studies. For example, Llamosas et al. used genome editing technology to knock out the *SYNGAP1* gene in neurons derived from iPSCs, to

model the molecular mechanisms of neurodevelopmental disorders [29]. After confirming that the *SYNGAP1* gene was mutated, they used the KaryoStat assay to confirm that the karyotype of the cells remained normal. Ahn et al. analyzed the function of the *ACTL6B* gene in iPSC-derived neurons from patients with early infantile epileptic encephalopathy (EIEE-76), and they used the KaryoStat assay to confirm that the induced cells had a normal karyotype [30]. Because iPSCs are enormously flexible, there is great interest in performing high-throughput iPSC studies. Boussaad et al. developed a sophisticated automated platform for the maintenance, expansion, and differentiation of iPSCs and neural progenitor cells [31]. Part of their workflow involved karyotyping the cells at various passages with the KaryoStat assay.

Assessing pluripotency and differentiated states

iPSCs are derived from differentiated cells that have been reprogrammed by a set of genes that cause them to enter an embryonic-like, pluripotent state. Over the course of any iPSC study, it is necessary to confirm that the cells are actually pluripotent after such manipulation. It is also desirable to confirm that the cells lose pluripotency after differentiation. Pluripotency has traditionally been assessed by injecting human pluripotent stem cells (hPSCs) into immunocompromised mice and analyzing the teratomas that develop for cells that are derived from the endodermal, mesodermal, and ectodermal germ layers. The presence of all three cell types demonstrates pluripotency. However, molecular testing, instead of using mice, is a much more attractive and cost-effective option. Molecular tests for pluripotency and differentiation are performed to analyze the expression patterns of specific genes involved in these pathways. The Applied Biosystems™ GeneChip™ PrimeView™ Global Gene Expression Profile Assay enables pluripotent gene expression profiling with an emphasis on established, well-annotated content. The Thermo Scientific™ PluriTest™ Assay is a bioinformatics assay that enables the transcriptional profile of a sample to be compared to a reference set of more than 450 cell and tissue types. The Applied Biosystems™ TaqMan® hPSC Scorecard™ Panel includes 94 predefined TaqMan® Gene Expression assays that enable rapid confirmation of pluripotency, prediction of differentiation potential, and determination of lineage bias for embryonic stem (ES) and iPSC cell lines [34].

These tools have been used by researchers in various studies. To establish iPSCs that could be used for research on autism spectrum disorder (ASD), Walker et al. derived iPSCs from cells obtained from ASD patients. They then used the TaqMan hPSC panel to confirm that the cells could differentiate into all three germ layer-derived tissues [32]. In their study on psoriasis gene expression, Ali et al. used the TaqMan hPSC panel to verify that the iPSCs could differentiate into the correct lineage with concomitant loss of pluripotency [19]. In a study on amyotrophic lateral sclerosis (ALS), Fumagalli et al. created iPSCs with cells obtained from ALS patients, differentiated them into motor neurons, and showed that a hexanucleotide repeat expansion in the C9orf72 gene inhibited microtubule-based axonal transport [33]. Before differentiating them into neurons, they used the TaqMan hPSC panel to confirm the pluripotency of their iPSCs.

Analyzing potentially pathogenic mutations

Another concern with cells grown in culture is that they may acquire new mutations that give them a growth advantage. These mutations can make understanding the biology more complicated and can cause grave concerns about any therapeutic research model. Cells manipulated *in vitro* should thus be checked to make sure there are no mutations in *TP53*, *KRAS*, or any other known oncogene. In a study on iPSC development with viral transduction, MacArthur et al. used a variety of tools for iPSC characterization and quality control [34]. They used the Ion Torrent™ Oncomine™ Comprehensive Assay to detect potentially oncogenic mutations in the cells. Although variants were detected, the variants were also seen in the parental lines, and none of them were in hotspot (COSMIC) locations. The Oncomine Comprehensive assay can quickly verify that no new mutations have emerged during a study involving induced pluripotent cells (Figure 4).

Hotspot genes			
AKT1	*ESR1*	*KIT*	*PDGRFB*
AKT2	*EXH2*	*KNSTRN*	*PIK3CA*
AKT3	*FGFR1*	*KRAS*	*PIK3CB*
ALK	*FGFR2*	*MAGOH*	*PPP2R1A*
AR	*FGFR3*	*MAP2K1*	*PTPN11*
ARAF	*FGFR4*	*MAP2K2*	*RAC1*
AXL	*FLT3*	*MAP2K4*	*RAF1*
BRAF	*FOX2*	*MAPK1*	*RET*
BTK	*GATA2*	*MAX*	*RHEB*
CBL	*GNA11*	*MDM4*	*RHOA*
CCND1	*GNAQ*	*MED12*	*ROS1*
CDK4	*GNAS*	*MET*	*SF3B1*
CDK6	*H3F3A*	*MTRO*	*SMAD4*
CHEK2	*HIST1H3E*	*MYC*	*SMO*
CSF1R	*HNF1A*	*MYCN*	*SPOP*
CTNNB1	*HRAS*	*MYD88*	*SRC*
DDR2	*IDH1*	*NFE2L2*	*STAT3*
EGFR	*IDH2*	*NRAS*	*TERT*
ERBB2	*JAK1*	*NTRK1*	*TOP1*
ERBB3	*JAK2*	*NTRK2*	*U2AF1*
ERBB4	*JAK3*	*PDGFRA*	*XPO1*
ERCC2	*KDR*		

Full-length genes		
ARID1A	*FBXW7*	*PTEN*
ATM	*MLH1*	*RAD50*
ATR	*MRE11A*	*RAD51B*
ATRX	*MSH2*	*RAD51C*
BAP1	*MSH6*	*RAD51D*
BRCA1	*NBN*	*RB1*
BRCA2	*NF1*	*RNF43*
CDK12	*NF2*	*SETD2*
CDKN1B	*NOTCH1*	*SLX4*
CDKN2A	*NOTCH2*	*SMARCA4*
CDKN2B	*NOTCH3*	*SMARCB1*
CHEK1	*PALB2*	*STK11*
CREBBP	*PIK3R1*	*TP53*
FANCA	*PMS2*	*TSC1*
FANCD2	*POLE*	*TSC2*
FANCI	*PTCH1*	

Copy number genes	
AKT1	*FGFR4*
AKT2	*FLT3*
AKT3	*IGFR1*
ALK	*KIT*
AR	*KRAS*
AXL	*MDM2*
BRAF	*MDM4*
CCND1	*MET*
CCND2	*MYC*
CCND3	*MYCL*
CCNE1	*MYCN*
CDK2	*NTRK1*
CDK4	*NTRK2*
CDK6	*NTRK3*
CDKN2A	*PDGFRA*
CDKN2B	*PDGFRB*
EGFR	*PIK3CA*
ERBB2	*PIK3CB*
ESR1	*PPARG*
FGF19	*RICTOR*
FGF4	*TERT*
FGFR1	*TSC1*
FGFR2	*TSC2*
FGFR3	

Gene fusions (inter- and intragenic)		
AKT2	*FGFR2*	*NUTM1*
ALK	*FGFR3*	*PDGFRA*
AR	*FGR*	*PDGFRB*
AXL	*FLT3*	*PIK3CA*
BRAF	*JAK2*	*PPARG*
BRCA1	*KRAS*	*PRKACA*
BRCA2	*MDM4*	*PRKACB*
CDKN2A	*MET*	*PTEN*
EGFR	*MYB*	*RAD51B*
ERBB2	*MYBL1*	*RAF1*
ERBB4	*NF1*	*RB1*
ERG	*NOTCH1*	*RELA*
ESR1	*NOTCH4*	*RET*
ETV1	*NRG1*	*ROS1*
ETV4	*NTRK1*	*RSPO2*
ETV5	*NTRK2*	*RSPO3*
FGFR1	*NTRK3*	*TERT*

Figure 4. Determining the oncogenic potential of manipulated cells. The Oncomine Comprehensive Assay v3 can be performed to ensure that iPSCs and other derived clones are devoid of variants associated with known human cancers. This NGS-based multiple biomarker assay detects single nucleotide variants, copy number variants, gene fusions, and indels from 161 unique cancer driver genes.

Guidelines for the use of cell lines in biomedical research

Ex vivo cultured cells will continue to play a crucial role in biomedical research. However, misidentification, contamination, genomic instability, and phenotypic instability continue to plague research utilizing cultured cells. Geraghty et al. published a set of guidelines in 2014 for controlling these variables in research involving immortalized cultured cells [35]. The guidelines include the following:

- Freeze a small portion of the starting tissue or isolated cells to serve as a reference (section 1.1.1).
- Generate a reference profile that can be used to authenticate cell lines derived from the sample. STR profiling is recommended for this purpose (section 1.1.1).
- Perform cell line authentication using a DNA-based method upon receipt of a stock from another laboratory or a frozen stock (section 1.2.2). The cell bank or laboratory of origin should be able to provide an STR profile for authentication purposes.
- Confirm that the cell line has and maintains the appropriate genome structure characteristics for the study by periodically karyotyping the cell line (section 1.2.3).

Pluripotent stem cells form specialized subsets of cultured cells. They have enormous potential for research and may ultimately have significant therapeutic benefits. There is growing interest in establishing guidelines that are specific to iPSCs used for research. The International Society for Stem Cell Research recently published updated guidelines for stem cell research [36,37]. The following recommendations are included in the guidelines [36]:

- The provenance of stem cell lines must be easily verifiable, and data establishing the identity of the cell line should be provided in transfer agreements (section 2.4.3).
- Culture composition, the purity of the desired phenotype, and the extent of undifferentiated progenitors (when applicable) should be carefully evaluated (section 3.2.2.2).
- Testing for genetic abnormalities should be performed if or when there is an extensive expansion *in vitro* (section 3.2.2.5).

It has also been recommended that *ex vivo* cultured cells be tested for contamination by mycoplasmas or other organisms. Testing cultured cells for mycoplasmas is routine, and a variety of methods can be used to screen for this organism [38,39,43]. Certain mixed cell growth systems, such as human–mouse xenografts [40], human cells grown on a mouse cell substrate [41], and organoids grown using medium conditioned with a mouse cell line [42], may be contaminated with DNA from the non-human component. Applied Biosystems™ TaqMan® probes can be used to test for the presence of non-human cells.

Conclusion

Research on *ex vivo* human cells, including iPSCs and CAR T cells, has advanced remarkably in the last decade, and some cell-based therapies have already been developed. However, much remains to be done to realize the full potential of these therapies. Key aspects of this research are to ensure that cells are correctly identified, have the correct karyotype and differentiation potential, lack oncogenic mutations, and are free of non-human contaminants. Thermo Fisher Scientific has a complete portfolio of tools for each of these important quality control measures, which should be integral parts of preclinical research experiments. These robust workflows will ultimately provide a comprehensive quality control solution for investigating human cells and will be critical for establishing *ex vivo* manipulation methods that comply with clinical research.

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