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STANDARD OPERATING PROCEDURE (SOP)	Document Num.: S-007
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Rat Liver Perfusion and Hepatocytes Isolation	Effective date: March 2013
	Version: 1
	First draft: THCT

Materials / Equipment:

1. Lab coat
2. Electric and blade razor
3. Sterile surgery devices and bulldog clamp
4. Sterile surgery tray and needle/tape
5. Sharp container
6. Carbogen gas bottle
7. Sterile silicon tubing for carbogen gas
8. Sterile three ways tube connector
9. Aervent gas filter
10. Sterile silicon tubing for perfusion with bubble trap
11. Sterile small tea strainer
12. Sterile 250 and 600 mL beaker glass
13. Sterile tweezers and forceps
14. Digital balance
15. Centrifuge
16. Cell counting chamber with cover
17. 10 cm sterile petri dish
18. Pipettor, pipette boy

Solutions / Media:

1. Ketamil injection (Ilium, Troy laboratories pty limited Australia)
2. Xylazil (Ilium, Troy laboratories pty limited Australia)
3. Iodine
4. Washing Buffer (see appendix)
5. 0.5 mM EGTA buffer (see appendix)
6. 50 U/mL Collagenase type IV (Gibco #17104-019) solution in low glucose DMEM media with Ca/Mg (Biowest cat # L0064-500) contain or completed with 2 mM L-Glutamin (Sigma Cat # G7513), 1 mM Na-pyruvat (Sigma Cat # 8636) and 1% AB/AM (Sigma Cat #A5955) (See appendix)
7. William E media culture completed with 2 mM L-Glutamin (Sigma Cat # G7513), 1 mM Na-pyruvat (Sigma Cat # 8636) and 1% AB/AM.
8. Hepatocytes Basal Medium (HBM™ Lonza Cat# CC-3199) with HCM™ Single Quots Kit (Lonza Cat #CC-4182)
9. CASO agar

10. 0.36% and 0.22% Trypan Blue solution (Sigma cat# T8154)

Consumables:

1. Sterile nitrile gloves
2. Surgery mask and surgery hat
3. 1 mL syringe with needle
4. IV catheter (wing needle) 27G
5. Sterile cotton balls
6. Sterile aluminium foil
7. Small and big autoclavable bags
8. Used newspaper
9. Sterile gauge
10. 70 % ethanol spray
11. Sterile three ways stop cock
12. Sterile 15 and 50 mL centrifuge tube
13. Sterile 2 ml, 5 ml, 10 ml and 25 ml serological pipet
14. 10 ml wide mouth serological pipet
15. Sterile 1.5 ml microtubes
16. 100 um nylon cell sieve
17. Micropipette and tips

Work sheet:

Date : _____
Name of officer : _____
Type/weight of rat : _____
Start at : _____

sticker

Researcher :

A. ANESTHESIA AND SHAVING

- ☐ Anesthesia rat with Ketamil (80 mg/kg) ____ (target: 0.15 ml) and Xilazil 2% (5 mg/kg weight body) ____ (target: 0.05 ml) intraperitoneally.
- ☐ Shave the rat's abdominal hair then place the rat on the surgical pad (*Note: Transfer to Operation Room*).

B. PERFUSION AND LIVER ORGAN PROCUREMENT

- ☐ Decontaminate the operation area with providone iodine solution. Cut abdominal skin and inner muscle with a scissor, starting from the bottom, make diagonal cut to the left and right side of the animal (diagonally), up to but not through the diaphragm. Pull the abdominal skin and muscle over the top of the animal, exposing the abdomen.
- ☐ Using sterile fingers, sweep the intestine and stomach to the right side of the animal, exposing the veins. Cover the bottom and left side of the animal with sterile gauze or cloth.
- ☐ Inject the heparin solution _____ (target: 0.3 ml), to avoid blood coagulation, into the Inferior Vena Cava (IVC) using 1 ml syringe. Hint: Pull back the IVC while injecting the needle into the vein. Wait for one minute before performing the perfusion.
- ☐ Construct the sterile perfusion equipment according to the following scheme. Switch on the peristaltic pump to start pumping 0.5 mM EGTA Solution, pre-warmed (37°C) and bubbled with carbogen gas, target volume ____ (target: 60 ml), from the lowest rate _____ (target: 3 ml/min). Start: _____

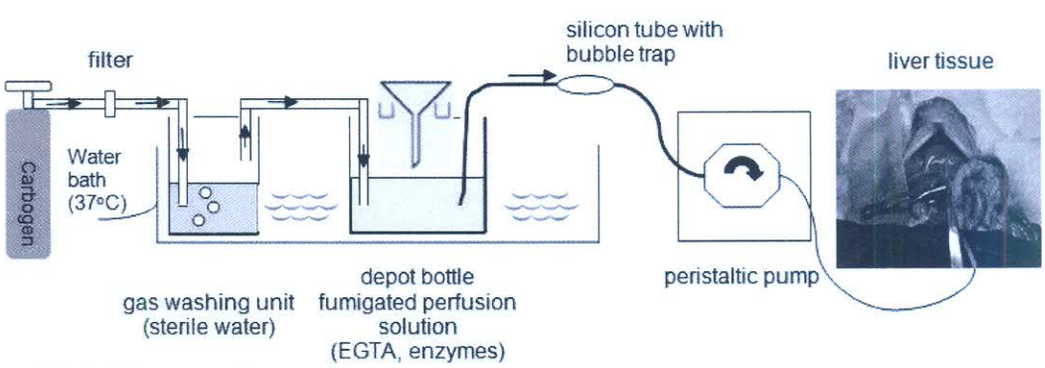

Fig. 1. Schematic drawing of the perfusion equipment.

- ☐ Insert the IV cat needle (size: 27G)) into the portal vein and fix the iv cat needle position with a *Bulldog Clamp*.
- ☐ Cut the Inferior Vena Cava (critical point!).
- ☐ Increase the rate to _____ (target: 10 ml/min) and then to _____ (target: 15 ml/min). The liver should change from red to light/pale yellow color rapidly – note a splotchy looking liver means the animal died prior to perfusion and may result in poor cell count, carefully massage liver to flush red spots if necessary.
- ☐ Rat's Liver Observation:

- ☐ Pipette warm washing buffer into the abdominal area carefully to flush clotting blood pools and keep organ warm during perfusion.
- ☐ Switch the perfusion tubing into freshly prepared pre-warmed 50 U/ml Collagenase Solution (CS) _____ (target: 70 ml) and continue the perfusion with rate _____(target: 10 ml/min) Start: _____
- ☐ Pipette warm DMEM into the abdominal area carefully to flush the blood and to keep the organ warm during perfusion. Block the IVC (1-2 times) to make sure the perfusion went well, the liver organ will swell.
- ☐ Liver should start to look mushy after 20 mls or so. Finish all CS: _____
  (*Tips: Total perfusion time should be less than 10-12 minutes*).
- ☐ When the perfusion is completed, remove the *bulldog clamp* and IV cat needle and carefully excise the liver.
- ☐ Carefully place the liver on chilled petri dish containing 20-30 ml cold CS and head to the tissue culture hood in ice box. Start: _____
- ☐ After organ procurement, the abdomen wall is closed (using PDS II 5-0 and silk 4-0 running sutures

C. HEPATOCYTES ISOLATION AFTER PERFUSION

- ☐ Weigh and perform sterility test on CASO agar for the liver organ and the media _____ gr
- ☐ Still in the petri dish containing cold CS, gently pull off the lobular capsule membranes with forceps and shake the tissue to disperse the hepatocytes into the solution.
- ☐ Sieve the cells through tea strainer (do not squeeze).
- ☐ SIeve cell dispersion by pipetting through a 100um Nylon Mesh Filter into a 50 ml conical tube.
- ☐ Spin at 60G for 5 min at 4°C.
- ☐ While spinning, remove and weigh the large central tree of connective and vascular tissue, and any undigested tissue or connective tissue.
  Discarded tissue: _____ gr
- ☐ Discard supernatant and re-suspend cells in 30 ml cold complete William E media (without FBS) containing 1% Ab/Am.
- ☐ Spin at 80G for 5 min at 4°C.

- ☐ Discard supernatant and re-suspend cells in 30 ml cold complete William E media (without FBS) containing 1% Ab/Am.
- ☐ Spin at 80 or 100G (depends on cells pellet obtained) for 5 min at 4°C.
- ☐ Discard supernatant and re-suspend cells in adequate volume of cold complete William E containing 1% Ab/Am and 10% FBS.
- ☐ Count the cells with tryphan blue exclusion method.
- ☐ Culture the cell in flask T25/T75
- ☐ Incubate at 37°C, 5% CO_2 for 3-4 hours
- ☐ Then change the medium with pre-warmed complete fresh medium (William E containing 1% Ab/Am and 10% FBS or Hepatocytes Basal Medium (HBM™) complete with 20% FBS)
- ☐ Incubate at 37°C, 5% CO_2, until further use

C. Cells counting in a Neubauer-Tuerk improved chamber:

- ☐ Mix tryphan blue and cell suspension dilution: ____ ul Tr.B + ____ ul cell suspension (1: __)
- ☐ Leave the Tr.B-cell suspension for 1-2 mins before counting.
- ☐ Use trypan blue 0.22% for 10x dilution and 0.36% for 2x dilution. If debris is too much that can interfere the counting, dilute for the second time the Tr.Blue-cells mixture with PBS into higher dilution.

  PBS re-dilution: _____ ul Tr.B & cell susp mix + ____ ul PBS* (1: __)
- ☐ **Counting formula**

  ***Cells per ml** = (viable + dead) cells/square x dilution factor x common factor (Improved Neubauer: 10^4)

  ***Total cells** = cells / ml x total volume

  ***Viability** = viable cells / (viable + dead) cells x 100%
- ☐ **Isolated cells (IC) counting**

  Counter I : ☐☐

  Total volume of cells suspension : _____ ml

  Dilution factor : _____

  Viable cells : _____/_____ squares

  Dead cells : _____/_____ squares

  Viable + dead cells : _____/_____ squares

  Hepatocytes per ml : _____

  Total hepatocytes : _____

  Viability : _____%

Counter II : ☐☐

Total volume of cells suspension : _____ ml
Dilution factor : _____
Viable cells : _____/_____ squares
Dead cells : _____/_____ squares
Viable + dead cells : _____/_____ squares

Hepatocytes per ml : _____
Total hepatocytes : _____
Viability : _____%

Resume
Hepatocytes per ml : _____
Total hepatocytes obtained : _____
Viability : _____ **%**

APPENDIX: **BUFFER AND SOLUTIONS**

Washing buffer

☐ Weigh required salts in accordance with the target volume;

Salts	Final concentration	Quantity / 1 L vol.	Quantity / ___ L vol
NaCl (Sigma cat# S9625)	137 mM	8 g	_____ g
KCl (Sigma cat# P4504)	2.68 mM	0.2 g	_____ g
$Na_3PO_4 12H_2O$ (Sigma cat# S7778)	0.7 mM	0.266 g	_____ g
Glucose (Sigma cat# G8270)	10 mM	1.8 g	_____ g

☐ Pour all salts in a beaker glass, add sterile Millipore water up to 90% of target volume: _____ L

0.5 mM Ethylene Glycol Tetraacetic Acid / EGTA Solution (ES)

☐ Add _____mL of 10 mM sterile EGTA solution in _____mL sterile washing buffer

☐ Add _____mL AB/AM for 1% final concentration

☐ Add _____mL of 1 M Hepes for 5-10 mM final concentration

50 U/ml COLLAGENASE SOLUTION (CS)

☐ Weight collagenase type IV _____ mg to get 50 U/mL for _____ mL complete low glucose DMEM media (depends on units content on each batch)

☐ Add collagenase into 50 mL centrifuge tube containing 20 mL DMEM media. Pipet and mix until completely dissolve.

☐ Sterilize the mixture through 0.22 uM syringe filter into 250 mL glass bottle containing _____ mL sterile DMEM media.

☐ Add _____ mL AB/AM (final conc 1%) and store at 4°C

☐ Add _____mL of 1M Hepes for 5-10 mM final concentration and store at 4°C

Note: It's better to use freshly prepared or at least one day before of collagenase solution

REPUBLIK INDONESIA
KEMENTERIAN HUKUM DAN HAK ASASI MANUSIA

SURAT PENCATATAN
CIPTAAN

Dalam rangka pelindungan ciptaan di bidang ilmu pengetahuan, seni dan sastra berdasarkan Undang-Undang Nomor 28 Tahun 2014 tentang Hak Cipta, dengan ini menerangkan:

Nomor dan tanggal permohonan	:	EC00201810578, 30 April 2018
Pencipta		
Nama	:	**Siufui Hendrawan**
Alamat	:	Jl. Letjen S. Parman No.1, Jakarta, Dki Jakarta, 11440
Kewarganegaraan	:	Indonesia
Pemegang Hak Cipta		
Nama	:	**Siufui Hendrawan**
Alamat	:	Jl. Letjen S. Parman No.1, Jakarta, Dki Jakarta, 11440
Kewarganegaraan	:	Indonesia
Jenis Ciptaan	:	**Buku Panduan/Petunjuk**
Judul Ciptaan	:	**Rat Liver Perfusion And Hepatocytes Isolation**
Tanggal dan tempat diumumkan untuk pertama kali di wilayah Indonesia atau di luar wilayah Indonesia	:	2 November 2017, di Jakarta
Jangka waktu pelindungan	:	Berlaku selama hidup Pencipta dan terus berlangsung selama 70 (tujuh puluh) tahun setelah Pencipta meninggal dunia, terhitung mulai tanggal 1 Januari tahun berikutnya.
Nomor pencatatan	:	000107024

adalah benar berdasarkan keterangan yang diberikan oleh Pemohon.
Surat Pencatatan Hak Cipta atau produk Hak terkait ini sesuai dengan Pasal 72 Undang-Undang Nomor 28 Tahun 2014 tentang Hak Cipta.

a.n. MENTERI HUKUM DAN HAK ASASI MANUSIA
DIREKTUR JENDERAL KEKAYAAN INTELEKTUAL

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