A guide for human-relevant tissue culture without the use of animal-derived biomaterial

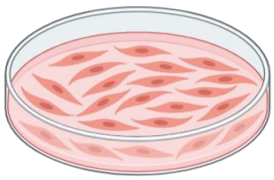
Laura R Bramwell[1†]*, Samantha J Gould[1†]*, Merlin Davies[1], Conor McMullan[2], Emily C Trusler[2] & Lorna W Harries[1]

[1]*RNA-Mediated Mechanisms of Disease group, College of Medicine and Health, Institute of Biomedical and Clinical Science, University of Exeter*

[2]*Technical Services, College of Medicine and Health, Institute of Biomedical and Clinical Science, University of Exeter*

[†]these authors contributed equally

*corresponding author



Correspondence and author details

Correspondence address:

Dr Laura Bramwell & Dr Sam Gould
Institute of Biomedical and Clinical Sciences
University of Exeter College of Medicine and Health
RILD Building
Royal Devon and Exeter Hospital
Barrack Road
Exeter
Devon
EX2 5DW
United Kingdom

Correspondence email:

TeamRNA@group.exeter.ac.uk

ORCID numbers:

LRB - 0000-0003-4069-3959, SJG - 0000-0001-9634-5772, MD - 0000-0002-6951-9762,
CM - 0000-0002-3329-1559, ECT - 0009-0008-9485-0204 & LWH - 0000-0001-7791-8061.

Website:

http://teamrna.wixsite.com/harrieslab

Twitter:

@miRNAgirl, @LauraBramwell1, @TeamRNA, @Merlin_Davies

Using this guide

This guide is designed to provide practical advice on the removal of animal-derived biomaterials (ADBs) from tissue culture models. We detail an overall process for "humanisation" (improving the human relevance of a tissue culture model) that can be adapted for trialling "humanised" or animal component-free (ACF) models for different cell types. We discuss the stages of the process from identification of ADBs through to validation. We also review the reasons for embarking on humanising a tissue culture model, common problems and general points that need considering ahead of humanisation. Additionally, we try to include common swaps, tips and tricks to help researchers to humanise their culture models.

Contribute to this guide?

If you would like to add anything to this guide, particularly to the appendices, please contact the co-authors and the RNA-Mediated Mechanisms of Disease group at the University of Exeter.

Version History

Current version: 1.0

March 2023: Information first discussed in the thesis of Dr L Bramwell. [1]

August 2023: Version 1.0 published online.

Contents

Correspondence and author details 2

Correspondence address: 2

Correspondence email: 2

ORCID numbers: 2

Website: 2

Twitter: 2

Using this guide 3

Contribute to this guide? 3

Version History 3

Contents 4

Introduction and aims: 6

Rationale 6

Background: The use of animal-derived biomaterial in tissue culture 7

The history of tissue culture 7

Foetal bovine serum 7

Alternatives to FBS 7

Bovine Pituitary Extract 8

Xeno-free nutrient sources 9

Growth factors 9

Overview of the removal process: 10

1: Research 10

Reducing complexity 10

Systematic approaches 13

Liaising with suppliers 14

Databases and resources 15

2: Planning & Preparation 15
Reducing complexity 15
Preparation of stocks 16
Experimental design 17
Designing criteria for a successful model 19
Not a one-size fits all approach 20
3: Assessment and Validation 21
1: Proliferation rate 21
2: Size 22
3: Morphology 22
4: Phenotypic markers 23
5: Application 24
Common replacements, tips and tricks for the replacement of animal-derived biomaterials 25
Quick reference tables 26
Human serum 27
Serum-free tissue culture 29
Growth factors 30
Coating reagents 31
Detachment reagents 31
General recommendations to the community 32
Discussion and concluding remarks 33
Bibliography 35
Appendices 37
Appendix 1: Human Pulmonary Fibroblast Culture 37

Introduction and aims:

Rationale

Removing animal-derived biomaterials from basic *in vitro* research is desirable for multiple reasons. Not only is it more ethical to source biomaterials from human-derived, recombinant, or synthetic sources, fitting with the initiatives of funding bodies/charities such as NC3Rs and Animal Free Research UK, it also allows for better reproducibility and translatability of research.

It has been successfully demonstrated that the culture of human primary cells in animal component-free (ACF) media allows the conduct of *in vitro* research that is at least as reproducible, representative, and reputable as its animal component containing counterparts. [2,3] Additionally, in recent years, there has been an increased call for serum-free and synthetic tissue culture media to enhance the reproducibility of *in vitro* research due to comprehensively defined media. Altering established cell culture practices to remove animal-derived biomaterials may seem like much effort for little gain, especially with technologies emerging that may potentially outcompete basic two-dimensional cell culture, such as organoids and organ-on-a-chip models. However, cell culture models are still used daily and will continue to be used long term in many areas of basic research. If a model can be improved quickly and easily, then this may help to bridge the gap in translation before emerging technologies can change the paradigm.

T guide aims to provide practical advice for researchers looking to remove animal derived- biomaterial from their tissue culture practice. We discuss the benefits and drawbacks that we encountered while deriving ACF media for human primary cells and illustrate the process. We aim to provide a realistic narrative of the challenges faced when humanising your tissue culture practice, in the hope to support animal-free researchers in their research endeavours, as well as encourage the wider research community to consider making their tissue culture more human relevant by removing animal-derived biomaterial.

Background: The use of animal-derived biomaterial in tissue culture

The history of tissue culture

Since the first cells were successfully cultured, balanced salt solutions have been used to enable their survival, and animal proteins, including sera, or fractions thereof, have enabled their further maintenance and expansion. [4] Cell culture media was initially developed for amphibious and avian animal cells in the late 19th century, only moving onto mammalian cells in the early 20th century. In 1951, owing to the isolation of the HeLa cell line from the cervical carcinoma of Henrietta Lacks, human cells could be consistently cultured for the first time. [5] Due to the widespread availability of this first immortalised human cell line, it was possible to further explore, optimise, and better define culture media.

Whilst basal media has become better defined and is often now entirely synthetic, serum is still a common nutrient source in basic cell culture. Serum contains a rich, but variable, cocktail of hormones, growth factors, amino acids, vitamins, salts, carbohydrates, lipids and proteins that are conducive to cellular metabolism, viability and proliferation. [6]

Foetal bovine serum

Foetal bovine serum (FBS) has been traditionally used in cell culture since the technique was first developed, and consequently its use is omnipresent in most tissue culture facilities. FBS, also known as foetal calf serum (FCS) is commonly used for its accessibility and success in supporting a wide range of cell types. Due to the variation of individual animals, batch-to-batch variation of FBS is a well acknowledged issue. [7–9] Batch-testing and maintaining batch consistency mitigates this issue within individual experiments for the most part; however, reproducibility on a wider scale is often still hindered. [8] Additionally, the use of FBS poses concerns surrounding viral contaminants and supply availability. [10]

Alternatives to FBS

When considering alternatives to FBS for the purposes of improving the human-relevance of a model, we must also consider if its replacement is susceptible to the same problem. Interference due to animal components can be more pronounced when culturing highly specialised cell types, or when investigating nuanced and highly species-specific processes. As well as for improving reproducibility, there is a call for entirely synthetic media for the culture of some specialised cell types. Depending on their origin, primary cells can be highly variable in their growth rates, and an imbalance of growth factors could alter cellular proliferation, viability, and phenotype and therefore increasing culture variability and reducing physiological relevance. Serum-free or low serum media is often used for such cell types.

Bovine Pituitary Extract

In the absence of sera, animal-derived supplements such as bovine pituitary extract (BPE) are often used to promote proliferation. Such media are frequently proprietary and costly due to their specialised nature. Additionally, whilst BPE is less variable than FBS, its active components are not well-reported. BPE promotes the survival and mitogenic activity of mammalian cells to a greater degree and in a more potent capacity than those cultured using FBS, but it can influence the culture in other ways, e.g. BPE is known to possess proteins that provide antioxidant activity. [11]

Whilst exchanging FBS for BPE may reduce the overall volume of animal components used within cell culture media, it is unlikely to reduce batch-to-batch variability entirely. Although certain factors must remain consistent to enable the proliferation of any cell type, the exact composition of growth factors and hormones present in BPE may vary. Many researchers in the 1980-1990s attempted to determine which components of BPE were required to reproduce its mitogenic activity in a variety of cell types. From inhibiting growth factors, to blocking receptors, to introducing growth factors individually, candidates such as fibroblastic growth factor (FGF) and certain hormones were proposed as the predominant mitogenic factors in BPE. However, results appeared to be cell type dependent, and it was often concluded that additional unknown components contained within the BPE also contributed to cellular proliferation. [12–15] From this early exploratory work, no singular component of BPE was found to harnesses its full mitogenic potential.

Xeno-free nutrient sources

Whether for translatability, reproducibility or ethical purposes, there are many reasons why it may be desirable to omit FBS and BPE from cell culture entirely. Products derived from human sera may be suitable to permit the growth of human cells in culture, but synthetic serum alternatives may be a better solution. [16] However, synthetic xeno-free alternatives are reliant upon sufficient characterisation of the mitogenic components in traditional sera to maintain basic cellular functions and permit proliferation. Regardless of whether the xeno-free alternative is human-derived or entirely synthetic, the suitability of such supplements to support effective cell culture is likely to be cell-type dependent.

Growth factors

In addition to FBS or BPE, specialised culture media will typically contain a cocktail of growth factors that are specific to the maintenance of a particular cell phenotype. Due to the growing market of recombinant growth factors, it is now very possible to replace growth factors with animal-free alternatives. Purchasing lyophilised or “carrier-free” growth factors is often necessary as, when purchased in solution, even recombinant human growth factors are still commonly stabilised with bovine serum albumin (BSA), unless specifically stated otherwise. [17]

Overview of the removal process:

The process of removal ("humanisation") of any ADB can be broken down into several phases (as summarised in Figure 1): reviewing literature, planning, preparing stocks of cells, transitioning cells into ACF media and validating the new media. Often guides for changing media only detail the transition phase, but the earlier stages are crucial and can be underdiscussed.

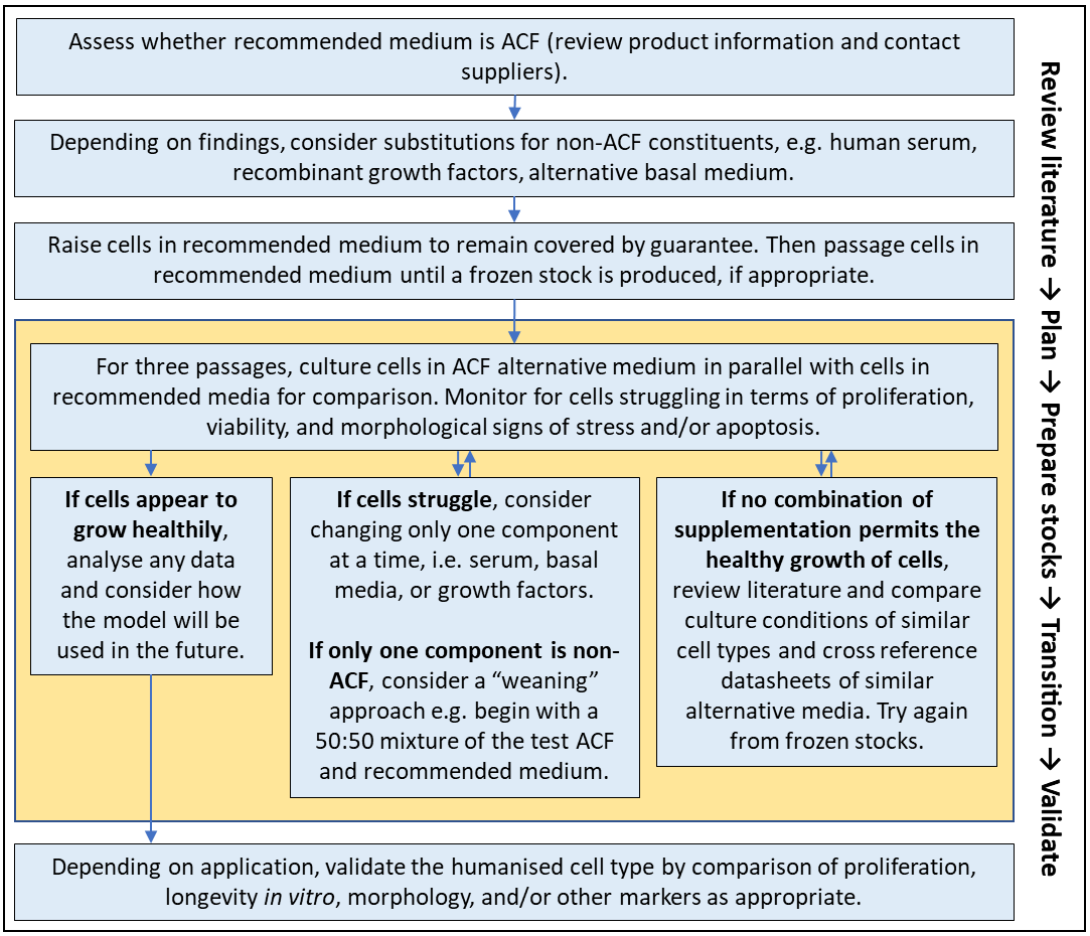


FIGURE 1: A FLOWCHART OUTLINING THE PROCESS OF TRANSITIONING HUMAN CELLS FROM A RECOMMENDED MEDIA CONTAINING ANIMAL-DERIVED COMPONENTS TO AN ANIMAL COMPONENT-FREE (ACF) MEDIA. FIGURE REPRODUCED FROM THE THESIS OF DR L BRAMWELL. [1]

1: Research

Reducing complexity

Often a researcher's first instinct is to try to replace every ADB in a medium with an ACF-alternative, however it is worth considering if every component is strictly necessary in the first instance. For example, if a recommended basal medium needs additional ADB supplements, but a basic medium such as DMEM needs only one supplement, it may be simplest to opt for the more basic recipe to start humanising. One of the most effective approaches we identified was to exchange FBS with Human serum at the same concentration (unless defined as low serum media, then the concentration was raised to 5-10%). Additionally, for many cell types such as fibroblasts, more traditional basal media such as DMEM was effective, as evidenced by the literature. By following the process indicated in the below flowchart Figure 2, we addressed identifying suitable ACF alternatives in a systematic way. For the final media outcomes, please see the protocols available in the **Appendices** of this document. An in-depth systematic review of media in the literature is often not necessary but for several cell types that had more complex growth requirements and less well-defined recommended media, the approach detailed in figure 2 and the following section provide a working example of this process.

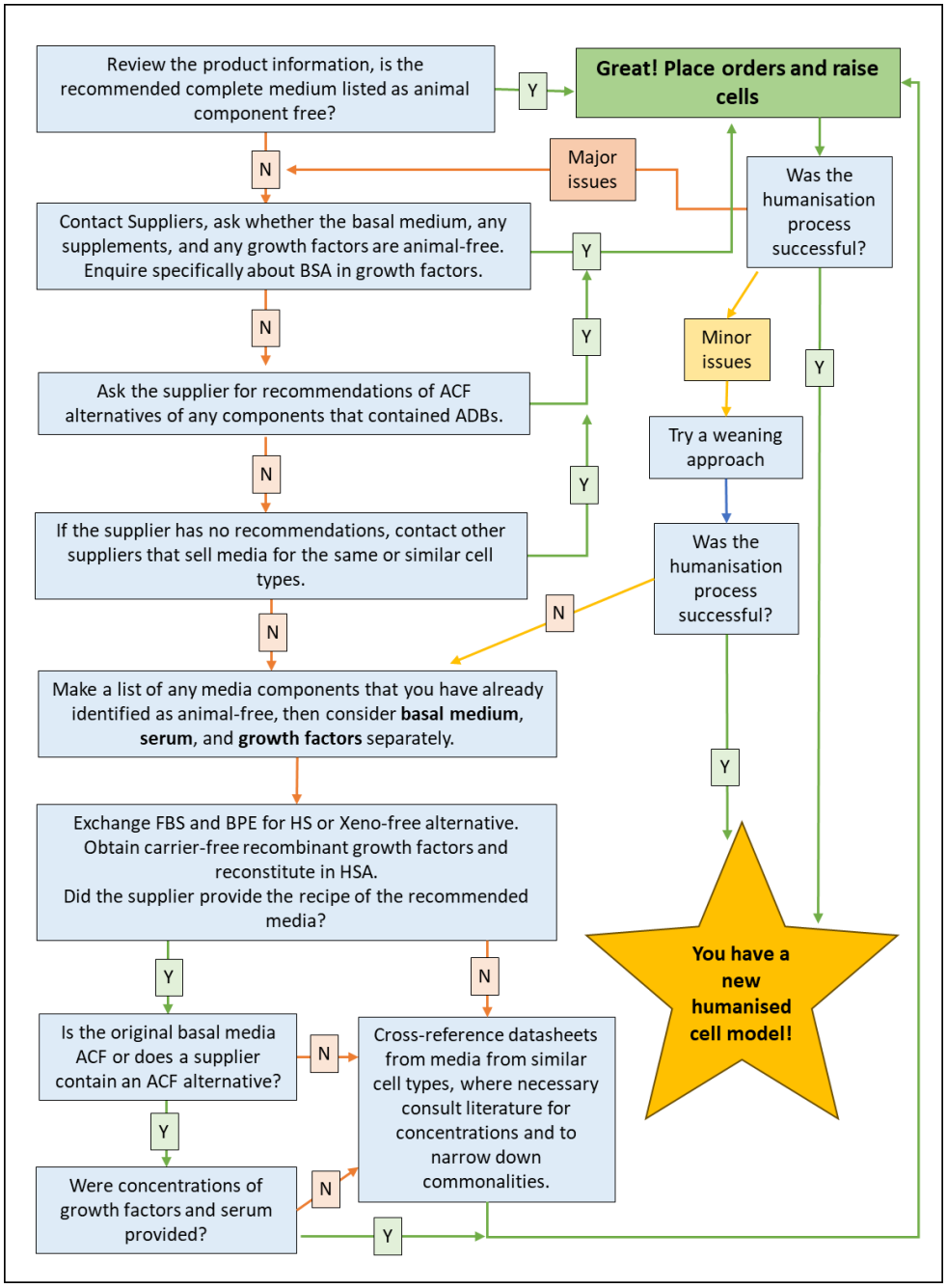


FIGURE 2: A FLOWCHART FOR PRACTICAL TROUBLESHOOTING OF THE HUMANISATION PROCESS.

Systematic approaches

Despite attempts to keep experiments simple, in some cases, a more systematic approach is needed. Here we present a worked approach used for RECs. Table 1 shows a comparison of growth factors that were supplemented across different media.

TABLE 1: A WORKED EXAMPLE IDENTIFYING THE PRESENCE OF GROWTH FACTORS (YES/NO) IN COMMERCIAL MEDIA SUPPLEMENTS FOR RETINAL ENDOTHELIAL CELL (REC) CULTURE.

Growth factor	Cell Biologics (Human endothelial cell)	R&D Systems (Endothelial cell growth)	Lonza (Endothelial cell growth medium-2)	Thermofisher (Primary microvascular endothelial)	Promocell MV (Endothelial growth medium)	Promocell MV2 (Endothelial growth medium)
EGF	Y	Y	Y	Y	Y	Y
FGF	Y	Y	Y	Y	N	Y
IGF	N	Y	Y	N	N	Y
Heparin	Y	Y	Y	Y	Y	N
Hydrocortisone	Y	Y	Y	Y	Y	Y
VEGF	Y	Y	Y	N	N	Y
Ascorbic acid	N	Y	Y	N	Y	Y
Dibutyryl cyclic AMP	N	N	N	Y	N	N
Endothelial cell growth supplement	N	N	N	N	Y	N

The growth factors and basal media indicated in green were selected for the initial ACF trial media recipe. The doses used were selected to correlate with that of the Promocell MV2 basal medium, which itself was selected as the basal medium itself is ACF. Cells were at first cultured without IGF, Heparin, Dibuturyl cyclin AMP, or endothelial cell growth supplement as these were not included in all endothelial cell media that was cross-referenced. The requirement of Heparin or IGF was unsure as their roles have overlap and one or the other was present in the supplements for all of the basal media

cross-referenced. VEGF was included as these cells were intended for use in 3D culture, and therefore VEGF would be necessary to drive vessel formation. bFGF was also included as it was present in all media with the exception of Promocell MV and it was therefore presumed that the endothelial growth supplement in this provided enough bFGF or alternative. RECs grew well in the initial humanised trial medium, they were comparable to their ADB containing counterparts and therefore no additional supplements were added.

This demonstrates that several considerations were made for the selection of growth factors to include in appropriate alternative media:

1) What growth factors do media for this cell type include?
2) Which are included in all cases?
3) What is the role of those that are excluded in some cases?
4) Is there an alternative growth factor that fulfils the same role?
5) Is this necessary for my application?

Liaising with suppliers

Whilst the assessment of the ACF status of the current cell culture reagents was usually straightforward, not all suppliers were forthcoming regarding whether, let alone which, contents were animal-derived within proprietary media/products. In some cases, a vendor would refuse to even offer yes or no in answer to whether a product contained any animal-derived biomaterial meaning that we had to assume the product was not ACF.

Many growth factors, supplements and media contain small amounts of Bovine Serum Albumin (BSA) to act as a stabilising agent for proteins. [17] Fifty percent of the Promocell basal media enquired about in personal correspondence with company representatives contained animal-derived biomaterial. Often, whilst company representatives understood the need for ACF media to be FBS free, when asked about animal components in general, the presence of BSA would often be overlooked by representatives unless a specific enquiry was made. Due to this, much like when reagents are recombinant, and/or carrier free, it would be useful if a standardised ACF labelling system were to be implemented on commercial laboratory reagents. Several suppliers have recently taken this feedback on board, including Promocell who now make the donor / synthesised origin of their products freely available, but there is still much room for improvement.

Databases and resources

Databases such as the FCS-free database and Cellosaurus can provide resources on cell lines and media. [18,19] Geraghty *et al.* list a selection of vendors for serum-free and xeno-free media. [20] Vendors such as Merck and Fisher Scientific often have information on alternative cell culture supplements. Several approaches exist for transitioning a cell culture model into serum-free media, however the decision-making process involved in choice of medium can often be unclear. Resources, such as those mentioned above, often have guidance on methods for switching from one medium to another, but they often lack effective advice on the selection of an initial approach and troubleshooting.

Although several invaluable literature banks exist, such as the FCS-free database, they can be difficult to search, often have many similar entries to search through, and often don't contain the information you are searching for about media and/or growth factors. Although a medium may be free from FBS, it does not mean that it is ACF and the databases do not adequately make the distinction. Similarly, information about individual growth factors can be difficult to find. Clearer, more coherent and more comprehensive information on the components of media, the role of cell culture supplements, and data surrounding their inclusion and/or exclusion in the culture of specific cell types would be very useful to researchers in the future.

Recently, Promocell have produced a helpful resource and have started to indicate the contents of their media if they are serum free, chemically defined, defined formulation, animal-component free, xeno-free and protein free; the definitions of these can be found at: https://promocell.com/wp-content/uploads/2021/04/Guidance-media-specifications-table.pdf.

2: Planning & Preparation

Reducing complexity

As previously discussed, simplicity is desirable when approaching humanising a new cell type. It can be easy to plan an all-encompassing study that validates a new media thoroughly, only for cells to die off early, so we recommend small simple experiments as an initial approach. Then one must consider

which downstream applications that the cells will be needed for. The outcome measures from the humanisation side-by-side comparison will be dependent on this. For example, for all applications it is important that the cells remain viable and phenotypically relevant. However, proliferation rate and cell size may not be of concern providing that the cell model produces adequate stocks for any desired downstream experimentation. For studies of cell identity changes, it is of importance to ensure that the newly humanised control cells have well-characterised expression of the expected phenotypic markers. Essentially, consider what you need from your cell model. Often, the same validation should be performed as would be performed for ADB-containing cell culture if cells were to be transitioned away from the media guaranteed by the manufacturer.

Preparation of stocks

To ensure that the newly obtained, often expensive cells are covered by the manufacturer's guarantee it will often be necessary to raise the cells in original media, which may contain ADBs. In the case of laboratories that endeavour to remove ADBs entirely for ethical purposes, or researchers funded by organisations with such goals, we recommend stringently planning the first steps of this in order to keep the use of media containing ADBs to an absolute minimum. It may be most sensible to rely on others for animal reagents so that the use is kept to a minimum and no new purchase is made, however it is worth noting that the use of borrowed reagents relies on the wider network of non-animal free researchers and may represent an additional barrier for animal-free method uptake to smaller/younger laboratories. Once cells are raised in accordance with the manufacturers' protocols, a frozen stock should be made if possible, of at least three vials ASAP, this may take 1-2 passages in the original media. Then, the humanisation process can begin.

Should the use of ADBs be entirely prohibited due to ethical or funding purposes, it may be possible to acquire cells from colleagues that already have frozen stocks, and cells may immediately be raised in humanised media. However, validation of this model may be difficult. It may be possible to compare the cellular characteristics to that of already published data, or well-kept lab notes of colleagues. However, this is likely not to convince reviewers, or other researchers of the success of the model as it has not been run in parallel.

We hope that by performing initial robust humanisation experiments, using minimal ADB-containing media (e.g. one bottle only) to act as a control, and publishing their outcomes, we may overall drastically reduce the use of ADBs overall in human primary cell culture by providing evidence-based protocols of humanised cell culture practices that appeal to all researchers. After all, this process needs only occur once for each cell type.

Experimental design

Figure 3 shows a basic experimental design for adherent cells cultured in T75 flasks. This protocol can be adapted for proliferative suspension cell types.

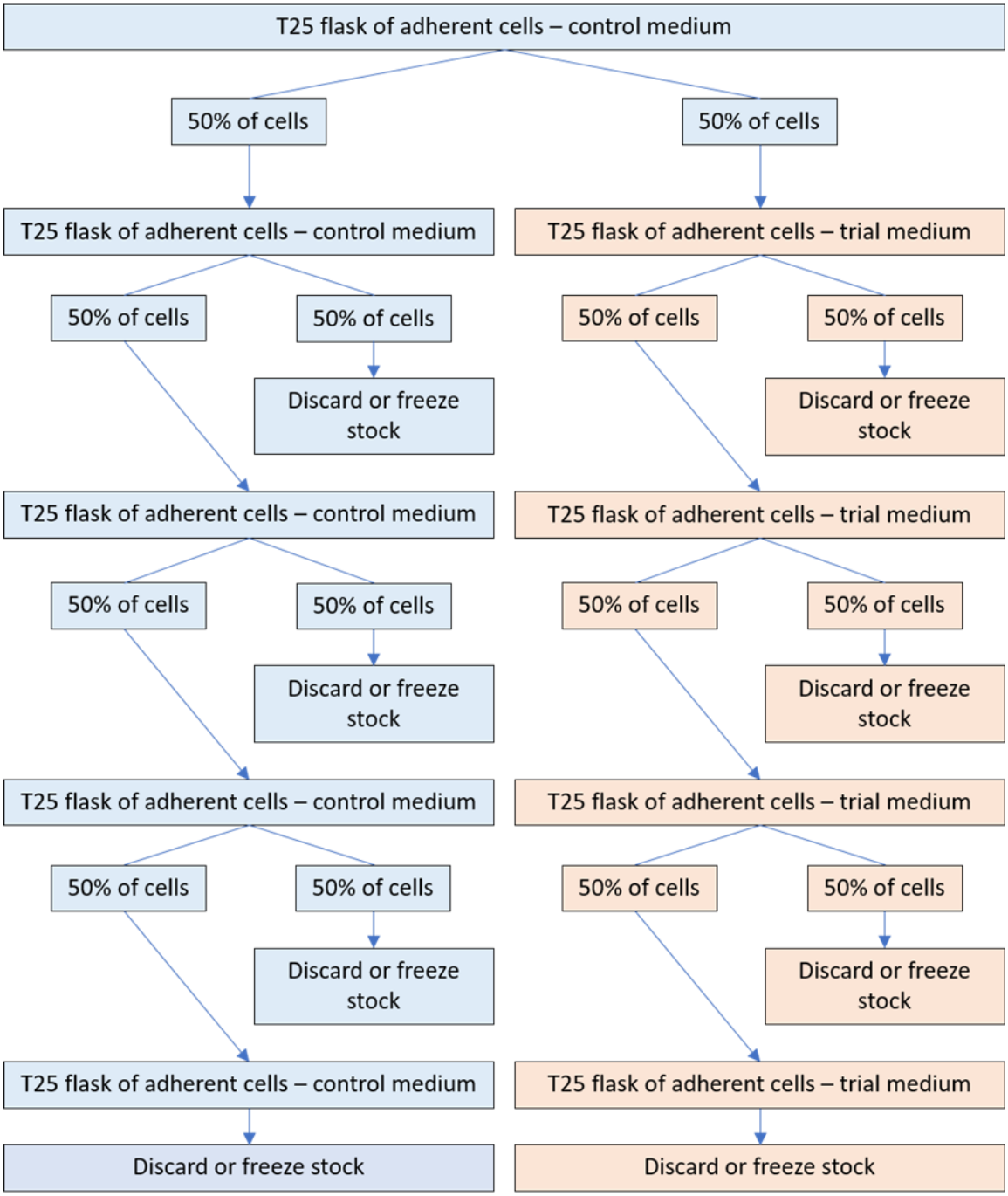


FIGURE 3: EXPERIMENTAL SET UP FOR TRANSITION OF CELLS TO ANIMAL-FREE CULTURE CONDITIONS. A FLOWCHART OUTLINING A BASIC SIDE-BY-SIDE COMPARISON EXPERIMENT OF HUMAN ADHERENT CELL LINES TO ANIMAL FREE CULTURE CONDITIONS IS PROVIDED ABOVE. CELLS WERE COUNTED AND MEASURED FOR CELL SIZE AS DESCRIBED ABOVE AT EACH PASSAGE. FIGURE REPRODUCED FROM THE THESIS OF DR L BRAMWELL. [1]

Designing criteria for a successful model

Whilst physiological relevance in research is strongly desirable, the very nature of *in vitro* research brings inherent limitations. Cells normally exist in a complex network in a three-dimensional structure within a tissue. The growth rate of cells *in vivo* is likely to be slower, and their morphology and polarisation is likely to vary from their *in vitro* counterparts. A cell culture model must be able to support enough expansion for experiments, meaning it may be considered beneficial for cells to expand more rapidly *in vitro*.

Considering that the cellular microenvironment can influence cellular phenotype including size, shape, and gene expression, it is important to determine which attributes are critical to maintain, and which may be permitted to alter in any given model. Whilst this consideration is not new nor specific to the humanisation process, changing culture media in any cell model requires a cost benefit analysis determined by the desired application. Donor age, species, batch, as well as additional supplementation, and choice of basal media all alter the *in vitro* microenvironment.

A successful cell culture model is defined by its uses, so the main consideration for changing to non-ACF media is the downstream applications of the model itself. In this guide, the cell types were to be used for medical research into the basic science behind ageing, diabetes and immunological responses in humans. These diseases have nuances in humans that are not replicated in animal studies, so it was in our interest to ensure these models were human-relevant. [2] As such, it was not imperative to ensure that the newly developed humanised models were identical to their animal containing predecessors, so long as they were applicable as a cell model.

It was necessary to ensure that cells remained viable, morphologically relevant, and where appropriate proliferative. Therefore, we monitored cells for changes in PD time, cell size and any obvious visual morphological changes. Cells were grown in the ACF media for experiments once cells grown in trial media had satisfied these criteria.

The cell type and the model's future use determines the criteria that a trial medium must meet in order to be used for research. For example, a cell type in our laboratory was to be used for replicative

senescence models. Preparing these models involves continuously growing cells to the point at which the culture's PD time slows and the culture begins to accumulate senescent cells. The vendor guarantees this cell type will grow for more than 15 population doublings in their recommended conditions. Post transition to ACF medium, the cells were grown in ACF medium for a further 38 population doublings; during which time their morphology and viability remained akin to counterparts grown in non-ACF medium. This represented a successful humanisation process for this model's uses.

Not a one-size fits all approach

Depending on the desired application, more stringent criteria may be required of a cell model than the maintenance of morphology and proliferation. For example, a model may need to be carefully checked for differences in epithelial to mesenchymal transition, or certain biomarkers to ensure a particular cell retains its original identity that it needs to provide the correct model for the experiment.

Suspension cultures of non-adherent cells require some minor considerations that differ to adherent cultures. Adequate gas exchange must be simulated by daily gentle shaking/stirring, with growth easily tracked using samples of the suspension counted at regular intervals. Without cell adhesion, enzymatic or chemical dissociation is not required when passaging. It is also worth noting that suspension culture cells such as primary peripheral blood mononuclear cells are not suitable for long term culture expansion, which places a different emphasis on utilisation of growth supplementation used for humanisation. That is to say, culture medium for these cells needs to support short term survival, but does not need to allow for long term expansion.

For most cell types, the initial approach for transitioning away from medium containing FBS and other animal-derived biomaterial was a simple direct swap for an ACF supplement. This approach worked for several straightforward cell types. However, we found in cells such as Human Aortic Endothelial Cells (HAoECs) that a direct swap of human serum in place of FBS did not always support growth or even caused cell death.

For certain cell types, a transition experiment is not possible due to limited lifespan of the cells. In these cases, it is advantageous to develop the trial medium using a similar cell type. For example, we

have used the medium worked up for normal human dermal fibroblasts successfully on diseased dermal fibroblasts. The diseased cells do not grow enough in normal conditions to permit a side-by-side comparison, so the medium used in normal cells was simply trialled from the start of the culture of diseased cells. In spite of the disease state, the cells grew successfully and as expected in the ACF medium. For other difficult cell types, a "weaning" approach may work. Guidance on this type of approach can sometimes be found on vendor websites.

3: Assessment and Validation

The assessment and validation process will be dependent on the cell type, intended use, and capacity of the laboratory. As previously discussed, ACF cell models often need to be assessed for their proliferative capacity to ensure they can produce a sufficient cell stock for experimentation, and for validation purposes, for comparison to their non-ACF counterparts. Size and morphology are easy to monitor, but may not be 100% necessary to maintain similarity depending on the model's future uses. Other characteristics/phenotypic markers will be cell type-specific e.g. expression of a particular protein. It is important to decide if a particular criterion is essential or optional for any new ACF model. This should be carefully considered to ensure that experimental results have the best chance of translatability, and at this stage also to ensure that the research community can see the validity of new cell culture models.

1: Proliferation rate

In our experimentation, we found it helpful to monitor cell proliferation when validating the new ACF model. Whilst proliferation didn't need to remain exactly the same between the original and the new media, monitoring proliferation provided an initial indication as to whether the cells were being adequately supported by new media. A sudden drop in proliferation was an indication that the media recipe may need to be tweaked. Additionally, for proliferative cells, we set the requirement for each cell type to have undergone three serial passages in new media prior to considering the cells successfully humanised. This allowed us to expand humanised cell stocks, and also to ensure that mitosis was not being driven by residual biomaterial from the original media. Proliferation was necessary in order for any new cell model to meet this criterion. However, we decided to not be

concerned if proliferation varied slightly between the humanised and original cells providing the populations were continuing to expand and there were no other causes for concern.

Cell proliferation can be measured in multiple manual or automated ways. Upon passaging the cells, using an automated cell counter or haemocytometer is the easiest way to do this. It is important to ensure that cells are kept in a continuous growth phase and not allowed to become over-confluent as this may skew data. Calculating population doubling times can provide an easy way to visualise the cells rate of proliferation.

2: Size

We observed cell size when transitioning cells from their original media into ACF media. Again, it is not of necessity for cell size to remain exactly the same across both groups. However, a distinct change might indicate apoptosis, premature senescence due to stress or de/differentiation of the cells into a different phenotype. Providing proliferation rate and morphology were in a similar range between the ACF and original cells, it was not of great concern if cells varied in size. After all, who is to say which of the cell sizes are the most physiologically relevant in 2D cell culture anyway.

Cell size can be determined using an automated cell counter such as the cell drop, this however measures the cells only after detachment. Cell size can also be determined post-fact after imaging, using software to measure the cells with use of scale bars. This can be more time consuming and in the case of irregular cells may require much user input. More expensive microscope technologies have been developed that are capable of live-imaging cells in culture. These are also capable of interpreting confluence, size, proliferation etc.

3: Morphology

Cellular morphology was of importance to us when considering new ACF media. As changes in cellular morphology can be indicative of stress, senescence, activation, and de/differentiation we closely monitored this as we cultured the cells in their new media. We found it helpful to take images of the cells at each passage, and at interim stages. We kept a close eye on cellular morphology over time as

well as between cells cultured in their original media and cells in new ACF media to make informed comparisons.

Visualisation and imaging are the most reliable way to compare cells in the humanisation process. Cells should be compared at the same time at the same passage as cells may undergo morphological changes as they age. Referring to the manufacturers images as well as images in the literature can be helpful when raising new cells for the first time.

4: Phenotypic markers

Rarely did we observe phenotypic markers in our studies as many were being used as senescence models for screening potential novel therapeutics and the kinetics used to determine therapeutic efficacy did not include such cell markers. For the retinal endothelial cells, we did stain for the endothelial cell marker CD31 when the humanised cells were placed in a collagen matrix. These did stain positively and formed endothelial tubes in collagen. This was indicative that these cells maintained their phenotypes. We observed certain types of fibroblasts to be quite hardy, and they demonstrated little change regardless of their media, these were not stained for phenotypic markers. Retinal pigmented epithelial (RPEs) cells were humanised, and as with their non ACF counterparts, after several passages they began to show signs of epithelial-mesenchymal transition. This was monitored morphologically initially, and it was found that increasing the seeding density reduced the re-occurrence of this. In future, confluent RPEs will be stained for ZO-1 as a control alongside experimentation to ensure that the cells being used are phenotypically relevant as it is critical that these cells maintain their RPE phenotype in order to employ them to model the retina.

Phenotypic markers will be cell type-dependent. Often these can be identified on the product datasheet when acquiring a new cell type, or by conducting a literature search. The monitoring of phenotypic markers may not always be necessary but could be particularly important if looking for signs of epithelial-mesenchymal transition, or when culturing cells that are prone to de-/differentiation. This can be conducted using ICC, IF, Flow Cytometry etc.

5: Application

Most importantly is your new, humanised cell model suitable for its intended purpose? The detail in this assessment and validation section is intended to summarise some of the considerations we made when determining what steps needed to be taken to assure us and the wider community that our ACF models are just as good as the original cell culture models from which they were derived. As with all cell culture models, the intended application will determine how stringent this process needs be.

Common replacements, tips and tricks for the replacement of animal-derived biomaterials

We have successfully derived ACF alternatives to the medium traditionally used for several different primary human cell types. We have substituted human serum in place of FBS in the medium for dermal fibroblasts, uterine fibroblasts, retinal endothelial cells, and peripheral blood mononuclear cells. An ACF alternative, GroPro, was able to act as a substitute in PBMC culture. Below we detail the exchanges we made in which to facilitate the successful culture and downstream applications of these cells.

Quick reference tables

TABLE 2: TABLE OF COMMONLY USED ANIMAL COMPONENT-FREE (ACF) ALTERNATIVES TO NON-ACF REAGENTS USED IN TISSUE CULTURE.

*Please check with suppliers prior to ordering any of these in case recipes have changed since our enquiries

Animal-derived biomaterial or non-ACF reagent	**ACF alternative**	**Link to example**
Foetal Bovine Serum (FBS)	-Human Serum -GroPro	https://www.sigmaaldrich.com/GB/en/substance/humanserum1234598765?gclid=Cj0KCQjw3JanBhCPARIsAJpXTx5CSkXUWz5gDlJkM_omG1eSIHfAFNb6qSPsoT-xZkZ_6kBSTaJmwVUaAkH-EALw_wcB&gclsrc=aw.ds https://www.bioscience.co.uk/product~723875
Bovine Serum Albumin (BSA)	-Human serum albumin (HSA)	https://www.sigmaaldrich.com/GB/en/substance/albuminfromhumanserum1234570024907?gclid=Cj0KCQjw3JanBhCPARIsAJpXTx4U6r6CvP6vvB-RbgKMvYTUQNgR2lClhjjL3jX_dWHC-X4qbG-LP4saAh5HEALw_wcB&gclsrc=aw.ds
Growth factors	-Carrier-free recombinant human growth factors re-constituted in ACF buffers	https://www.ptglab.com/products/humankine-human-cell-expressed-proteins/
Attachment reagents	-Human fibronectin	https://www.rndsystems.com/products/human-fibronectin-protein-cf_1918-fn?gclid=Cj0KCQjw3JanBhCPARIsAJpXTx4LlaQjw8coU8v496-

		LD3_4h7l5j1Bqd5nv_ZKxaG1YPyUaxQfHpy4aArmFEALw_wcB&gclsrc=aw.ds
Trypsin	• TryPLE Express	https://www.thermofisher.com/order/catalog/product/12604013?gclid=Cj0KCQjw3JanBhCPARIsAJpXTx43w5EhgjOTuh1im30XVVgefUhmGl3Yh9_crJzPApxMBuoPt4K2TScaAsw5EALw_wcB&ef_id=Cj0KCQjw3JanBhCPARIsAJpXTx43w5EhgjOTuh1im30XVVgefUhmGl3Yh9_crJzPApxMBuoPt4K2TScaAsw5EALw_wcB:G:s&s_kwcid=AL!3652!3!523795782217!!!g!!!1597425078!122399684093&cid=bid_clb_scl_r01_co_cp0000_pjt0000_bid00000_0se_gaw_dy_pur_con

Human serum

Although human serum has proved useful as an alternative to FBS, it is not without its problems. Although commercially available as a heat-sterilised and filtered product (e.g. H3667, Merck), human serum is often turbid with large proteins and lipids. The turbidity can make it difficult to view cells under the microscope and, more importantly, some cells in our lab (dermal fibroblasts) have grown much more slowly in medium with unfiltered human serum in compared to filtered. The vendor notes that they are not able to filter it more due to the lipid content, however we have managed to do this. Lipids and proteins typically clog a standard vacuum filter (double layered with a 0.4 µm mesh layer and 0.22 µm layer) relatively quickly, but serum can be poured through a nylon mesh as a first pass to remove larger debris. This makes it easier to filter in a vacuum filter unit (see Figure 4), but not all filter units are equal. Our experience notes Nalgene Rapid Flow (10370311, Fisher Scientific) as being the most suitable of the units tried. We have also explored centrifugation before filtration, but a concern is that necessary smaller molecules would be removed alongside the larger molecules. Alternatively, medium containing serum can be filtered, but this also clogs very quickly.

Human-derived alternatives to animal serum are derived from pooled human donors and is obtained in small volumes, pooled, and filtered/treated before sale. Commercially available human serum is obtained under informed consent from adults, nonetheless, there remain understandable ethical implications surrounding commercialisation of person's biomaterial. Additionally, as human serum is from adult donors, it is likely to be inherently less mitogenic than its foetal-derived bovine counterpart. It could therefore be expected that cells may be less proliferative when this is used as the main source of mitogenic factors, however in our experience that is not necessarily the case. More positively, this alternative may be more representative of adult human *in vivo* physiology.

As human serum products are typically obtained in small volumes, from pooled donors, batch-to-batch variability may prove additionally challenging when compared with the larger volumes of single donor FBS available. Theoretically, the preparation methods of human serum deemed appropriate for laboratory use, namely heat inactivation and obtaining samples from healthy AB+ donors, minimise the risk of human serum from reacting with downstream assays. However, it is yet to be determined whether there is a difference between the immunogenicity, stimulatory effects, or downstream assay interference, instigated by the use of human serum in cell culture. Regardless, as with FBS, batch testing should help to negate these issues, particularly when implemented for immunological study. However, additional consideration may be required when ensuring enough of the selected batch is available for a comprehensive set of experiments. As always, medium-only controls account for the majority of issues that may arise from batch-to-batch variability in basic experimentation.

A consideration that is important to note is that use of pooled human serum will contain human specific antibodies and proteins. As such, experiments detecting extracellular production of human-specific antibodies/proteins are not suitable to use human serum - especially considering higher batch-to-batch variation issues.

FIGURE 4: HUMAN SERUM IN A VACUUM FILTER UNIT.

Serum-free tissue culture

Serum-free media could be a viable alternative to both FBS and human serum, but unfortunately media advertised as serum-free often appears to contain BPE as a growth factor source.

Fully synthetic xeno-free media and supplements are fully characterised and therefore do not exhibit batch-to-batch variation in the same way as biological products. However, these synthetic substitutes pose their own issues. Xeno-free supplements are not necessarily a comprehensive replacement for serum. Whilst GroPro did enable the survival of the mononuclear cells, it was not able to support nHDF culture. Other issues - due to the factors available within the growth supplement - include small, white, fibrous formations among the culture media. The supplier acknowledges this limitation and assures users that this is common and does not impact the culture viability – however, it may not be suitable for all downstream cell applications post-culture. A next step for optimising this supplement for fibroblasts would be to add synthetic growth factors to the mix. It is also worthwhile noting here that unless they are specifically ACF, synthetic growth factors often contain small amounts of BSA in the same way that any medium (unless specified as ACF) may contain some animal-derived biomaterial.

In addition to culture media and sera, other reagents commonly used in cell culture (and in downstream characterisation, experimentation and analyses) often contain animal-derived biomaterial. More recently, as demand increases, recombinant and synthetic alternatives are becoming more available and affordable. Recombinant proteins are widely available but are often reconstituted in BSA. Human serum albumin (HSA) may be substituted in place of BSA in which to reconstitute carrier-free proteins and may also serve as an appropriate blocking buffer for many protein assays. Certain primary cells may require coated culture dishes to promote cellular attachment during culture. Gelatin and animal-derived fibronectin are commonly used reagents for this purpose, however human cell-derived and synthetic alternatives are available and are ACF. Such alternatives are increasing in popularity, and if an ACF alternative is not available it is beneficial to report this to suppliers that provide equivalent products and discuss future research needs.

Growth factors

Whilst recombinant growth factors are often more expensive than alternatives, providing they are not reconstituted in BSA, they are simple to exchange into cell culture. Obtaining carrier-free growth factors that can be re-constituted in house by something such as HSA further ensures the ACF nature of such reagents. The Protein tech Humankine range were found to be an affordable and reliable source throughout this work https://www.ptglab.com/products/humankine-human-cell-expressed-proteins/.

Depending on the supplier of the recommended medium, the concentrations of growth factors included may or not be available. Should they be available, growth factors can be exchanged at the exact concentration. To their credit, Promocell were particularly transparent about the contents of their media. Looking for the contents of the supplement “kit” rather than purchasing complete media was a helpful way to ascertain which growth factors were contained in the recommended medium of a cell type, suppliers such as Lonza often provide growth factors in this kit form. However, even if a supplier would real which growth factors were included, they would not necessarily reveal the concentrations.

On this occasion, we often found it helpful to cross reference the kits from multiple suppliers to see if any contained the same supplements of similar cell types and note the concentrations. Additionally,

the literature was consulted to look for peers that had successfully published in alternative media to ascertain the concentrations of growth factors used. Sometimes, it was informative to look at early literature that would carefully detail the process in which the successful culture of a cell type was achieved for the first time. It is important to critically evaluate the outcomes of such papers and ensure that the alternative media was appropriate, justified, and that cells behaved typically of their phenotype. If a range of concentrations were used of a particular growth factor among the literature, the first trial concentration was selected only after considering the role of this growth factor in the culture of the cell type in question. If this provided no further guidance, a mid-range concentration would be selected.

Coating reagents

Some cells require coated culture dishes to promote cellular attachment during culture. Gelatin and animal-derived fibronectin are commonly used reagents for this purpose, however human cell-derived and synthetic alternatives are available and are ACF.

Detachment reagents

More recently, as demand increases, recombinant and synthetic alternatives are becoming more available and affordable. In tissue culture, trypsin, commonly derived from the pancreas of livestock, is used to detach adherent cells from their culture vessel. The alternatives CTS TrypLE™ and CTS™Versene™ are now readily available and highly effective. These alternatives provide the added benefit of being consistent, and also often gentler on cells, and therefore can be used on a wider variety of cell types at the same concentration.

General recommendations to the community

- Keep it simple!
- Ask suppliers, make requests for what you need, drive the ACF market!
- Publish your ACF protocols so that the humanisation process only needs to be performed once for each cell type. E.g. attached to this guide.
- Share your ACF downstream protocols, ACF kits are hard to obtain at present and taking time to individually source reagents can be time consuming.
- Publish negative data, if something doesn't work, let people know!
- Reach out to your ACF community for help.
- Constantly question your laboratory practices and encourage others to do so too.

Discussion and concluding remarks

Ultimately all tissue culture models have inherent limitations, meaning it can be difficult to determine what is physiologically relevant enough for a model. Cells normally exist in a complex network in a three-dimensional structure within a tissue. The growth rate of cells *in vivo* is likely to be different to their *in vitro* counterparts. Donor age, species, batch, as well as additional supplementation, and choice of basal media all alter the *in vitro* microenvironment. FBS has been traditionally used in cell culture since the technique was first developed, and consequently is heavily relied upon for *in vitro* research. Considering that the local microenvironment influences cellular phenotype, including size, shape, and gene expression, a simple first step towards physiological relevance could be the culture of human cells in species specific or human biomimetic reagents.

Bioreactors, hydrogels and mechanical stress systems permit gas exchange, provide space and exhibit mechanical pressures that are more akin to the *in vivo* environment. [21,22] The necessity and utility of these technologies depend upon the cell type, disease model, and outcome measures of the research. Cost and availability are other factors that may influence the ability to culture cells under physiologically relevant conditions. However, it may be argued that tradition is the main reason that the use of animal-derived biomaterials is seen as a "gold standard" in human cell culture, but moving away from this practice may provide a very simple, yet often overlooked step towards physiological relevance, and hence the translatability of cell culture.

Animal *in vivo* studies have their own well-described drawbacks, and the species-specific nature of certain biological processes could mean that a human cell culture model may actually be more representative of what happens *in vivo* in humans. [8,9,23,24] Although any tissue culture experiment will have control groups, the presence of animal-derived biomaterials represents an unknown in cell culture medium which can alter the microenvironment and could mask or change a response. Similarly, human serum could represent an unknown factor, but, arguably, putting a human-derived biomaterial into a human cell model of human disease may represent a better approach than animal-derived biomaterial.

The humanisation of cell culture represents a small step towards more translatable science. It is worth considering that the cost in time and resources may be small and may pay dividends in the future, helping to reduce the poor rate of translation of medical research into new, human, clinical therapies. Cell culture models are not perfect representations of what happens *in vivo* and we must bear this in mind when considering humanisation: while it is not perfect, it can be worth improving in a small way to ensure that research findings are more applicable to humans.

Bibliography

(1) Bramwell, L. R. Mechanisms of Cellular Senescence and the Use of Repurposed Drugs as Senotherapeutic Compounds., University of Exeter, 2023.

(2) Jeffery, N.; Richardson, S.; Chambers, D.; Morgan, N. G.; Harries, L. W. Cellular Stressors May Alter Islet Hormone Cell Proportions by Moderation of Alternative Splicing Patterns. *Hum. Mol. Genet.* **2019**, *28* (16), 2763. https://doi.org/10.1093/HMG/DDZ094.

(3) Martin, E.-R.; Gandawijaya, J.; Oguro-Ando, A. A Novel Method for Generating Glutamatergic SH-SY5Y Neuron-like Cells Utilizing B-27 Supplement. *Front. Pharmacol.* **2022**, No. October, 1–20. https://doi.org/10.3389/fphar.2022.943627.

(4) Yao, T.; Asayama, Y. Animal-cell Culture Media: History, Characteristics, and Current Issues. *Reprod. Med. Biol.* **2017**, *16* (2), 99. https://doi.org/10.1002/RMB2.12024.

(5) Beskow, L. M. Lessons from HeLa Cells: The Ethics and Policy of Biospecimens. *Annu. Rev. Genomics Hum. Genet.* **2016**, *17*, 395. https://doi.org/10.1146/ANNUREV-GENOM-083115-022536.

(6) Subbiahanadar Chelladurai, K.; Selvan Christyraj, J. D.; Rajagopalan, K.; Yesudhason, B. V.; Venkatachalam, S.; Mohan, M.; Chellathurai Vasantha, N.; Selvan Christyraj, J. R. S. Alternative to FBS in Animal Cell Culture - An Overview and Future Perspective. *Heliyon* **2021**, *7* (8), e07686. https://doi.org/10.1016/J.HELIYON.2021.E07686.

(7) Zheng, X.; Baker, H.; Hancock, W. S.; Fawaz, F.; McCaman, M.; Pungor, E. Proteomic Analysis for the Assessment of Different Lots of Fetal Bovine Serum as a Raw Material for Cell Culture. Part IV. Application of Proteomics to the Manufacture of Biological Drugs. *Biotechnol. Prog.* **2006**, *22* (5), 1294–1300. https://doi.org/10.1021/BP060121O.

(8) Valk, J. van der; Gstraunthaler, G. Fetal Bovine Serum (FBS) — A Pain in the Dish? *ATLA* **2017**, *45* (6), 329–332. https://doi.org/10.1177/026119291704500611.

(9) van der Valk, J.; Bieback, K.; Buta, C.; Cochrane, B.; Dirks, W. G.; Fu, J.; Hickman, J. J.; Hohensee, C.; Kolar, R.; Liebsch, M.; Pistollato, F.; Schulz, M.; Thieme, D.; Weber, T.; Wiest, J.; Winkler, S.; Gstraunthaler, G. Fetal Bovine Serum (FBS): Past - Present - Future. *ALTEX* **2018**, *35* (1), 99–118. https://doi.org/10.14573/altex.1705101.

(10) Gstraunthaler, G.; Lindl, T.; Van Der Valk, J. A Plea to Reduce or Replace Fetal Bovine Serum in Cell Culture Media. *Cytotechnology* **2013**, *65* (5), 791. https://doi.org/10.1007/S10616-013-9633-8.

(11) Kent, K. D.; Bomser, J. A. Bovine Pituitary Extract Provides Remarkable Protection against Oxidative Stress in Human Prostate Epithelial Cells. *Vitr. Cell. Dev. Biol. - Anim.* **2003**, *39* (8–9), 388–394. https://doi.org/10.1290/0311082.

(12) Galy, A.; Jolivet, M.; Jolivet-Reynaud, C.; Hadden, J. Fibroblast Growth Factor (FGF) and an FGF-like Molecule in Pituitary Extracts Stimulate Thymic Epithelial Cell Proliferation. *Thymus* **1990**, *15* (4), 199–211.

(13) Hadden, M. Factor Induces Proliferation Thymic Epithelial in Vitro. **1989**, *159*, 149–159.

(14) Tanigaki-Obana, N.; Ito, M. Can Fibroblast Growth Factors Substitute for Bovine Pituitary Extracts in Culture Systems for Hair Apparatus Cells? *Arch Dermatol Res* **1994**, *286*, 484–489.

(15) Hammond, S. L.; Ham, R. G.; Stampfert, M. R. Serum-Free Growth of Human Mammary Epithelial Cells: Rapid Clonal Growth in Defined Medium and Extended Serial Passage with Pituitary Extract (Prostaglandin El/Cycic AMP/Ethanolamine/Insulin/Epidermal Growth Factor). *Cell Biol.* **1984**, *81*, 5435–5439.

(16) Muraglia, A.; Nguyen, V. T.; Nardini, M.; Mogni, M.; Coviello, D.; Dozin, B.; Strada, P.; Baldelli, I.; Formica, M.; Cancedda, R.; Mastrogiacomo, M. Culture Medium Supplements Derived from Human Platelet and Plasma: Cell Commitment and Proliferation Support. *Front. Bioeng. Biotechnol.* **2017**, *5* (NOV). https://doi.org/10.3389/FBIOE.2017.00066.

(17) Chang, B.; Mahoney, R. Enzyme Thermostabilization by Bovine Serum Albumin and Other Proteins: Evidence for Hydrophobic Interactions. *Biotechnol. Appl. Biochem.* **1995**, *22* (2), 203–214. https://doi.org/10.1111/J.1470-8744.1995.TB00346.X.

(18) *Fetal Calf Serum Free Database*. https://fcs-free.org/ (Accessed: 5th October 2022).

(19) Bairoch, A. The Cellosaurus, a Cell-Line Knowledge Resource. *J. Biomol. Tech.* **2018**, *29* (2), 25–38. https://doi.org/10.7171/JBT.18-2902-002.

(20) Geraghty, R. J.; Capes-Davis, A.; Davis, J. M.; Downward, J.; Freshney, R. I.; Knezevic, I.; Lovell-Badge, R.; Masters, J. R. W.; Meredith, J.; Stacey, G. N.; Thraves, P.; Vias, M. Guidelines for the Use of Cell Lines in Biomedical Research. *Br. J. Cancer* **2014**, *111* (6), 1021–1046. https://doi.org/10.1038/bjc.2014.166.

(21) Stephenson, M.; Grayson, W. L.; Ma, T.; Geris, L.; Leuven, K. U. Recent Advances in Bioreactors for Cell-Based Therapies. *F1000Research 2018 7517* **2018**, *7*, 517. https://doi.org/10.12688/f1000research.12533.1.

(22) Caliari, S. R.; Burdick, J. A. A Practical Guide to Hydrogels for Cell Culture. *Nat. Methods* **2016**, *13* (5), 405. https://doi.org/10.1038/NMETH.3839.

(23) Witzeneder, K.; Lindenmair, A.; Gabriel, C.; Höller, K.; Theiß, D.; Redl, H.; Hennerbichler, S. Human-Derived Alternatives to Fetal Bovine Serum in Cell Culture. *Transfus. Med. Hemotherapy* **2013**, *40* (6), 417–423. https://doi.org/10.1159/000356236.

(24) Gstraunthaler, G. Alternatives to the Use of Fetal Bovine Serum: Serum-Free Cell Culture. *ALTEX* **2003**, *20* (4), 275–281. https://doi.org/10.14573/altex.2003.4.257.

Appendices

Appendix 1: Human Pulmonary Fibroblast Culture

Harries' Lab Humanised Cell Culture Protocol

Disclaimer: This document is intended as a basic SOP from which to develop your own protocol.

Human Pulmonary Fibroblast Culture

Raising cells

1. In a clean laminar flow hood / class-2 safety cabinet prepare reagents ahead of raising cells.

Original		**Humanised**	
Media		**Media**	
FGM 2	Promocell C-23220	DMEM	Gibco-31885023
Supplement kit	Promocell C-39325	10 % Human Serum	Sigma Aldrich- H3667
		1 ng / ml bFGF	Proteintech-HZ-1285
Stop solution		**Stop solution**	
dPBS	Any	dPBS	Any
10 % FBS	Any	10 % Human Serum	Sigma Aldrich- H3667

* Re-constitute growth factors according to the manufacturer's instructions but exchange HSA for BSA.

2. Place 9 ml of stop solution into a 15 ml falcon tube and warm to 37 degrees Celsius.
3. Place 6 ml of media into a T25 flask or 13 ml of media into a T75 flask and warm to 37 degrees Celsius.
4. Raise cells from liquid nitrogen, defrost rapidly using a bead bath or in the palm of a gloved hand.
5. Transfer cells immediately to the 9ml of stop solution once defrosted.
6. Centrifuge at 500 x g for 3 minutes.
7. Re-suspend in 1ml of pre-warmed media and gently pipette up and down.
8. Transfer to flask and swirl gently.
9. Incubate at 37 degrees Celsius 5% CO_2.
10. Change media every 2-3 days until cells reach ~80% confluent.

Passaging cells

1. In a clean laminar flow hood / class-2 safety cabinet prepare reagents ahead of raising cells.

Original		Humanised	
Media		**Media**	
FGM 2	Promocell C-23220	DMEM	Gibco-31885023
Supplement kit	Promocell C-39325	10 % Human Serum	Sigma Aldrich- H3667
		1 ng / ml bFGF	Proteintech-HZ-1285
Stop solution		**Stop solution**	
dPBS	Any	dPBS	Any
10 % FBS	Any	10 % Human Serum	Sigma Aldrich- H3667
Disassociation reagent		**Disassociation reagent**	
Trypsin	Any	TrypLE	Gibco- 12604013

2. Pre-warm media, PBS, stop solution and the disassociation reagent.
3. Remove cells from the incubator and in a clean hood, discard the media to Virkon unless it is being kept for experimentation.
4. Wash the cells once in ~7 ml of PBS.
5. Add 2 ml of disassociation reagent for a T25, or 3 ml for a T75.
6. Place in the incubator and check every 2 minutes for detachment by viewing under the microscope.
7. Once cells have detached add at least an equal volume of stop solution to the flask and transfer the contents to a labelled 15ml falcon tube.
8. Centrifuge at 500g for 3 minutes.
9. Discard the supernatant.
10. Re-suspend the cell pellet in 1 ml of culture media.
11. Transfer the culture media at the desired ratio into a new flask/s containing pre-warmed media.

Freezing cells

1. Follow the passaging cells protocol until step 9.
2. Re-suspend cells in 50% Serum, 40% media and 10% DMSO.
3. Freeze 1ml per cryovial in a Mr Frosty at -80 degrees Celsius.
4. Transfer to liquid nitrogen the next day.

SG 22/08/2023