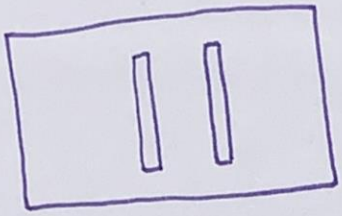
LABORATORY NOTEBOOK

028

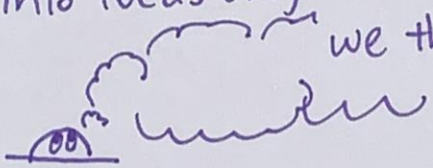
先人後己的無我心
先捨後得的慈悲心
珍愛大地萬物的惜福心
當下就行就做的實踐行動

大地不生無用之物
斷枝落葉也可以是藥材，為眾生所用



when you are the observer, you can change events that have already happened.

everything is fundamentally blurry. objects exist in a small state of probability and snap into focus only when observed.



before you look at an object, it is not in any definite location.

an object's behavior depends on whether or not someone is looking at it! 😮😮

Love is the most powerful force
Imagination is the richest wealth

"What we sow in this life,
we reap in our future lives.
This is our karma." XWP S4E15

LABORATORY NOTEBOOK

Company Name: CMRL

Assigned To: Denise Itsu

Department: BME

Notebook No: 028

INSTRUCTIONS

1. This notebook and all the information recorded therein are the sole property of this Company. The contents of this notebook are strictly confidential and may be disclosed to others only with the written permission of the Company. The employee must return this notebook upon request or termination of employment. Keep this notebook in a protected place to prevent loss. In the event of loss, notify your supervisor immediately and draft a written statement describing the contents of the notebook and the manner in which it was lost.

2. This notebook is intended to be a permanent record of your lab or field work. In order to fully protect your work and achieve the desired recognition, either academic or economic, you will need to pay careful attention to the manner in which you record entries in this notebook.

3. Write in concise and clear language and write everything down. Draw and diagram directly on these pages. To include a printout: label the printout, attach it securely to the notebook page, and then write a brief description of the printout in the notebook directly below the place where it is attached. All note should be made in the book, not on loose pages stuck inside the notebook. Be clear and safeguard your work.

4. Use only ink when marking in this notebook. Pencil markings should be avoided. To delete an error: draw a single line through the error and place your initials and the date close by.

5. The title, date and project name should be recorded at the start of each entry. Make sure you write down your full name, lab location and company or institution in the front of the notebook.

6. Begin your entries by explaining in chronological order exactly what procedure were used and in what order you completed the various steps. Date your entries. Specify the equipment and consumable materials that were used, preferably by manufacturer and part number. Describe completely and accurately exactly what results were achieved, and at what stages, including the time. It is always better to include too much detail in your entries, than too little. If you make a scientific discovery, or invent a product, these details may be very important to proving any new discoveries.

7. Witnesses are important in cases where new concepts or approaches are determined. Also, where new discoveries are made, or where the potential exists for a patent. In those cases, at least one witness who is not a co-discoverer should sign and date in the indicated space at the bottom of the relevant work sheets. The witness needs to be able to understand and describe the basic procedures and the results they observed.

8. Patentable subject matter may appear in the course of your work. Any new and surprising product, composition or method may be patentable. Be especially alert for patentability when results appear strange, interesting or of commercial importance. Protect the results by writing in your notebook: a) what the result is, b) why the result is significant, and c) how the result was produced.

9. The time between the actual experiment or procedure and the time that you record your findings should be minimized. Act immediately in order to fully record your findings.

10. Separate your notes for each long term project into separate notebooks. Do not use a single page to record observations or procedures from more than one subject.

Assigned To _____ Date_____ Notebook No._____

Returned To _____ Date_____ By _____

Transferred To _____ Date_____ By _____

Continued From Notebook Number _____Date _____

Continued To Notebook Number _____ Date _____

6/10/2019: Bioreactor tissue RNA extraction
Static PSIS 5 scaffolds
PGAPLLA 3 scaffolds
Bioreactor PSIS 3 scaffolds
PGAPLLA 3 scaffolds

RNA Concentrations:

PSIS Static (ng/μL)

①	3	3.4	3.2	] 4.7
②	1.9	4.7	3.3	
③	3.4	4.2	7.6	

PSIS Bioreactor (ng/μL)

①	2.9	2.1	2.5	] 4.05
②	4.7	6.5	5.6	

PGA-PLLA Static

①	4.9	5.3	5.1	] 5.925
②	9.2	4.3	6.75	

PGA-PLLA Bioreactor

①	40	4.3	4.15	] 17.06
②	30.3	31.5	30.9	

6/12/2019: Heated up RNA to 60°C for 10-15 minutes. Remeasured RNA concentration (suggested by AB)

RNA Requantification

PSIS Static (ng/μL) (μL left)

①	24.9	24.6	24.75	13
②	13.5	14.6	14.05	14
③	14.6	14.4	14.5	14

PSIS Bioreactor (ng/μL) (μL left)

①	10.4	10.9	10.65	14
②	49.8	56.2	53	14

PGA-PLLA Static

①	15.7	13.7	14.7	14
②	12.1	12.4	12.25	14

PGA-PLLA Bioreactor

①	14.6	17.6	16.1	14
②	136.6	154.7	145.65	12

COMBINED CONCENTRATION & VOLUME

PGA PLLA Static 377.3
13.475 ng/μL & 28 μL

PSIS Static 707.25
17.25 ng/μL & 41 μL

PGA PLLA Bioreactor 1973.14
75.89 ng/μL & 26 μL

PSIS Bioreactor 891.1
31.825 ng/μL & 28 μL

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Targets: GAPDH, FzD2, MCL1F, KLf2A, αSMA, PECAM1, Col1a1, ELN, B3GAT3, BMP2
Samples: PSIS Static, PSIS Bioreactor, PGAPLLA Static, PGAPLLA Bioreactor
# of replicates: 1

10 targets × 4 samples × 1 replicate(s) = 40 wells

	1	2	3	4	5	6	7	8
A	PSIS, S GAPDH	PSIS, S Col1a1	PSIS, B GAPDH	PSIS, B Col1a1	PGA, S GAPDH	PGA, S Col1a1	PGA, B GAPDH	PGA, B Col1a1
B	PSIS, S FzD2	PSIS, S ELN	PSIS, B FzD2	PSIS, B ELN	PGA, S FzD2	PGA, S ELN	PGA, B FzD2	PGA, B ELN
C	PSIS, S MCL1F	PSIS, S B3GAT3	PSIS, B MCL1F	PSIS, B B3GAT3	PGA, S MCL1F	PGA, S B3GAT3	PGA, B MCL1F	PGA, B B3GAT3
D	PSIS, S KLf2A	PSIS, S BMP2	PSIS, B KLf2A	PSIS, B BMP2	PGA, S KLf2A	PGA, S BMP2	PGA, B KLf2A	PGA, B BMP2
E	PSIS, S αSMA		PSIS, B αSMA		PGA, S αSMA		PGA, B αSMA	
F	PSIS, S PECAM1		PSIS, B PECAM1		PGA, S PECAM1		PGA, B PECAM1	

PSIS, Static

$20\,ng = 17.25\ ng/\mu L \times V_1$

$V_1 = \frac{20}{17.25} = 1.159\ \mu L$

PSIS, Bioreactor

$20\,ng = 31.825\ ng/\mu L \times V_2$

$V_2 = \frac{20}{31.825} = 0.628\ \mu L$

PGAPLLA, Static

$20\,ng = 13.475\ ng/\mu L \times V_3$

$V_3 = \frac{20}{13.475} = 1.484\ \mu L$

PGAPLLA, Bioreactor

$20\,ng = 75.89\ ng/\mu L \times V_4$

$V_4 = \frac{20}{75.89} = 0.264\ \mu L$

1. RNA to C_T RT MM
   5.0 µL Power SYBR Green RT-PCR Mix (2×)
   0.08 µL RT enzyme
   5.0 µL × 44 wells (40+4) = 220 µL SYBR Green
   0.08 µL × 44 wells = 3.52 µL RT Enzyme

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2. Primers - Use 1 uL of the 10 uM stock for both FWD & REV
1 replicate × 4 samples = 4 primer pairs = 8 uL
1 uL FWD + 8 uL DEPC H_2O = 10 uL total
1 uL REV
Make primer tubes @ 10 uL total (forward + reverse) for:

GAPDH	KLF2A	Col1a1	BMP2
Fz D2	αSMA	ELN	
MCL1F	PECAM1	B3GAT3	

3. RNA Samples (for 10 uL reactions)
5.0 uL SYBR Green
0.08 uL RT Enzyme
1 uL FWD primer
1 uL REV primer
7.08 uL
→ 10 - 7.08 = 2.92 uL
this is the amount of RNA + DEPC H_2O

PSIS, Static (10+1)

1.159 uL × 11 wells = 12.749 uL
7.08 + 1.159 = 8.239 uL (fill the rest w/ DEPC H_2O up to 10 uL)
10 - 8.239 = 1.761 uL DEPC H_2O per well
1.761 × 11 wells = 19.371 uL DEPC H_2O
✡ 12.749 uL [PSIS, Static] + 19.371 uL DEPC H_2O

PSIS, Bioreactor

0.628 uL × 11 wells = 6.908 uL
7.08 + 0.628 = 7.708 uL
10 - 7.708 = 2.292 uL DEPC H_2O per well
2.292 × 11 wells = 25.212 uL DEPC H_2O
✡ 6.908 uL [PSIS, Bioreactor] + 25.212 uL DEPC H_2O

PGAPLLA, Static

1.484 uL × 11 wells = 16.324 uL
7.08 + 1.484 = 8.564 uL
10 - 8.564 = 1.436 uL DEPC H_2O per well
1.436 × 11 wells = 15.796 uL DEPC H_2O
✡ 16.324 uL [PGAPLLA, Static] + 15.796 uL DEPC H_2O

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PGAPLLA, Bioreactor

0.264 µL × 11 wells = 2.904 µL
7.08 + 0.264 = 7.344 µL
10 − 7.344 = 2.656 µL DEPC H_2O
2.656 × 11 wells = 29.216 µL DEPC H_2O

✡ 2.904 µL [PGAPLLA, Bioreactor] + 29.216 µL DEPC H_2O

TUBE

1. 12.749 µL [PSIS, S] + 19.371 µL DEPC H_2O
2. 6.908 µL [PSIS, B] + 25.212 µL DEPC H_2O
3. 16.324 µL [PGAPLLA, S] + 15.796 µL DEPC H_2O
4. 2.904 µL [PGAPLLA, B] + 29.216 µL DEPC H_2O
5. 220 µL SYBR Green + 3.52 µL RT Enzyme
6. GAPDH: 1 µL FWD + 1 µL REV + 8 µL DEPC H_2O
7. FzD2
8. MCL1F
9. KLF2A
10. αSMA
11. PECAM1
12. Col1a1
13. ELN
14. B3GAT3
15. BMP2

Pipette Volume (µL)
2.92
5.08
2

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StepOne™ Software v2.1

le Edit Instrument Analysis Tools Help

New Experiment Open... Save Close | Send Experiment to Instrument... Download Experiment from Instrument... | Export... Print Report...

Experiment Menu

Setup

Run

Analysis

Amplification Plot

Gene Expression

Multicomponent Plot

Melt Curve

Raw Data Plot

QC Summary

Multiple Plots View

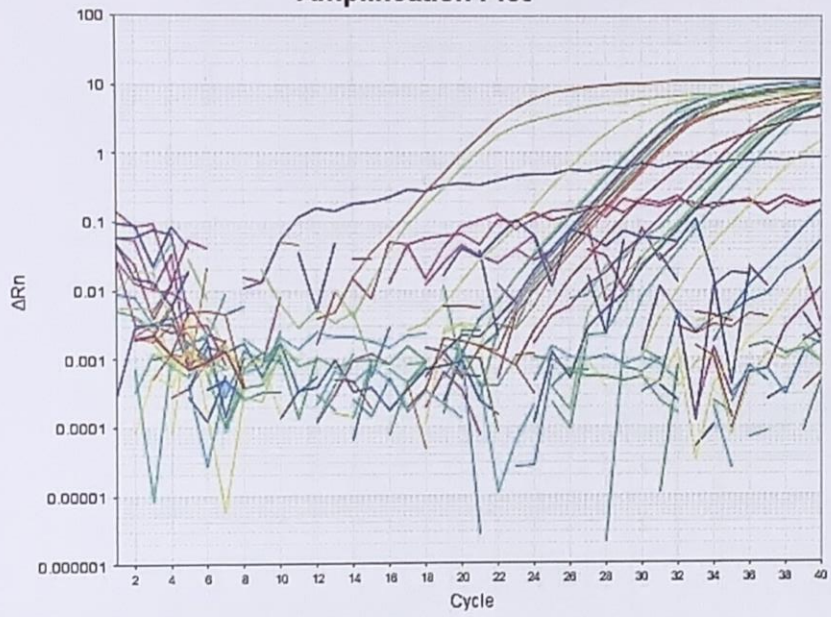
Experiment: DH_Bioreactor_06-13-2019 Type: Comparative CT (ΔΔCT) Reagents: SYBR® Green Reagents Analyze Analysis Settings

Amplification Plot

Plot Settings

Plot Type: ΔRn vs Cycle Graph Type: Log Plot Color: Well

Save current settings as the default



Legend A B C D E F

Options

Target: All Threshold: Auto Auto Baseline

Show: Threshold Baseline Start: Well Target Baseline End: Well Target

View Plate Layout | View Well Table

Select Wells With: - Select Item - - Select Item -

Show in Wells View Legend

	1	2	3	4	5	6	7	8
A	PSIS, S GAPDH CT: 22.6	PSIS, S Col1a1 CT: Undete.	PSIS, BR GAPDH CT: Undete.	PSIS, BR Col1a1 CT: Undete.	PGA, S GAPDH CT: 11.03	PGA, S Col1a1 CT: 30.69	PGA, BR GAPDH CT: 21.17	PGA, BR Col1a1 CT: 29.9
B	PSIS, S FzD2 CT: Undete.	PSIS, S ELN CT: Undete.	PSIS, BR FzD2 CT: 36.95	PSIS, BR ELN CT: Undete.	PGA, S FzD2 CT: 25.07	PGA, S ELN CT: 29.89	PGA, BR FzD2 CT: 19.07	PGA, BR ELN CT: 36.34
C	PSIS, S MCL1F CT: Undete.	PSIS, S B3GAT3 CT: Undete.	PSIS, BR MCL1F CT: Undete.	PSIS, BR B3GAT3 CT: Undete.	PGA, S MCL1F CT: 35.89	PGA, S B3GA... CT: 30.7	PGA, BR MCL1F CT: 35.26	PGA, BR B3GA... CT: 36.95
D	PSIS, S KLF2A CT: Undete.	PSIS, S BMP2 CT: Undete.	PSIS, BR KLF2A CT: Undete.	PSIS, BR BMP2 CT: Undete.	PGA, S KLF2A CT: 29.9	PGA, S BMP2 CT: 24.56	PGA, BR KLF2A CT: Undete.	PGA, BR BMP2 CT: 37.12
E	PSIS, S aSMA CT: Undete.	PSIS, S GAPDH CT: Undete.	PSIS, BR aSMA CT: Undete.	PSIS, BR GAPDH CT: Undete.	PGA, S aSMA CT: 35.9	PGA, S GAPDH CT: 7.9	PGA, BR aSMA CT: 29.94	PGA, BR GAPDH CT: 33
F	PSIS, S PECAM1 CT: Undete.	PSIS, S GAPDH CT: Undete.	PSIS, BR PECAM1 CT: Undete.	PSIS, BR GAPDH CT: 13.87	PGA, S PECA... CT: 31.89	PGA, S GAPDH CT: Undete.	PGA, BR PECA... CT: 35.81	PGA, BR GAPDH CT: 15.12

Wells: U 48 Unknown N 0 Negative Control 0 Empty

Analysis Summary: Total Wells in Plate: 48 Wells Set Up: 48 Wells Omitted Manually: 0 Wells Flagged: 32 Wells Omitted by Analysis: 0 Samples Used: 4 Targets Used: 1

Home DH_Bioreactor_06-13-2019.eds

Local Instrument : connected.

start StepOne™ Software ...

7/12/2019: RNA extraction (samples F1 & F3)
Nanodrop quantification

	Read 1	Read 2	(ng/μL)
F1	46.2	45.1	
F3	337.3	340.0	

Email from William (UM RNA Seq contact) indicated F1 & F3 were out of range. Submitted the above two replacements to UM on 7/15.

8/26/2019: RNA Seq. data from UM (Raw Data) received
9/13/2019: Slaughterhouse VEC/VIC harvest, porcine cells
Two 3-cm plates of VEC. P0. in endothelial media
9/17/2019: changed media (Porcine VEC, P0)
9/20/2019: changed media (Porcine VEC, P0)
* cell type to be determined. They look elongated, might not be VEC
9/23/2019: VEC? passaged P0 → P1, in regular DMEM
Aliquotted into 3 wells in a 24-well plate for immunostaining
9/27/2019: Passaged P1 → P2 (1 plate → 4 plates), regular DMEM
PAVEC? PAVIC? (TBD)
10/1/2019: PAVIC (or could be VEC) P2 changed media (regular DMEM)
The above cells are used for Cell/Tissue class (Fall 2019)

11/5/2019: Received RooserBio kit
HBMSCs 1 vial (MSC-031)
Media 1 bottle 500mL (SU-005)
Supplement 1 bottle 10 mL (SU-016)
} details on following pages
Protocols for RooserBio HBMSC culture can be found on pg. 95.
Plated 6 flasks of HBMSCs (T75's)
11/8/2019: Media change 6 flasks (T75)
11/11/2019: Trypsinized 6 T75 flasks containing HBMSCs in P2
Counted # of cells in 2 flasks:
Count 1: 1.88×10^6 cells/mL
Count 2: 1.35×10^6 cells/mL
AVG: 1.615×10^6 cells/mL
} total volume of cell suspension: 11 mL
Want 2.2 million cells in T75 for static group for 48 hours in T75.
2,200,000 cells = 1,615,000 cells/mL × V

$$V = \frac{2.2}{1.615} = 1.36 \text{ mL}$$

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7/12/2019
Date

RoosterBio®
RoosterVial™-hBM-1M-XF
Part: MSC-031 Lot: 00227
Store at -150 to -196°C
For Research Use Only
Made in the USA
www.roosterbio.com

↑ sticker from vial

RoosterBio®

CERTIFICATE OF ANALYSIS

Product Name: **RoosterVial™-hBM-1M-XF**
Part Number: **MSC-031**

Product Information	
Lot Number	00227
Tissue Origin	Human Bone Marrow
Donor Age	29
Donor Sex	Female
Storage Conditions	LN_2 Vapor Phase
Date of Manufacture	23 OCT 2018
Date of Expiration	23 OCT 2021

Assay	Method	Specification		Result	
Mycoplasma	Detection by PCR	Negative		Negative	
Sterility	Direct Inoculation per USP <71>	No Growth		No Growth	
Viable Cell Count	Automated Count	≥ 1 million viable cells		Conforms	
Performance Test	Cell Growth Promotion	≥ 10-fold expansion within 7 days		Conforms	
Surface Marker Expression	Multiplex Flow Cytometry	CD34	≤ 10%	CD34	0.2%
		CD45	≤ 10%	CD45	0.0%
		CD90	≥ 90%	CD90	100.0%
		CD166	≥ 90%	CD166	99.9%
Adipogenic Differentiation	Oil Red O Staining	Positive Staining		Positive Staining	
Osteogenic Differentiation	Alizarin Red Staining	Positive Staining		Positive Staining	

For Research Use Only. Not intended for diagnostic or therapeutic use.
RoosterVial™-hBM-1M-XF contains human sourced material; treat as potentially infectious.

The test results cited above meet all quality specifications.

Approved by: _____ 18Dec2018
Quality Assurance Date

RoosterBio®

CERTIFICATE OF ANALYSIS

Product Name: **RoosterBasal™-MSC**
Part Number: **SU-005**

Product Information	
Lot Number	00241
Storage Conditions	2°C to 8°C (protect from light)
Date of Manufacture	13 MAR 2019
Date of Expiration	13 MAR 2020
Volume	500 mL

Assay	Method	Specification	Result
Sterility	Membrane Filtration	Negative	Negative
Endotoxin	LAL	≤ 1.00 EU/mL	< 0.050 EU/mL
pH	Measurement	6.8 – 7.3	7.1
Osmolality	Measurement	283 – 309 mOsm/kg H_2O	298 mOsm/kg H_2O
Performance Test	Cell Growth Promotion	Pass	Pass

For Research Use Only. Not intended for diagnostic or therapeutic use.

RoosterBasal™ -MSC contains human source material; treat as potentially infectious.

The test results cited above meet all quality specifications.

Approved by: _____
Quality Assurance

11Apr2019
Date

Read and Understood By

Signed _____ Date _____

Signed _____ Date 11/5/2019

RoosterBio®

CERTIFICATE OF ANALYSIS

Product Name: **RoosterBooster™-MSC-XF**
Part Number: **SU-016**

Product Information	
Lot Number	00259
Date of Manufacture	01 AUG 2019
Date of Expiration	01 AUG 2021
Storage Conditions	-20°C (protect from light)
Volume	10 mL

Assay	Method	Specification	Result
Sterility	Membrane Filtration per USP <71>	No Growth	No Growth
Performance Test	Cell Growth Promotion	≥ 10-fold expansion within 7 days	Pass

For Research Use Only. Not intended for diagnostic or therapeutic use.
RoosterBooster™-MSC-XF contains human sourced material; treat as potentially infectious.

The test results cited above meet all quality specifications.

Approved by: _____
Quality Assurance

28 Aug 2019
Date

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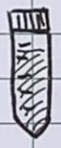
_____ _____
Signed Date

_____ 11/5/2019
Signed Date

11/11/2019 (con'd): Plated 3 T75's at 1.5 mL cell suspension each.
Filled the flasks w/ fresh DMEM to 10 mL total per flask
DMEM used for experimentation:

Hyclone DMEM/HIGH GLUCOSE
+ 4.00 mM L-Glutamine
+ 4500 mg/L Glucose
- Sodium Pyruvate

Cat No: SH 30022.01
Lot No: AE27902269
Bottle No: 03286
Volume: 500 mL
EXP DATE: MAY/2020



BCS (10%) - 50 mL
LOT: C98036

P/S (1%) - 5 mL

Plated 6.5 mL of cell suspension across 2 Bioflux plates (8 channels per plate, so 16 channels in total.) Each channel had 400 µL of cell suspension, in addition to 5% of gelatin (20 µL of gelatin per channel).
Passaged the rest of the 4 T75 flasks into 9 T75's. (P2 → P3)
Of which 4 was cultured in regular DMEM (same DMEM as above)
3 was cultured in Biorooster media w/ Biorooster protocol (protocol indicated to place cell suspension in fridge of 1 hr before re-plating - see pg. 94 for details)
2 was cultured in Biorooster media without fridge placement.
P4

11/12/2019: Started Bioflux experiment
0.25 OSI Start time: 6:00pm
11/13/2019: Froze 2 2-mL vials of regular DMEM T75 flasks (4 flasks)
Froze 2 2-mL vials of RoosterBio no fridge T75 flasks (2 flasks)
5:40pm collected static cultures in TRIzol (n=3)
6:00pm Repipetted media into input wells
11/14/2019: Trypsinized 2 flasks (out of 3 that was cultured in Biorooster media)

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and splitted them into 6 T75 flasks. (P3 → P4)
Plated the last T75 in two Bioflux plates
Cell count 1: 1.75×10^6 cells/mL
count 2: 9.66×10^5 cells/mL > AVG = 1.358×10^6 cells/mL
Want: 2,200,000 cells per Bioflux plate
2,200,000 cells = 1,358,000 cells/mL × V

$$V = \frac{2.2}{1.358} = 1.62 \text{ mL}$$

Plated 2 Bioflux plates @ 8:30pm. Placed the rest in 1 T125.
8:30pm terminated 0.25 OSI, placed in 3 vials of TRIzol.
9:00pm started steady flow (0 OSI).

11/15/2019: 8:00am redistributed media from output wells to input wells.
3:00 pm redistributed media
9:00 pm redistributed media

11/16/2019: 9:00pm stopped steady flow, collected RNA samples in TRIzol
10:00pm started 0.50 OSI

11/18/2019: 9:00am repipetted media into input wells.
9:05am resumed flow
11:00am Froze 4 T75 flasks in Mr. Frosty
Freezing media: gibco Exp 2020-04-30
Cat No. 12646-010
Lot No. 2090206

11/18/2019: 4:00pm suspended 2 T75's
Cell count 1: 6.84×10^5 cells/mL > AVG: 7.625×10^5 cells/mL
Cell count 2: 8.41×10^5 cells/mL
Want: 2,200,000 cells per Bioflux plate
2,200,000 cells = 7.625×10^5 cells/mL × V

$$V = \frac{2,200,000}{762,500} = 2.88 \text{ mL} \approx 3 \text{ mL per plate}$$

$$\frac{3 \text{ mL}}{8 \text{ channels}} = 0.375 \text{ mL per channel.}$$

Plated the rest in 4 T75's (P5)
9:00pm collected 0.50 OSI RNA in TRIzol.
10:00pm started 0.10 OSI.

11/19/2019: 7:00pm redistributed wells.
7:05pm resumed 0.10 OSI flow

萬千
里江
無雲
萬水
里千
天月

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Signed _____ Date _____ Signed _____ Date 11/14/2019

11/20/2019: 10:00pm terminated 0.1 OSI experiment
stored RNA in TRIzol in -80°C
11/21/2019: 9:30am started 0.2 OSI experiment
passaged T175 flask P4 → P5
11/22/2019: 9:00am repipetted media into input wells
9:05am resumed flow (0.20 OSI)
9:00pm repipetted media into input wells & resumed flow @ 0.20 OSI
11/23/2019: 10:am stopped 0.20 OSI experiment. Stored RNA in TRIzol in -80°C
plated 1 Bioflux plate (trypsinized 1 T75 & 1 T175)

T75 < cell count 1: 7.49×10^5 cells/mL
cell count 2: 7.92×10^5 cells/mL > 7.705×10^5 cells/mL

T175 < cell count 1: 5.26×10^5 cells/mL
cell count 2: 3.8×10^5 cells/mL > 4.53×10^5 cells/mL

Average All: 6.1175×10^5 cells/mL

total volume = 4mL. $4 \times 6.1175 \times 10^5 = 24.47 \times 10^5$ cells/mL
pipetted 800 µL of cell suspension per channel
Bioflux plate seeding time: 11/23/2019 @ 5pm.
Started 0.30 OSI w/a previously seeded plate @ 6:00pm.

11/24/2019: 10:00am redistributed media, resumed flow
9:00pm redistributed media, resumed flow
11/25/2019: 3:00pm media change on T175 flask
passaged 1 T75 → 2 T75
froze 2 T75 w/ cryostor (CoA attached on next page)
placed in Mr. Frosty in -80
11/25/2019: 9:00pm collected 0.3 OSI RNA in TRIzol, stored in -80°C.
10:00pm started 0.4 OSI in Bioflux.
11/27/2019: 10:00pm stopped 0.4 OSI in Bioflux. Collected RNA in TRIzol, stored in -80°C
changed media. 1 T175 & 2 T75 flasks
↑ regular DMEM ↑ Rooster Bio media
12/1/2019: Passaged two T75's (P5) → five T75's (P6)
12/4/2019: changed media 1 T175
12/5/2019: Froze 5 T75's into 6mL of Cryostor, placed in 3 vials in -80 in Mr. Frosty.
12/6/2019: Moved frozen vials into Liq. N_2

Continued on Page

Read and Understood By

_____ Signed _____ Date

_____ Signed 11/20/2019 Date

BIOLIFE SOLUTIONS®
BIOPRESERVATION TOOLS FOR CELLS, TISSUES, AND ORGANS

3303 Monte Villa Parkway, Suite 310
Bothell, WA 98021 USA
425.402.1400 | 425.402.1433
BioLifeSolutions.com

CERTIFICATE OF ANALYSIS AND RELEASE

Product	CryoStor® CS5	Fill Volume/Packaging	100mL Bottle
Lot Number	19140	Part Number	205102
Manufacture Date	May 2019	Expiration Date	May 2021

Test Results

Test	Method	Acceptance Criteria	Results
Appearance	Visual Inspection	Clear, colorless to slightly yellow liquid with no visible particulates	Conforms
pH	TM 5110 USP <791>	7.5 to 7.7	7.6
Metabolic Activity Assay	TM 5100	Cell viability is 75% to 200% of cells preserved in the internal standard at Day 1 recovery following preservation.	109
Endotoxin	Kinetic Chromogenic USP <85>	≤ 0.5 EU/mL	Conforms
Sterility	Membrane Filtration USP <71>	Sterile	Conforms
Identification	TM 5111 FT-IR	Conforms to CryoStor CS5 Reference Standard	Conforms
Osmolality	TM 5112 USP <785>	1360-1390 mOsm/kgH_2O	1379
Specific Gravity	TM 5114	1.055 – 1.063	1.059
DMSO Content	Gas Chromatography (FID)	4.0% - 7.0%	5.3

Material was manufactured under cGMP, meets all requirements and is approved for release.

BioLife Solutions Quality Assurance

13JUN2019
Date

Continued on Page

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Signed 11/25/2019
Date

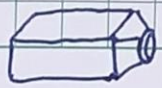
1/8/2020: Received Rat valve endothelial cells from Celprogen (P 1)
Contents: Rat VEC in T25 flask. lot# 20010301. cat# 12128-20-T25
Rat valvular Endothelial Primary Cell Culture Complete Growth Media w/ Serum & Antibiotics
CAT# M12128-20S
LoT# 20010359 03-01
EXP. Jan. 2021
Rat Valvular Endothelial Cell Culture Extracellular Matrix T75 Flasks
CAT# E12128-20-T75
LoT# 20010315-05
EXP: Jan. 2021
Plated the Rat VEC's in 1 T75 flask

1/10/2020: Passaged Rat VECs, Seeded 2 Bioflux Plates (16 channels), started "static" conditions for 48 hrs. (start time: 3 pm)
cell Count 1: 7.47×10^6 cells/mL
Count 2: 7.44×10^6 cells/mL
} AVG: 7.455×10^6 cells/mL
seeded 2,200,000 cells per Bioflux plate (275,000 cells/channel)
seeded 800,000 cells per T75 flask for static conditioning (3 flasks)
Passaged the rest into 3 T75's (P2)

1/11/2020: 4pm started 0.50 OSI
Media used for experiment
HyClone DMEM/HIGH GLUCOSE
+4.00mM L-Glutamine CAT: SH30022.01
+4500 mg/L Glucose LOT: AE29422280
- Sodium Pyruvate EXP: 09/2020
Fetal Bovine Serum (10%) EXP: 03/2021
Cat# S1195
Lot# C16036
} Atlanta Biologicals
Pen/Strep (1%)
Fisher Scientific
Lot# 1989509

1/12/2020: 11 am paused 0.50 OSI, redistributed media. resumed at 11:05 am
4pm terminated static conditioning. Stored RNA in TRIzol in -80. and conditioned media.

1/13/2020:



→ froze 2 vials (CryoStor CS5 pg. 13)
→ passaged into 3 new T75's (P3)
→ plated 2 Bioflux plates

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Cell count of two confluent flasks
Count 1: 5.98×10^6 cells/mL
Count 2: 6.58×10^6 cells/mL
> 6.28×10^6 cells/mL

7pm: terminated 0.50 OSI. Stored RNA in TRIzol in -80.
stored conditioned media (centrifuged) in -80.
started 0.25 OSI.

1/14/2020: Plated 2 Bioflux from 1 confluent T75
Avg cell count: 5.68×10^6 cells/mL

1/15/2020: Aborted 0.25 OSI experiment. Media crawled up Bioflux tube.
Replaced filters and rinsed system, restarted 0.25 OSI @ 2:30pm.
Froze 2 confluent flasks into 4 vials.
Passaged 1 confluent flask into 3 T75's. (P3 → P4)
9:30pm paused 0.25 OSI, redistributed media, resumed flow at 9:35pm.

1/16/2020: Paused and redistributed 0.25 OSI. Resumed at 7:05 pm.

1/17/2020: From 6 confluent T75's, passaged 1 → 3 T75's. Put 1 into Static
Seeded 2 into two Bioflux plates
Froze 3 into 3 vials w/ CryoStor CS5 freeze media.
5:00 terminated 0.25 OSI, stored and centrifuged conditioned media.
5:30pm started steady flow (0 OSI)

1/18/2020: 9:00am redistributed media, resumed @ 9:05am.

1/19/2020: Terminated static group x1 & steady flow x1. Stored RNA in TRIzol in -80
Passaged 1 flask into 3 flasks. Froze 2 flasks in 3 vials.
Started 0.50 OSI @ 6pm.

1/20/2020: Froze 1 T75 flask into 1 vial.

1/21/2020: Terminated 0.50 OSI, stored RNA in TRIzol in -80. started 0.25 OSI @ 8:00 pm.

1/22/2020: 9:00 am redistributed media, resumed 0.25 OSI flow
3 T75 confluent flasks. Passaged 2 → 2 T75's + 3 static conditions
Seeded 1 into 2 Bioflux plates (P6 → P7)
Received Rat VIC cells from Innoprot
Cultured Rat VIC in regular DMEM
Cryopreserved Valvular Interstitial Cells
Cat #: P10462
# of cells: 5×10^5 cells/vial (1 mL)

Innoprot P10432
RAT AORTIC VALVE INTERSTITIAL CELLS
Batch # 200119 - Qty: >5 x 10^5 cells

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Read and Understood By

Signed Date Signed 1/13/2020 Date

Project

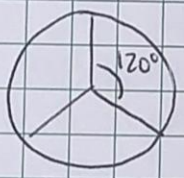
Continued from Page

P7→P8

1/24/2020: RAT VEC
- Three confluent flasks, passaged 1 T75 → 3 T75, 2 flasks into 2 Bioflux plates. Terminated 0.25 OSI, stored RNA in TRIzol in -80. Started 0 OSI steady flow @ 12:00 noon.
- Collected Static conditions into three vials in TRIzol, stored in -80.
- changed Rat VIC media.

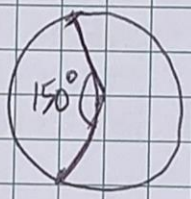
1/26/2020: Terminated 0 OSI steady flow. Started 0.50 OSI.
Passaged Rat VICs P1→P2.
Collected vivitro data for CorMatrix 26mm PSIS (2-ply) values.
n=3 for mitral & n=1 for aortic. Each "n" had 3 runs, each run consisted of 10 cardiac cycles, and each cycle contained 256 data points.
Waveform: S35 from Vivitest
Nominal conditions: ~~[illegible]~~
70 Beats per minute, SV = 71.4 ml/s
Mean arterial pressure = 100 mmHg
Pressure probe placement: Ventricular, Atrial, Aortic
Flow probe placement: mitral OR Aortic

~~AORTIC~~



three posts

MITRAL



two posts

valves were tubular shapes of diameter = 26 mm, length was 36 mm. They were sutured on two/three posts (mitral/aortic) and around the annulus on the other end.

1/27/2020: Flow of 0.50 went through pipe. Stopped flow and disposed plate. Washed tubings & restarted 0.50 OSI.

1/29/2020: Terminated 0.50 OSI, stored RNA in TRIzol in -80.
Started 0.25 OSI @ 9:00 pm.

1/31/2020: Flow went through pipe. Stopped flow and disposed plate. Restarted 0.25 OSI. Passaged Rat VICs P2→P3.

2/2/2020: Terminated 0.25 OSI, collected RNA in TRIzol, stored in -80.
Started 0 OSI (steady flow) @ 10:00 pm.

2/4/2020: Terminated 0 OSI, started 0.25 OSI @ 10:00 pm.
Collected 0 OSI VEC RNA in TRIzol, stored in -80.
Rat VEC, froze 3 vials (P8). Rat VIC, froze 1 vial (P3)
Changed Rat VIC media x 9 T75's.

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2/6/2020: Terminated 0.25 OSI (slightly early b/c media was pushed into tube)
Collected Rat VEC in TRIzol, stored in −80.
2/7/2020: Rat VICs changed media
2/10/2020: Froze 3 vials (P4) - VICs
2/11/2020: Media change (8 T75's) - VICs
2/14/2020: Media change (Rat VIC's) - 8 T75's by YM
2/17/2020: Rat VIC media change (8 T75's) by YM
2/18/2020: Froze 4 T75 flasks in 4 vials (P4)
Passaged 4 T75 flasks to 12 ~~(P4 → P5)~~ (P3 → P4)

RAT VIC CONDITIONS

Pro-calcifying Media — Rathan, Swetha et al. 2014 } combined recipe
DMEM, 5% FBS, 1% P/S — Goto, Shinji et al. 2019 }
1.8mM $CaCl_2$
3.8mM NaH_2PO_4
0.4 units (4ml per 10mL) inorganic Pyrophosphate (IP)

GROUP	CULTURE MEDIA CONTENT (7 days, 1 media change)
1	Regular DMEM
2	Pro-calcifying Media
3	Static from VEC + Regular DMEM
4	0 OSI (steady) from VEC + Regular DMEM
5	0.25 OSI from VEC + Regular DMEM
6	0.50 OSI from VEC + Regular DMEM
7	Static from VEC + Pro-calcifying media
8	0 OSI (steady) from VEC + Pro-calcifying media
9	0.25 OSI from VEC + Pro-calcifying media
10	0.50 OSI from VEC + Pro-calcifying media

30 T25's + three 12-well plates = 40mL of media per group

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Signed _____ Date _____ Signed _____ Date 2/6/2020

2/22/2020: Prepared Group 1 ~ Group 10 media, 40 mL each.
T25 seeding density (minimum): 700,000 cells, 4 mL
12-well plate seeding density: 100,000 cells, 1.5 mL per well
30 × (700,000 + 100,000) = 24,000,000 cells needed.
Trypsinized 4 T75 flasks (P4)
Count 1: 2.18×10^6 cells/mL
Count 2: 2.41×10^6 cells/mL
} AVG = 2.295×10^6 cells/mL
Volume = 35 mL.
Total cell count = $2.295 \times 10^6 \times 35 = 80,000,000$ cells.
Seeded 30 T25's @ 1,000,000 cells each and 110,000 cells per well.

GROUP	CONTENTS
1	Regular Media (RM1)
2	Pro-Calcific Media (PM1)
3	Static from VEC + (RM2)
4	0 OSI + RM2
5	0.25 OSI + RM2
6	0.50 OSI + RM2
7	Static + PM2
8	0 OSI + PM2
9	0.25 OSI + PM2
10	0.50 OSI + PM2

Molecular weights
Ca: 40 g/mol
Cl: 35.45 g/mol
} $CaCl_2 \Rightarrow 110.9$ g/mol

Na: 23 g/mol
H: 1 g/mol
P: 31 g/mol
O: 16 g/mol
} $NaH_2PO_4 \Rightarrow 120$ g/mol

* $1\ M = 1\ \frac{mol}{L}$

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RM 1 (40mL)
2 FBS
0.4 P/S
37.6 DMEM

PM 1 (40 mL)
2 FBS
0.4 P/S
4.8 mL × 4 IP = 19.2 mL (Amanda)
18.4 DMEM
7.98 mg of $CaCl_2$ ⇒ 0.008 g
18.24 mg of NaH_2PO_4 ⇒ 0.01824 g

RM 2 (20mL) ← make 4
0.2 P/S
19.8 DMEM

PM 2 (20 mL) ← make 4
0.2 P/S
4.8 mL × 4 IP = 19.2 mL
0.6 DMEM
7.98 mg $CaCl_2$ ⇒ 0.008 g
18.24 mg of NaH_2PO_4 ⇒ 0.01824 g

Want:

1.8 mM $CaCl_2$ ⇒ 0.0018 M $CaCl_2$

$0.0018 \frac{mol}{L} CaCl_2 \times 110.9 \frac{g}{mol} = 0.19962 \frac{g}{L}$

$0.19962 \frac{g}{L} = \frac{X\ g}{0.04\ L}$

$X = 0.0079848\ g = 7.98\ mg$ of $CaCl_2$

3.8 mM NaH_2PO_4 ⇒ 0.0038 M NaH_2PO_4

$0.0038 \frac{mol}{L} NaH_2PO_4 \times 120 \frac{g}{mol} = 0.456 \frac{g}{L}$

$0.456 \frac{g}{L} = X \frac{g}{0.04L}$

$X = 0.01824\ g = 18.24\ mg$ of NaH_2PO_4

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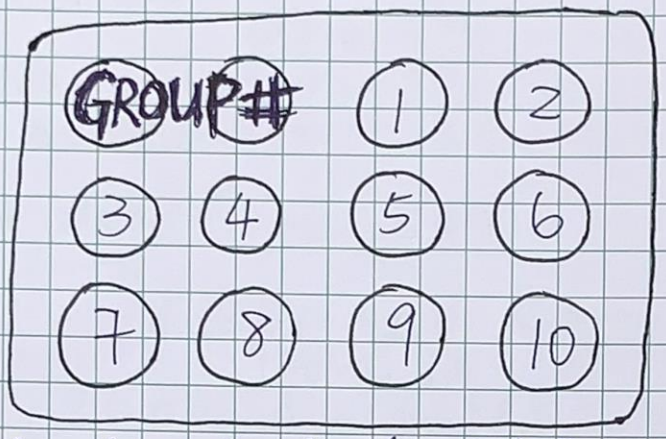
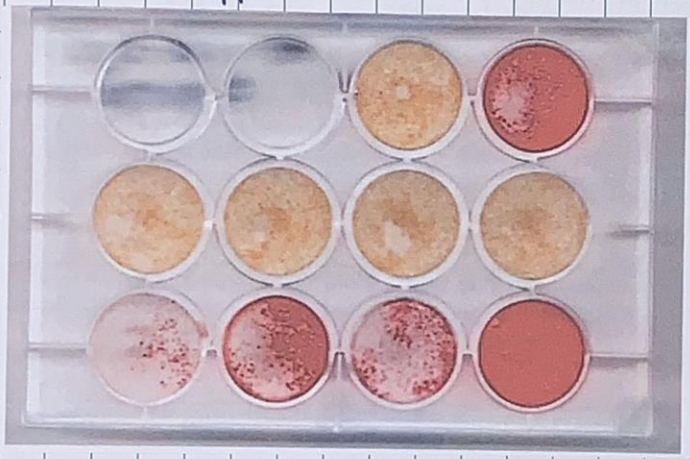
3 T25's per group (n=3)



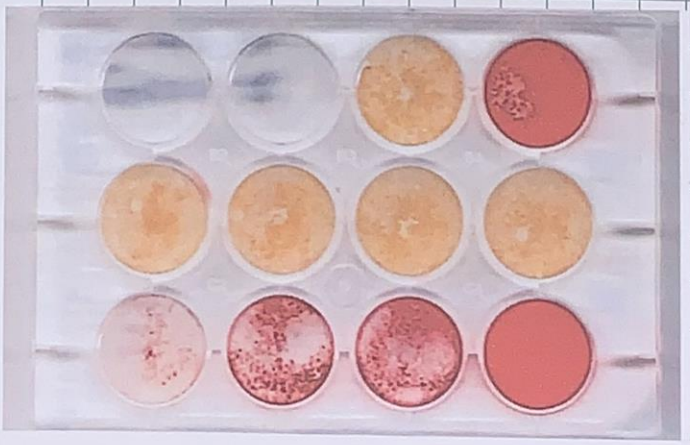
Three 12-well plates (for Alizarin Red Staining)

2/26/2020: Changed media (4 T75's Rat VICs)
(All ten groups @ n=3, 30 samples)
2/28/2020: Passaged 4 T75's into 14 T75's (P5 → P6)
2/29/2020: Collected all 30 samples in TRIzol, stored in -80°C
Applied Alizarin Red in 3 12-well plates



n=1

n=2



n=3

Scanner in EIC:
1. Windows Fax & Scan
2. New Scan
3. 600 dpi
4. TIF File
5. Right click → save as

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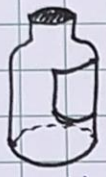
Date

3/3/2020: Froze 8 T75's into 8 vials (P7)
Changed media in 5 flasks
Passaged 1 T75 into 4 T75's (P6 → P7)
3/7/2020: Changed media in 4 T75's
3/9/2020: Received new bottle of inorganic pyrophosphatase (IP)



Catalog # I1643-100UN
Supplier: Sigma-Aldrich
Batch #: SLBX7623
Lot #: SLBX7623

Added 1000 µL (1 mL) DMEM, equivalent to 100 units.
1 unit = 10 µL

Using pro-calcific media (recipe found on page 17)
Reconditioned groups 2, 7, 8, 9, and 10.

For 10 mL Solutions:

* Molecular weight found on page 18.

1.8 mM $CaCl_2$ = 0.0018 M $CaCl_2$

$C = \frac{n}{V} = 0.0018\ M = \frac{\#\ of\ mols}{0.01\ L}$ ← 10 mL

# of moles of $CaCl_2$ = 0.0018 × 0.01 = 0.000018 moles $CaCl_2$

0.000018 mols × 110.9 g/mol = 0.002 grams of $CaCl_2$

3.8 mM NaH_2PO_4 = 0.0038 M NaH_2PO_4

$C = \frac{n}{V} = 0.0038\ M = \frac{\#\ of\ mols}{0.01\ L}$ ← 10 mL

# of moles of NaH_2PO_4 = 0.0038 × 0.01 = 0.000038 moles NaH_2PO_4

0.000038 mols × 120 g/mol = 0.00456 grams of NaH_2PO_4

Calculated for 10 mL solutions

For 30 mL Solution media:

GROUP 2 pro-calcific media

300 µL P/S
28.2 mL DMEM
1.5 mL FBS
12 µL IP
0.006 g $CaCl_2$
0.0137 g NaH_2PO_4

30 mL

GROUP 7
GROUP 8
GROUP 9
GROUP 10

30 mL total.
50% conditioned media from Rat VECs + 50% fresh Pro-calcific media

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Conditioned media (mL)	15, Static group (7)	15, 0 OSI group (8)	15, 0.25 OSI group (9)	15, 0.50 OSI group (10)
P/S (μL)	150	150	150	150
DMEM (mL)	15	15	15	15
$CaCl_2$ (g)	0.006	0.006	0.006	0.006
NaH_2PO_4 (g)	0.0137	0.0137	0.0137	0.0137

48 μL IP
600 μL P/S
60 mL DMEM
0.024 g $CaCl_2$
0.05472 g NaH_2PO_4

÷ 4 & evenly distribute to ⑦, ⑧, ⑨, ⑩ conicals.

Conditioned groups ②, ⑦, ⑧, ⑨, ⑩ in T25 flasks, with each group consisting of 3 flasks. (Total 15 T25's)
Each flask was seeded w/ 1,000,000 cells

Cell Counts: 2.74×10^6 cells/mL, 3.76×10^6 cells/mL } 3.25×10^6 cells/mL

$\times 25$ mL
$= 81.25 \times 10^6$ cells in total.

Also seeded three 12-well plates (groups ① ~ ⑩) at 200,000 cells per well. 12-well plate configuration is same as page 20.

3/16/2020: Collected all RNA samples, stored in TRIzol in -80.
Applied Alizarin Red to 3 12-well plates. (see pg. 23 for results)
Extracted RNA from VEC (Rat) n=1.
RNA quantification:

	ng/μL		AVERAGE ng/μL
Static	493.2	602.1	547.65
Steady 0 OSI	1447.1	1348.0	1397.55
0.25 OSI	248.3	222.9	235.6
0.50 OSI	716.6	528.7	622.65

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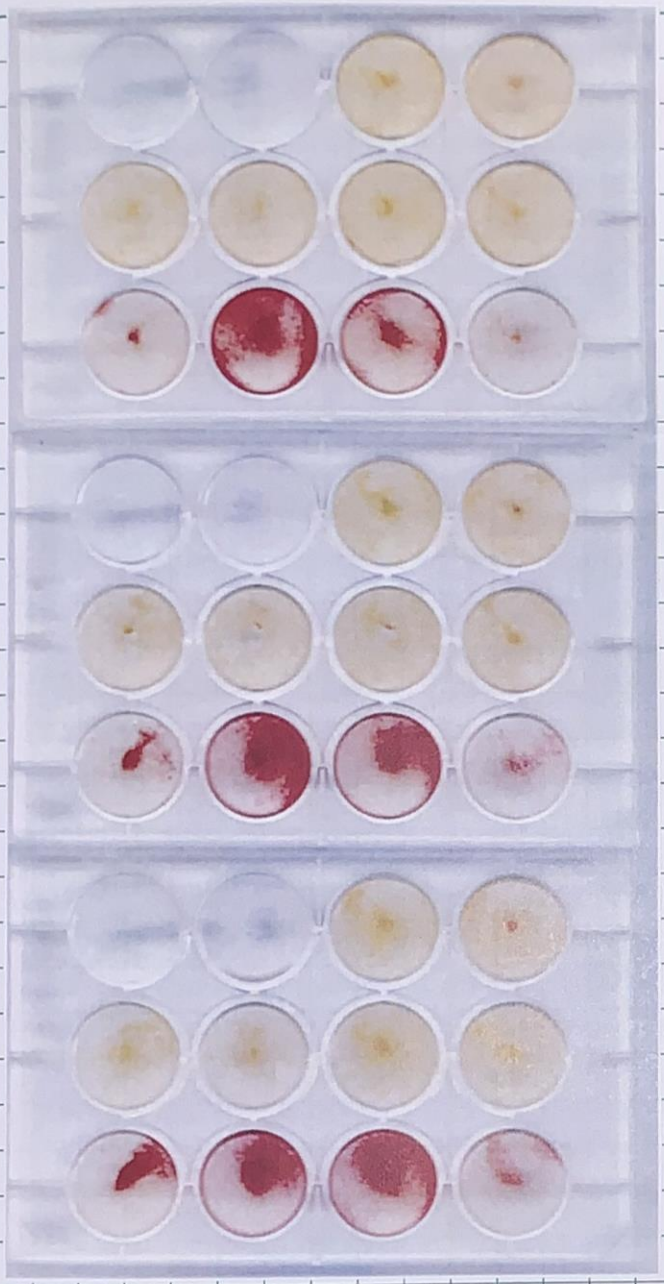

3/17/2020: Extracted another set of VEC rat RNA and a full set of HBMSC RNA. Quantification shown below:

Rat VEC

Group	ng/uL		AVG
Static	1376.1	1386.9	1381.5
Steady	1182.5	1227.6	1205.05
0.25 OSI	1586.3	1725.7	1656
0.50 OSI	1344.4	1297.4	1320.9

HBMSC

Group	ng/uL		AVG
Static	1727.1	1728.7	1727.9
Steady	1488.8	1475.5	1482.15
0.10 OSI	1102.2	1092.5	1097.35
0.20	1632.6	1632.9	1632.75
0.30	1662.4	1552.5	1607.45
0.40	1570.6	1677.2	1623.9
0.50	1606.9	1163.7	1385.3

VEC PRIMERS

CDKN1B: housekeeping for rodent myocardium tissue
ELN: elastin marker
NOTCH1: Valve development
Cathepsin S: ECM degradation & fibrin total dissolution
vWF: Endothelial marker

Made 100uM stocks w/DEPC water.

HBMSC PRIMERS

COL1A1: type I collagen
COL2A1: type II collagen
COL3A1: type III collagen
EXT1: GAG Synthesis
GNS: GAG Degradation

ELN: Elastin
ELN Variant 1: Tropoelastin protein sequence corresponds to Ref. Seq var. 1
COL9A2: FACIT collagen *

* Fibril Associated Collagens w/ Interrupted Triple helices
Cartilage, associated w/ type II collagen.

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made 100uM stocks w/DEPC water.

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Project _____

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Want: 100 ng RNA per well (Rat VEC)

Static

$V = \frac{100\ ng}{547.65\ ng/\mu L} = 0.18\ \mu L$

Steady Flow

$V = \frac{100\ ng}{1397.55} = 0.07\ \mu L$

0.25 OSI

$V = \frac{100\ ng}{235.6\ ng/\mu L} = 0.42\ \mu L$

0.50 OSI

$V = \frac{100\ ng}{622.65\ ng/\mu L} = 0.16\ \mu L$

TARGETS: CDKN1B, ELN, vWF, NOTCH1
Samples: Static, Steady, 0.25 OSI, 0.50 OSI
# of replicates: 3

	1	2	3	4	5	6	7	8
A	Static CDKN1B	Static vWF	Flow CDKN1B	Flow vWF	0.25 CDKN1B	0.25 vWF	0.50 CDKN1B	0.50 vWF
B	Static CDKN1B	Static vWF	Flow CDKN1B	Flow vWF	0.25 CDKN1B	0.25 vWF	0.50 CDKN1B	0.50 vWF
C	Static CDKN1B	Static vWF	Flow CDKN1B	Flow vWF	0.25 CDKN1B	0.25 vWF	0.50 CDKN1B	0.50 vWF
D	Static ELN	Static NOTCH1	Flow ELN	Flow NOTCH1	0.25 ELN	0.25 NOTCH1	0.50 ELN	0.50 NOTCH1
E	Static ELN	Static NOTCH1	Flow ELN	Flow NOTCH1	0.25 ELN	0.25 NOTCH1	0.50 ELN	0.50 NOTCH1
F	Static ELN	Static NOTCH1	Flow ELN	Flow NOTCH1	0.25 ELN	0.25 NOTCH1	0.50 ELN	0.50 NOTCH1

RNA Samples for 10 µL reactions:

Contents per well
- 5.0 SYBR Green
- 0.08 RT Enzyme
- 1 FP
- 1 RP

7.08 µL

10 − 7.08 = 2.92 µL ↑ total amount of RNA + DEPC H_2O

Static

(12+1)

0.18 × 13 wells = 2.34 µL of [S]
7.08 + 0.18 = 7.26 µL
10 − 7.26 = 2.74 µL DEPC H_2O per well
2.74 × 13 = 35.62 µL of DEPC H_2O
Static tube:
2.34 µL of [S] + 35.62 DEPC H_2O

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Steady Flow

0.07 × 13 wells = 0.91 µL of F
7.08 + 0.07 = 7.15 µL
10 − 7.15 = 2.85 µL DEPC H_2O per well
2.85 × 13 = 37.05 µL DEPC H_2O
Steady tube:
0.91 µL of F + 37.05 µL DEPC H_2O

0.25 OSI

0.42 × 13 wells = 5.46 µL of 0.25
7.08 + 0.42 = 7.50 µL
10 − 7.50 = 2.50 µL DEPC H_2O
2.50 × 13 = 32.5 µL DEPC H_2O
0.25 OSI Tube:
5.46 µL of 0.25 + 32.5 µL DEPC H_2O

0.50 OSI

0.16 × 13 = 2.08 µL of 0.50
7.08 + 0.16 = 7.24 µL
10 − 7.24 = 2.76 µL DEPC H_2O
2.76 × 13 = 35.88 µL DEPC H_2O
0.50 tube:
2.08 µL 0.50 + 35.88 µL DEPC H_2O

TUBE #:

1. Static RNA: 2.34 µL of S + 35.62 µL DEPC H_2O
2. Steady RNA: 0.91 µL of F + 37.05 µL DEPC H_2O
3. 0.25 RNA: 5.46 µL of 0.25 + 32.5 µL DEPC H_2O
4. 0.50 RNA: 2.08 µL of 0.50 + 35.88 µL DEPC H_2O
5. RT MM: 260 µL SYBR Green + 416 µL RT Enzyme
6. CDKN1B: 1 µL FP + 1 µL RP + 24 µL DEPC
7. ELN:
8. VWF:
9. NOTCH1:

Pipette Volume (µL)
2.92
↓
5.08
2
↓

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File Edit Instrument Analysis Tools Help
New Experiment Open... Save Close Send Experiment to Instrument... Download Experiment from Instrument... Export... Print Report...
Experiment Menu
Setup
Run
Analysis
Amplification Plot
Gene Expression
Multicomponent Plot
Melt Curve
Raw Data Plot
QC Summary
Multiple Plots View
Experiment: AT Rat VEC 1 Type: Comparative CT (ΔΔCT) Reagents: SYBR® Green Reagents
Analysis Settings
Amplification Plot
Plot Settings
Plot Type: ΔRn vs Cycle Graph Type: Log Plot Color: Well
Amplification Plot
ΔRn
Cycle
Options
Target: All Threshold:
Show: Threshold Baseline Start: Well Target Baseline End: Well Target
View Plate Layout
View Well Table
Select Wells With: - Select Item - - Select Item -
Show in Wells
View Legend
Wells: 48 Unknown 0 Negative Control 0 Empty
Analysis Summary: Total Wells in Plate: 48 Wells Set Up: 48 Wells Omitted Manually: 0 Wells Flagged: 47 Wells Omitted by Analysis: 0 Samples Used: 4 Targets Used: 4
Home AT Rat VEC 1.eds
Local Instrument : connected
Wells: 48 Unknown 0 Negative Control 0 Empty
Analysis Summary: Total Wells in Plate: 48 Wells Set Up: 48 Wells Omitted Manually: 0 Wells Flagged: 42 Wells Omitted by Analysis: 0 Samples Used: 4 Targets Used: 4
Home Rat VEC 2020-...7 quickrun.eds
Local Instrument : connected
Wells: 48 Unknown 0 Negative Control 0 Empty
Analysis Summary: Total Wells in Plate: 48 Wells Set Up: 48 Wells Omitted Manually: 0 Wells Flagged: 38 Wells Omitted by Analysis: 0 Samples Used: 4 Targets Used: 4
Home AT_DH_Rat VEC 2020-03-18.eds AT_DH_Rat VEC...8 quickrun.eds
Local Instrument : connected
Wells: 48 Unknown 0 Negative Control 0 Empty
Analysis Summary: Total Wells in Plate: 48 Wells Set Up: 48 Wells Omitted Manually: 0 Wells Flagged: 44 Wells Omitted by Analysis: 0 Samples Used: 4 Targets Used: 4
Home AT_DH Rat VEC 2020-03-19.eds
Local Instrument : connected

3/19/2020: Want 200 ng of RNA per well. 200ng = Concentration × Volume

Volume = $\frac{200\text{ ng}}{\text{concentration}}$ HBMSC ↑ from pg. 23

Static $V = \frac{200}{1727.9} = 0.116\ \mu L$

Flow $V = \frac{200}{1482.15} = 0.135\ \mu L$

0.1 $V = \frac{200}{1097.35} = 0.182\ \mu L$

0.2 $V = \frac{200}{1632.75} = 0.122\ \mu L$

0.3 $V = \frac{200}{1607.45} = 0.124\ \mu L$

0.4 $V = \frac{200}{1623.9} = 0.123\ \mu L$

0.5 $V = \frac{200}{1385.3} = 0.144\ \mu L$

TARGETS: RPLP13A(HK), ELN, ELN var.1, COL1A1, COL2A1, EXT1
Samples: static, Flow, 0.1, 0.2, 0.3, 0.4, 0.5 OSI's
# of replicates: 1

	1	2	3	4	5	6	7	8
A	static RPLP13A	steady	0.1	0.2	0.3	0.4	0.5 →	
B	static ELN	steady	0.1	0.2	0.3	0.4	0.5 →	
C	static ELN V.1	steady	0.1	0.2	0.3	0.4	0.5 →	
D	static COL1A1	steady	0.1	0.2	0.3	0.4	0.5 →	
E	static COL2A1	steady	0.1	0.2	0.3	0.4	0.5 →	
F	static EXT1...	steady	0.1	0.2	0.3	0.4	0.5 →	

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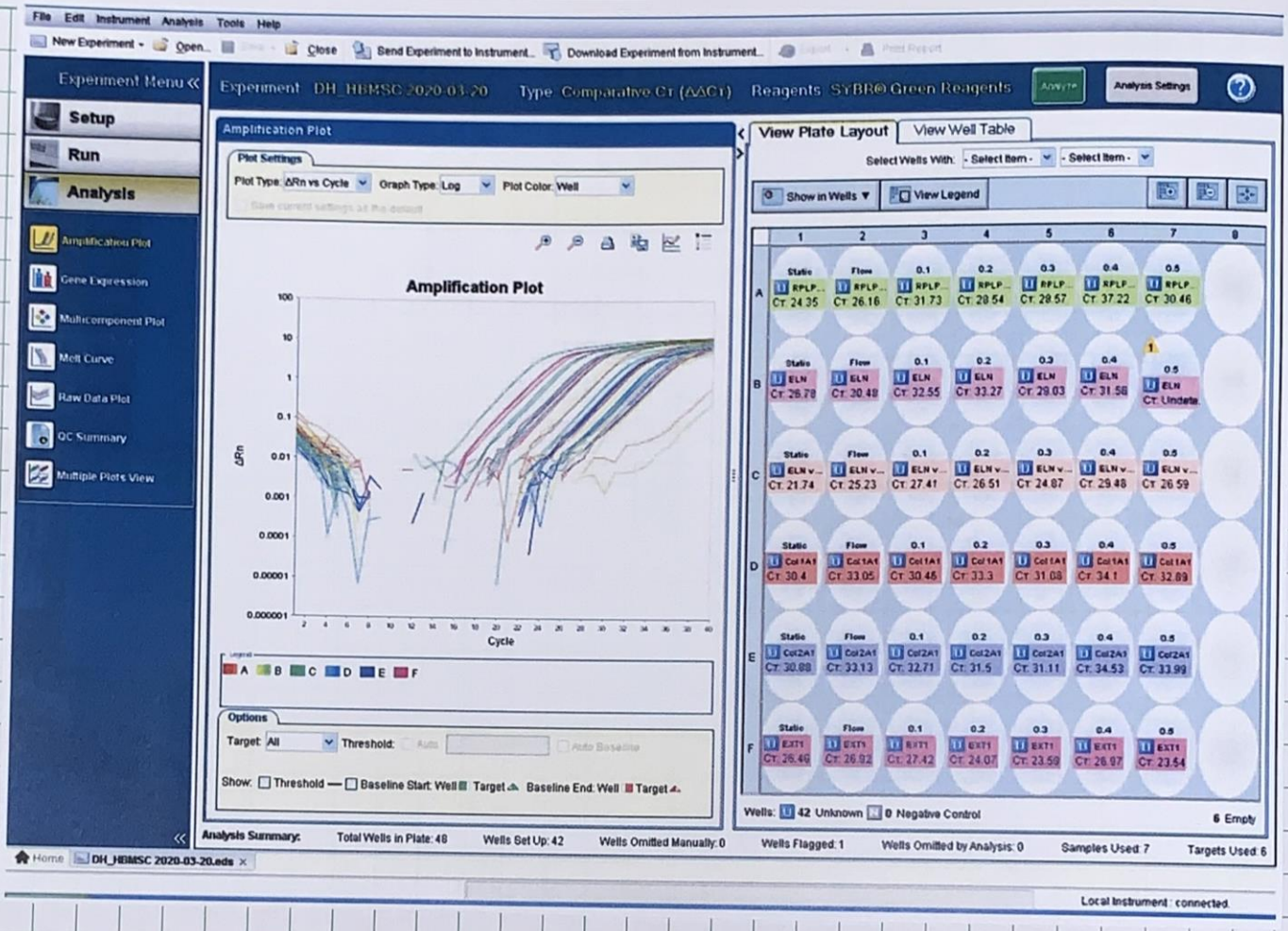

Tube # 200 ng RNA per well, 12 wells of RNA

1. Static RNA: 1.508 µL [Static] + 36.452 µL DEPC
2. Steady RNA: 1.742 µL [Steady] + 36.218 µL DEPC
3. 0.1 RNA: 2.392 µL [0.1 OSI] + 35.568 µL DEPC
4. 0.2 RNA: 1.586 µL [0.2 OSI] + 36.374 µL DEPC
5. 0.3 RNA: 1.612 µL [0.3 OSI] + 36.348 µL DEPC
6. 0.4 RNA: 1.612 µL [0.4 OSI] + 36.348 µL DEPC
7. 0.5 RNA: 1.872 µL [0.5 OSI] + 36.088 µL DEPC
8. RT MM: 5 × (42+4) SYBR Green + 0.08 × (42+4) RT Enzyme = 230 µL SYBR Green + 3.68 µL RT Enz.
9. RPLP13A (HK): 1 µL FP + 1 µL RP + 15 µL DEPC
10. ELN:
11. ELN var. 1:
12. COL1A1:
13. COL2A1:
14. EXT1:

Pipette Volume (µL)

2.92 ↓

5.08 / 2 ↓

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pg. 26 combined (n=1) Rat VEC RT-qPCR

Static

CDKN1B	VWF	ELN	NOTCH1
37.03	34.84	31.36	33.81
34.85	34.41	33.44	33.57
	25.38	31.95	39.96

Steady 0 OSI

CDKN1B	VWF	ELN	NOTCH1
34.8	32.47	35.97	36.79
34.81		34.74	34.81
35.57	33.88	33.42	33.61

0.25 OSI

CDKN1B	VWF	ELN	NOTCH1
32.47	36.31	36.79	37.14
	36.22	34.81	
33.88	35.99	33.60	

0.50 OSI

CDKN1B	VWF	ELN	NOTCH1
38	30.87	33.68	31.53
	30.58	30.98	31.89
40	32.7	27.05	34.83

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3/21/2020: Rat VIC RNA extraction & quantification

GROUP	CONCENTRATION (ng/μL)		AVERAGE
1-1	504.2	447.8	476.0
2-1	1195.0	1439.7	1317.35
3-1	1301.9	1617.4	1459.65
4-1	1710.5	1680.3	1695.4
5-1	1571.2	1511.7	1541.45
6-1	1506.0	1553.1	1529.55
7-1	1698.5	1327.9	1513.2
8-1	292.5	453.0	372.75
9-1	106.7	83.5	95.1
10-1	61.6	57.2	59.4

4/23/2020: VIC calcification quantification
ARS area scanning at 405 nm w/microplate reader.
Samples used are from page 20.

4/24/2020: Extracted ARS dye & quantified using acetic acid & ammonium hydroxide. Protocol details are on page 84 & 83. Samples used are from page 20.

5/5/2020: Performed ALP assay on conditioned media that was collected from Rat VICs (on day 7). ARS for these VICs are on page 23.

ARS Dye Extraction & Quantification

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.167	0.165	0.159	0.801	0.8	0.762	0.206	0.206	0.205	0.178	0.178	0.174
B	1.583	1.605	1.592	3.029	3.243	2.951	0.847	0.87	0.835	0.186	0.187	0.183
C	0.187	0.188	0.189	0.198	0.197	0.198	1.268	1.281	1.244	0.235	0.233	0.231
D	0.204	0.206	0.205	1.428	1.517	1.426	2.442	2.425	2.184	1.055	1.074	1.027
E	0.195	0.194	0.228	0.176	0.175	0.174	0.195	0.2	0.188	0.596	0.595	0.596
F	0.191	0.191	0.19	0.18	0.18	0.175	1.315	1.338	1.333	3.018	3.099	2.819
G	0.199	0.194	0.194	0.241	0.241	0.239	0.181	0.182	0.182	0.053	0.053	0.053
H	0.896	0.931	0.925	0.174	0.175	0.169	0.18	0.181	0.175	0.053	0.053	0.053

ALP Assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.055	1.795	1.295	1.409	1.525	0.836	0.305	0.251	0.675	0.905	0.056	0.054
B	1.154	1	1.243	0.898	1.055	1.235	1.25	0.882	0.804	0.914	0.054	0.053
C	1.232	1.844	1.122	1.914	1.009	1.147	0.81	1.141	1.204	1.136	0.054	0.054
D	0.888	1.255	1.428	1.465	0.762	1.211	0.826	1.292	1.306		0.054	0.055
E		1.674	1.324	1.612	1.503	0.717	1.236	1.134	0.688	1.203	0.055	0.053
F	0.184	0.815	0.369	0.779	0.8	0.836	0.577	0.474	0.541	0.513	0.053	0.053
G	0.053	0.054	0.055	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.053	0.054
H	0.047	0.397	0.716	1.061	1.363	1.757	0.053	0.06	0.053	0.054	0.054	0.053

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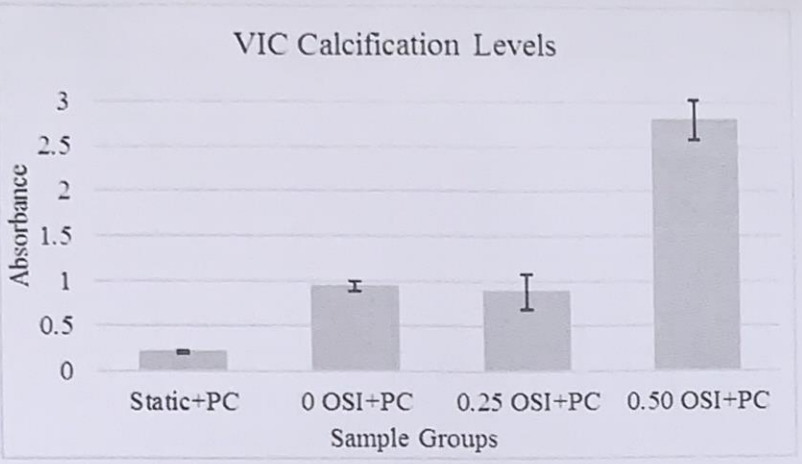
ARS Analysis



Oneway

[DataSet0] C:\Users\HP\Desktop\RatVIC_ARS_Pvalue.sav

ANOVA

Absorbance

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11.127	3	3.709	52.217	.000013
Within Groups	.568	8	.071		
Total	11.695	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Absorbance

Tukey HSD

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Static	SteadyFlow	-.728555333*	.217605381	.040754	-1.42540436	-.03170630
	0.25 OSI	-.671111000	.217605381	.059069	-1.36796003	.02573803
	0.50 OSI	-2.58966633*	.217605381	.000011	-3.28651536	-1.89281730
SteadyFlow	Static	.728555333*	.217605381	.040754	.03170630	1.42540436
	0.25 OSI	.057444333	.217605381	.993047	-.63940470	.75429336
	0.50 OSI	-1.86111100*	.217605381	.000125	-2.55796003	-1.16426197
0.25 OSI	Static	.671111000	.217605381	.059069	-.02573803	1.36796003
	SteadyFlow	-.057444333	.217605381	.993047	-.75429336	.63940470
	0.50 OSI	-1.91855533*	.217605381	.000100	-2.61540436	-1.22170630
0.50 OSI	Static	2.589666333*	.217605381	.000011	1.89281730	3.28651536
	SteadyFlow	1.861111000*	.217605381	.000125	1.16426197	2.55796003
	0.25 OSI	1.918555333*	.217605381	.000100	1.22170630	2.61540436

*. The mean difference is significant at the 0.05 level.

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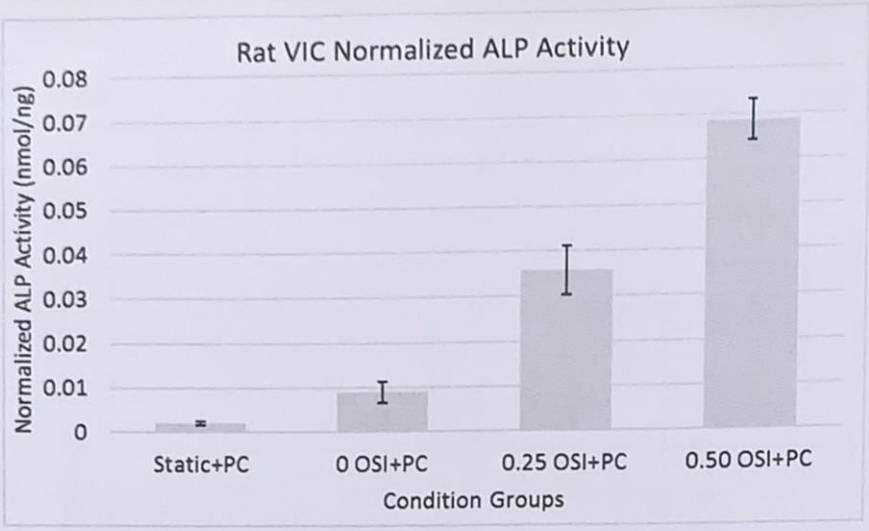

Oneway

[DataSet1] C:\Users\HP\Desktop\SPSS_RatVIC_ALPAssay_Pvalue.sav

ANOVA

Normalized_Concentration

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.008	3	.003	65.250	.000
Within Groups	.000	8	.000		
Total	.009	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Normalized_Concentration

Tukey HSD

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Static	SteadyFlow	-.006902744	.0052784813	.5834523	-.023806299	.0100008116
	0.25 OSI	-.033625899*	.0052784813	.0009767	-.050529454	-.016722344
	0.50 OSI	-.066376756*	.0052784813	.0000070	-.083280311	-.049473201
SteadyFlow	Static	.0069027437	.0052784813	.5834523	-.010000812	.0238062989
	0.25 OSI	-.026723155*	.0052784813	.0042855	-.043626711	-.009819600
	0.50 OSI	-.059474012*	.0052784813	.0000162	-.076377568	-.042570457
0.25 OSI	Static	.033625899*	.0052784813	.0009767	.0167223437	.0505294543
	SteadyFlow	.026723155*	.0052784813	.0042855	.0098196001	.0436267106
	0.50 OSI	-.032750857*	.0052784813	.0011649	-.049654412	-.015847302
0.50 OSI	Static	.066376756*	.0052784813	.0000070	.0494732007	.0832803113
	SteadyFlow	.059474012*	.0052784813	.0000162	.0425704571	.0763775676
	0.25 OSI	.032750857*	.0052784813	.0011649	.0158473017	.0496544123

*. The mean difference is significant at the 0.05 level.

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9/3/2020: RT-PCR Rat VIC (concentration below), from page 30

Group	Description	Concentration (ng/μL)
1-1	Regular DMEM	476
2-1	PC	1317.35
3-1	Regular static from VEC	1459.65
4-1	Regular Steady from VEC	1695.4
5-1	Regular 0.25 from VEC	1541.45
6-1	Regular 0.50 from VEC	1529.55
7-1	PC static from VEC	1513.2
8-1	PC Steady from VEC	372.75
9-1	PC 0.25 from VEC	95.1
10-1	PC 0.50 from VEC	59.4

Targets: ACTB, TNAP, αSMA, MMP2
Samples: 10 → Housekeeping
# of replicates: 1

1. RNA to CT Master Mix (for 10μL reactions)
   5.0 μL SYBR Green
   0.08 μL RT Enzyme
   5.0 × 43 = 215 μL of SYBR green
   0.08 × 43 = 3.44 μL of RT Enzyme

2. Primers - Use 1 μL of the 10 μM stock for both FWD & REV
   1 replicate × 10 samples = 10 primer pairs
   Make primer tubes for 11 wells
   Each well needs 1 μL FWD + 1 μL REV
   2 μL per well × 11 wells = 22 μL

1 μL of 10 μM FWD
1 μL of 10 μM REV
} + 20 μL DEPC H_2O

Primer tube (22 μL each) ACTB, TNAP, MMP2, αSMA

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3. RNA Samples. Want 40 ng of RNA per well

1-1: $40\,ng = 476\,ng/\mu L \times V_{1-1} \Rightarrow V_{1-1} = 0.084\,\mu L$

2-1: $40\,ng = 1317.35 \times V_{2-1} \Rightarrow V_{2-1} = 0.030\,\mu L$

3-1: $40\,ng = 1459.65 \times V_{3-1} \Rightarrow V_{3-1} = 0.027\,\mu L$

4-1: $40\,ng = 1695.4 \times V_{4-1} \Rightarrow V_{4-1} = 0.0236\,\mu L$

5-1: $40\,ng = 1541.45 \times V_{5-1} \Rightarrow V_{5-1} = 0.026\,\mu L$

6-1: $40\,ng = 1529.55 \times V_{6-1} \Rightarrow V_{6-1} = 0.026\,\mu L$

7-1: $40\,ng = 1513.2 \times V_{7-1} \Rightarrow V_{7-1} = 0.026\,\mu L$

8-1: $40\,ng = 372.75 \times V_{8-1} \Rightarrow V_{8-1} = 0.107\,\mu L$

9-1: $40\,ng = 95.1 \times V_{9-1} \Rightarrow V_{9-1} = 0.421\,\mu L$

10-1: $40\,ng = 59.4 \times V_{10-1} \Rightarrow V_{10-1} = 0.673\,\mu L$

1-1 $0.084\,\mu L \times 5$ wells $= 0.42\,\mu L$ of [1-1]

$7.08 + 0.084 = 7.164\,\mu L$

$10 - 7.164 = 2.836\,\mu L$ of DEPC H_2O per well

2.836×5 wells $= 14.18\,\mu L$ of DEPC H_2O

1-1 tube:

$0.42\,\mu L$ of [1-1] + $14.18\,\mu L$ of DEPC H_2O

2-1 $0.030\,\mu L \times 5$ wells $= 0.15\,\mu L$ of [2-1]

$7.08 + 0.03 = 7.11\,\mu L$

$10 - 7.11 = 2.89\,\mu L$ DEPC H_2O

$2.89 \times 5 = 14.45\,\mu L$ DEPC H_2O

2-1 tube:

$0.15\,\mu L$ of [2-1] + $14.45\,\mu L$ DEPC H_2O

3-1 $0.027\,\mu L \times 5 = 0.135\,\mu L$ [3-1]

$7.08 + 0.027 = 7.107\,\mu L$

$10 - 7.107 = 2.893\,\mu L$ DEPC H_2O

$2.893 \times 5 = 14.465\,\mu L$ DEPC

3-1 tube:

$0.135\,\mu L$ of [3-1] + $14.465\,\mu L$ DEPC H_2O

4-1 $0.0236 \times 5 = 0.118\,\mu L$ [4-1]

$7.08 + 0.0236 = 7.1036\,\mu L$

$10 - 7.1036 = 2.8964$

$2.8964 \times 5 = 14.482\,\mu L$ DEPC H_2O

4-1 tube:

$0.118\,\mu L$ of [4-1] + $14.482\,\mu L$ DEPC H_2O

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5-1 $0.026 \times 5 = 0.13\ \mu L$ [5-1]
$7.08 + 0.026 = 7.106\ \mu L$
$10 - 7.106\ \mu L = 2.894\ \mu L$ DEPC H_2O
2.894×5 wells $= 14.47\ \mu L$ DEPC H_2O
5-1 tube:
$0.13\ \mu L$ [5-1] $+ 14.47\ \mu L$ DEPC H_2O

6-1 Same as 5-1
6-1 tube:
$0.13\ \mu L$ [6-1] $+ 14.47\ \mu L$ DEPC H_2O

7-1 Same as 5-1
7-1 tube:
$0.13\ \mu L$ [7-1] $+ 14.47\ \mu L$ DEPC H_2O

8-1 $0.107 \times 5 = 0.535\ \mu L$ [8-1]
$7.08 + 0.107 = 7.187\ \mu L$
$10 - 7.187 = 2.813\ \mu L$ DEPC H_2O
$2.813 \times 5 = 14.065\ \mu L$ DEPC H_2O
8-1 tube:
$0.535\ \mu L$ [8-1] $+ 14.065\ \mu L$ DEPC H_2O

9-1 $0.421 \times 5 = 2.105\ \mu L$ [9-1]
$7.08 + 0.421 = 7.501\ \mu L$
$10 - 7.501 = 2.499\ \mu L$ DEPC
$2.499 \times 5 = 12.495\ \mu L$ DEPC H_2O
9-1 tube:
$2.105\ \mu L$ [9-1] $+ 12.495\ \mu L$ DEPC H_2O

10-1 $0.673 \times 5 = 3.365\ \mu L$ [10-1]
$7.08 + 0.673 = 7.753\ \mu L$
$10 - 7.753 = 2.247\ \mu L$ DEPC H_2O
2.247×5 wells $= 11.235\ \mu L$ DEPC H_2O
10-1 tube:
$3.365\ \mu L$ [10-1] $+ 11.235\ \mu L$ DEPC H_2O

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Tube #:

1. 0.42 µL of [1-1] + 14.18 µL DEPC H_2O
2. 0.15 µL of [2-1] + 14.45 µL DEPC H_2O
3. 0.135 µL of [3-1] + 14.465 µL DEPC H_2O
4. 0.118 µL of [4-1] + 14.482 µL DEPC H_2O
5. 0.13 µL of [5-1] + 14.47 µL DEPC H_2O
6. 0.13 µL of [6-1] + 14.47 µL DEPC H_2O
7. 0.13 µL of [7-1] + 14.47 µL DEPC H_2O
8. 0.535 µL of [8-1] + 14.065 µL DEPC H_2O
9. 2.105 µL of [9-1] + 12.495 µL DEPC H_2O
10. 3.365 µL of [10-1] + 11.235 µL DEPC H_2O
11. 107.5 µL SYBR Green + 1.72 µL RT Enzyme
12. 107.5 µL SYBR Green + 1.72 µL RT Enzyme
13. ACTB: 1 µL FWD + 1 µL REV + 20 µL DEPC H_2O
14. TNAP:
15. αSMA:
16. MMP2:

Pipette Volume Per Well (µL)

2.92

5.08

5.08

2

RAW Ct Values

GROUP	GENE	Ct VALUE
1-1	ACTB, TNAP, αSMA, MMP2	24.93, 34.91, 31.85, 22.96
2-1		28.25, Und, 37.11, 33.75
3-1		23.16, 28, 27.39, 19.42
4-1		23.17, 29.31, 28.71, 23.59
5-1		23.91, 34.56, 29.83, 19.28
6-1		24.31, 31.31, 26.75, 22.55
7-1		24.84, 34.29, 32.41, 24.59
8-1		24.66, Und, 31.2, 26.13
9-1		28.87, Und, 33.66, 30.05
10-1		32.76, Und, Und, Und

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9/4/2020: Repeated the same RT-PCR as 9/3 but w/ the following genes:
ACTB, Col7A1, BMP2, and Runx2.
↑ Housekeeping

RAW C_T Values

GROUP	GENE	C_T VALUE	one more gene ←	C_T Value
1-1	ACTB, Col7A1, BMP2, Runx2	25.01, 22.71, 36.93, und	dSMA	28.1
2-1	↓	29.16, 33.81, und, 38.9	↓	36.96
3-1		22.76, 22.3, 34.91, 36.92		28.3
4-1		22.23, 22.47, 35.63, 36.38		29
5-1		21.94, 22.08, 34.49, 35.84		28.15
6-1		23.92, 23.48, 35.08, und		30.13
7-1		25.96, 26.7, und, 38.48		32.52
8-1		26.02, 28.71, 36.8, 38.26		32.54
9-1		29.82, 31.55, und, und		x
10-1		35.1, und, und, und		x

9/5/2020: RNA Extraction & Quantification
Rat VIC

Group	Read 1	Read 2	AVERAGE (ng/uL)
1-2	1801.3	1392.8	1597.05
2-2	327.8	465	396.4
3-2	887.4	443.4	665.4
4-2	1033.9	954.6	994.25
5-2	991.3	1257.3	1124.3
6-2	871.9	665.6	768.75
7-2	145.8	133.5	139.65
8-2	376.6	399.2	387.9
9-2	386.2	399.7	392.95
10-2	87.6	132.7	110.15

Continued on Page

Read and Understood By

_____ Signed _____ Date

_____ Signed 9/4/2020 Date

9/6/2020: RNA Extraction & quantification
Rat VIC

Group	Read 1	Read 2	AVERAGE (ng/uL)
1-3	1274.7	1059.9	1167.3
2-3	595.3	639.9	617.6
3-3	1128.5	1245.6	1187.05
4-3	1374.0	963.3	1168.65
5-3	1085.7	1139.3	1112.5
6-3	1384.9	1314.4	1349.65
7-3	643.6	651.9	647.75
8-3	496.2	461.3	478.75
9-3	753.2	743.6	748.4
10-3	426.7	371.3	399.0

9/7/2020: qPCR

Raw Ct Values

GROUP	Gene	Ct Values
1-2	ACTB, TNAP, αSMA, MMP2	21.63, 34.59, 30.19, Und
2-2		27.73, Und, 36.59, 28.9
3-2		24.92, 34.92, 30.63, 20.63
4-2		21.85, Und, 31.74, 22.6
5-2		19.66, 35.86, 31.75, 20.97
6-2		20.16, Und, 30.89, 21.84
7-2		26.98, Und, 37.09, 28.98
8-2		25.97, Und, 34.49, 27.33
9-2		33.45, Und, Und, 35.9
10-2		Und, Und, Und, Und.

9/8/2020: qPCR

Group	Gene	Ct Values
1-2	ACTB, Col1A1, BMP2, Runx2	25.55, 21.2, 37.04, Und
2-2		27.6, 34.92, Und, Und
3-2		21.22, 18.95, 30.81, 35.36
4-2		24.61, 24.89, 34.33, 37.07
5-2		23.92, 21.25, 35.85, 33.74
6-2		23.29, 20.88, 33.52, 34.57
7-2		25.1, 27.25, Und, Und
8-2		27.73, 29.6, Und, Und
9-2		35.68, Und, Und, Und
10-2		33.86, Und, Und, Und

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9/11/2020: qPCR

GROUP	Gene	Ct Values
1-2	ACTB, TNAP, αSMA, MMP2	23.24, 35.32, 32.46, 21.42
2-2		29.09, Und, 35.93, 28.9
3-2		25.96, 36.16, 31.59, 22.15
4-2		25.6, 34.14, 32.65, 23.98
5-2		23.97, 37.02, 32.16, 20.59
6-2		24.55, 34.93, 31.69, 21.97
7-2		26.99, Und, 32.97, 26.54
8-2		28.4, Und, 33.49, 28.14
9-2		36.96, Und, Und, 36.96
10-2		Und, Und, Und, Und

9/12/2020: qPCR

Group	Gene	Ct Values
1-2	ACTB, Col1A1, BMP2, RunX2	26.37, 27.78, Und, 37.04
2-2		30.92, 36.95, Und, Und
3-2		25.94, 24.39, 36.89, Und
4-2		27.14, 25.98, 37.01, Und
5-2		25.8, 24.77, Und, Und
6-2		26.39, 27.4, Und, Und
7-2		29.15, 32.46, Und, Und
8-2		28.79, 36.48, Und, Und
9-2		Und, Und, Und, Und
10-2		Und, Und, Und, Und

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9/14/2020: Rat VIC RNA extraction & quantification (PC groups only)

GROUP	CONCENTRATION (ng/uL)	"	AVERAGE
2-1	624.0	703.5	663.8
2-2	308.2	160.3	234.3
2-3	1062.9	1038.4	1050.7
7-1	1203.7	1148.5	1176.1
7-2	907.9	927.6	917.8
7-3	844.0	868.7	856.4
8-1	759.8	762.8	761.3
8-2	818.1	807.7	812.9
8-3	899.4	845.3	872.4
9-1	977.7	1001.9	989.8
9-2	882.4	690.0	786.2
9-3	583.4	930.2	756.8
10-1	495.3	1336.2	915.8
10-2	881.7	1446.8	1164.3
10-3	841.8	1166.3	1004.1

10/5/2020: Combined data from 10/1, 10/2, and 10/4 (qPCR runs on PC's)

GROUP	ACTB	TNAP	aSMA	MMP2	Col1a9	BMP2	Runx2
PC	23.42	34.22	32.74	24.48	25.24	33.78	34.06
Static-PC	23.10	35.41	33.68	24.01	26.52	34.54	36.97
Steady-PC	23.15	34.79	33.62	23.17	26.82	33.42	36.03
0.25-PC	22.39	34.25	32.77	23.04	22.90	33.23	35.25
0.50-PC	22.59	33.90	32.57	21.90	23.98	32.12	34.86

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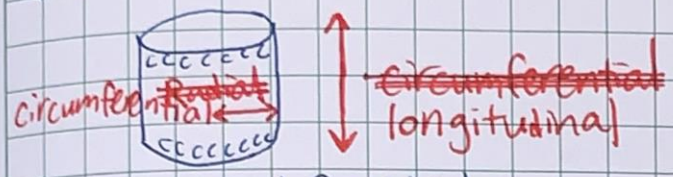
11/30/2020: Raw PSIS mechanical testing w/Bose System



Cylindrical PSIS Valve

Tested n=3 cylindrical valves longitudinal
Each n consisted of 3 ~~circumferential~~ and 3 ~~radial~~ circumferential technical replicates.

YOUNG'S MODULUS

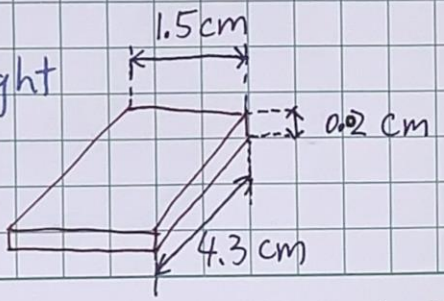
~~Circumferential~~ Longitudinal

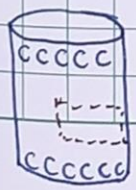
N	1	2	3
Replicate 1	1.083168	2.589133	1.421704
Replicate 2	1.287824	2.575204	1.323609
Replicate 3	1.605165	1.466868	1.678577
Average	1.325386	2.210402	1.47463
AVERAGE ALL	1.670139		

Circumferential
~~Radial~~

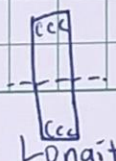
N	1	2	3
Replicate 1	0.110978	0.243861	0.097321
Replicate 2	0.172159	0.420038	0.146241
Replicate 3	0.090404	0.284412	0.147129
Average	0.124514	0.316104	0.13023
AVERAGE ALL	0.190283		

1/11/2021: Placed raw PSIS in 10% formalin overnight
1/12/2021: Embeded samples in OCT

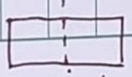




Cylindrical Valve



Longitudinal
~~Circumferential~~
Strips



~~Radial~~ circumferential
Strips

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11/30/2020
Date

Project

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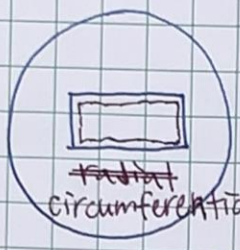
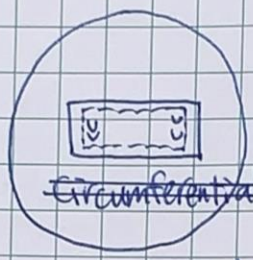
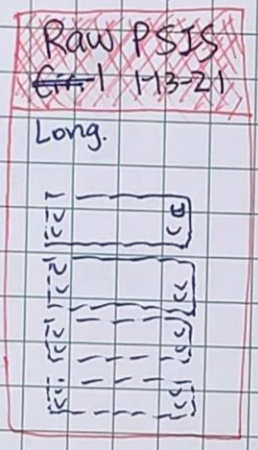


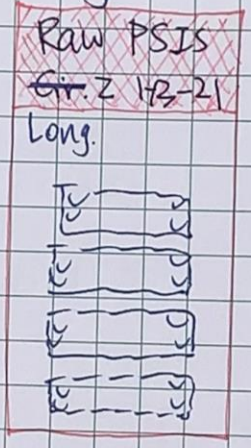
OCT mold

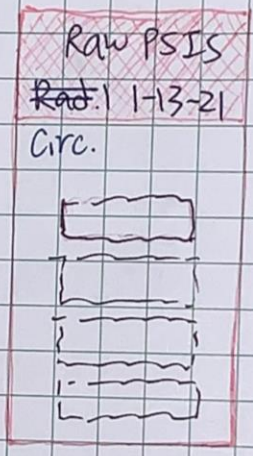


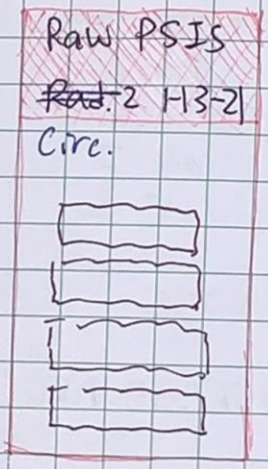
OCT mold

1/13/2021: Cryostat sectioning









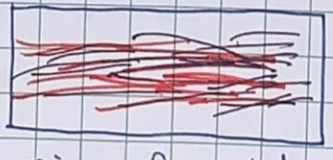
glass slides

1/16/2021: Russell-MOVAT PENTACHROME STAIN KIT
LOT 085764
a/m American MasterTech
Scientific laboratory supplies

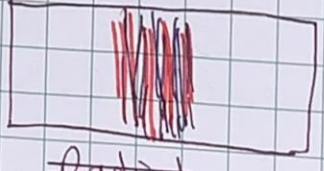
staining images

1/17/2021: Imaging MOVAT Stains

Observed Fiber alignment:



~~circumferential~~
Longitudinal



~~Radial~~
Circumferential

Observations: Bose system pulling in the direction of fiber alignment resulted in higher yield stress

Continued on Page

* Note (Jan. 31, 2021): Change original "circumferential" to "Longitudinal"
Change original "radial" to "circumferential"

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Date

1/12/2021

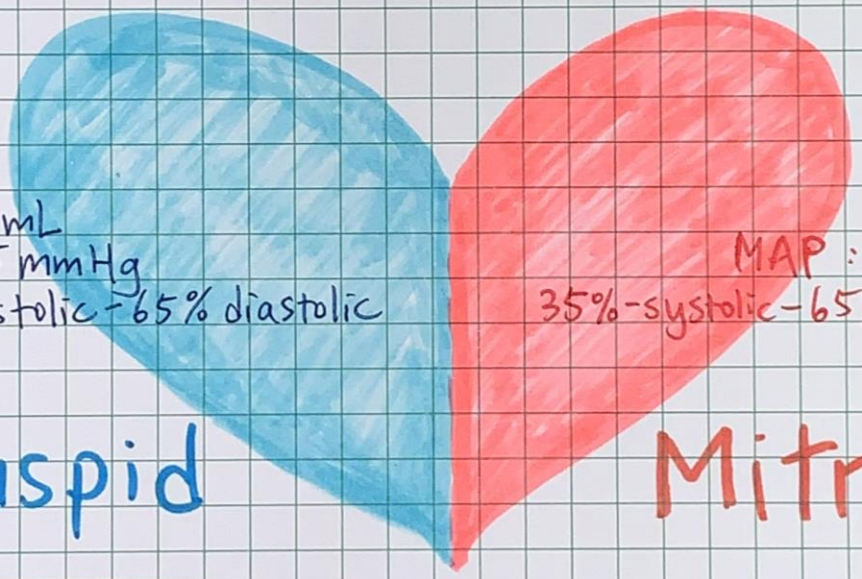
Signed

Date

2/9/2021: Vivitro Valvetesting 26-mm PSIS valves from CorMatrix
2/11/2021: Tri-leaflet configuration in
2/18/2021: tricuspid & mitral positions.
One suture, one mount (for both conditions we used the same valve)

P125824 CMCTV – 026
Cook Biotech Inc
1425 Innovation Place
W. Lafayette, IN 47906
765 – 497 – 3355
PROTOTYPE
NOT FOR USE IN HUMANS OR IN VITRO DIAGNOSTICS
COOK BIOTECH CONFIDENTIAL PROPRIETARY DEVICE

Right side heart conditions
Mandapaka, S. et al
"Simultaneous Measurement of Left and Right Ventricular Volumes and Ejection..."
Noordegraaf, A.V. et al
"Pathophysiology of the right ventricle and of the pulmonary..."

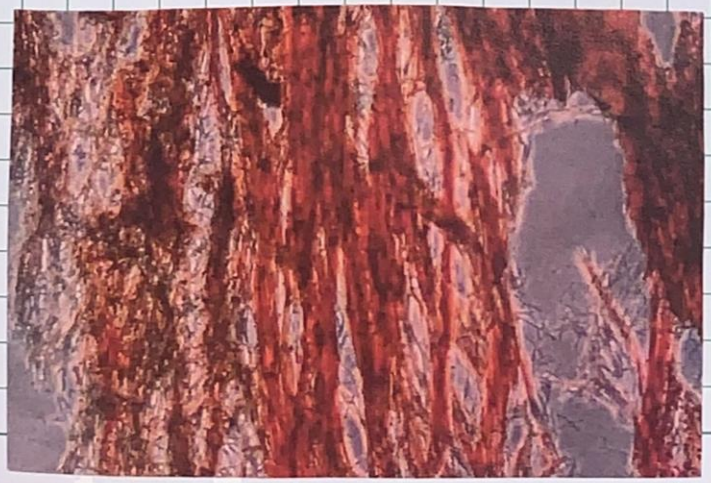


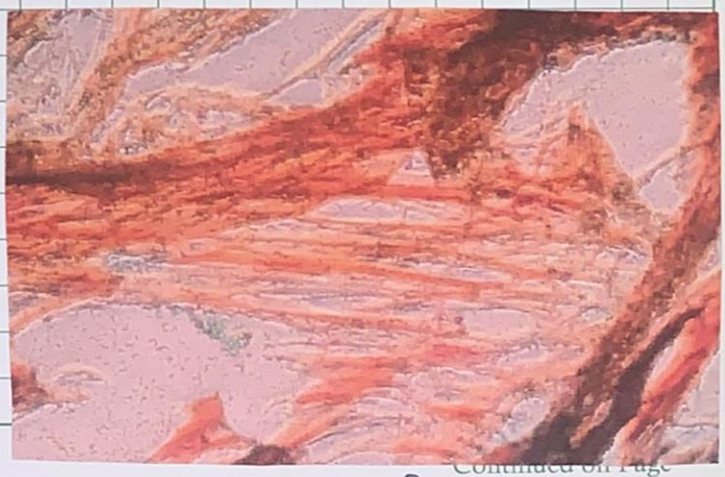
70 bpm
SV: 50 mL
MPP: 15 mmHg
35%-systolic - 65% diastolic

Tricuspid

70 bpm
SV: 71 mL
MAP: 100 mmHg
35%-systolic - 65% diastolic

Mitral





Longitudinal

Direction of pull

Circumferential

Continued on Page

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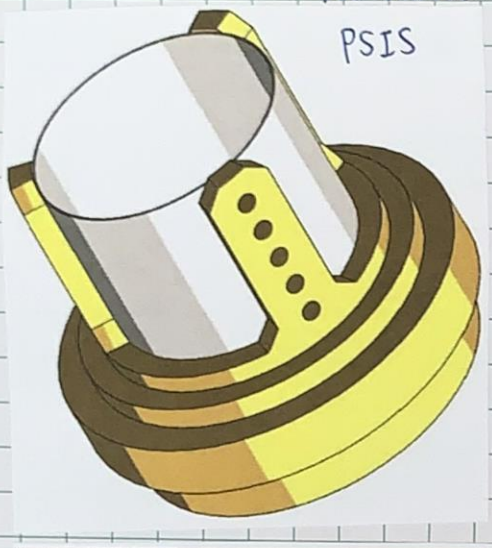
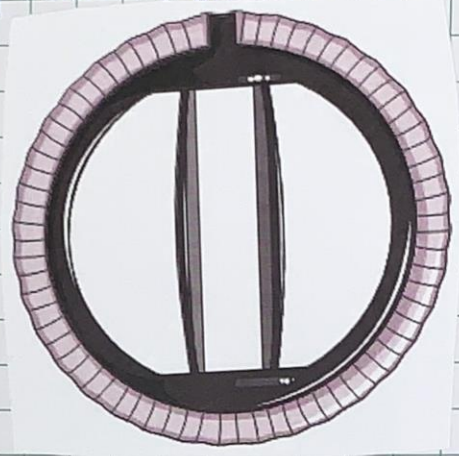
Signed Date Signed 2/9/2021 Date

Project

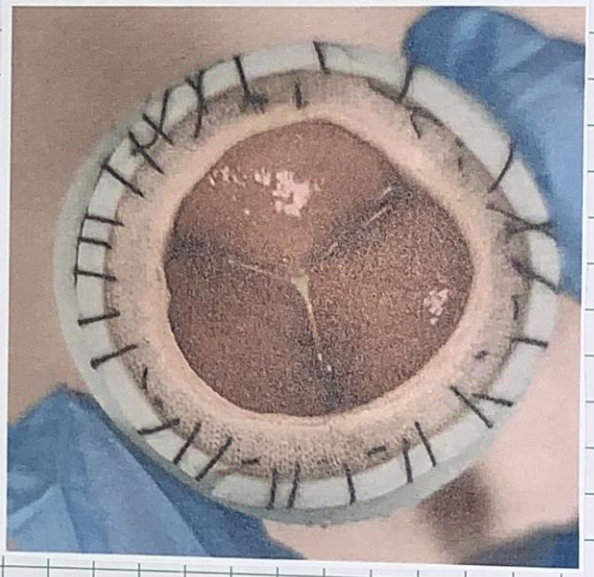
Continued from Page

2/18/2021: Vivitro tests - N=4 CorMatrix 26-mm PSIS cylindrical valves
Data used for HVS 2021 Conference
Controls: clinically available bi-leaflet mechanical valve and tri-leaflet bioprosthetic valve of similar diameter.

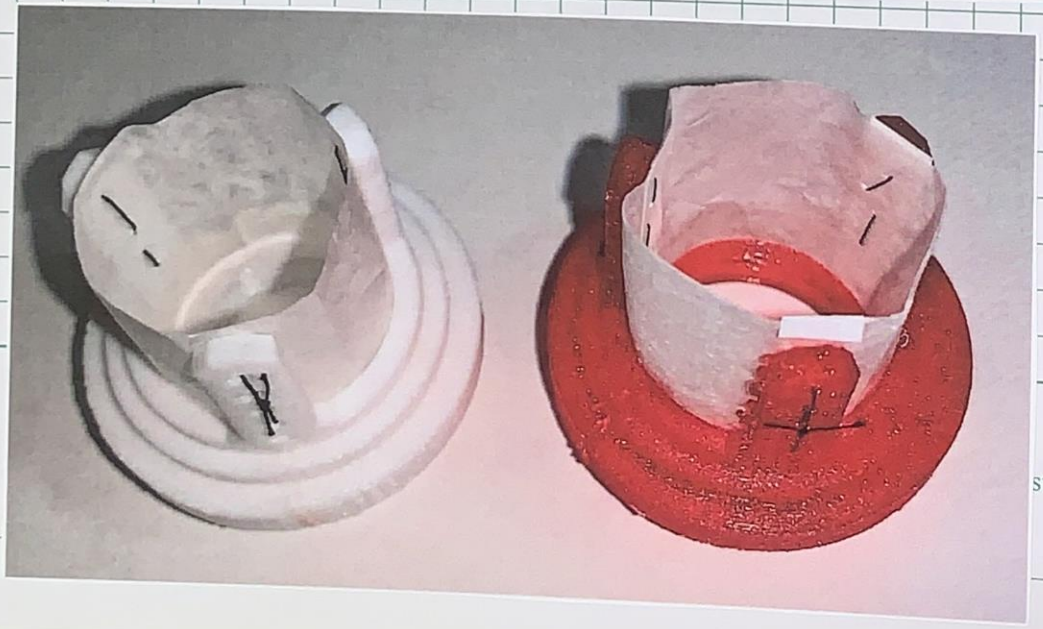




mechanical valve



bioprosthetic valve



cylindrical PSIS valve

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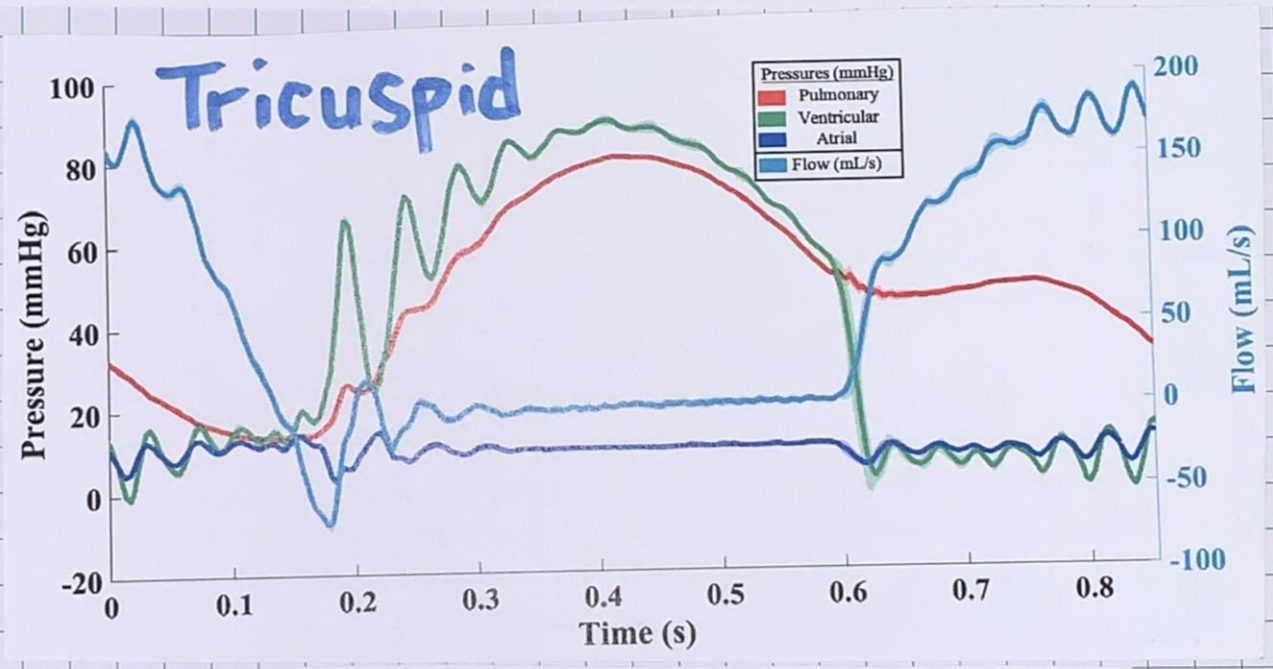

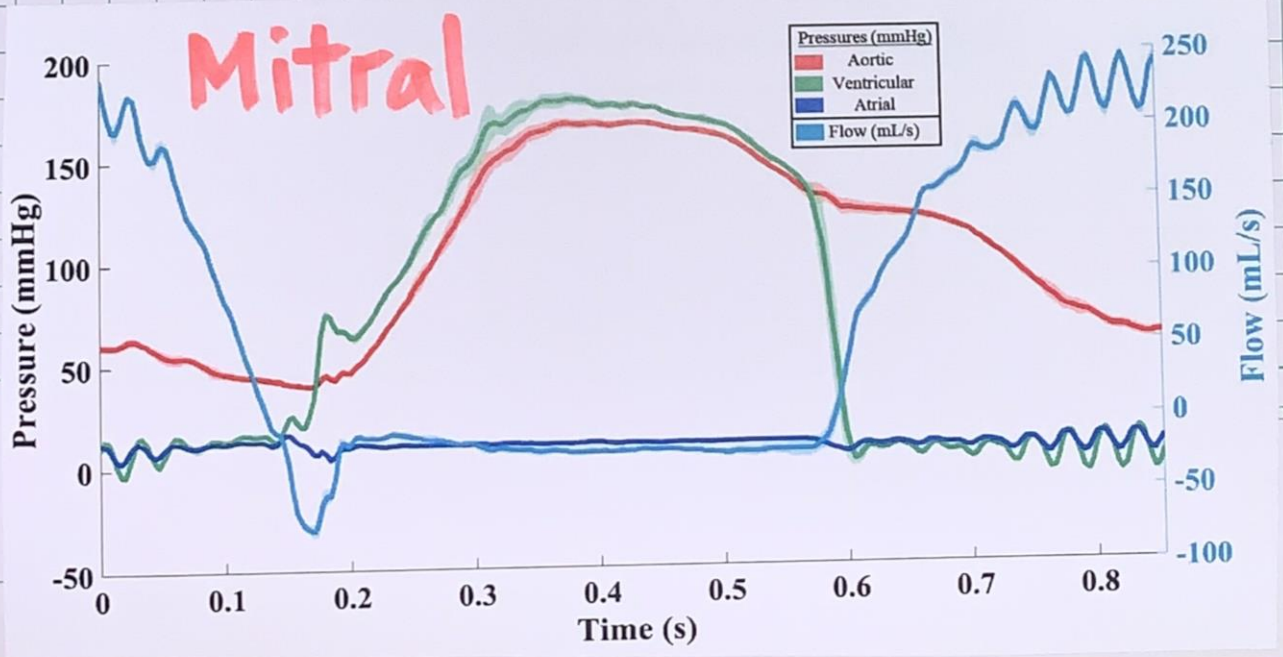

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2/18/2021
Date

Flow and Pressure Comparison

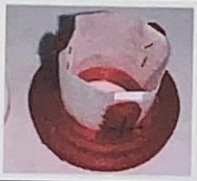
TRICUSPID				
Valve Type	Regurgitation Factor (%)	ΔP (mmHg)	Q_{RMS} (mL/s)	EOA (cm^2)
*PSIS (N=4)	6.84 ± 0.62	2.47 ± 0.92	72.75 ± 1.05	1.11 ± 0.24
Mechanical	4.91	1.89	74.7	1.05
Bioprosthetic	8.73	0.8	72.76	1.57
Stenotic	> 30%	> 5	> 225	< 1

*Mean ± SEM

MITRAL				
Valve Type	Regurgitation Factor (%)	ΔP (mmHg)	Q_{RMS} (mL/s)	EOA (cm^2)
*PSIS (N=4)	13.75 ± 0.71	0.395 ± 0.13	101.75 ± 0.44	2.48 ± 0.35
Mechanical	3.71	2.08	101.42	1.36
Bioprosthetic	10.41	1.88	103.37	1.46
Stenotic	> 30%	> 10	> 712	< 1

*Mean ± SEM

Baumgartner, H. et al.
Zoghbi, W. A. et al.

Valve Type	Parameter	Mitral	Tricuspid	Overall Performance
Cylindrical PSIS 	Regurgitation		√	Mitral ≥ Tricuspid
	ΔP	√		
	EOA	√		
Mechanical 	Regurgitation	√		Mitral ≥ Tricuspid
	ΔP		√	
	EOA	√		
Bioprosthetic 	Regurgitation		√	Tricuspid > Mitral
	ΔP		√	
	EOA		√	

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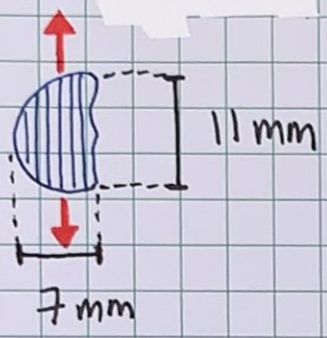
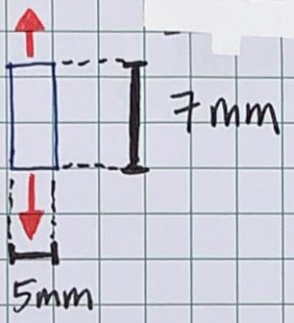
Signed _____ Date 2/18/2021

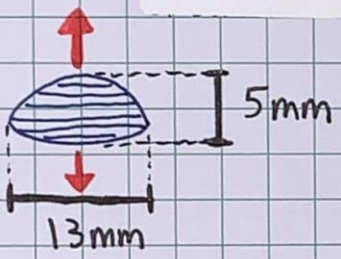
Aortic 1 Circumferential (C)



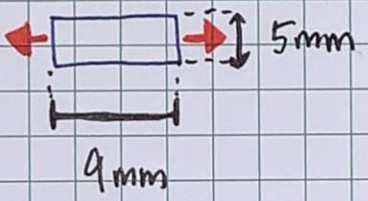
Mitral 1 (C2):



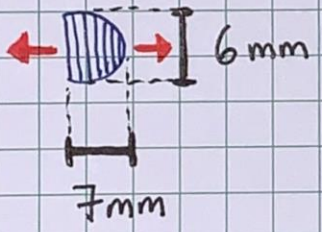
Aortic 2 Radial (R1)



Mitral 2 Radial (R)



Aortic 3 Radial (R2)




Direction of pull

Thickness: 0.1 mm

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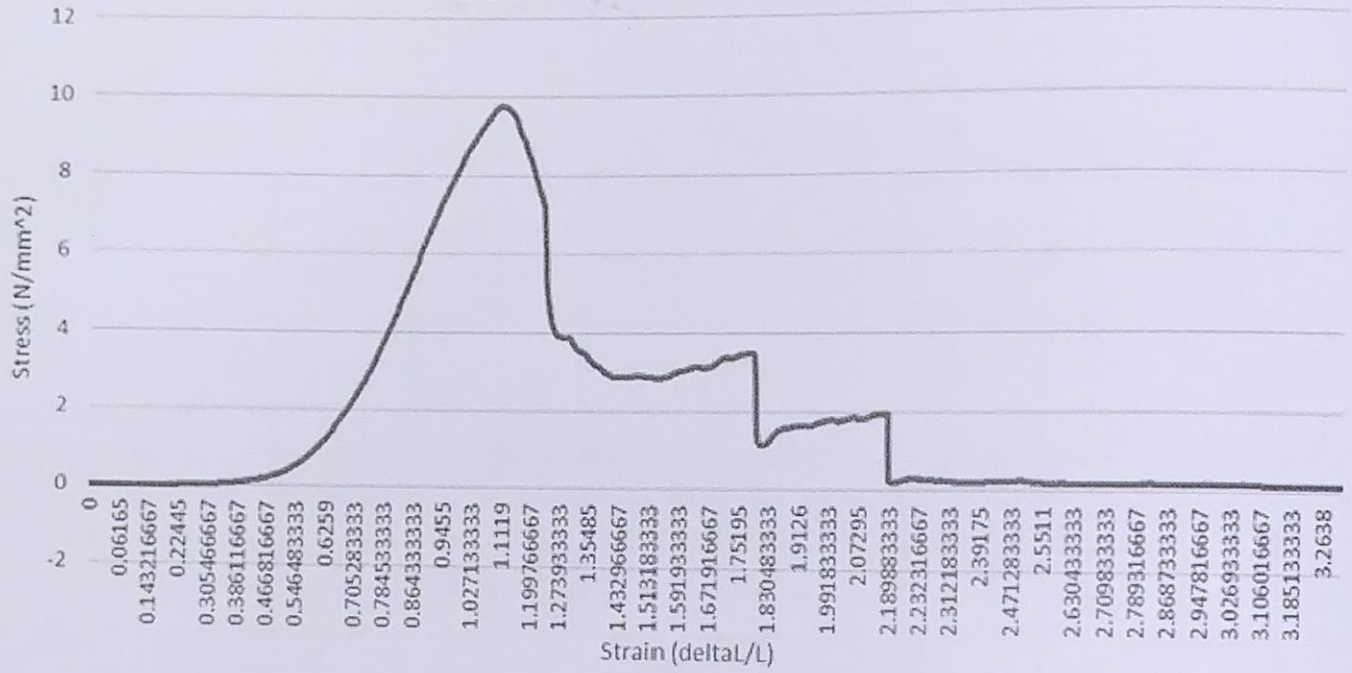
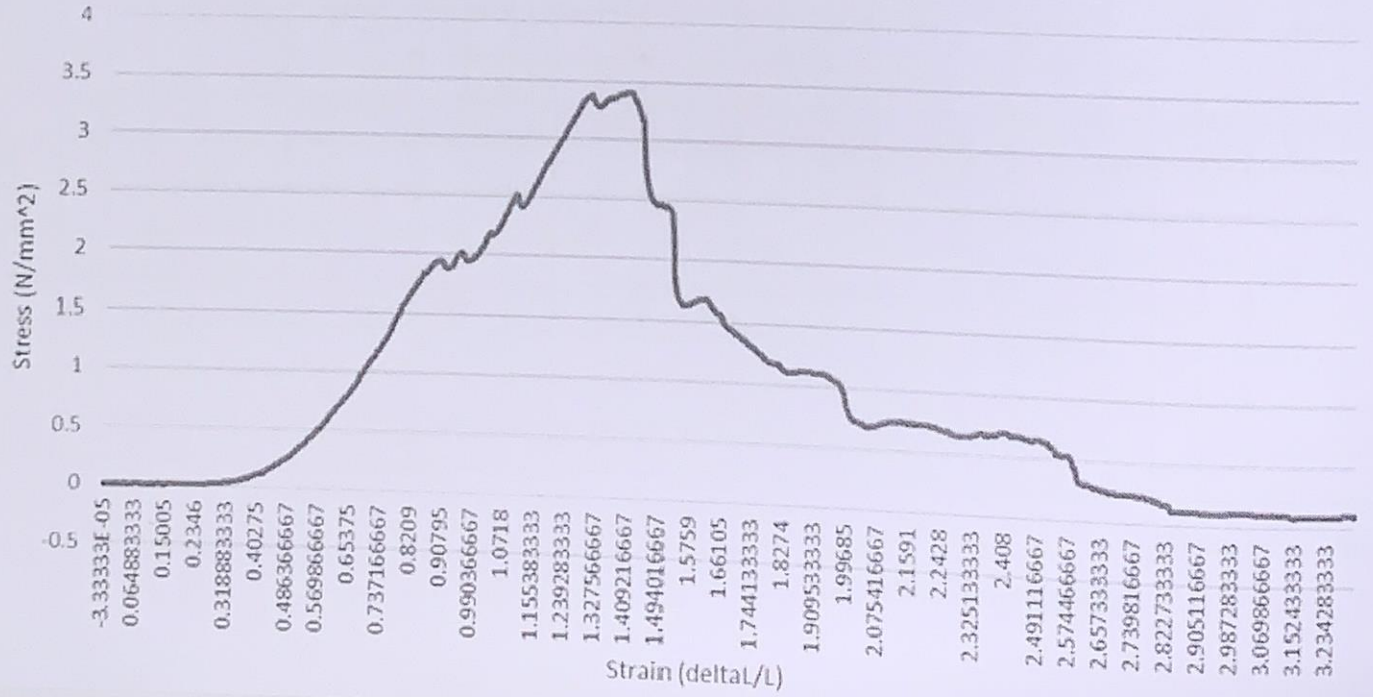
Date

Young's Moduli (N/mm^2)
Mechanical Test Results
Circumferential: 11.61332
Radial: 3.355208
} Aortic Valves

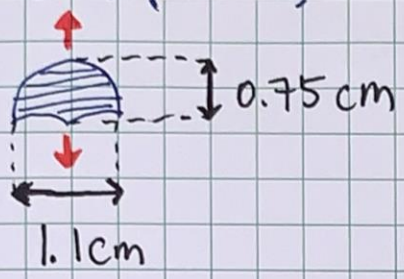
Aortic Valve
Native Porcine Circumferential: Stress-Strain Curve



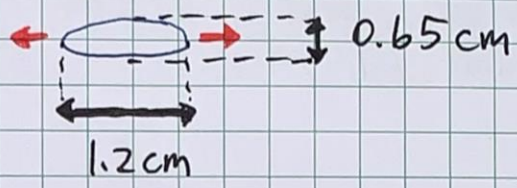
Native Porcine Aortic Valve Radial 1: Stress-Strain Curve



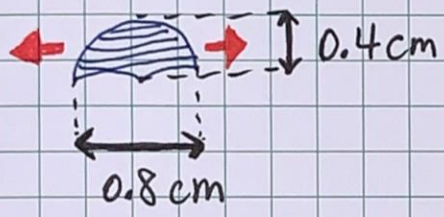
Aortic #1 (Radial)



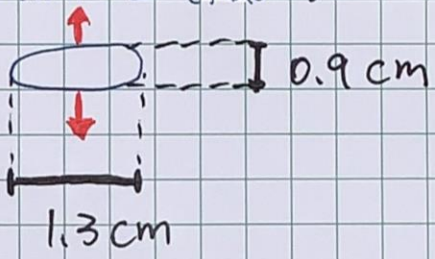
Mitral #1 (Cir 1)



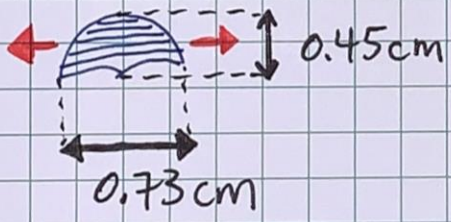
Aortic #2 (Cir 1)



Mitral #2 (Rad 1)



Aortic #3 (Cir 2)




Direction of pull

thickness: 0.1 mm = 0.01 cm

Continued on Page

Read and Understood By

Signed Date Signed 4/22/2021 Date

Project _____

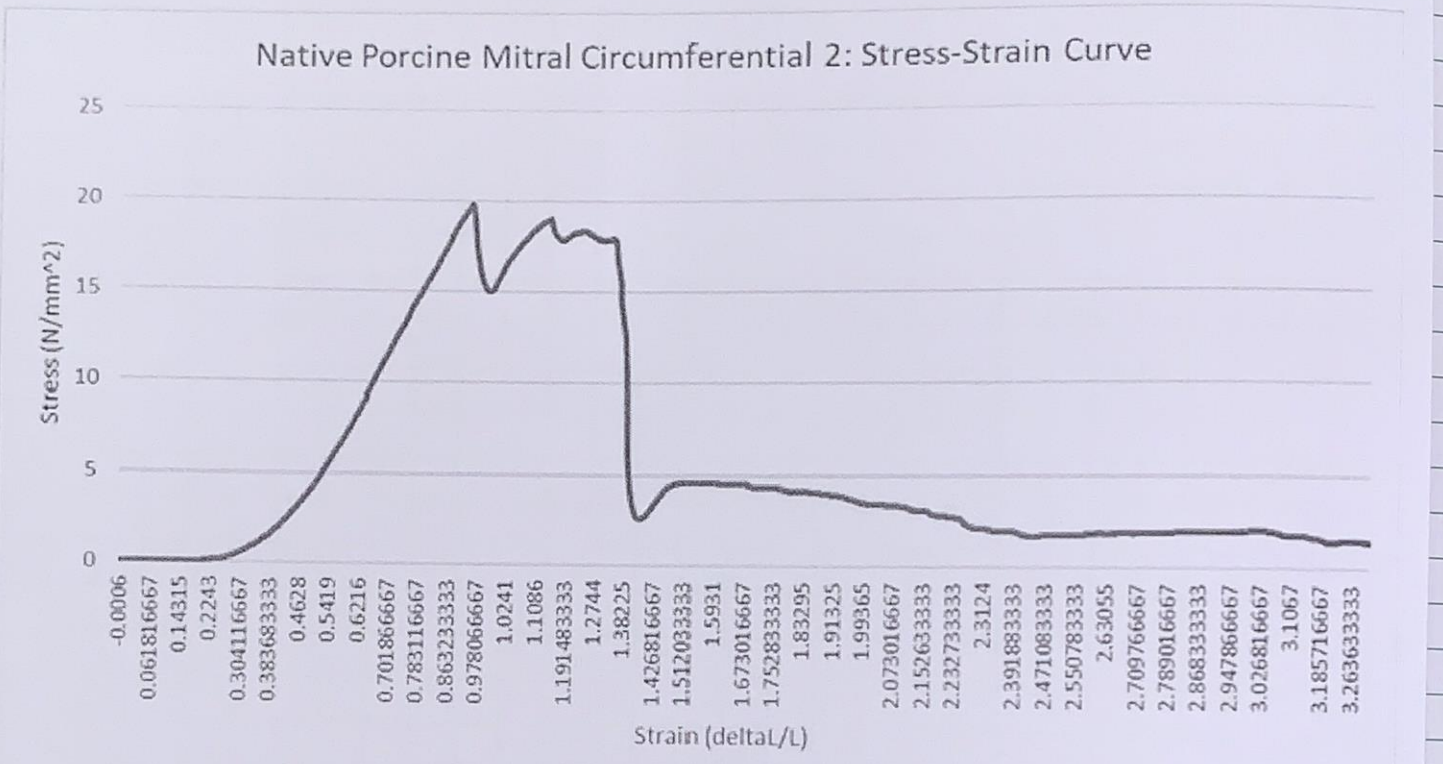
Continued from Page _____

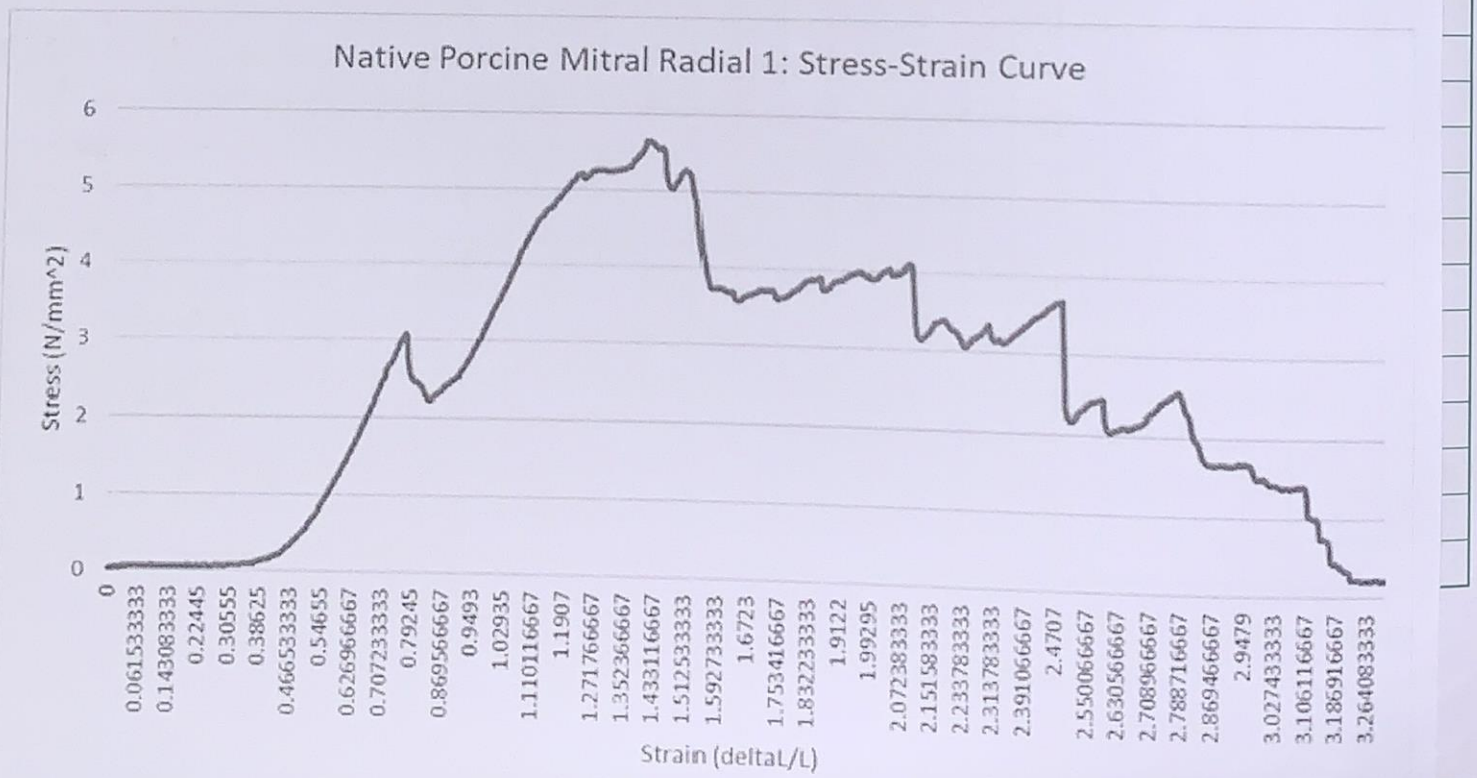
Young's Moduli (N/mm^2)

Mechanical Test Results } Mitral Valves

Circumferential: 16.46789

Radial: 6.652912





hAOVEC - Aortic Valve Endothelial Cells (hVEC)
Catalog # 00225975
Quantity ordered: 2 vials @ 500,000 cells per vial
Lot #: 1F5027

Catalog #: CC-3202

hVEC culture media

Culture system containing EBM™-2 Basal Medium (CC-3156) and EGM™-2 MV Microvascular Endothelial Cell Growth Medium SingleQuots™ supplements (CC-4147) required for growth of Microvascular Endothelial Cells
https://bioscience.lonza.com/lonza_bs/US/en/Primary-and-Stem-Cells/p/0000000000000185321/EGM--2-MV-Microvascular-Endothelial-Cell-Growth-Medium-2-BulletKit

Order No.	Order Date	Customer Order No.	Customer Contact
33504745	07-Apr-2021	MATRIX	Sharan Ramaswamy - 410 812 1734

Line Item	Product Code/ Description	Order Qty	Ship Qty	UOM	Lot No./Ser.Nr.	Expiration date
010	CC-3202 hVEC media EGM-2 MV BulletKit (CC-3156 & CC-4147)	2.000	2.000	KT		
020	CC-3156 EBM-2 Basal Medium 500 ml	2.000	2.000	BOT	0000966095	14-Jan-2022
030	CC-4147 EGM-2 MV SingleQuot Kit Suppl. & Growth Factors	2.000	2.000	KT	0000974197	10-Feb-2022

FBS
CAT. NO.: CC-4102B 25 ML
FETAL BOVINE SERUM
CELL CULTURE TESTED
LOT NO.: 0000974188 EXP.: 08 MAR 2025
STORE AT -20 °C
FOR RESEARCH USE ONLY
(rev. 6/08)

rhEGF
CAT. NO.: CC-4317B 0.5 ML
EPIDERMAL GROWTH FACTOR HUMAN, RECOMB. IN A BUFFERED BSA SALINE SOLUTION
LOT NO.: 0000974195 EXP.: 05 MAR 2022
STORE AT -20 °C
FOR RESEARCH USE ONLY
(REV. 6/08)

ASCORBIC ACID
CAT. NO.: CC-4116B 0.5 ML
IN AQUEOUS SOLUTION
CELL CULTURE TESTED
LOT NO.: 0000974194 EXP.: 15 FEB 2022
STORE AT -20° C
FOR RESEARCH USE ONLY
(REV. 6/08)

VEGF
CAT. NO.: CC-4114B 0.5 ML
ENDOTHELIAL GROWTH FACTOR VASCULAR HUMAN RECOMBINANT
LOT NO.: 0000974191 EXP.: 11 FEB 2022
STORE AT -20° C
FOR RESEARCH USE ONLY
(REV. 6/08)

rhFGF-B
CAT. NO.: CC-4113B 2.0 ML
r HUMAN FIBROBLAST GROWTH FACTOR-B
LOT NO.: 0000974190 EXP.: 17 FEB 2022
STORE AT -20° C
FOR RESEARCH USE ONLY
(REV. 6/08)

R³-IGF-1
CAT. NO.: CC-4115B 0.5 ML
RECOMB. LONG R INSULIN-LIKE GROWTH FACTOR-1 IN AQUEOUS SOLUTION
CELL CULTURE TESTED
LOT NO.: 0000974193 EXP.: 26 FEB 2022
STORE AT -20° C
FOR RESEARCH USE ONLY
(REV. 6/08)

GA-1000
CAT. NO.: CC-4381B 0.5 ML
GENTAMICIN SULFATE AMPHOTERICIN-B
CELL CULTURE TESTED
LOT NO.: 0000974196 EXP.: 16 FEB 2022
STORE AT -20° C
FOR RESEARCH USE ONLY SEE MSDS
(REV. 6/08)

HYDROCORTISONE
CAT. NO.: CC-4112B 0.2 ML
WARNING!: R11 HIGHLY FLAMMABLE
CELL CULTURE TESTED
LOT NO.: 0000974189 EXP.: 10 FEB 2022
STORE AT -20° C
FOR RESEARCH USE ONLY
(REV. 6/08)

Continued on Page

4/29/2021
Date

Lonza

Certificate of Analysis

Florida International University
Denise Hsu
Engineering Ctr Rm EC2614
10555 West Flagler Street
Miami FL 33174

Despatch Date: 19-Apr-2021
Customer Order: MATRIX
Delivery: 72095399
Sales Order: 33504740

Product Name: hAoVEC-Aortic Valve Endothelial Cells
Material Number: 00225975
Batch No: 1F5027
Quantity: 2.000 AMP
Manufacturing Date: 01-Feb-2021

Test	*RESULT*	*SPECIFICATION MIN*	*MAX*	*UNIT*
DONOR INFORMATION				
Age	37			
Sex	FEMALE			
VIRUS TESTING				
HIV	Not Detected			
Hepatitis B	Not Detected			
Hepatitis C	Not Detected			
SAFETY TESTING				
Sterility Test	Negative	Negative		
Mycoplasma	Negative	Negative		
CELL STRAIN CALCULATIONS				
Viability	91	For Information Only		Target: >= 70%
%				
Cell Count (cells/amp)	1601700	>= 5x10E5 cells/vial		
Seeding Efficiency	56	For Information Only		9999999 %
Doubling Time	17	For Information Only		9999999 hrs
CELL STAINING				
Factor VIII Expression	90	For Information Only		9999999 %
Alpha Smooth Muscle Actin Expression	0	For Information Only		%

This lot has been reviewed by Quality Assurance in compliance with requirements of Lonza's Quality System. This document was generated from a validated Part 11-compliant electronic system and thus handwritten signatures are not required.

For Technical Assistance, call 1-800-521-0390

Lonza
523 Davis Drive Suite 400B
Morrisville, NC, 27560

Signed Date Signed Date

4/29/2021

Certificate of Analysis

Florida International University
Denise Hsu
Engineering Ctr Rm EC2614
10555 West Flagler Street
Miami FL 33174

Despatch Date: 19-Apr-2021
Customer Order: MATRIX
Delivery: 72095399
Sales Order: 33504740

Product Name: hAoVEC-Aortic Valve Endothelial Cells
Material Number: 00225975
Batch No: 1F5027
Quantity: 2.000 AMP
Manufacturing Date: 01-Feb-2021

Test	*RESULT*	*SPECIFICATION MIN*	*MAX*	*UNIT*

Additional Information:
These cells were isolated from donated human tissue after obtaining permission for their use in research applications by informed consent or legal authorization. This product is for research use only. Details concerning the use of our cell and media products can be downloaded from our website at www.lonza.com/cell-protocols.

Cheryl Kitchen
Electronically signed by Cheryl Kitchen
Date: 11-MAR-2021 14:39:00 EST
RELEASE (Inspection Lot: Usage Decision)

This lot has been reviewed by Quality Assurance in compliance with requirements of Lonza's Quality System. This document was generated from a validated Part 11-compliant electronic system and thus handwritten signatures are not required.

For Technical Assistance, call 1-800-521-0390

Lonza
523 Davis Drive Suite 400B
Morrisville, NC, 27560

Signed Date

Signed Date 4/29/2021

Lonza

Certificate of Analysis

Florida International University
Denise Hsu
Engineering Ctr Rm EC2614
10555 West Flagler Street
Miami FL 33174

Despatch Date: 22-Apr-2021
Customer Order: MATRIX
Delivery: 72100557
Sales Order: 33504745

Product Name: EBM-2 Basal Medium
500 ml
Material Number: CC-3156
Batch No: 0000966095
Quantity: 2.000 BOT
Manufacturing Date: 14-Jan-2021
Expiration Date: 14-Jan-2022

Test	*RESULT*	*SPECIFICATION MIN*	*MAX*	*UNIT*
pH Test (Undiluted)	7.8	7.6	8.0	
Osmolality (mOsm/kg H2O)	273	260	290	mOs/kg
Endotoxin-Media (EU/ml)	< 0.500	Test & Report	***	
Sterility	Negative	Negative	***	

Additional Information:
This product was manufactured aseptically according to the requirements of ISO:9001 using a validated sterile filtration method and tested where appropriate using USP/EP methodology or approved alternative methods. This product is intended for research use only. It is the end user#s responsibility to ensure that the final product meets the requirements of the application for which it is to be used. Test results are determined using Lonza#s currently approved protocols. If animal origin materials are used, these materials are sourced from suppliers considered low risk according to the #Note for Guidance on minimizing the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products" (EMEA/410/01) as put in force by directive 1999/82/EC.

SAPNA PATEL
Electronically signed by SAPNA PATEL
Date: 05-FEB-2021 19:02:31 CET
RELEASE (Inspection Lot: Usage Decision)

This lot has been reviewed by Quality Assurance in compliance with requirements of Lonza's Quality System. This document was generated from a validated Part 11-compliant electronic system and thus handwritten signatures are not required.

For Technical Assistance, call 1-800-521-0390

Lonza Walkersville Inc.
8830 Biggs Ford Road
Walkersville, MD 21793 8415
Tel (301) 898 7025
Fax (301) 845 4024

Signed _____ Date _____

Signed _____ Date 4/29/2021

AoVEC 41911
WARNING HUMAN SOURCE MATERIAL
Human Aortic Valvular Endothelial Cells
≥500,000 CELLS/ML STORE AT −180 C
DATE CRYOPRESERVED: 01 FEB 2021
CAT. #: 00225975
LOT NO.: 1F5027
FOR RESEARCH USE ONLY
04689
(rev. 1/18)

4/29/2021: Plated human valve endothelial cells in 1 T25 + 1 T75 (protocol on pg. 77, seeding density 5,000 cells/cm²)
4/30/2021: Changed hVEC media
5/2/2021: Passaged hVEC P3 → P4 Count 1: 7.92×10^5 cells/mL
Count 2: 9.44×10^5 cells/mL > 8.68×10^5 cells/mL
Replated @ 5000 cells/cm² At 5mL → 4.34 million cells
5/4/2021: Trypsinized hVEC Count 1: 2.33×10^6 cells/mL
count 2: 2.20×10^6 cells/mL > 2.265×10^6 cells/mL (5mL)
Plated 3.2 million in 1 T75 static start time 5:30pm
seeded 3.2 million in 8 channels (400,000 per channel) in 1 Bioflux plate
Bioflux media: DMEM 1000 mL
5% FBS 50 mL
1% P/S 10 mL
Plated the rest in 3 T125's (P5)
5/5/2021: Started steady flow (0 OSI) in Bioflux
5/6/2021: Trypsinized 1 T175 flask Count 1: 5.81×10^5 cells/mL > 5.43×10^5 cells/mL
Count 2: 5.05×10^5 cells/mL
Total of 6mL → 3.258×10^6 cells. Seeded 1 Bioflux plate
Collected static media & hVEC static RNA, stored in −80°C.
Changed hVEC media in 2 T175's.
5/7/2021: Terminated steady flow 0 OSI, stored conditioned media & RNA in −80°C
Started 0.25 OSI. (9pm)
5/8/2021: Trypsinized 1 T175 Count 1: 9.28×10^5 cells/mL > 10.54×10^5 cells/mL
Count 2: 1.18×10^6 cells/mL
× 6 mL → 6.3×10^6 cells.
Seeded 1 Bioflux plate @ 400,000 cells per channel (381 µL)
plated the rest in 3 T175 flasks.
Froze 1 T175 into 6 vials.
5/9/2021: Froze 1 T175 & 2 T75 into 7 vials.
5/10/2021: Terminated 0.25 OSI, stored conditioned media & RNA in −80°C
Started 0.50 OSI. (10pm)
5/12/2021: Terminated 0.50 OSI, stored conditioned media & RNA in −80°C
5/13/2021: Froze 6 vials of hVEC in P6.
5/20/2021: Ultracentrifuged hVEC media

Continued on Page

Read and Understood By

Signed _____ Date _____ Signed _____ Date 4/29/2021

Conditioning groups:

- Fresh PC - Pro-calcific media
- Fresh N - Normal media

→ 18 wells

(2)

- hVEC-Static
- hVEC-Steady
- hVEC-0.25 OSI
- hVEC-0.50 OSI

ORG (4)

- hVEC-Static-PC
- hVEC-Steady-PC
- hVEC-0.25 OSI-PC
- hVEC-0.50 OSI-PC

ORG PC (4)

- hVEC-Static-EX-PC
- hVEC-Steady-EX-PC
- hVEC-0.25 OSI-EX-PC
- hVEC-0.50 OSI-EX-PC

EX PC (4)

- hVEC-Static-CY-PC
- hVEC-Steady-CY-PC
- hVEC-0.25 OSI-CY-PC
- hVEC-0.50 OSI-CY-PC

CY PC (4)

Ultra-centrifuge

After UC, remove supernatant. Pipette into new Eppendorf tube. Add PC ingredients and label it according to flow group + CY + PC.

Supernatant / cytokines (CY Group)

Pellet / exosomes (EX Group)

Resuspend pellet w/ fresh PC media. Label according to flow group + EX + PC.

24-well plate

FRESH PC		STATIC ORG	STEADY ORG	0.25 ORG	0.50 ORG
FRESH N		STATIC ORG PC	STEADY ORG PC	0.25 ORG PC	0.50 ORG PC
		STATIC EX PC	STEADY EX PC	0.25 EX PC	0.50 EX PC
		STATIC CY PC	STEADY CY PC	0.25 CY PC	0.50 CY PC

Continued on Page

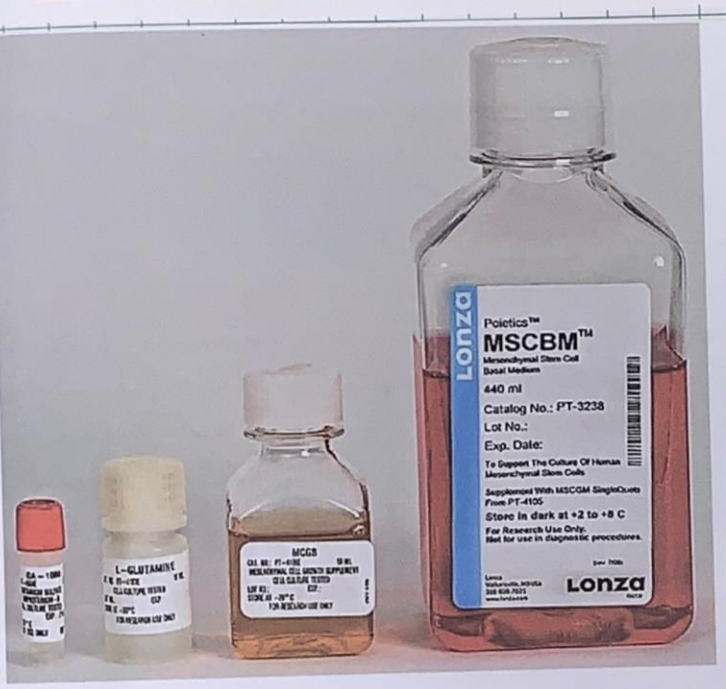
Read and Understood By

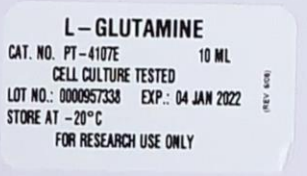
Signed Date Signed 5/20/2021 Date

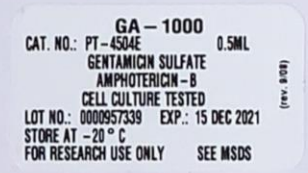
Order No.	Order Date	Customer Order No.	Customer Contact
33504740	07-Apr-2021	MATRIX	Sharan Ramaswamy - 410 812 1734

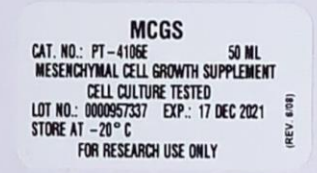
Line Item	Product Code/ Description	Order Qty	Ship Qty	UOM	Lot No./Ser.Nr.	Expiration date
010	00225974 hVIC-Valvular Interstitial Cells	2	2	AMP	1F5026	
020	00225975 hAoVEC-Aortic Valve Endothelial Cells	2	2	AMP	1F5027	

hVIC culture media









added on 5/17/2021

Catalog #: PT-3001

Culture system containing MSCBM™ Basal Media (PT-3238) and MSCGM™ SingleQuots Supplement Kit (PT-4105) required for proliferation of human bone marrow derived mesenchymal stem cells.
https://bioscience.lonza.com/lonza_bs/US/en/Primary-and-Stem-Cells/p/0000000000000186709/MSCGM-Mesenchymal-Stem-Cell-Growth-Medium-BulletKit

Order No.	Order Date	Customer Order No.	Customer Contact
33504745	07-Apr-2021	MATRIX	Sharan Ramaswamy - 410 812 1734

Line Item	Product Code/ Description	Order Qty	Ship Qty	UOM	Lot No./Ser.Nr.	Expiration date
009	REFRIG REFRIGERATION (hVIC media)	1.000	1.000	EA		
040	PT-3001 MSCGM BulletKit (PT-3238 & PT-4105)	2.000	2.000	KT		
050	PT-3238 MSCGM hMSC Basal Medium, 440 ml	2.000	2.000	BOT	0000937155	05-Oct-2021
060	PT-4105 MSCGM hMSC SingleQuot Kit	2.000	2.000	KT	0000957340	15-Dec-2021

Signed Date Signed Date 4/29/2021

Lonza

Certificate of Analysis

Florida International University
Denise Hsu
Engineering Ctr Rm EC2614
10555 West Flagler Street
Miami FL 33174

Despatch Date: 19-Apr-2021
Customer Order: MATRIX
Delivery: 72095399
Sales Order: 33504740

Product Name: hVIC-Valvular Interstitial Cells
Material Number: 00225974
Batch No: 1F5026
Quantity: 2.000 AMP
Manufacturing Date: 08-Feb-2021

Test	*RESULT*	*SPECIFICATION MIN*	*MAX*	*UNIT*
DONOR INFORMATION				
Age	37			
Sex	FEMALE			
VIRUS TESTING				
HIV	Not Detected			
Hepatitis B	Not Detected			
Hepatitis C	Not Detected			
SAFETY TESTING				
Sterility Test	Negative	Negative		
Mycoplasma	Negative	Negative		
CELL STRAIN CALCULATIONS				
Viability	95	For Information Only	Target: >= 70%	
%				
Cell Count (cells/amp)	595800	>= 5x10E5 cells/vial***		
Seeding Efficiency	90	For Information Only	9999999	%
Doubling Time	70	For Information Only	9999999	hrs
CELL STAINING				
Factor VIII Expression	0	For Information Only	9999999	%
Alpha Smooth Muscle Actin Expression	90	For Information Only		%

This lot has been reviewed by Quality Assurance in compliance with requirements of Lonza's Quality System. This document was generated from a validated Part 11-compliant electronic system and thus handwritten signatures are not required.

For Technical Assistance, call 1-800-521-0390

Lonza
523 Davis Drive Suite 400B
Morrisville, NC, 27560

Signed _____ Date _____

Signed _____ Date 4/29/2021

Certificate of Analysis

Florida International University
Denise Hsu
Engineering Ctr Rm EC2614
10555 West Flagler Street
Miami FL 33174

Despatch Date: 19-Apr-2021
Customer Order: MATRIX
Delivery: 72095399
Sales Order: 33504740

Product Name: hVIC-Valvular Interstitial Cells
Material Number: 00225974
Batch No: 1F5026
Quantity: 2.000 AMP
Manufacturing Date: 08-Feb-2021

Test	*RESULT*	*SPECIFICATION MIN*	*MAX*	*UNIT*

Additional Information:
These cells were isolated from donated human tissue after obtaining permission for their use in research applications by informed consent or legal authorization. This product is for research use only. Details concerning the use of our cell and media products can be downloaded from our website at www.lonza.com/cell-protocols.

Cheryl Kitchen
Electronically signed by Cheryl Kitchen
Date: 11-MAR-2021 14:52:35 EST
RELEASE (Inspection Lot: Usage Decision)

This lot has been reviewed by Quality Assurance in compliance with requirements of Lonza's Quality System. This document was generated from a validated Part 11-compliant electronic system and thus handwritten signatures are not required.

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Lonza
523 Davis Drive Suite 400B
Morrisville, NC, 27560

Signed _____ Date _____ Signed _____ Date 4/29/2021

Lonza

Certificate of Analysis

Florida International University
Denise Hsu
Engineering Ctr Rm EC2614
10555 West Flagler Street
Miami FL 33174

Despatch Date: 12-Apr-2021
Customer Order: MATRIX
Delivery: 72086716
Sales Order: 33504745

Product Name: MSCBM hMSC Basal Medium, 440 ml
Material Number: PT-3238
Batch No: 0000937155
Quantity: 2.000 BOT
Manufacturing Date: 05-Oct-2020
Expiration Date: 05-Oct-2021

Test	*RESULT*	*SPECIFICATION MIN*	*MAX*	*UNIT*
Sterility	Negative	Negative	***	
pH Test (Undiluted)	7.25	7.00	7.70	
Osmolality (mOsm/kg H2O)	316	306	346	
Endotoxin-Media (EU/ml)	< 0.500	Test & Report	***	

Additional Information:
This product was manufactured aseptically according to the requirements of ISO:9001 using a validated sterile filtration method and tested where appropriate using USP/EP methodology or approved alternative methods. This product is intended for research use only. It is the end user#s responsibility to ensure that the final product meets the requirements of the application for which it is to be used. Test results are determined using Lonza#s currently approved protocols. If animal origin materials are used, these materials are sourced from suppliers considered low risk according to the #Note for Guidance on minimizing the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products" (EMEA/410/01) as put in force by directive 1999/82/EC.

Valerie Williams
Electronically signed by Valerie Williams
Date: 16-NOV-2020 12:15:54 EST
RELEASE (Inspection Lot: Usage Decision)

This lot has been reviewed by Quality Assurance in compliance with requirements of Lonza's Quality System. This document was generated from a validated Part 11-compliant electronic system and thus handwritten signatures are not required.

For Technical Assistance, call 1-800-521-0390

Lonza Walkersville Inc.
8830 Biggs Ford Road
Walkersville, MD 21793 8415
Tel (301) 898 7025
Fax (301) 845 4024

Signed Date Signed Date 4/29/2021

5/18/2021: Plated a frozen vial of hVIC in two T 75's.
Made 50 mL of fresh Pro-Calcific media (PC media)
50 mL DMEM, 5% FBS, 1% PS
1.8 mM $CaCl_2$
3.8 mM NaH_2PO_4
0.4 units/mL of inorganic pyrophosphate

hVIC 41911
WARNING HUMAN SOURCE MATERIAL
Human Aortic Valve Interstitial Cells
≥500,000 CELLS/ML STORE AT −180 C
DATE CRYOPRESERVED: 08 FEB 2021
CAT. #: 00225974
LOT NO.: 1F5026
FOR RESEARCH USE ONLY
04689

MW:
NaH_2PO_4 = 119.98 g/mol
$CaCl_2$ = 110.98 g/mol

Made 50 mL of PC
Want 0.0038 M = 0.0038 $\frac{\text{moles}}{\text{liter}}$ of NaH_2PO_4

$$\frac{0.0038 \text{ moles}}{1 \text{ Liter}} = \frac{X \text{ moles}}{0.05 \text{ Liter}}$$

$X = 0.00019$ moles of NaH_2PO_4

$$\frac{1 \text{ mole}}{119.98 \text{ g}} = \frac{0.00019 \text{ mole}}{Y}$$

$Y = 0.0228$ g of NaH_2PO_4

Want 0.0018 M = 0.0018 $\frac{\text{moles}}{\text{liter}}$ of $CaCl_2$

$$\frac{0.0018}{1} = \frac{A}{0.05} \Rightarrow A = 0.00009 \text{ moles of } CaCl_2$$

$$\frac{1 \text{ mole}}{110.98 \text{ g}} = \frac{0.00009}{B} \Rightarrow B = 0.009988 \text{ g of } CaCl_2$$

Stock solution = 1 M $CaCl_2$

$$0.0018 \text{ mole/liter} \times 0.05 \text{ liter} = 1 \text{ mole/liter} \times ? \text{ volume}$$

$? = 0.00009$ Liter
$= 0.09$ mL
$= 90$ µL of $CaCl_2$ Stock Solution

Continued on Page

Read and Understood By

Signed Date Signed Date

5/18/2021

IP stock: 100 units = 1000 μL IP solution
Want: 0.4 units/mL

$$\frac{0.4 \text{ units}}{\text{mL}} = \frac{X \text{ units}}{50} \Rightarrow X = 20 \text{ units}$$

1 unit = 10 μL
20 units = 200 μL of IP stock solution

5/19/2021: Plated 1 frozen hVIC vial P3 in two T75's
5/20/2021: changed media
5/24/2021: changed media
5/28/2021: Passaged P3 → P4
6/1/2021: changed media
6/4/2021: Passaged P4 → P5
6/8/2021: Trypsinized 10 T75's
Count 1: 2.34×10^6 cells/mL
Count 2: 1.73×10^6 cells/mL
> 2.035×10^6 cells/mL (3 mL)
Seeded 1 24-well plate (layout on page 56) at 0.1×10^6 cells per well
Seeded 1 6-well plate at 0.5×10^6 cells per well in 4 wells
Plated the rest in 1 T75 (P6)

Static	Steady	X
0.25	0.50	X

6/12/2021: changed media in 24-well and 6-well plates
Split 1 T75 → 3 T75 hICs P6 → P7
6/15/2021: ARS staining on 24-well plate
collected RNA from 6-well plate
6/17/2021: Area scan of 24-well plate (ARS) 9×9

	1	2	3	4	5	6
A	0.882	0.065	0.517	0.376	0.416	0.341
B	0.368	0.071	1.685	1.85	1.124	1.43
C	0.063	0.096	1.239	1.078	0.831	0.846
D	0.066	0.063	1.507	2.318	0.87	1.207

Continued on Page

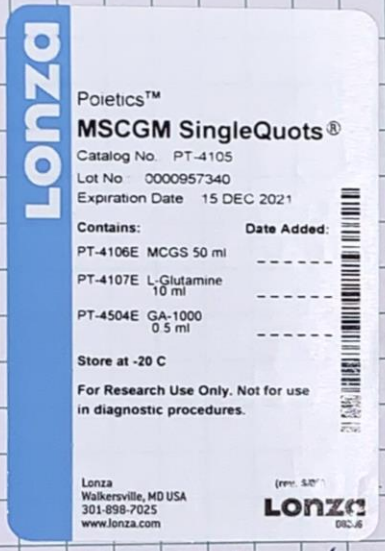
Read and Understood By

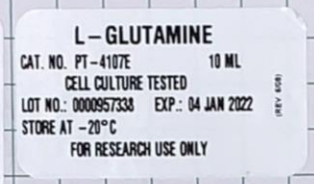
Signed _____ Date _____ Signed _____ Date 5/20/2021

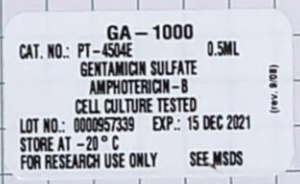
6/24/2021: RNA extraction & quantification (ng/μL)

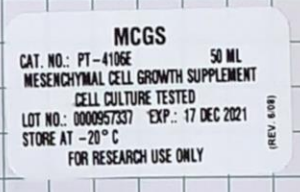
				AVG
HVEC	Static	112.2	116.8	114.5
	Steady	70.1	40.9	55.5
	0.25	21.6	19.1	20.35
	0.50	30.8	29.4	30.1
HVIC	Static	54.4	67.0	60.7
	Steady	163.2	246.7	204.95
	0.25	81.6	67.0	74.3
	0.50	55.1	61.2	58.15

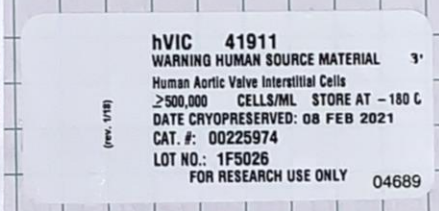
8/11/2021
8/12/2021





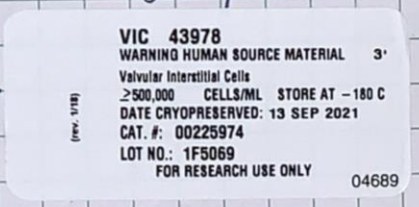






made hVIC media
plated 1 frozen vial of
hVIC in 1 T75.

8/13/2021: hVIC media change
8/17/2021: hVIC passage P4 → P5 (three T75's)
8/24/2021: Re-plated hVICs, instead of using trypsin, switched to TrypLE.
Suggested trypsin concentration <0.025% (from Lonza) to prevent cell detachment.
10/27/2021: Received a replacement hVIC vial from Lonza.
Plated new vial in 1 T75 flask. AGE 41 → SEX FEMALE



10/28/2021: Changed media
10/31/2021: Changed media
11/3/2021: Passaged P4 → P5 hVIC
11/5/2021: Changed media

media volume (0.5-1mL per well) 24-well seed density: 0.05×10^6 cells/well
18 wells in total → 900,000 cells
2 plates → 1.8 million cells

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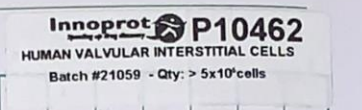
Signed 6/24/21 Date



12/6/2021: Innoprot hVIC & media + supplements
Media: FM (PLUS) Refer #P60166
Fibroblast Medium Batch # 4280921
Plated in 1 T75 P1
1 T25 P1
Coated w/ 0.2% w/v gelatin

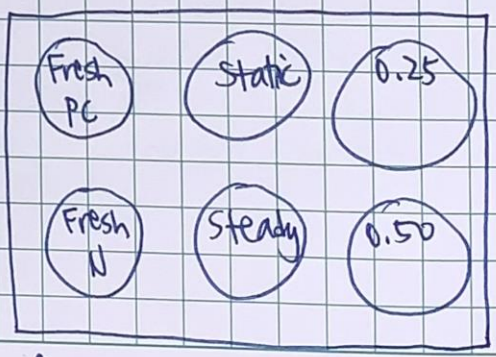
12/8/2021: Changed media

12/9/2021: Passaged hVIC P1 → P2, placed 37500 cells on glass coverslip
37500 per coverslip, made 3 coverslips in total 22293232
Steps for preparing coverslip: Fisherbrand Microscope Cover Glass 12CIR-2
1. Place coverslip in well, add 500 uL pure ethanol.
2. Incubate in UV for 30 minutes.
3. Remove ethanol, wash well w/coverslip using PBS or media.
4. Add 0.2% w/v gelatin onto coverslip in well.
5. Incubate in hood for 1 hr.
6. Remove gelatin without touching coverslip.
7. Wash well with PBS or media, gently.
8. Once dry, plate w/coverslip can be stored in fridge or in incubator. When using, pipette cell suspension directly onto glass. For 24-well plate size, pipette 25000 ~ 50000 cells per well. Move plate orbitally to evenly distribute cell suspension.

12/11/2021: Started experiment (HVEC conditioned media on hVICs) n=2
froze 4 vials. Placed 37500 cells on glass coverslip per well. Made 3 wells in total (for Dani's characterization)

x2

Seeded 50,000 cells per well in 24-well plates



x2

for RNA qPCR

Seeded 300,000 cells per well in 6-well plates

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Signed 12/6/2021 Date

12/10/2021: ARS staining (24-well plates) + collected hVIC RNA in 6-well plates using TRIzol.

FIBROBLAST MEDIUM-II

Product Type: Fibrobast Medium-II
Catalog Number: P60166

Product Description

Fibroblast Medium-II (FM-II) is a complete medium designed for optimal growth of normal human cardiac and kidney fibroblasts in vitro. It is a sterile, liquid medium which contains essential and non-essential amino acids, vitamins, organic and inorganic compounds, hormones, growth factors, trace minerals and a low concentration of fetal bovine serum (5%). The medium is HEPES and bicarbonate buffered and has a pH of 7.4 when equilibrated in an incubator with an atmosphere of 5% CO_2/95% air. The medium is formulated (quantitatively and qualitatively) to provide a defined and optimally balanced nutritional environment that selectively promotes proliferation and growth of normal human cardiac fibroblasts in vitro.

Components

- 500 ml of Basal Medium
- 25 ml of Fetal Bovine Serum (FBS)
- 5 ml of Fibroblast Growth Supplement 2 (FGS-2)
- 5 ml of penicillin/streptomycin solution (P/S solution)

Prepare for use

Thaw FGS-2, FBS and P/S solution at 37°C. Gently tilt the FGS tube several times during thawing to help the contents dissolve. **Make sure the contents of the supplement are completely dissolved into solution before adding to the medium.** Rinse the bottle and tubes with 70% ethanol, and then wipe to remove excess. Remove the cap, being careful not to touch the interior threads with fingers. Add FGS, FBS and P/S solution into basal medium in a sterile field, mix well and then the reconstituted medium is ready for use. Since several components of this medium are light-labile, it is recommended that the medium not be exposed to light for lengthy periods of time. If the medium is warmed prior to use, do not exceed 37°C. When stored in the dark at 4°C, the reconstituted medium is stable for one month.

Caution

If handled improperly, some components of the medium may present a health hazard. Take appropriate precautions when handling it, including the wearing of protective clothing and eyewear. Dispose of properly.

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REF: P10462

CARDIAC CELL SYSTEM INNOPROFILE™
HUMAN CARDIAC VALVULAR INTERSTITIAL CELLS

Product Type:	Cryo-preserved Valvular Interstitial Cells
Catalog Number:	P10462
Source:	Human Heart Valves
Number of Cells:	5 x 10^5 Cells / vial (1ml)
Storage:	Liquid Nitrogen

Human Valvular Interstitial Cells (HVIC) provided by Innoprot have been derived from heart valves that are explanted in culture. Human valvular interstitial cells are from a single donor. They are cryopreserved at primary culture and can be cultured and propagated at least 10 population doublings in the conditions provided by Innoprot.

These cells are positive for smooth muscle actin. These cells enable researchers to study the role of cardiac valves in vitro. Human valvular interstitial cells may be used for various types of valve replacement, stimulus contraction and transplantation studies into normal or diseased systems. In addition, they may be used for tissue engineering applications.

Product Use

THESE PRODUCTS ARE FOR RESEARCH USE ONLY. Not approved for human or veterinary use, for application to humans or animals, or for use in vitro diagnostic or clinical procedures

Recommended Medium

- Fibroblast Medium II Kit (Reference: P60166)

Product Characterization

Immunofluorescent method

- α-smooth muscle actin
- Vimentin

The cells test negative for HIV-1, HIV-II, HBV, HCV, mycoplasma, bacteria, yeast and fungi

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INSTRUCTIONS FOR CULTURING CELLS

IMPORTANT: Cryopreserved cells are very delicate. Thaw the vial in a 37 °C waterbath and return them to culture as quickly as possible with minimal handling!

Set up culture after receiving the order:

1. Prepare a poly-L-lysine coated flask (2 µg/cm², T-75 flask is recommended). Add 10 ml of sterile water to a T-75 flask and then add 150 µl of poly-L-lysine stock solution (1 mg/ml, Innoprot cat. no. PLL). Leave the flask in incubator overnight (minimum one hour at 37°C incubator).

2. Prepare complete medium: decontaminate the external surfaces of medium and medium supplements with 70% ethanol and transfer them to sterile field. Aseptically open each supplement tube and add them to the basal medium with a pipette. Rinse each tube with medium to recover the entire volume.

3. Rinse the poly-L-lysine coated flask with sterile water twice and add 20 ml of complete medium to the flask. Leave the flask in the hood and go to thaw the cells.

4. Place the vial in a 37°C waterbath, hold and rotate the vial gently until the contents are completely thawed. Remove the vial from the waterbath immediately, wipe it dry, rinse the vial with 70% ethanol and transfer it to a sterile field. Remove the cap, being careful not to touch the interior threads with fingers. Using 1 ml eppendorf pipette gently resuspend the contents of the vial.

5. Dispense the contents of the vial into the equilibrated, poly-L-lysine coated culture vessels. A seeding density of 5,000 cells/cm² is recommended.

Note: Dilution and centrifugation of cells after thawing are not recommended since these actions are more harmful to the cells than the effect of DMSO residue in the culture. It is also important that fibroblasts are plated in poly-L-lysine coated culture vessels that promote cell attachment.

6. Replace the cap or cover, and gently rock the vessel to distribute the cells evenly. Loosen cap if necessary to permit gas exchange.

7. Return the culture vessels to the incubator.

8. For best result, do not disturb the culture for at least 16 hours after the culture has been initiated. Change the growth medium the next day to remove the residual DMSO and unattached cells, then every other day thereafter.

Maintenance of Culture:

1. Change the medium to fresh supplemented medium the next morning after establishing a culture from cryopreserved cells..

2. Change the medium every three days thereafter, until the culture is approximately 70% confluent.

3. Once the culture reaches 70% confluence, change medium every other day until the culture is approximately 90% confluent.

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Subculture:

1. Subculture the cells when they are over 90% confluent.

2. Prepare poly-L-lysine coated flasks (2 µg/cm²) one day before subculture.

3. Warm medium, trypsin/EDTA solution (T/E, cat. no. 0103), trypsin neutralization solution (TNS, cat. no. 0113), and DPBS to room temperature. We do not recommend warming the reagents and medium at 37°C waterbath prior to use.

4. Rinse the cells with DPBS.

5. Add 8 ml of DPBS first and then 2 ml of trypsin/EDTA solution into flask (in the case of T-75 flask); gently rock the flask to make sure cells are covered by trypsin/EDTA solution; incubate the flask at 37°C incubator for 1 to 3 minutes or until cells are completely rounded up (monitored with inverted microscope). During incubation, prepare a 50 ml conical centrifuge tube with 5 ml of fetal bovine serum; transfer trypsin/EDTA solution from the flask to the 50 ml centrifuge tube (a few percent of cells may detached); continue incubate the flask at 37°C for 1 minutes (no solution in the flask at this moment); at the end of trypsinisation, one hand hold one side of flask and the other hand gently tap the other side of the flask to detach cells from attachment; check the flask under inverted microscope to make sure all cells are detached, add 5 ml of trypsin neutralization solution to the flask and transfer detached cells to the 50 ml centrifuge tube; add another 5 ml of TNS to harvest the residue cells and transfer it to the 50 ml centrifuge tube. Examine the flask under inverted microscope to make sure the cell harvesting is successful by looking at the number of cells left behind. There should be less than 5%.

Note: DPBS, trypsin/EDTA solution & trypsin neutralization solution are included in the "Primary Cells Detach Kit provided by Innoprot (Cat. N° P60305).

6. Centrifuge the 50 ml centrifuge tube (harvested cell suspension) at 1000 rpm (Beckman Coulter Allegra 6R centrifuge or similar) for 5 min; re-suspend cells in growth medium.

7. Count cells and plate cells in a new, poly-L-lysine coated flask with cell density as recommended.

Caution: *Handling human derived products is potentially bioharzadous. Although each cell strain testes negative for HIV, HBV and HCV DNA, diagnostic tests are not necessarily 100% accurate, therefore, proper precautions mush be taken to avoid inadvertent exposure.*
Always wear gloves and safety glasses when working these materials. Never mouth pipette. We recommend following the universal procedures for handling products of human origin as the minimum precaution against contamination [1].

[1]. Grizzle, W. E., and Polt, S. S. (1988) Guidelines to avoid personal contamination by infective agents in research laboratories that use human tissues. *J Tissue Culture Methods*. 11(4).

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PACKING LIST (PURCHASE ORDER # PO-67602021)

SHIPPER	CONSIGNEE
Jorge Gamiz	Att. Dr. Denise Hsu
Innovative Technologies in Biological Systems S.L. Parque Tecnológico de Bizkaia Edificio 502-1ª planta 48.160 Derio-Vizcaya Spain Phone number: +34944005355 E-mail: aaldecocea@innoprot.com Tax ID/VAT number: ESB95481909	Florida International University Biomedical Engineering Department 10555 West Flagler Street - Suite EC2600 Miami, FL 33174 USA E-mail: chsu013@fiu.edu Phone Number: 305 348 6717

Nºpackages	Nº units	Unit of Measure	Country of origin	Description of goods/Harmonized Tariff
2	2	kit	Spain	Fibroblast Medium Kit - II
	2	kit	Spain	Hu. Valvular Interstitial Cells
				MANUFACTURER: INNOPROT (SPAIN)
				Cell Culture Medium for stable cell lines Non-hazardous, non-toxic, non-infectious. For laboratory research only. For invitro research purposes only.

Date: 11/29/2021	Signature:

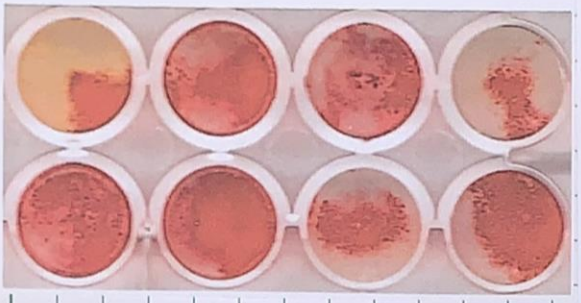
Signed Date Signed Date

Project

Continued from Page

12/14/2021: Froze 3 vials in P4, plated three coverslips at 37500 cells per slip.
12/22/2021: Plated a frozen vial P4 in 2 T75's
12/24/2021: Changed media
12/26/2021: Seeded another N of EX-PC & CY-PC in 24-well plate. Froze rest of cells.
1/2/2022: ARS staining. Imaged N=3 (from 12/18) hVICs in ARS.

C



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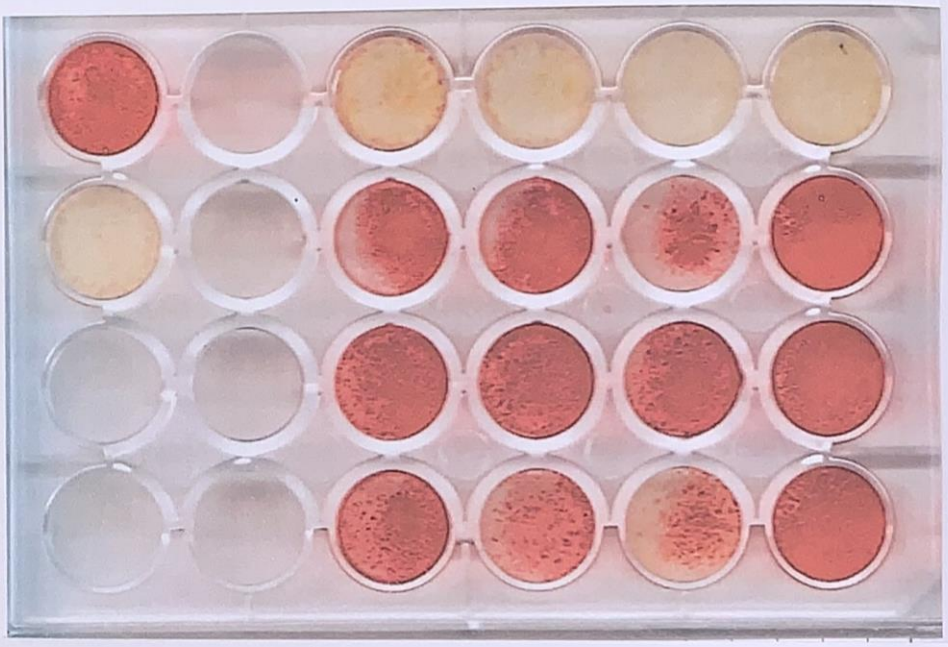
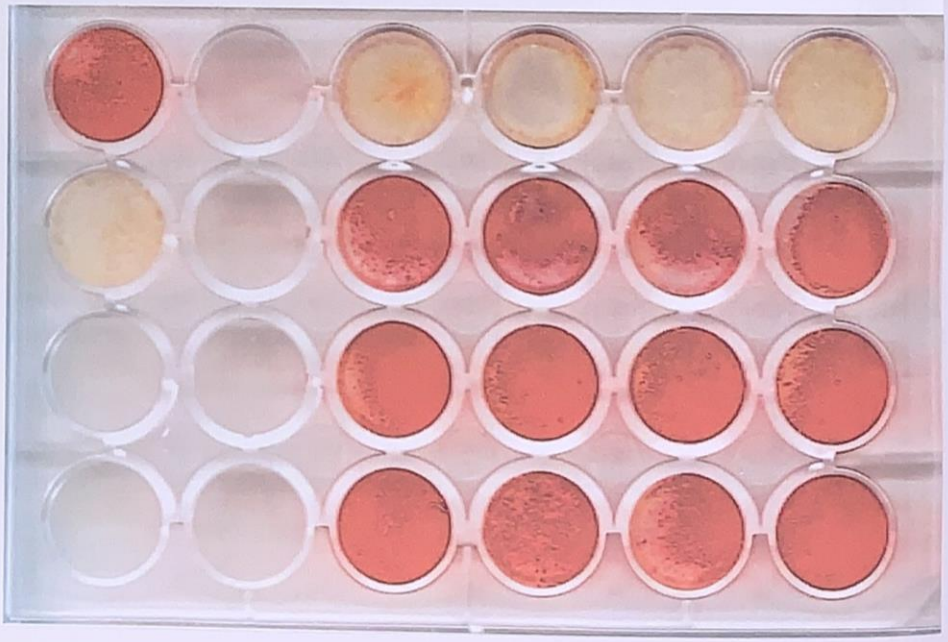


A



1/5/2022: ARS dye extraction & quantification
1/6/2022: BCA Protein assay (for ARS normalization)
Acetic acid (1.67 M) - 10% v/v per well to digest dye in the 24-well plates: 300 uL ← this was pipetted in triplicates at 100 uL per well in 96-well plate.
After ARS dye digestion, 220 uL of RIPA was added to each well in the 24-well plates to scrape off remaining tissue/cell/protein. Of the 220 uL, 25 uL was used per well in 96-well plate for quantification, in addition to 200 uL of working solution from BCA assay kit.
Plate 1 on page 72.

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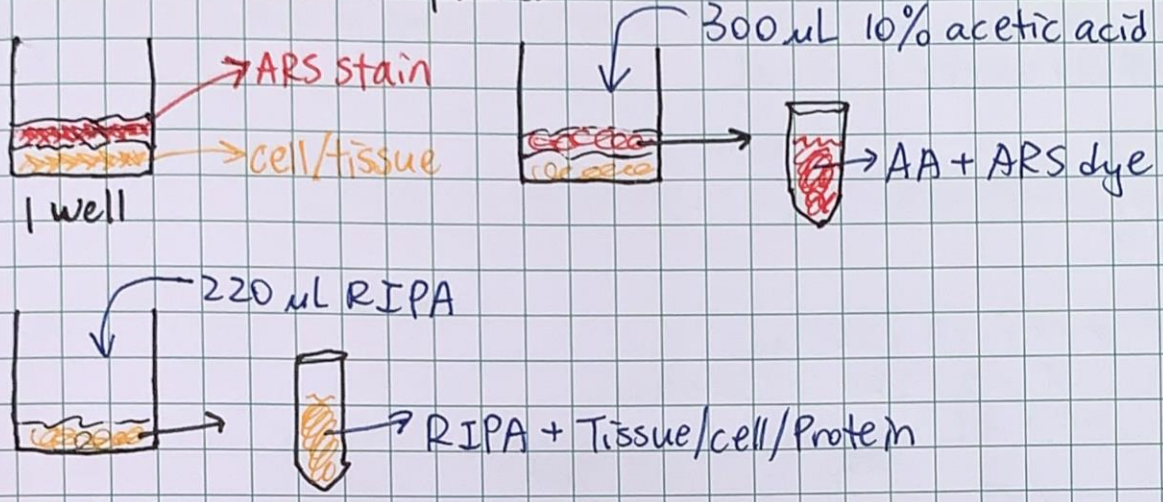
ARS stained hVIC 24-well plates



ARS Standards

Solute: ARS 1% w/v, MW: 342.253 g/mol
solvent: AA

Tube #	Volume of ARS (µL)	Volume of AA (µL)	Molarity (M) mol/L
1	0	100	0
2	3.125	96.875	0.000913067
3	6.25	93.75	0.001826134
4	9.375	90.625	0.002739202
5	12.5	87.5	0.003652269
6	15.625	84.375	0.004565336
7	18.75	81.25	0.005478403
8	21.875	78.125	0.006391471

1 % w/v: Every 100 mL of solvent, 1 g of ARS salt is dissolved.

Stock:

$$\frac{1\ g}{342.253\ g/mol} = 0.00292 \text{ moles of ARS salt.}$$

ARS Stock Concentration: 0.0292 M

$$\text{Concentration} = \frac{\#\text{ of moles}}{\text{Volume}} = \frac{0.00292\ \text{mols}}{0.10\ L} = 0.02921815\ \frac{\text{moles}}{\text{liter}}$$

BSA standards

Solute: BSA (2 mg/mL)
Solvent: RIPA

Tube #	
1	2000 µg/mL ← BSA concentration
2	1500
3	1000
4	500
5	250
6	125
7	25
8	0

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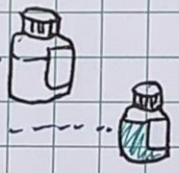
BCA/BSA quantification
(# standards + # unknowns) × (# replicates) × (volume of WR)
Volume of WR per sample = 200 uL

standards: 8
unknowns: 13 + 13 + 13 + 8 + (4 extra) = 51
replicates: 3

Total WR = (8 + 51) × 3 × 200 uL
= (59) × 3 × 200 = 35400 uL
= 35.4 mL

WR: 50 parts of (A) ...
1 part of (B) .....

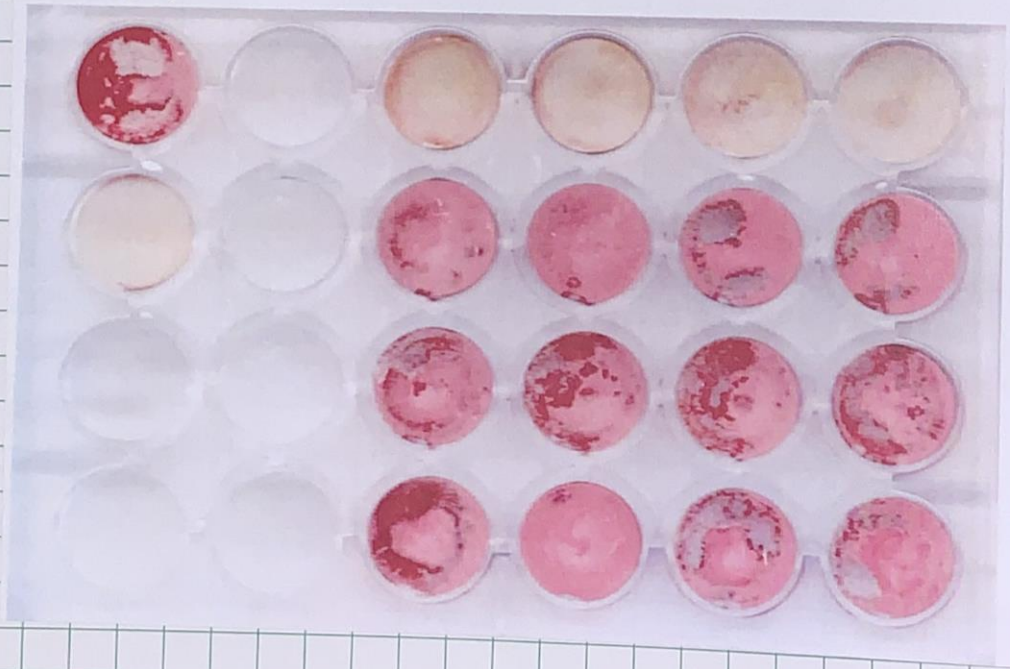


WR:
$\frac{35400}{51} = 694.118$ uL (Reagent B)

35400 − 694.118 = 34705.88 (Reagent A)

WR: 34.705 mL of Reagent A
694 uL of Reagent B

Plate 1



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ANOVA in SPSS

Analysis of Variance

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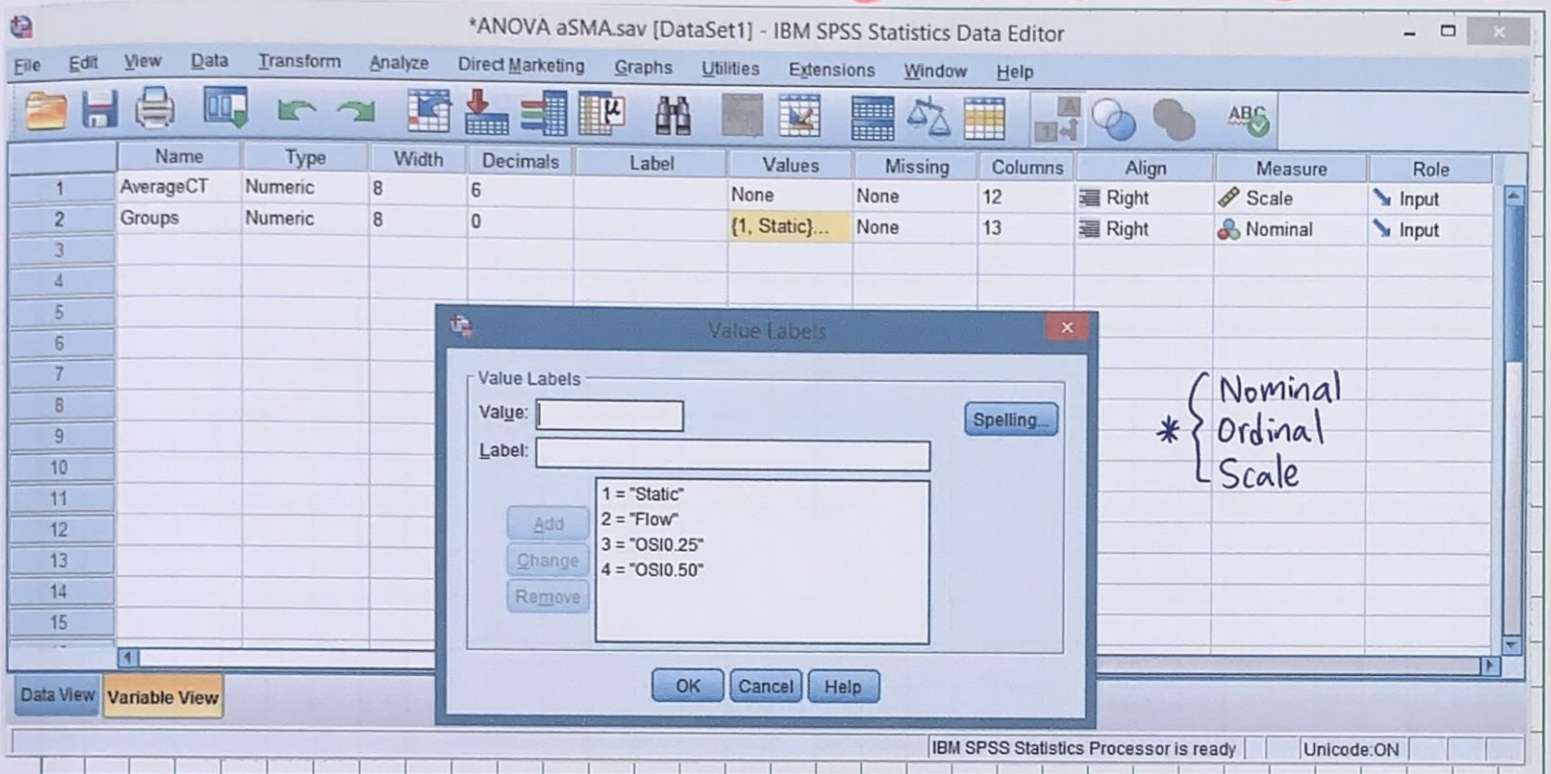
PROTOCOLS



* { Nominal / Ordinal / Scale

Nominal: labeling variables without quantitative value, no numerical significance

eg. gender: M-male, F-female] a sub-type of nominal scale with only two categories is also known as "dichotomous"

hair color: Brown, black, blond, gray, other

place: north of the equator
south of the equator
neither (international space station)

Ordinal: Measures non-numeric concepts like satisfaction, happiness, discomfort, etc. On a scale of 1-5, how do you feel today?

1- Very unhappy	1- Very unsatisfied
2- Unhappy	2- Unsatisfied
3- OK	3- Neutral
4- Happy	4- Satisfied
5- Very happy	5- Very satisfied

Scale: An interval, a ratio
A number that specifies a magnitude. It can be distance, weight, age, or a count of something.

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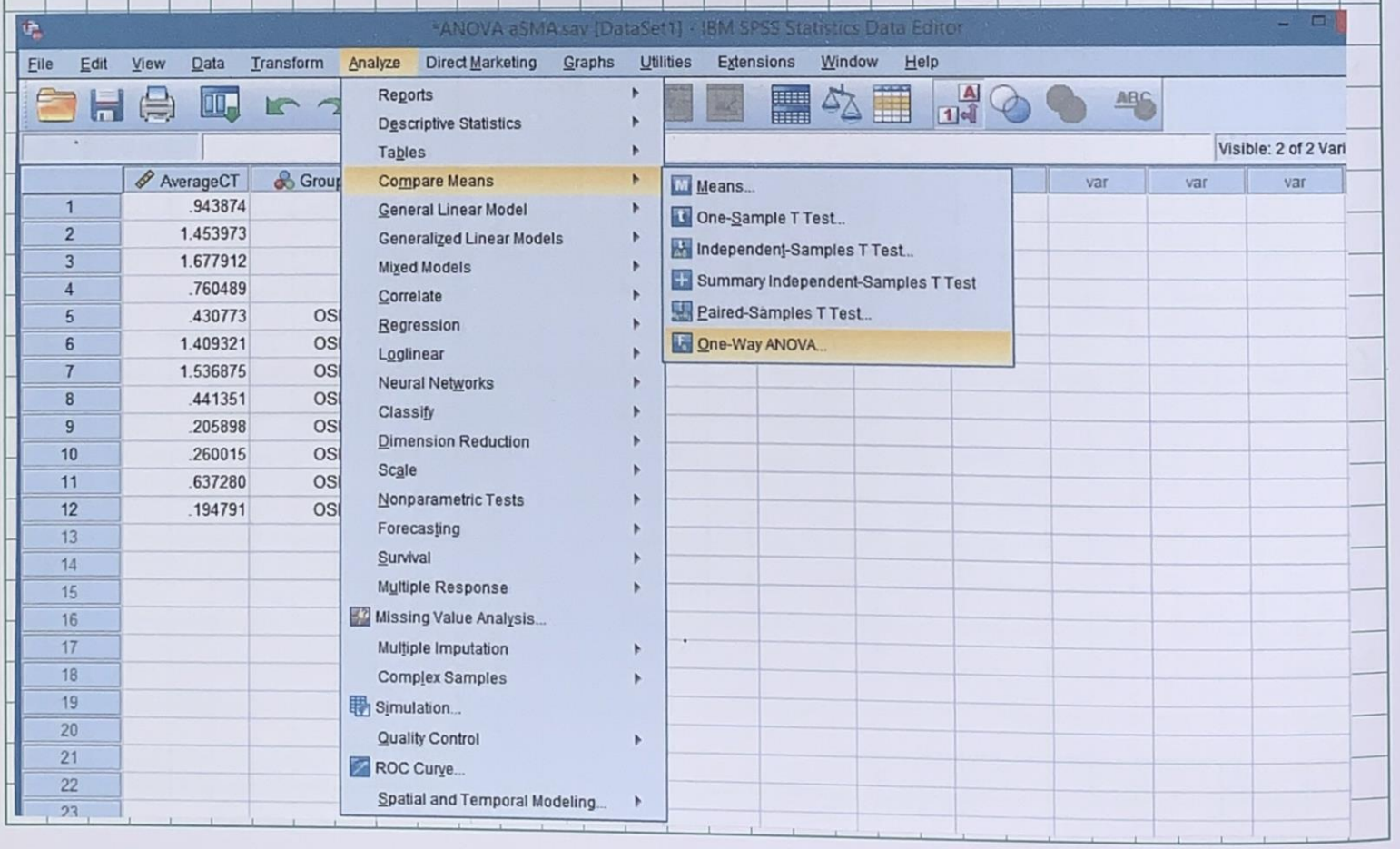
Provides:	Nominal	Ordinal	Interval	Ratio
The "order" of values is known		✔	✔	✔
"Counts," aka "Frequency of Distribution"	✔	✔	✔	✔
Mode	✔	✔	✔	✔
Median		✔	✔	✔
Mean			✔	✔
Can quantify the difference between each value			✔	✔
Can add or subtract values			✔	✔
Can multiple and divide values				✔
Has "true zero"				✔



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*ANOVA aSMA.sav [DataSet1] - IBM SPSS Statistics Data Editor

File Edit View Data Transform Analyze Direct Marketing Graphs Utilities Extensions Window Help

Visible: 2 of 2 Variables

	AverageCT	Groups	var	var	var	var	var	var	var	var
1	.943874	Flow								
2	1.453973	Flow								
3	1.677912	Flow								
4	.760489	Flow								
5	.430773	OSI0.25								
6	1.409321	OSI0.25								
7	1.536875	OSI0.25								
8	.441351	OSI0.25								
9	.205898	OSI0.50								
10	.260015	OSI0.50								
11	.637280	OSI0.50								
12	.194791	OSI0.50								
13										
14										

One-Way ANOVA

Dependent List: AverageCT

Factor: Groups

Contrasts... Post Hoc... Options... Bootstrap...

OK Paste Reset Cancel Help

Data View Variable View

IBM SPSS Statistics Processor is ready Unicode:ON

One-Way ANOVA: Post Hoc Multiple Comparisons

Equal Variances Assumed

LSD S-N-K Waller-Duncan

Bonferroni Tukey Type I/Type II Error Ratio: 100

Sidak Tukey's-b Dunnett

Scheffe Duncan Control Category: Last

R-E-G-W F Hochberg's GT2 Test

R-E-G-W Q Gabriel 2-sided < Control > Control

Equal Variances Not Assumed

Tamhane's T2 Dunnett's T3 Games-Howell Dunnett's C

Significance level: 0.05

Continue Cancel Help

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Oneway

[DataSet1] C:\Users\chsu013\Desktop\ANOVA aSMA.sav

ANOVA

AverageCT

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.659	2	.829	4.223	.051
Within Groups	1.768	9	.196		
Total	3.427	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: AverageCT

Tukey HSD

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Flow	OSI0.25	.254481811	.313391630	.705	-.62050938	1.12947300
	OSI0.50	.884565829*	.313391630	.048	.00957464	1.75955702
OSI0.25	Flow	-.254481811	.313391630	.705	-1.12947300	.62050938
	OSI0.50	.630084018	.313391630	.165	-.24490718	1.50507521
OSI0.50	Flow	-.884565829*	.313391630	.048	-1.75955702	-.00957464
	OSI0.25	-.630084018	.313391630	.165	-1.50507521	.24490718

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

AverageCT

Tukey HSD[a]

Groups	N	Subset for alpha = 0.05	
		1	2
OSI0.50	4	.32449611	
OSI0.25	4	.95458013	.95458013
Flow	4		1.20906194
Sig.		.165	.705

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000

ViVitro AMPack Calibration

Appendix I – AmPack Calibration

Each amplifier module and pressure transducer may be calibrated following the procedure given below. You will need a reference pressure gauge. A manometer with a range of at least 250 mmHg and accuracy of at minimum ±0.025% is suitable. You will need a means of reading amplifier output between -10.00 and +10.00 volts DC to an accuracy ≥ than the pressure gauge accuracy.

Use the supplied trim-pot tool to adjust the 15-turn potentiometers for **GAIN** & **SHIFT**. The extreme position, indicated by a click, may be located by rotation of at least 15 turns clockwise or anti-clockwise.

Ensure the AmPack has been on for at least 30 minutes prior to calibration to prevent drift.A two point pressure transducer calibration can be made as follows:

1. Connect amplifiers to pressure transducers and switch amplifier chassis power ON.
2. Use a syringe to fill the pressure transducers with distilled water.
3. Adjust the **SHIFT** control to give the desired voltage output at **0 mmHg** gage pressure (atmospheric).
   a. -5.9 to -6.1 VDC for Pulse Duplicator
   b. -0.9 to -1.1 VDC for HiCycle/RWT
4. Apply a pressure of **+200 mmHg** to transducers and adjust **GAIN** for the target voltage output.
   a. +5.9 to +6.1 VDC for Pulse Duplicator
   b. +6.9 to +7.1 VDC for HiCycle/RWT
5. Repeat steps 3 and 4 the desired sttings at both limits are achieved.
6. Below are the approximate values that will be achieved from the calibrations described above (**CALIBRATION** = Δ voltage/ Δ pressure):
   a. 0.06 V/mmHg for Pulse Duplicator
   b. 0.04 V/mmHg for HiCycle/RWT

The GAIN and SHIFT settings may need to be changed depending on the range of pressure generated in the specific application. Different settings may be needed for monitoring the ViVitro Labs Inc. pulse duplicator and the Hi-Cycle. tester. Changing GAIN will also change the output at atmospheric pressure. It may be necessary to re-adjust SHIFT until the required outputs are obtained at the two calibration pressures.

The factory preset values shown above are based on the following:

1. Application: Hydrodynamic Testing (probable pressure range -50 to 250 mmHg)
2. Application: Durability Testing (probable pressure range -200 to 200 mmHg)

SHIFT, 0 mmHg, -5.9 ~ -6.1 VDC

GAIN, +200 mmHg, +5.9 ~ +6.1 VDC

Protocol for RoosterVial™-hBM-1M-XF EXPANSION

Expansion of RoosterBio XF hBM-MSC to yield 10 million cells	
RoosterVial-hBM-1M-XF Lot No:	Date/Time:

1.0 MATERIALS

ITEM	VENDOR	PART No*	EXP DATE
RoosterVial-hBM-1M-XF, (1 million (M) cells)	RoosterBio	MSC-031	
CTS TrypLE Select Enzyme	Life Technologies	A1285901	
DPBS (without Ca^{++}, Mg^{++})			
DPBS (without Ca^{++}, Mg^{++}) + hPL or SPENT MEDIA for quench			
hMSC High Performance Media Kit XF	RoosterBio	KT-016	
2 x T225 CELLBIND flasks OR 6 x T75 CELLBIND flasks	Corning		

* Vendors and part numbers are included for critical items.

2.0 MEDIA PREPARATION & CELL EXPANSION

2.1 Bring hMSC High Performance Media Kit XF to room temperature. Add 1 vial hMSC Media Booster XFM (SU-016) to 500 mL hMSC High Performance Basal Media (SU-005).

2.2 Obtain RoosterVial-hBM-1M-XF from liquid nitrogen dewar and immediately thaw in 37°C water bath. Monitor the process and remove from water bath once a small bit of ice is remaining (2-3 min).

2.3 Spray vial well with 70% isopropyl alcohol before transferring into biosafety cabinet.

2.4 Aseptically transfer cells into a 50 mL centrifuge tube.

2.5 Slowly (dropwise) add 4 mL of culture media to the cells.

2.6 Centrifuge at 200 x *g* for 10 min.

2.7 Carefully remove the supernatant without disturbing the cell pellet.

2.8 Resuspend the cells in 5 mL of culture media. When cells are resuspended bring volume up to 30 mL with culture media.

2.9 Mix well and seed cells equally into two T225 or into six T75 vessels, and add media to bring volume up to final volume:

Type of culture vessel (X)	Total volume of cell suspension per vessel	Cells/ cm^2	Final Volume/ flask
T75 x 6 ☐		2,222	15ml
T225 x 2 ☐		2,222	45ml

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2.10 Transfer vessel(s) into 37°C incubator and ensure that the surfaces are covered with media.

2.11 Microscopically observe culture everyday from day 3 onwards to determine percentage confluency.

2.12 If culture is less than 50% confluent on day 3, perform a media change. Completely remove the spent media from the vessel, and replace with same volume of fresh culture media. Transfer vessel back into incubator.

2.13 When culture is >80% confluent, prepare to harvest the following day.

Day	Confluency (%)
3	
4	
5	
6	
7	

3.0 CELL HARVESTING

3.1 For harvesting, transfer vessel into biosafety cabinet and remove spent media.
*Collect ~10 mL spent media in sterile container if using to quench harvest enzyme.

3.2 Remove media and add 10 mL of TrypLE to T225 or 3 mL TrypLE to each T75 flask, and incubate in 37°C incubator.

3.3 Check culture every 5 min until cells are detached from surface. Gently tap to dislodge remaining cells from surface.

Total time required for cells detachment

3.4 Add equal volume of quench or spent media to stop the TrypLE activity.

3.5 Transfer the cell suspension into a 50 mL centrifuge tube.

Total volume of cell suspension (=A)

3.6 Centrifuge at 200 x *g* for 10 min.

3.7 Aspirate the supernatant and resuspend cells with 4-5 mL of fresh media.

3.8 Measure the total volume of cell suspension.

3.9 Mix well and transfer 0.1 mL of cells into microcentrifuge tubes for cell counts.

3.10 Dilute cells to 0.5 mL with /DPBS to get counts in the range of 0.1-1 x 10^6 cells/mL.

3.11 Mix well and cells are ready for counts with cell counting device.

Raw data		Adjusted data	
Dilution Factor (=B)	Viable Cell Concentration (=C)	Cell concentration (D)=B*C	Total cells at harvest (E)=D*A

3.12 Place harvested cell suspension in 4°C refrigerator for use within 1 hour of harvest.

CALIBRATION Vivitro System

CAUTIONS

1. Always turn the Flowmeter to "OFF" before draining the system
2. Always add fluid to the system before turning the Flowmeter to "+" or "-"
3. Use distilled water in the left ventricle chamber (fluid outside the sac)

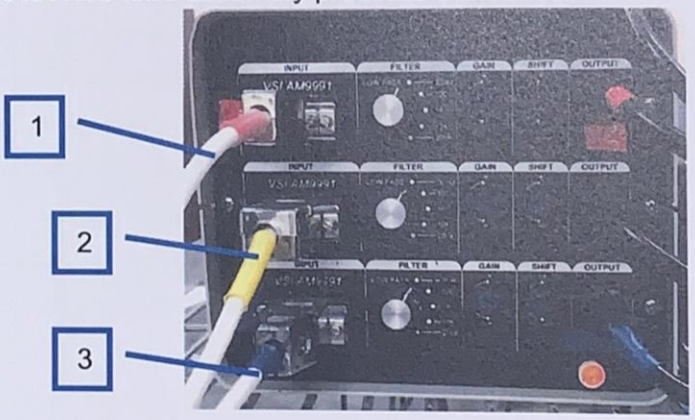
EQUIPMENT LIST

Flowmeter Box

1. Flowmeter probe
2. Flowmeter ground

AM Pack (Pressure Transducers Box)

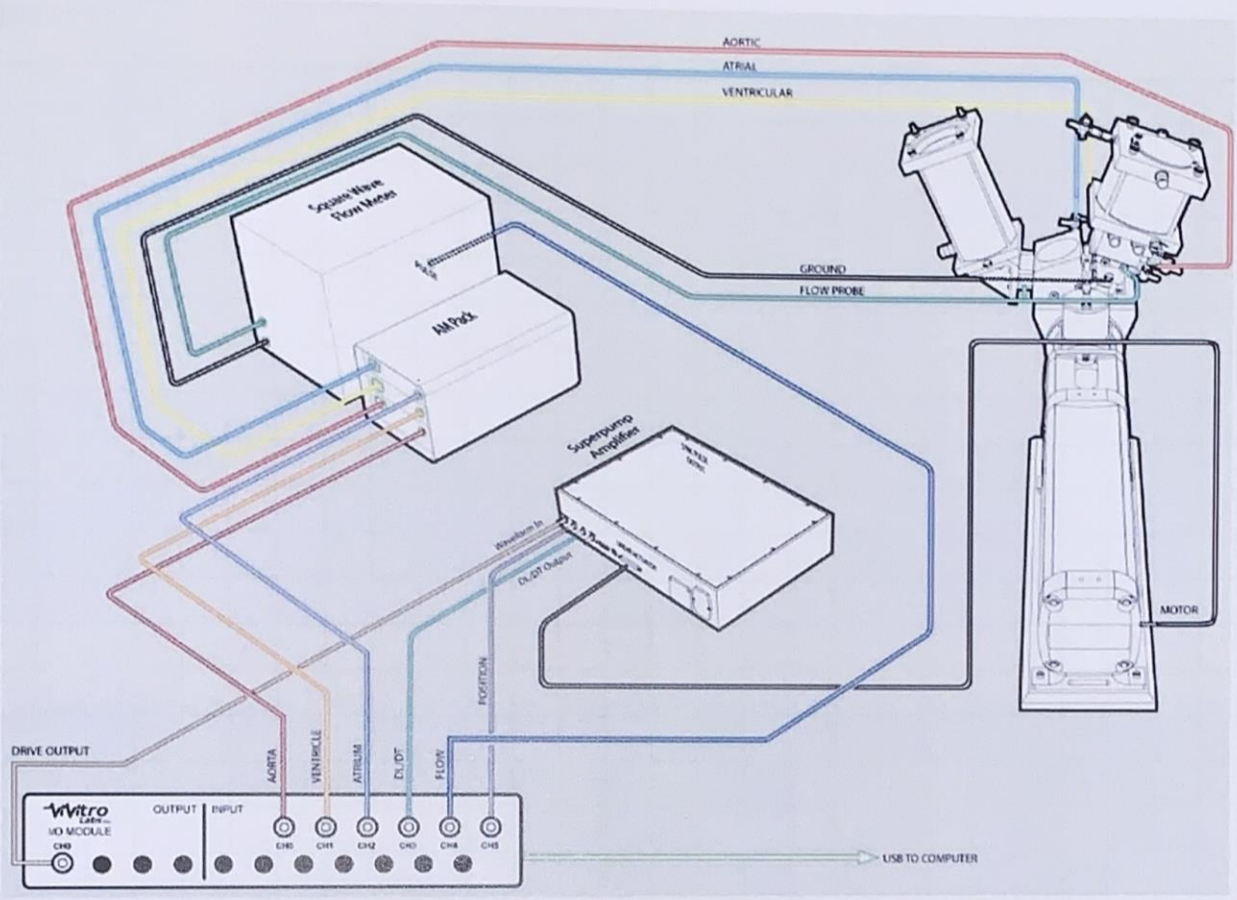
1. Left ATRIAL pressure transducer
2. Left VENTRICULAR pressure transducer
3. AORTIC load assembly pressure transducer



I/O Module Box

3/18/2020

Connection Schematic

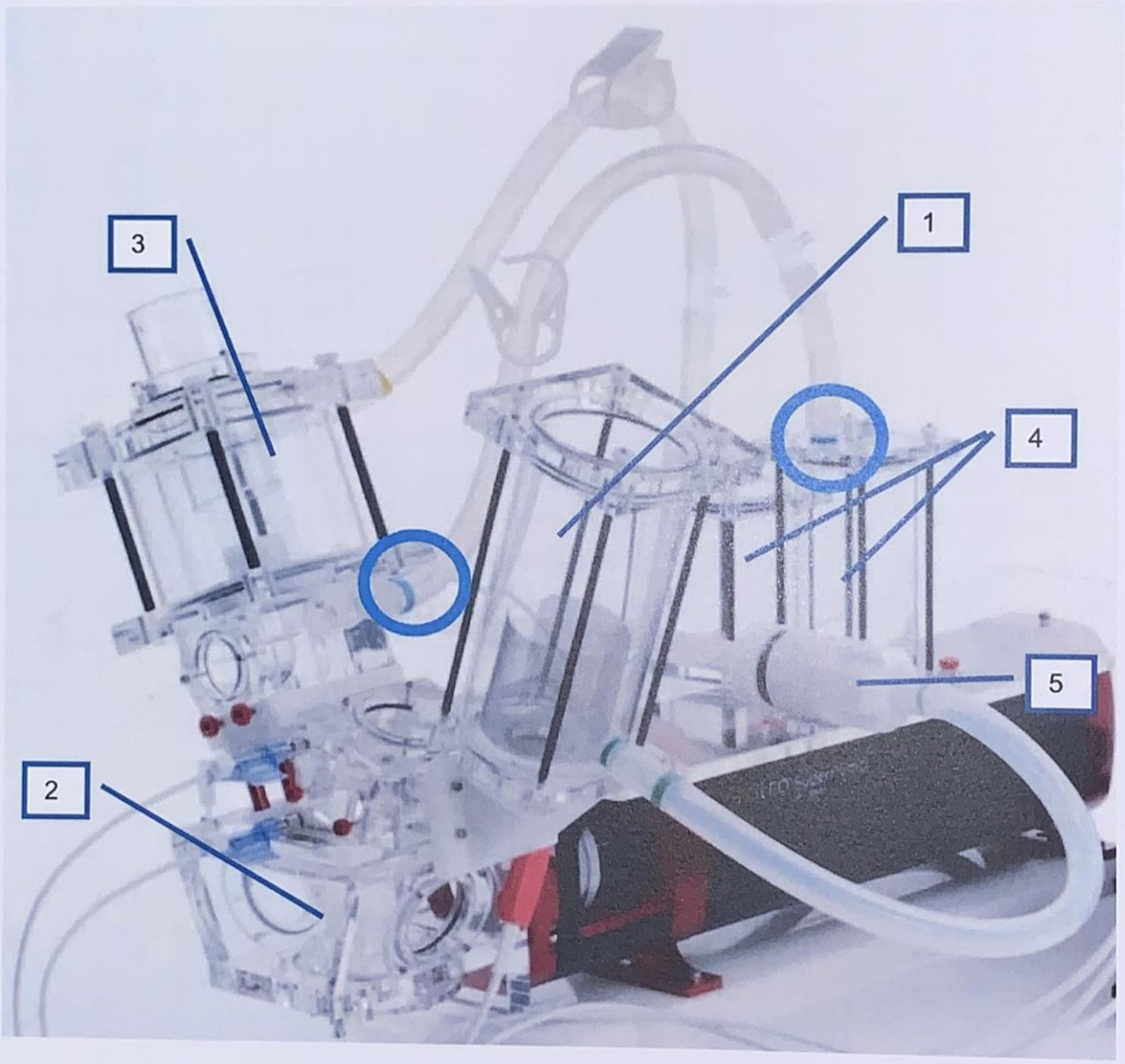


Superpump Controller Box

3/18/2020

Apparatus

1. Left atrium chamber
2. Left ventricle membrane sac and chamber
3. Aortic load assembly
4. Compliance chambers x2
5. Restrictor



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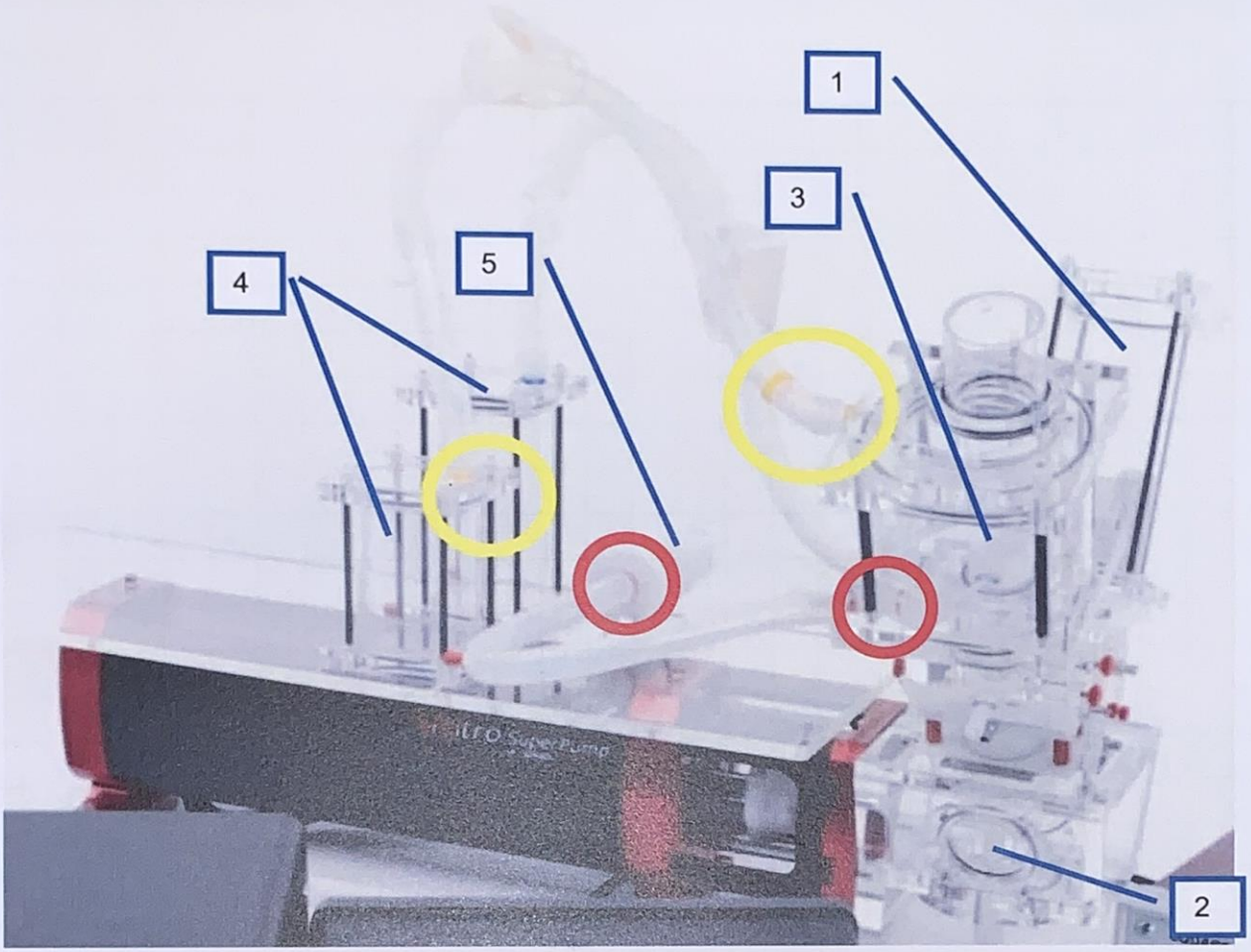

Software

Vivitest

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TURNING ON EQUIPMENT

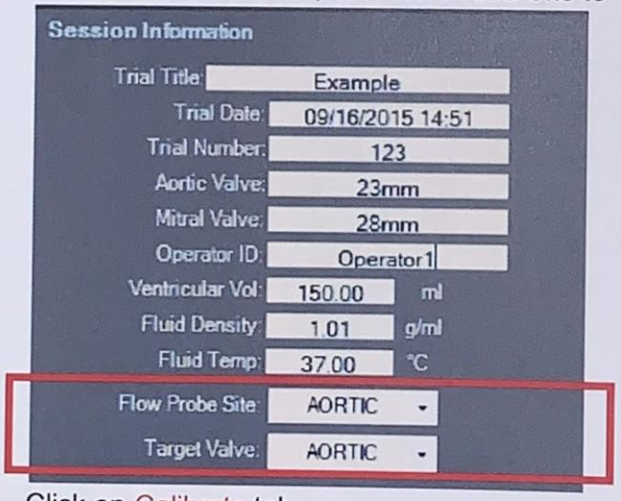
1. Turn on power for all boxes and let them run for 30 minutes
    a. Flowmeter Box (ensure sure probe is in off position (not "+")
    b. AM Pack (Pressure Transducers Box)
    c. I/O Module Box
    d. Superpump Controller Box
2. Press the "ALARM" button on Flowmeter Box to turn off red light
3. Connect all tubing and chambers
4. Run Vivitest software as "administrator" (right click -> run as administrator)

FLOWMETER CALIBRATION

1. For mitral valve flow calibration, insert a blank seal under the aortic load assembly and an open ring under the left atrium chamber (Note: Flowmeter probe should always be placed with the open ring, and a blank seal in the other chamber)

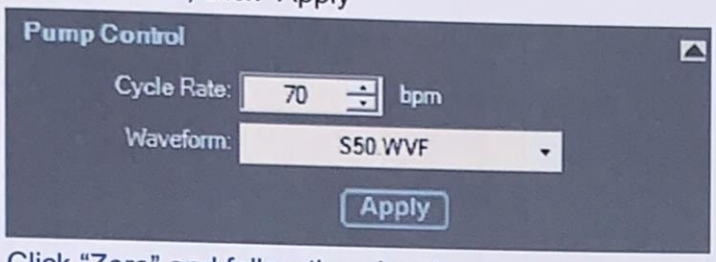
(Left: open ring, Right: blank seal)

2. Clamp flow loop tube to prevent water from flowing between the left atrium chamber and the aortic load assembly
3. Pour water into left atrium chamber (Or aortic load assembly for aortic flow calibrations)
4. For mitral calibration remove air from under the blank seal located on the aortic side
5. Attach flowmeter ground
6. Go to Vivitest software, set Flow Probe Site to "MITRAL" and Target Value to "MITRAL"



7. Click on Calibrate tab

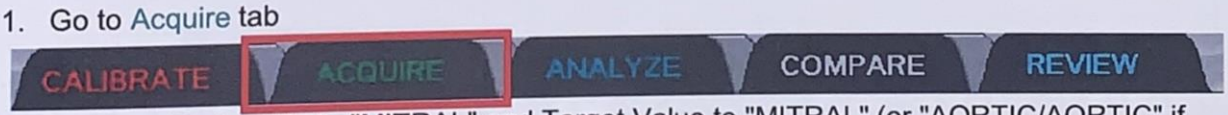
8. On the right hand bottom corner, set Pump Control Cycle Rate to "70 BPM" and Waveform to "S50.WVF", click "Apply"



9. Click "Zero" and follow the wizard
    a. Click "Proceed" to start a new calibration, or "Accept" to accept the previous calibration
    b. With Superpump power "On" and amplitude at "zero", open all pressure transducers to atmosphere, Flow meter set to "+"
    c. Click "proceed" to zero all sensors to atmosphere.
10. Click "Flow" and follow the wizard
    a. Set Flowmeter Box as per manual
        i. Knob at OFF -> Display should be 0.00, if not, use the "ZERO" knob to dial it to zero
        ii. Knob at NULL -> Get the lowest possible value by dialing the "NULL" knob
        iii. Knob at BAL -> Turn "BALANCE" knob to get 0.00 on display (Ideally a healthy probe shows 0.00 on display when the "BALANCE" knob is set to 5.0)
        iv. Knob at "+" -> Turn "BALANCE" knob to get 0.00 on display
    b. Ensure Pump Control is set to Cycle Rate = "70 BPM" and Waveform = "S50.WVF"
    c. Remove air from under the blank seal in the aortic load assembly with syringe
    d. Make sure there's fluid in flow probe chamber, turn Flowmeter to "+"
    e. Turn pump amplitude knob to Stroke 50-60 mL for a few cycles to dislodge bubbles (stroke volume can be obtained from the bottom left corner of software screen)
    f. Stop the pump and click "Proceed" to verify sensor values, click "Proceed" again
    g. Restart pumping to a stroke of 70-80 mL, click "Proceed" to observe graph (Ideally Pump Velocity, dL/dt, should be equivalent to Flowmeter for MITRAL/MITRAL settings, and symmetrical with respect to the x-axis for AORTIC/AORTIC settings). Flow and dL/dt should each have 3 zero crossings and these should line up with each other.
    h. Click "Proceed" to capture ten cycles
    i. Compare with previous calibrations, confirm calibration gains, save data

To Double Check Flow Calibration…

1. Go to Acquire tab



2. Set Flow Probe Site to "MITRAL" and Target Value to "MITRAL" (or "AORTIC/AORTIC" if doing aortic valve tests)
3. Make sure there's fluid in the flow probe chamber, turn Flowmeter to "+", open all pressure transducers to atmosphere, Superpump power "On" and amplitude at "zero"

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4. Click "Re-zero sensors", "OK", and "Re-zero done" (With Superpump power "On" and amplitude at "zero", open all pressure transducers to atmosphere, Flow meter set to "+")
5. Turn pump amplitude to Stroke 50 mL (or otherwise), click "capture 10 cycles"
6. Dial pump amplitude back to zero
7. Go to Analyze tab

8. Click "Turn on all waveforms"
9. Only display the "FLOWMETER"
10. Reorder F1, F2, F3, F4 from left to right to match the measured stroke
    a. Check V3 (closing volume), V4 (leakage volume). Note: If Flow Probe Site and Target Value settings are set to AORTIC/AORTIC, the order will show V1, V3, and V4. V1 (forward volume) can be obtained by clicking on the table icon on the top menu.
    b. Volume of positive curve should match volume of negative curve, which should also match the stroke volume set on the pump.

PRESSURE CALIBRATION

1. Replace both valves with open rings
2. Remove aortic load assembly top cover
3. Click on "Pressure" in the Calibrate tab
4. Follow the "Pressure" wizard
    a. Click "Proceed" to start a new calibration, or "Accept" to accept the previous calibration
    b. Remove all pressure transducers, attach them to manifold and manometer
    c. Check (on screen) that upper applied pressure is set to 200.0 mmHg
    d. Open the manifold to atmosphere, click "Next"
    e. Add pressure w/syringe and manometer to 200 mmHg, click "Proceed" at 200 mmHg for upper reference check
    f. Check the gains and click "Proceed"
    g. Connect the pressure transducers back with flow chambers
    h. Unclamp flow loop and fill system with fluid from left atrium
    i. Remove all air bubbles from transducers
    j. NOTE: Wizard skips from step 6 to step 10, retrace back to step 7 and continue
    k. Click "Proceed" to capture static head difference
    l. Click "Yes/Finish" to confirm static head

To Double Check Pressure Calibration…

1. Go to Acquire tab
2. Make sure there is fluid in the system and no air bubbles in pressure transducers
3. Make sure there's fluid in the flow probe chamber, turn Flowmeter to "+", open all pressure transducers to atmosphere, Superpump power "On" and amplitude at "zero"
4. Click "Re-zero pressure" -> "OK" -> "Re-zero done"
5. With transducers still open to atmosphere, uncheck the static head compensation in ViViTest, all the transducers should read zero.

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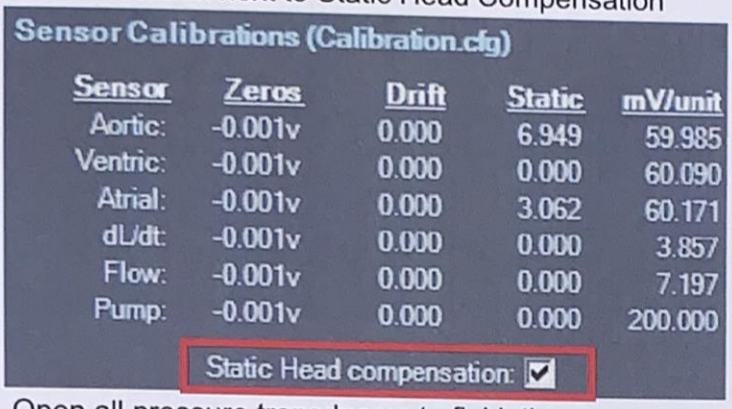
Continued from Page
Project

6. Click checkbox next to Static Head Compensation



7. Open all pressure transducers to fluid, the pressure curve should jump up on the screen. Starting with ventricular, then atrial, then aortic, all pressure lines should match the ventricular pressure when all transducers are open to fluid pressure.
8. Optional: On the same screen, remove transducer(s) and attach them on manifold with manometer, verify manometer value with wave pressure value (static head should be unchecked for this)

DATA COLLECTION

(For best results, collect data right after flow and pressure calibration. For best results, set all conditions first, re-zero all sensors, tune back all conditions immediately to capture 10 cycles. The key is data is collected as close to the zero as possible.)

Nominal Conditions for Valve Testing

1. 70 beats per minute
2. Systolic duration = 35%
    a. S35 waveform
    b. FDA waveform
3. Simulated cardiac output = 5 liters per minute
    a. Vivitro Cardiac Output = Forward Volume * Beat Rate
    b. Use Stroke (on screen) as approximation to obtain Forward Volume in data table
    c. Set Stroke to 71.4~75 mL using Superpump amplitude knob
4. Mean Aortic Pressure (MAP) = 100 mmHg
    a. Use restrictor to set MAP (decrease resistance - CCW, increase resistance - CW)

Steps for Valve Testing

1. In the Acquire tab, set Pump Control at bottom right corner of screen to Cycle Rate = "70 bpm" and Waveform = "S35.WVF" (35% systole waveform)
2. Set Flow Probe Site and Target Value to “MITRAL/MITRAL” or “AORTIC/AORTIC” accordingly
3. Make sure there’s fluid in system and all pressure transducers are debubbled
4. Re-zero sensors (Flowmeter on “+”, all pressure transducers open to atmosphere, Superpump power “On” and amplitude set to “zero”)
5. Open all pressure transducers to fluid

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6. Start pumping, note down amplitude turn dial digits when Stroke reaches 71.4 (71.4~75 mL), goal is to get a Forward Volume of <=71.4 (in Vivitro, the Forward Volume is necessary to calculate Cardiac Output)
7. Use restrictor to set MAP to 100 mmHg or to required back pressure
8. Dial pump amplitude to zero
9. Re-zero sensors (Flowmeter on "+", all pressure transducers open to atmosphere, Superpump power "On" and amplitude set to "zero")
10. Open all pressure transducers to fluid
11. Quickly dial pump back to the same position from step 6
12. Click "Capture 10 cycles"
13. Dial down pump frequency back to zero after cycles are captured. You can check if the flow meter drifted here.
14. Click on Analyze tab for data
15. Reorder F1, F2, F3, F4 from left to right to match the measured stroke. Pressure markers will also need setting (see user manual)
16. Check V3 (closing volume), V4 (leakage volume), V1 (forward volume) Note: If Flow Probe Site and Target Value settings are set to AORTIC/AORTIC, the order will show V1, V3, and V4. V1 can be obtained by clicking on the table icon on the top menu.

* AM Pack Gains & Shift Calibration info on page 96.

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Alizarin Red Staining

Read and Understood By

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STAINING

1. Aspirate condition media
2. Fix cells w/ 10% formalin for 15 minutes
3. Aspirate formalin
4. Wash w/ PBS, aspirate
5. Add alizarin red, incubate at room temperature for 30 minutes, shake gently.
6. Aspirate alizarin red
7. Wash plates once w/ H_2O (MilliQ)
8. Flip the plate to get extra liquid out
9. Image/Scan/Photograph

AREA SCANNING

Using the plate reader (Synergy HTX Multimode Reader)

1. Open Gen 3.00
2. New
3. 12-well plate (or however many wells)
4. Select "Absorbance", "Area Scanning", "Monochromators"
5. Enter wavelength 405
6. Default Scan setting is 5×5, or 25 dots per well, equally spaced. This can be modified.
7. Save scanned values in Excel.

	1	2	3	4	
A	0.063	0.06	0.608	0.539	Mean OD [405]
B	0.541	0.622	0.609	0.687	Mean OD [405]
C	0.23	0.501	0.323	1.455	Mean OD [405]

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This is for a 12-well plate. For other plates, make sure the entire surface is submerged in acid.

ARS Dye Extraction & Quantification

1. Add 500 µL 10% acetic acid into each well. Place plate in shaker for 30 min.
2. Collect and transfer all well components into Eppendorf tubes.
3. Vortex for 30 seconds.
4. Wrap tubes w/ parafilm and heat samples at 85°C for 10 minutes.
5. Incubate tubes on ice for 5 minutes.
6. Centrifuge at 20,000 g for 15 minutes.
7. Transfer 350 µL supernatant to a new tube.
8. Add 140 µL 10% ammonium hydroxide to neutralize the acid.
   Note: Volume may vary, make sure ratio of 10% acetic acid to 10% ammonium hydroxide is 500 : 200.
9. Aliquot 150 µL of the neutralized mixture into each well in a 96-well microplate in triplicates. (opaque-walled, transparent-bottomed)
10. Read the absorbance at 405 nm w/ plate reader.
    Software: Gen3.00 → New → 96 well plate → Read
    Select: Absorbance, Kinetic, Monochromators
    λ = 405 (Endpoint)

$$\frac{\text{Wanted v/v \%}}{\text{Current v/v \%}} = \frac{A}{\text{Total}} = \frac{A}{A+B}$$

A: Volume of current v/v %
B: Volume of diluent
Total: Total volume you want to make

centrifuged eppendorf
pic of microplate after pipetting
Excel after reading

ALP ASSAY

From BioVision Alkaline Phosphatase Activity Colorimetric Assay kit.

1. Dissolve 1 tablet of pNPP into 2.7 mL of ALP assay buffer at room temperature (RT) to make 5 mM working solution.
2. Dilute 40 uL of the pNPP working solution from part 1 w/160 uL ALP assay buffer to make 1 mM pNPP standards.
3. Make the following standards:

Concentration (nmol)	Assay Buffer (uL)	1mM pNPP Soln (uL)	Enzyme (uL) *add last*
0	120	0	10
4	116	4	10
8	112	8	10
12	108	12	10
16	104	16	10
20	100	20	10

4. For each sample:

Volume of sample (uL)	5mM pNPP Soln (uL)
80	50

5. Pipette all samples and standards into a 96-well microplate, shake well for 30 seconds, incubate plate at RT for 1 hour away from light.
6. Read the absorbance w/plate reader at 405 nm.
   Software: Gen 3.00 → New → 96 well plate → Read
   Select: Absorbance, Endpoint/Kinetic, Monochromators
   $\lambda = 405$ nm

5/5/2020

Mechanical Testing

Read and Understood By
Continued on Page

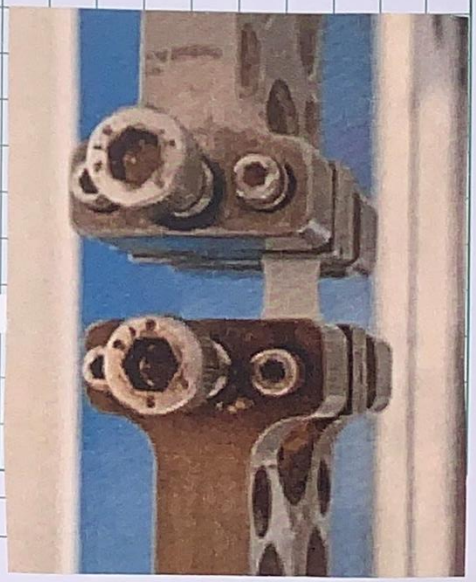
Bose System

1. Turn on Bose power supplies 1 and 2
2. Open WinTest 7 Software
3. File → Open → From Unsecure Workspace → Project Select Desktop → 32A034 Florida MT_MS_BG.PRJ file → Open → OK → OK
4. Check "Disp" under "Control Channel"
5. Click "Limits", check "Disp", Action → "Control Stop" → OK
6. Set Disp window to zero by right clicking → Properties → Auto → OK
7. Set Load window to zero by right clicking → Properties → Auto → OK
8. Waveform → Ramp tab → Level mm = 5 and Rate mm/sec = 0.1 (or some #) → OK
9. Mover Power → On → Close
10. Adjust Mover → -5 (under Current Command) → OK (the top clamp of the Bose machine should move after this step)
11. Standard Timed Data → Scan Time 0.1, Scan points 20, Number of Scans 1000. Set File Name → (type in a name under both fields) → Set Destination Folder (select where to save) → "Start" ~~(if Disp maxes out to 5mm, machine will automatically stop) Manually click "Stop" after the sample breaks.~~ (saved files will be in .txt and .tdf)
12. Clamp the samples, Re-zero "Load" (only Load)
13. Click "Run" → Start (if Disp maxes out to 5mm, machine will automatically stop). Manually click "Stop" after the sample breaks.
14. Mover Power Off. Close everything without re-writing the .PRJ file.

The test above is for tension. To test compression, switch the 5mm and -5mm.

Text to Columns

1. Highlight the column that contains your list.
2. Go to Data > Text to Columns.
3. Choose Delimited. Click Next.
4. Choose Comma. Click Next.
5. Choose General or Text, whichever you prefer.
6. Leave Destination as is, or choose another column. Click Finish.

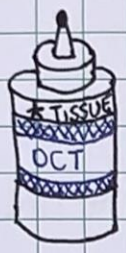


Tissue Embedding

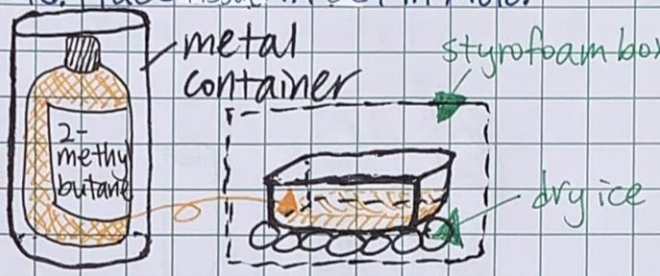
Continued on Page

Read and Understood By

1. Place tissue samples in 10% Formalin. Store up to 24 hours in 4°C.
2. Move samples out to PBS for 15 minutes.
3. Fill a small layer of OCT at bottom of mold. Place tissue in OCT in mold.

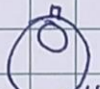


OCT Compound Tissue Plus



4. Place the embedded tissue in OCT in mold in liq. N_2 environment. The cold temperature solidifies OCT compound. Leave mold w/samples in liq. N_2 for 20 minutes. If liq. N_2 is not available, use dry ice and 2-methyl butane.
5. Wrap samples in Al foil, place in a box and store in -80°C.
6. Bring the sectioning kit w/samples to cryostat.
   Sectioning kit includes: OCT compound
   glass slides
   blades for cutting
   Paint brushes

Sectioning w/ Cryostat

1. Turn on slide dryer machine (should be @ 37°C)
2. Unlock cryostat by pressing [🔓] button.
3. Unlock the turning wheel on the RHS 
4. Set thickness to 16 μm (16~18 μm). Press the [−] TRIM [+] buttons.
5. Insert and lock blade 
6. Press [⇧] or [⇩] to move platform away from blade or toward, respectively
7. Rotate the turning wheel clockwise  to cut slices (sample moves toward blade)
   If turning wheel is rotated CCW, sample moves away from blade.
8. Use a paint brush to hold sectioned slices.
9. Attach cut slices on glass slides (few cuts may be needed before reaching the samples)
10. Each slide can hold up to 6 slices. Make sure each slide holds the same tissue sample.
11. Place slides w/slices into slide dryer.

Current cryostat location: AHC I Rm 208

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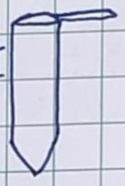
1/12/2021

Ultra Centrifugation (UC)

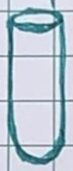
UC @ 100,000 g's Use specific eppendorf tubes
(Regular eppendorfs ≤ 30,000 g's) eg. Lot# P90617MPI
Case of 500 box

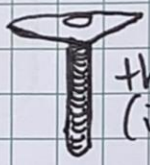


Beckman Coulter
REF 357448
1.5mL, Snap-on cap
Polypropylene Tube

Tubes are placed inside adaptors

adapters (re-usable)



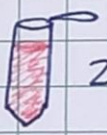


this screws into the center ● of the rotor.
(it's heavy)

Rotor has 8 compartments

1. From a conical of conditioned media Distribute media into 8 2-mL regular eppendorf tubes and centrifuge them at 10,000 g for 30 minutes.

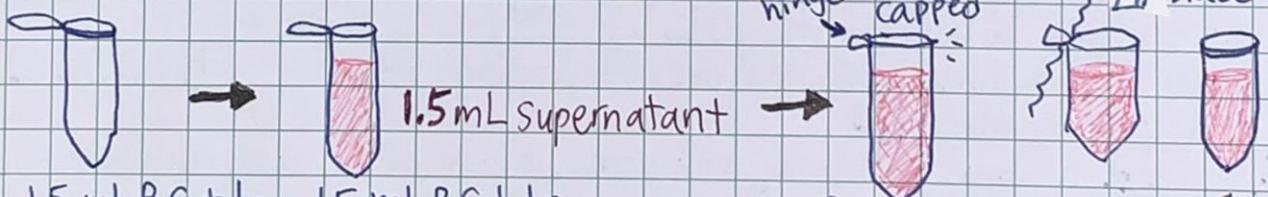
×8



2mL of media
Regular eppendorf

(B.C.)

2. Remove 1.5mL supernatant, place into 1.5mL Beckman Coulter microcentrifuge tubes. (Pellet from Step 1 contains cell debris while exosomes remain in the supernatant). Place the B.C. microcentrifuge tubes in the adapters, and place the adapters (w/tubes) in the UC. rotor. If the microcentrifuge tubes have cap hinges, cut the hinges before placing in the adapter.



adapter

Place the capped w/o hinge tubes in adapters

Place the adapter w/tubes in rotor

push center button to lock into UC machine

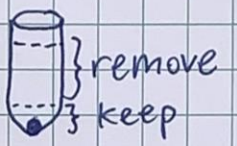
Tighten rotor with screw cap

Pre-Warm

3. Place cap onto rotor
   Put in Optima MAX-TL Ultracentrifuge
   50,000 RPM = 100,000g
   70 minutes
   4°C

Prewarm machine for 30 minutes
1. Press POWER button
2. Vacuum UP (1000 → 5 microns)
3. Press Vacuum UP again after 30 minutes (this releases air and completes warm up process)

4. When UC is done, press vacuum OFF, remove tubes with tweezers
5. Remove 1400 uL (1.4 mL) of supernatant

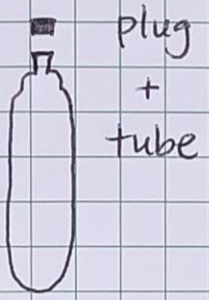


6. Pipette 1 mL of PBS into each tube
7. Vortex the tubes
8. Pipette 1 mL of the vortexed tubes into new UC microcentrifuge tubes
9. Cut hinges, place into adapters. Place adapters into rotor.
10. Ultracentrifuge (same settings as step 3) for 70 minutes.
11. Remove supernatant (950 uL) so there is only 50 uL left in tubes.
    (or remove any volume until there is only 50 uL left in tube)
12. Add complete RIPA buffer (200 uL) into each tube.

10 mL RIPA + 1 tablet protease inhibitor

(+ 1 phosphatase inhibitor) ← if looking at phosphorylized proteins

Beckman Coulter
OptiSeal
Polypropylene Centrifuge Tubes
13 x 48 mm
REF 361621
Designed for rotors TLA 100.4, Type 50.4 Ti, Type 50.3 Ti
Capacity: 4.7 mL per tube



3/23/2021

hVEC Cell Plating

Lonza

6. When preparing these BulletKit™ Media, it may not be possible to recover the entire volume listed for each vial. Small losses (up to 10%) should not affect the cell growth characteristics of the supplemented medium.
7. Transfer the label provided with each kit to the basal medium bottle(s) being supplemented (avoid covering the basal medium lot # and expiration date). Use it to record the date and amount of each supplement added. After SingleQuots™ Kit is added to basal medium, store at 2°-8°C and use within 1 month. Do not freeze medium.

NOTE: If there is concern that sterility was compromised during the supplementation process, the entire newly prepared growth medium may be re-filtered with a 0.2 µm filter to assure sterility. Routine re-filtration is not recommended.

X. Thawing of Cells / Initiation of Culture Process

NOTE: For proliferation of these cells, cells must be cultured at 37°C±1°C, 5% CO_2, 90%±2% humidity.

1. When initially plating endothelial cells from cryopreservation, the recommended seeding density is provided in the table below:

Cell Type	Recommended Seeding Density from Cryopreservation	Minimum Number of Flasks to Plate*
HUVEC	2,500 viable cells/cm²	≥5 x T-25 flasks OR ≥1 x T-75 flask
HUVEC-XL	2,500 viable cells/cm²	≥12 x T-225 flasks OR
Non-HUVEC Endothelial	5,000 viable cells/cm²	≥2 x T-25 flasks OR ≥1 x T-75 flask

*Calculations based on the minimum guaranteed cell count and viability. Please consult the product COA for exact cell count and viability when determining the appropriate number of flasks to plate. Alternative plate sizes/formats may be used so long as the appropriate seeding density is achieved.

2. To set up culture vessels, calculate the number of vessels needed based on the recommended seeding density as well as the surface area of the vessels being used.
3. Add the appropriate amount of medium to the vessels (1 ml/5 cm²) and allow the vessels to equilibrate in a 37°C±1°C, 5% CO_2, 90%±2% humidity incubator for at least 30 minutes.
4. Wipe cryovial with ethanol or isopropanol before opening. In a sterile field, briefly twist the cap a quarter turn to relieve pressure and then retighten. Quickly thaw the cryovial in a 37°C water bath being careful not to submerge the entire vial. Watch your cryovial closely; when the last sliver of ice melts, remove it. Do not submerge it completely. Thawing the cells for longer than 2 minutes results in less than optimal results.

NOTE: Centrifugation should **not** be performed to remove cells from cryoprotectant cocktail. This action is more damaging than the effects of DMSO residue in the culture.

5. Carefully mix the cell suspension using a micropipette. Dispense cells into the culture vessels set up in previous steps. Gently rock the culture vessel to evenly distribute the cells and return to the 37°C±1°C, 5% CO_2, 90%±2% humidity incubator.

NOTE: Endothelial cells tend to more strongly adhere to the cryovial than other cell types. Additional and/or more forceful trituration may be necessary to remove all cells.

6. Change the growth medium 16 to 24 hours after seeding.

XI. Maintenance

1. Change the growth medium 16 to 24 hours after seeding and every other day (every 48 hours) thereafter.
2. When cell confluence is 25-45%, increase the media volume to 1.5 ml/5 cm².
3. When cell confluence is greater than 45%, increase the media volume to 2 ml/5 cm².
4. Warm an appropriate amount of medium to 37°C in a sterile container. Remove the medium and replace it with the warmed, fresh medium and return the flask to the incubator.
5. Avoid repeated warming and cooling of the medium. If the entire contents are not needed for a single procedure, transfer and warm only the required volume to a sterile secondary container.

XII. Subculturing

NOTE: Lonza warrants its Clonetics™ Cells only if Lonza Subculturing Reagents are used. The recommended subculturing reagents for these cells are Trypsin/EDTA (CC-5012), Trypsin Neutralizing Solution (CC-5002), and HEPES Buffered Saline Solution (CC-5022). These reagents can be purchased individually or together as part of the Reagent Pack™ Subculture Reagents (CC-5034).

4/29/2021

hVIC Cell Plating

Lonza Walkersville, Inc.
bioscience.lonza.com
scientific.support@lonza.com
Scientific Support: 800-521-0390
Document # TS CC-2903 04/20
Walkersville, MD 21793-0127 USA

Cardiac Fibroblast Cell Systems

NHCF-A and NHCF-V

Introduction

Cardiac Fibroblast Cell Systems are available as Normal Human Atrial Fibroblasts (NHCF-A) and Normal Human Ventricular Fibroblasts (NHCF-V) with optimized media for their growth. Each system can quickly generate NHCF cultures for the study of disorders of human fibroblasts such as cardiac fibrosis. Cardiac Fibroblast Cell Systems are convenient and easy to use, allowing the researcher to focus on results. Cryopreserved NHCF are shipped in second passage. Lonza's Cells, Medium and Reagents are quality tested together and guaranteed to give optimum performance as a complete cell system.

Cell system components (need to be purchased separately)

- One cardiac fibroblast cell product—NHCF-A, NHCF-V (cryopreserved)
- One Fibroblast Medium BulletKit™ - 500 mL
  FGM-3™ BulletKit™ (CC-4526) contains one 500 mL bottle of fibroblast basal medium and the following growth supplements: hFGF-B, 0.5 mL; Insulin, 0.5 mL; FBS, 50 mL; GA-1000, 0.5 mL.
- One ReagentPack™ (CC-5034) containing:

Trypsin/EDTA	100 mL
Trypsin Neutralizing Solution	100 mL
HEPES Buffered Saline Solution	100 mL

Characterization of cells

Routine characterization of NHCF includes positive immunofluorescent staining for collagen I. Cardiac fibroblasts are negative for von Willebrand factor VIII related antigen.

Performance

Recommended seeding density for subculture	3,500 cells/cm²
Typical time from subculture to confluent monolayer	6 - 9 days
Additional population doublings guaranteed using Lonza's system	5

Quality control

All cells are performance assayed and test negative for HIV-1, mycoplasma, Hepatitis-B, Hepatitis-C, bacteria, yeast and fungi. Cell viability, morphology and proliferative capacity are measured after recovery from cryopreservation. Lonza's media are formulated for optimal growth of specific types of normal human cells. A Certificates of Analysis (COA) for each lot is shipped with each order. COA's for all other products are available upon request.

Ordering information

Cryopreserved cells

Cat no.	Description	Size
CC-2903	NHCF-A	≥ 500,000 cells
CC-2904	NHCF-V	≥ 500,000 cells
Proliferating cells – flasks and multiwell plates		
CC-2903T25	NHCF-A T-25	T-25 Flask
CC-2904T25	NHCF-V T-25	T-25 Flask
CC-2903W96	NHCF-A 96-well	96-well Plate
CC-2904W96	NHCF-V 96-well	96-well Plate

Other proliferating formats are available. Refer to the Lonza website for details.

4/29/2021

The following instructions are for a 25 cm² flask. Adjust all volumes accordingly for other size flasks.

1. Subculture the cells when they are 70%-85% confluent.
2. For each 25 cm² of cells to be subcultured:
   a. Thaw 2 ml of Trypsin/EDTA and allow to come to room temperature.
   b. Allow 7-10 ml of HEPES Buffered Saline Solution (HEPES-BSS) to come to room temperature.
   c. Allow 5 ml of Trypsin Neutralizing Solution (TNS) to come to room temperature.
   d. Remove growth medium from 4°C storage and allow warming to room temperature.
   e. Prepare new culture vessels.
3. Subculture one flask at a time. All flasks following the first flask will be subcultured following an optimization of this protocol (explained later in this procedure), based on calculated cell count, cell viability, and seeding density.

NOTE: The following steps must be performed in a sterile field.

4. Aspirate the medium from one culture vessel.
5. Rinse the cells with 5 ml of room temperature HEPES-BSS. DO NOT forget this step. The medium contains complex proteins and calcium that neutralize the trypsin.
6. Aspirate the HEPES-BSS from the flask.
7. Cover the cells with 2 ml of Trypsin/EDTA solution.
8. Place the culture vessels into a 37°C humidified incubator for 3-5 minutes. Periodically examine the cell layer microscopically and check for cell detachment.
9. Allow the trypsinization to continue until approximately 90% of the cells are rounded up.
10. At this point, tap the flask against the palm of your hand to release the majority of cells from the culture surface. If only a few cells detach, you may not have let them trypsinize long enough. Wait 30 seconds and tap again. If cells still do not detach, wait and tap every 30 seconds thereafter. This entire process should take no more than 5 minutes.

NOTE: If the majority of cells does not detach within 5 minutes, the trypsin is either not warm enough or not active enough to release the cells. Harvest the culture vessel as described below, and either re-trypsinize with fresh, warm Trypsin/EDTA solution or rinse with Trypsin Neutralizing Solution and then add fresh, warm medium to the culture vessel. Return to an incubator until fresh trypsinization reagents are available.

11. After cells are released, neutralize the trypsin in the flask with 5 ml of Trypsin Neutralizing Solution at room temperature.
12. Quickly transfer the detached cells to a sterile 15 ml centrifuge tube.
13. Rinse the flask with a final 2 ml of HEPES-BSS to collect residual cells, and add this rinse to the centrifuge tube.
14. Examine the harvested flask under the microscope to make sure the harvest was successful by looking at the number of cells left behind. This should be less than 5%.
15. Centrifuge the harvested cells at 200 x g for five minutes to pellet the cells.
    - Aspirate most of the supernatant, except for 100-200 µl
    - Flick the cryovial with your finger to loosen the pellet
16. Dilute the cells to a final volume of 2 to 3 ml of growth medium and note the total volume of the diluted cell suspension.
17. Determine cell count and viability using a hemacytometer and Trypan Blue. Make a note of your cell yield for later use.
18. If necessary, dilute the suspension with growth medium to achieve the desired "cells/ml" and re-count the cells.
19. Use the following equation to determine the total number of viable cells.

$$\text{Total \# of Viable Cells} = \frac{\text{Total cell count} \times \text{percent viability}}{100}$$

20. The number of flasks needed depends upon cell yield, cell type, seeding density, and application. The recommended seeding density when subculturing endothelial cells for further proliferation or angiogenesis is provided in the table below:

Cell Type/Application	Recommended Seeding Density after Subculture
HUVEC	2,500 viable cells/cm²
HMVEC-DNeo	2,000 viable cells/cm²
HMVEC-LLy	2,500 - 5,000 viable cells/cm²
All Other Endothelial	5,000 viable cells/cm²
Angiogenesis	65,000 – 80,000 viable cells/cm²

75

5/3/2021

Project

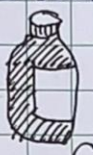
Immunofluorescent Staining

Continued on Page

Read and Understood By

1. Prepare sample on microscope slides (from embedding w/ OCT & sectioned)
   Use Super PAP PEN [redacted] hydrophobic slide marker to draw around sectioned samples.
2. 15 minutes PBS
3. 15 minutes Permeabilizing Solution
   - 0.2% Triton + PBS

   TRITON® X-100 Electrophoresis $C_{34}H_{62}O_{11}$

   For a 9-mL Permeabilizing Solution
   $\frac{0.2}{100} = \frac{x}{9000}$ $x = 18$ µL Triton X-100
   PBS ⇒ 8982 µL of PBS
4. 2 hours Blocking Solution
   - 1% w/v BSA (BSA is powder)
   - 2% goat serum (or donkey, depending on 2ndary)
   - 97% PBS

   For a 3-mL Blocking Solution: 30 mg BSA
   60 µL goat serum
   2000 + 940 µL PBS
5. 2 hours Primary antibody
   1:200
   4:800 • 4 µL ELN Antibody (OR other antibody) + 796 µL 1% BSA

   make 20 mg BSA in 2000 µL PBS
6. 5 minutes (x3) Wash with Permeabilizing Solution (3 times)
7. 1 hour Secondary antibody
   1:400
   • 2 µL Secondary antibody + 798 µL 1% BSA
8. 5 minutes (x3) Wash with Permeabilizing Solution (3 times)
9. 10 minutes DAPI solution (4',6-diamidino-2-phenylindole)
   1:40
   20:800 • 20 µL DAPI + 780 µL PBS
10. 5 minutes (x3) Wash with PBS
11. Mount Slide with Southern Biotech Fluoromount-G } or similar reagent

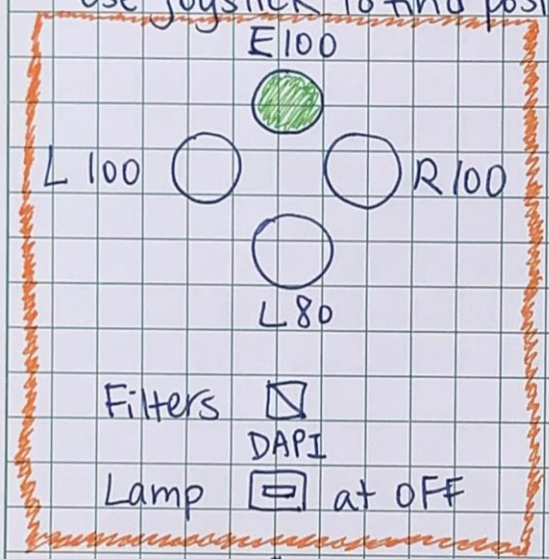
Continued from Page

09/17/2021

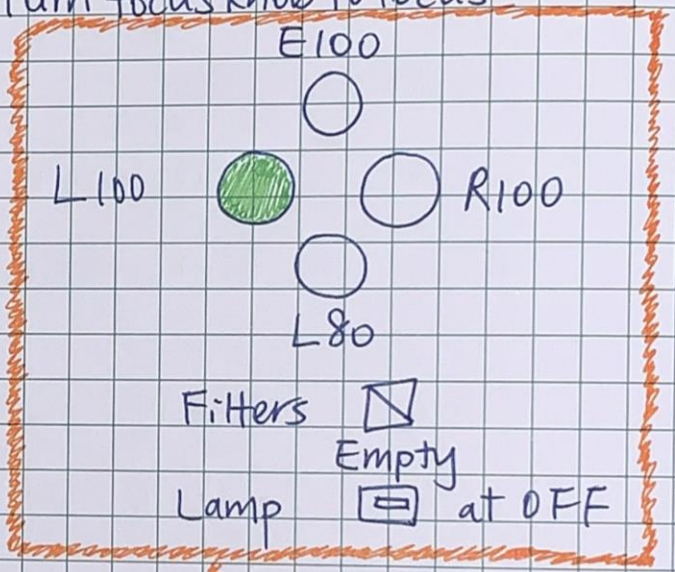
Confocal Imaging

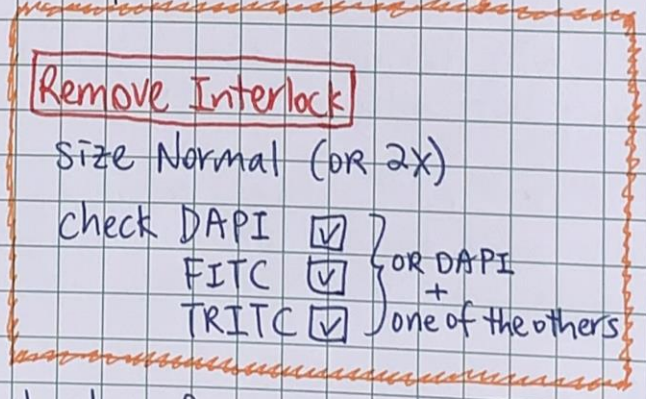
Read and Understood By
Continued on Page

1. Turn on machines in order from 1 → 7. (Turn off order is 7 → 1)
2. Computer sign in account: Ramaswamy. PW: sharan2017
3. Open NIS Elements AR 4.51.01 64-bit
4. Nosepiece 20X
   Lamps ▣ ON/OFF. Switch to ON to load slide
5. Load sample slide w/cover slip facing down
   Use joystick to find position, turn focus knob to focus

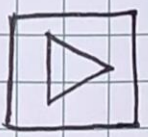


Use this setting to find sample using eyepiece.





these two combined click



6. Use mouse scroll wheel to focus image on screen
7. Stop scan, click 02X + ChSeries +  to capture image.

8. Note the scale bar. eg. 0.62 μm/pixel
   Save image as tiff.
   ↑
   Use ImageJ to process tiffs.

09/18/2021