Annual Review of Risk Assessment Made Under:
Genetically Modified Organisms (Contained Use) Regulations 2014

Department: Nuffield Division of Clinical Laboratory Sciences
Radcliffe Department of Medicine

Supervisor: Prof Stephen Hyde

Ref No: CBGM18

Title: Genetic Engineering Of Mammalian Cell Lines

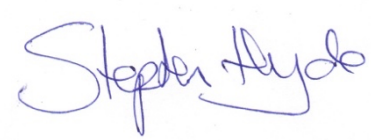
The Risk Assessment has been reviewed: YES
Key aspects: identification of any potentially harmful effects, characteristics of the proposed activity, the severity of any potentially harmful effects, the likelihood of them occurring and disposal of waste and effluent.

Appropriate containment measures have been confirmed: YES
Complete attached containment levels/measures table

Original containment level and risk classification remain valid: YES

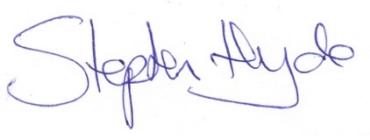
Classification and assignment of final control measures:
Containment Level: CL1
Risk Classification: 1

Reviewed By:
Date (YYYY-MM-DD):



Prof Stephen Hyde
2024-08-16

Approved By Genetic Modification Safety Committee
Agreed By One-Of DSO/BSO/HoD:
Date (YYYY-MM-DD):



Prof Stephen Hyde – NDCLS BSO
2024-10-02

Approved by Head of Department
Date (YYYY-MM-DD):

Prof Deborah Gill – NDCLS HoD
2024-10-02

Next Review Due:
Before end 2025

List Of Associated Transgenic Sequences:

Common Reporter Genes:
EGFP and similar proteins

Bacterial Proteins
Staphylococcus aureus Cas9 (saCas9) and similar proteins along with associated gRNA and similar sequences.

Target Loci:
Mammalian ion channels/transporters proteins:
Cystic fibrosis transmembrane conductance regulator (CFTR),
ATP-Binding Cassette, Sub-family A, Member 3 (ABCA3)

Mammalian secreted proteins:
Immuno-globulins
surfactant protein A to D (SFTPA-SFTPD)
alpha-1 anti trypsin (SERPINA1),
Decorin
DNAseI
TRIM72

Common Gene Editing Reporter Sites:
HEK3 Target and similar

Risk Assessment Users & Supervisor During Year To Review Date
Stephen Hyde
Emily Castells (Stephen Hyde)
Marina Cerezuela (Stephen Hyde)
Hamid Dolatshad (Stephen Hyde)
Arlene Glasgow (Stephen Hyde)
Omar Habib (Stephen Hyde)
Jakob Haldrup (Stephen Hyde)
Kamran Miah (Stephen Hyde)
Eoin Mac Reamoinn (Stephen Hyde)
Aimee Ruffle (Stephen Hyde)
Dwiantari Satyapertiwi (Stephen Hyde)
Shahzaib Tariq (Stephen Hyde)
Gavin Turnbull (Stephen Hyde)

Galina Boskh (Shijie Cai / Stephen Hde)

Visiting Students
Sanuba Khan (Stephen Hyde)
Alice Coffey (Stephen Hyde)

Table 1a Containment measures applicable to contained use involving micro-organisms in laboratories

Containment Measures		**Containment Levels**			
		CL1	CL2	CL3	CL4
Facilities					
1	Laboratory suite: isolation[1]	not required	not required	required	required
2	Laboratory: sealable for fumigation	not required	not required	required	required
Equipment					
3	Surfaces impervious to water, resistant to acids, alkalis, solvents, disinfectants and decontamination agents and easy to clean	required for any bench	required for any bench	required for any bench and floor	required for any bench, floor, ceilings and walls
4	Entry to laboratory via airlock[2]	not required	not required	required where and to extent the risk assessment shows it is required	required
5	Negative pressure relative to the pressure of the immediate surroundings	not required	not required	required except for activities where transmission does not occur by the airborne route	required
6	Extract and input air from the laboratory must be HEPA filtered	not required	not required	HEPA filters required for extract air except for activities where transmission does not occur by the airborne route	HEPA filters required for input and extract air[3]
7	Microbiological safety cabinet/ enclosure	not required	required where and to extent the risk assessment shows it is required	all procedures with infective materials required to be contained within a cabinet/ enclosure	required, and all procedures with infective materials required to be contained within a cabinet/ enclosure
8	Autoclave	required on site	required in the building	required in the laboratory suite[4]	double ended autoclave required in laboratory

Containment Measures		Containment Levels			
		CL1	CL2	CL3	CL4
System Of Work					
9	Access restricted to authorised personnel only	not required	required	required	required (via airlock key procedure)
10	Biohazard sign on door	not required	required	required	required
11	Specific measures to control aerosol dissemination	not required	required so as to minimise	required so as to prevent	required so as to prevent
12	Shower	not required	not required	required where and to extent the risk assessment shows it is required	required
13	Protective clothing	suitable protective clothing required	suitable protective clothing required	suitable protective clothing required; footwear required where and to extent the risk assessment shows it is required	complete change of clothing and footwear required before entry and exit
14	Gloves	not required	required where and to extent the risk assessment shows they are required	required	required
15	Efficient control of disease vectors (eg rodents and insects) which could disseminate GMMs	required where and to extent the risk assessment shows it is required	required	required	required
Waste					
16	Inactivation of GMMs in effluent from hand- washing sinks and showers and similar effluents	not required	not required	required where and to extent the risk assessment shows it is required	required
17	Inactivation of GMMs in contaminated material and waste	required by validated means where and to extent the risk assessment shows it is required	required by validated means	required by validated means, with waste inactivated within the laboratory suite	required by validated means, with waste inactivated within the laboratory

Containment Measures		Containment Levels			
		CL1	CL2	CL3	CL4
Other Measures					
18	Laboratory to contain its own equipment	not required	not required	required, so far as is reasonably practicable	required
19	An observation window or alternative is to be present so that occupants can be seen	required where and to extent the risk assessment shows it is required	required where and to extent the risk assessment shows it is required	required where and to extent the risk assessment shows it is required	required
20	Safe storage of GMMs	required where and to extent the risk assessment shows it is required	required	required	secure storage required
21	Written records of staff training	not required	required where and to extent the risk assessment shows it is required	required	required

1 "isolation" means, in relation to a laboratory, separation of the laboratory from other areas in the same building, or being in a separate building.

2 Entry must be through an airlock which is a chamber isolated from the laboratory. The clean side of the airlock must be separated from the restricted side by changing or showering facilities and preferably by interlocking doors.

3 Where viruses are not retained by the HEPA filters, extra requirements will be necessary for extract air.

4 Where the autoclave is outside the laboratory in which the contained use is being undertaken, but within the laboratory suite, there must be validated procedures for the safe transfer of material into that autoclave, which provide a level of protection equivalent to that which would be achieved by having an autoclave in that laboratory.

Table 1b Containment measures applicable to contained use involving micro-organisms in plant growth facilities (to be read with Table 1a)

Omitted as not relevant to NDCLS activities

Table 1c Containment measures applicable to contained use involving micro-organisms in animal units (to be read with Table 1a)

Omitted as not relevant to NDCLS activities

Containment Measures		Containment Levels				Additional / Modification
		CL1	CL2	CL3	CL4	
Facilities						
1	Isolation of animal unit[1]	required where and to extent the risk assessment shows it is required	required	required	required	modification
2	Animal facilities[2] separated by lockable doors	required where and to extent the risk assessment shows it is required	required	required	required	additional
3	Animal facilities (cages, etc) designed to facilitate decontamination (waterproof and easily washable material)	required where and to extent the risk assessment shows it is required	required where and to extent the risk assessment shows it is required	required	required	additional
4	Floor, walls and ceiling easily washable	required where and to extent the risk assessment shows it is required	required for floor	required for floor and walls	required for floor, walls and ceiling	Modification
5	Appropriate filters on isolators or isolated rooms[3]	not required	required where and to extent the risk assessment shows it is required	required	required	additional
6	Appropriate barriers at the room exit, and at drains or ventilation duct work	required	required	required	required	additional
7	Animals kept in appropriate containment facilities, such as cages, pens or tanks but not isolators	required where and to extent the risk assessment shows it is required	required where and to extent the risk assessment shows it is required	required where and to extent the risk assessment shows it is required	required where and to extent the risk assessment shows it is required	Additional
8	Animals kept in isolators	not required	required where and to extent the risk assessment shows it is required	required	required	modification

1 "animal unit" means a building, or separate area within a building, containing an animal facility and other areas including changing rooms, showers, autoclaves and food storage areas.

2 "animal facility" means a facility normally used to house stock, breeding or experimental animals or one which is used for the performance of minor surgical procedures on animals.

3 "isolators" means transparent boxes where small animals are contained within or outside a cage; for large animals, isolated rooms may be more appropriate

Risk Assessment made under the Genetically Modified Organisms (Contained Use) Regulations 2000

(Form GMM – for genetically modified micro-organisms and eukaryotic cell and tissue culture systems)

Department: NDCLS / RDM	**Supervisor:** Dr Stephen Hyde	**Ref. No: CBGM20**

Project Title:
Genetic engineering of mammalian cell lines for maximisation of viral vector production

Overview of Project:
(include aims and objectives)

The CRISPR/Cas9 system is a versatile tool for genome engineering. The system employs the type II prokaryotic CRISPR adaptive immune system, which uses a guide RNA (gRNA) to target the Cas9 nuclease to a specific 20nt genomic sequence upstream of a "protospacer adjacent motif" (PAM), which can take the form of NGG or NAG (Jinek M, et al., *Science,* 2012 **337**: 816-21). Cas9-induces double-stranded DNA breaks which are repaired either by imperfect nonhomologous end-joining (NHEJ) to generate insertions or deletions ("indels") (Barnes DE, *Curr Biol,* 2001 **11**: R455-7) or, if a repair template is provided, by homology directed repair (HDR) (Ran FA, et al., *Nat Protoc,* 2013 **8**: 2281-308).

We aim to use the CRISPR/Cas9 genome editing technology to knock-out genes that might be involved in viral vector production in order to increase current production levels. The gRNA will target mammalian genes found in the literature that may be either positive or negative factors for viral production.

Custom-designed 20 nt guide sequences targeting genomic regions overlapping with specific mutations observed in mammalian cell lines, will be inserted into suitable vectors such as the pSpCas9(BB)-2A-GFP bicistronic expression vector (PX458) (Addgene; https://www.addgene.org/48138/), which also encodes the Cas9 nuclease. Optionally, such vectors may include standard marker genes and antibiotic selection cassettes, such as GFP, Amp, Puro. Alternative vectors include, but are not limited to, pSpCas9(BB)-2A-Puro (PX459) (48139), pSpCas9n(BB)-2A-GFP (PX461) (48140) and pSpCas9n(BB)-2A-Puro (PX462) (48141).

CRISPR/Cas9/gRNA vectors will be delivered to mammalian cells by transfection, nucleofection or other similar methods. Transfected cells may be sorted by FACS. Successful insertion/deletion mutations may be assessed by Sanger sequencing, Surveyor nuclease assay, targeted next generation sequencing, or restriction enzyme digestion. Cells may be used in other projects, under other risk assessments, for the production of viral vectors.

Give details of Recipient/Host(s): *(specify if wild type or disabled)*	**Vector(s):**
Common, laboratory cultured mammalian cell lines.	These will include commonly available CRISPR/Cas9/gRNA vectors, such as pSpCas9(BB)-2A-GFP (PX458) (48138), pSpCas9(BB)-2A-Puro (PX459) (48139), pSpCas9n(BB)-2A-GFP (PX461) (48140) and pSpCas9n(BB)-2A-Puro (PX462) (48141) (all from Addgene)

Normal/expected biological action of inserted DNA/RNA or transcribed/translated gene product:

The bacterial Cas9 is known to cause a double strand break in the genome in the presence of a guide RNA (gRNA). In the absence of gRNA no effect is observed. The expected effect of knocking out of viral restriction factors will be to generate cell lines that when later transiently transfected with the plasmids necessary to generate viral vectors will produce higher viral titres. In the case of essential factors for viral production, they will serve as a proof of concept to show that viral titres can be affected by gene knock-out (i.e. decrease or absence of viral production).

Technique used to introduce insert or vector into host: Plasmids will be introduced into host cells using either common transfection method or electroporation methods such as nucleofection (Amaxa Nucleofection Kit, Lonza)	
Assessed By:	
Signature: 	Date: 16th July 2014
Risk Assessment approved by Genetic Modification Safety Committee	
Signature: 	Date: 16th July 2014
Biological Safety Officer	Dr Stephen Hyde
Permission granted by Head of Department for project to be undertaken	
Signature: 	Date 16th July 2014
Head Of Department	Prof Kevin Gatter

RISK ASSESSMENT FOR HUMAN HEALTH AND SAFETY	***GUIDANCE***
Human health hazard identification – (Identify any potential harmful properties of:) i) the recipient micro-organism *(for micro-organisms also give ACDP hazard group)* E.coli cells that we would be using to expand the relevant vectors are from commercial suppliers such as Oneshot Top 10 cells which have no known health or physical hazard to human. Minimal hazard from mammalian cell lines obtained from commercial sources – containment level 1.	*Potentially harmful effects include:* *disease to humans – consider all properties which may give rise to harm eg infection, toxins, cytokines, allergens, hormones etc* *alteration of existing pathogenic traits – consider alteration of tissue tropism or host range, alteration in susceptibility to human defence mechanisms etc* *adverse effects resulting from inability to treat disease or offer effective prophylaxis* *possibilities for any disablement or attenuation to be overcome by recombination or complementation* *adverse effects resulting from the potential for transfer of inserted genetic material to another micro-organism*
ii) the inserted (donated) genetic material The inserts code for the Cas9 nuclease from *Streptococcus pyogenes*is and include standard marker genes and antibiotic selection cassettes, such as GFP, Amp, Puro. The gRNAs's function is to guide/target the Cas9 nuclease to specific genomic sequences. Inserts are not expected to have harmful physiological or pharmacological properties or to affect pathogenicity of cloning host or normal human defence mechanisms.	
iii) the donor micro-organisms *(where used/appropriate)* The inserts code for the Cas9 nuclease from *Streptococcus pyogenes*.	
iv) the vector The expression vectors are standard laboratory derived or commercial plasmids and are considered non-pathogenic. We will use the pSpCas9(BB)-2A-GFP bicistronic expression vector (PX458) (Addgene), which also encodes the Cas9 nuclease or similar. This plasmid contains two expression cassettes, hSpCas9 and the chimeric guide RNA. The vector can be digested using BbsI, and a pair of annealed oligos can be cloned into the guide RNA. Current alternative vectors include pSpCas9(BB)-2A-Puro (PX459) (48139), pSpCas9n(BB)-2A-GFP (PX461) (48140) and pSpCas9n(BB)-2A-Puro (PX462) (48141).	
v) the resulting genetically modified micro-organism No significant hazards identified above, the resulting GMOs are therefore not expected to carry any additional risks to that of the un-modified recipients. E.coli strains used are disabled. Cell lines would be recognised as non-self by the immune system and be removed.	
Brenner Scheme values (*COMPLETION OPTIONAL and in any case for disabled E. coli only*)	

Access	Expression	Damage	Overall	

<u>Control measures</u> – Assign provisional containment level: **Containment Level: 1, for both bacterial and mammalian cell culture work** **with Good Microbiological Practice and Good Occupational Safety and Hygiene**	*Assign a provisional containment to control the hazards identified above taking account of severity of any consequence and likelihood of harm occurring. Select from 1,2,3 or 4*
NATURE OF WORK TO BE UNDERTAKEN	***GUIDANCE***

Give brief description of types of laboratory procedures including maximum culture volumes at any time (show as multiples of unit volumes) For E.coli work The procedures are standard laboratory practice for gene cloning and manipulation. Individual culture volumes will typically be ≤500mL. For Mammalian Cell And Tissue Culture Work The procedures are standard laboratory practice for mammalian cell and tissue culture. Individual culture volumes will typically be ≤100mL	*Consider any activities that may involve risks which require specific additional control measures such as:* *inoculation of animals or plants with GMMs* *the use of equipment or procedures likely to generate aerosols* *large scale work*
Provide details of any non-standard laboratory operations None	
Additional control measures required for specific risks: No known additional control measures are required for health and safety reasons when carrying out experiment	

RISK ASSESSMENT FOR ENVIRONMENTAL HARM **Environmental hazard identification -** Identify any potentially harmful properties of:	***GUIDANCE*** *Potentially harmful effects include:* *disease to animals including allergenic and toxic effects* *disease to animals and plants* *adverse effects resulting from inability to treat disease or offer effective prophylaxis* *adverse effects resulting from establishment or dissemination of the GMMs in the environment* *adverse effects resulting from the natural transfer of inserted genetic material to other organisms*
i) the recipient micro-organism None. No disease or other harmful effects to humans, other animals or plants.	
ii) the inserted (donated) genetic material None. The inserts code for the Cas9 nuclease from *Streptococcus pyogenes* and include standard marker genes or antibiotic selection cassettes, such as GFP, Amp and Puro. The gRNAs's function is to guide/target the Cas9 nuclease to specific genomic sequences. Inserts are not expected to have harmful physiological or pharmacological properties or to affect pathogenicity of cloning host.	
iii) the donor micro-organisms *(where used/appropriate)* None. The inserts code for the Cas9 nuclease from *Streptococcus pyogenes.*	
iv) the vector None. The vector is commercially available and has no noted harmful effects.	
v) the resulting genetically modified micro-organism None. The resulting GMO's and mammalian cells are not expected to have any significant risk compared to those of the unmodified cells. GMOs and mammalian cells would not survive outside laboratory conditions.	
Where potentially harmful effects are identified estimate:	
i) consequence/severity of effects Negligible	*select from:* *Severe/Medium/Low/Negligible*
ii) likelihood of effects being realised *(taking containment and control measures assigned above into account)* Negligible	*Select from:* *High/Medium/Low/Negligible*
iii) overall risk Effectively zero	*Select from:* *High/Medium/Low/Effectively zero*
<u>Additional control measures</u> required to reduce all risks to low/effectively zero:	
CLASSIFICATION AND ASSIGNMENT OF FINAL CONTROL MEASURES **Consider each item on Table 1a** indicate whether or not it is required taking account of the provisional containment level assigned to protect human health and safety and any additional control measures necessary to control specific activities and environment risks **Consider also Tables 1b and 1c where appropriate**	***GUIDANCE*** *Mark up table(s) by circling for each item the first correct answer reading across the table from left to right*
<u>Classification:</u> **Class: 1**	*The highest numbered column in which a control measure is required indicates the Class of the activity – circle class on table 1a*
<u>Assign corresponding level of containment:</u> **Containment Level: 1**	*The class number indicates the minimum containment level required*

specify any other control measures required After consideration of the procedures to be undertaken, no additional need was identified for additional control measures to protect human health and safety	

Table 1a: Containment Measures for Activities involving GMMs in Laboratories

Where an item is listed as "may be required" this indicates the item to be an option at that particular containment level and its requirement should be determined by the risk assessment for the particular activity concerned. Delete no or yes as indicated by risk assessment.

Containment Measures	**Containment Levels**			
	1	**~~2~~**	**~~3~~**	**4**
Isolated laboratory suite	not required	~~not required~~	~~required~~	~~required~~
Laboratory sealable for fumigation	not required	~~not required~~	~~required~~	~~required~~
Surfaces impervious, resistant and easy to clean	required for bench	~~required for bench~~	~~required for bench and floor~~	~~required for bench, floor, ceiling and walls~~
Entry to lab via airlock	not required	~~not required~~	~~may be required no / yes~~	~~required~~
Negative pressure relative to the pressure of the immediate surroundings	not required	~~may be required no / yes~~	~~required~~	~~required~~
HEPA filtered extract and input air	not required	~~not required~~	~~required for extract~~	~~required for input and extract~~
Microbiological safety cabinet/enclosure	not required	~~may be required no / yes~~	~~required~~	~~required (class 3)~~
Autoclave	required on site	~~required in the building~~	~~required in the lab suite~~	~~required in lab (double ended)~~
Access restricted to authorised personnel	not required	~~required~~	~~required~~	~~required~~
Specified measures to control aerosol dissemination	not required	~~required so as to minimise~~	~~required so as to prevent~~	~~required so as to prevent~~
Shower	not required	~~not required~~	~~may be required no / yes~~	~~required~~
Protective clothing	suitable protective clothing required	~~suitable protective clothing required~~	~~suitable protective clothing required~~	~~complete change of clothing and footwear~~
Gloves	not required	~~may be required no / yes~~	~~required~~	~~required~~
Control of disease vectors (eg rodents, insects) which could disseminate GMMs	may be required no / ~~yes~~	~~required~~	~~required~~	~~required~~
Specified disinfection procedures in place	may be required no / ~~yes~~	~~required~~	~~required~~	~~required~~
Inactivation of GMMs in effluent from handwashing sinks, showers etc	not required	~~not required~~	~~may be required no / yes~~	~~required~~
Inactivation of GMMs in contaminated material and waste	required by validated means	~~required by validated means~~	~~required by validated means, with waste inactivated in the laboratory suite~~	~~required by validated means, with waste inactivated within the laboratory~~
Laboratory to contain its own equipment	not required	~~not required~~	~~required~~	~~required~~
An observation window or alternative so that occupants can be seen	may be required no / ~~yes~~	~~may be required no / yes~~	~~required~~	~~required~~
Safe storage of GMMs	may be required no / ~~yes~~	~~required~~	~~required~~	~~secure storage required~~
Written records of staff training	not required	~~may be required no / yes~~	~~required~~	~~required~~

CLASSIFICATION	**CLASS 1**	**~~CLASS 2~~**	**~~CLASS 3~~**	**~~CLASS 4~~**