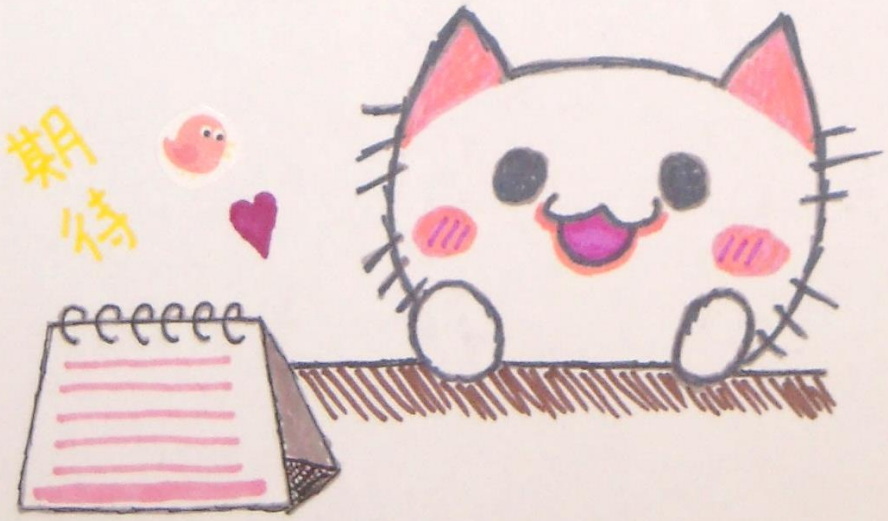
LABORATORY NOTEBOOK

我創造我的宇宙

我對發生在我身上的所有事情負責



LABORATORY NOTEBOOK

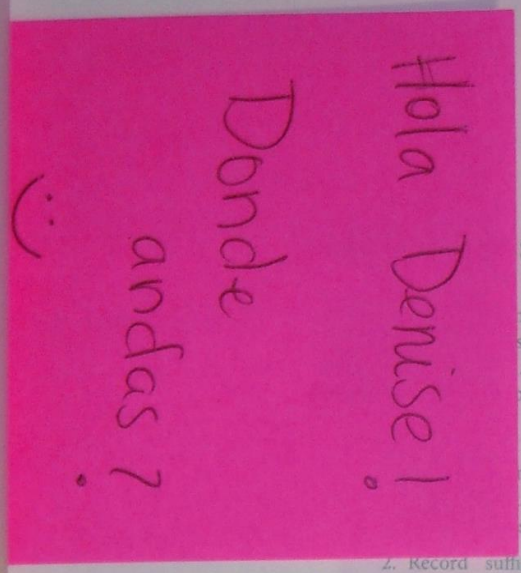
Company Name: CMRL

Department: Biomedical Engineering

Notebook No.: 021

Assigned to: Denise Hsu

Date: 9/5/2018



S FOR KEEPING RESEARCH RECORDS

ng a complete record of your laboratory work which can be understood self and others, this notebook has been designed to afford maximum . Several practices must be followed to give the notebook value as a legal patent litigation:

directly into this book; it is permanently bound with numbered pages so n not be substituted or deleted. Do not record data elsewhere for transfer . Write in ink. Never make erasures. Thus, the integrity of the records will stion.

2. Record sufficient information. All procedures, reagents, apparatus, sketches, conditions, references, etc., should be entered in the book as the work is done. The purpose and significance of the experiment as well as the observations, results and conclusion should be made clear. What may seem trivial at the time may later prove of critical importance. Your entries should be clear and complete enough for someone else who is "skilled in the art" to read and comprehend what has been accomplished. Avoid sweeping negative statements, e.g.: "This procedure is worthless," which could later limit the scope of your claims.

3. Not only is the conception of an invention important, but so is the diligence shown in making a working model or demonstrating that the idea works—"reducing the practice." These two elements of an invention, conception and reduction to practice, must be corroborated by a witness. The records of the inventor(s) are not enough. Thus, each page of the notebook should be read, witnessed, and dated (daily, if possible) by someone who is competent to understand it, but who does not claim to be a co-inventor. Charts, tables, etc., should be complete, and lines should be drawn through any blank spaces prior to witnessing. It may be wise to perform key experiments in front of one or more witnesses. Spectra, charts, etc., should be signed, dated, witnessed, and if they can not be permanently attached to the notebook, they should be referred to with an entry in the book and kept on file. Dates and witnesses can establish your priority of invention.

4. To delete an entry, draw a line through it so that it is still legible. Corrections should be made adjacent to the deleted entry, and they should be initialed and dated by you and the corroborating witness. Changes made after the page has been witnessed should also be initialed and dated by you and the witness. Always use the current date.

5. The notebook and its contents are to be considered confidential and of great value. Exercise every care in preserving them. Report the loss or theft of a research notebook to a group leader immediately.

6. Index the contents and return each book as completed (or when not in use) for filing.

7. New ideas must be recorded and witnessed as they occur to establish priority of invention. Even ideas which do not pertain to the project at hand should be documented in the book.

Keep your research records as if each project were to be patented. Even though the work contained in the book may not result in a patent application, observance of these practices will provide a clear record for reports, publication, or future reference.

Instructions Read and Understood by _____

Dated _____

Table of Contents	Page

All good things are coming to me today
I'm grateful to be alive
I see beauty all around me
I live with passion and purpose
I take time to laugh and play everyday
I'm awake, energized and alive
I focus on all the good things in life
and give thanks for them
I'm at peace and one with everything
I feel the love, the joy, the abundance
I am free to be myself
I am grateful to be ... me.
Today is the best day of my life.

09-23-2018 Perfection of life

Page

Table of Contents

Florida Heart
research foundation

所有的美好將在今天到來
我對活著表達感激
我處處看到了美
我生活充滿著熱情和目標
每天有歡笑、有快樂
我清醒著，充滿精力與活力
我專注於生命裡所有美好的事物
並對此深深感激
我與萬物合一、與萬物平靜
09-23-2018 生命的完美

我感受到愛
喜悅和豐足
自由自在地做自己
我很感恩我能夠
成為...我自己
今天是我生命中
最美好的一天

www.stopheartdisease.org

Coating Bioflux 24-well plates

2% gelatin in H_2O, Type B

1. Pipette 200 µL gelatin into each outlet well (total of 8 outlet wells)
2. Run plate from O → A well at ~5 dynes/cm² until volume in O and A are equal.
3. Incubate plate at 37°C for 30 minutes
4. Remove excess gelatin from all wells with micropipette
5. Pipette 200 µL sterile PBS into each outlet well ← if cell attachment is low, don't flush channels w/ PBS
6. Run plate from O → A until PBS starts accumulating in well A.
7. Remove excess PBS from all wells w/ micropipette.
8. Plate is now ready for cell seeding

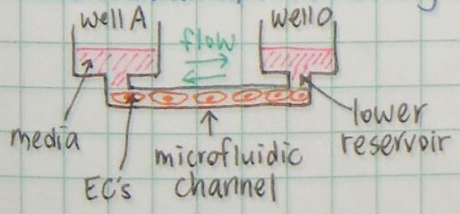
Cell Seeding in Bioflux 24-well Plates

NEED: Starting cell concentration per channel: 2×10^6 cells/mL
Minimum cell seeding volume per channel: 100 µL or 0.1 mL
Minimum cell count per channel for seeding: 2×10^6 cells/mL × 0.1 mL = 2×10^5 cells
Total # of channels: 8
Total Volume of cells per plate: 800 µL or 0.8 mL
Total # of cells needed per Bioflux 24-well plate (for seeding):

# of channels × cell count per channel
= 8 × 2×10^5
= 1,600,000 cells per plate or 1.6×10^6 cells per plate

Cell Type: Porcine aortic endothelial cells (EC)

1. Pipette 100 µL of cells at concentration 2×10^6 cells/mL into each outlet well.
2. Run plate from O → A at ~5 dynes/cm² until cells fill up the microfluidic channels. Use microscope to observe flow in the viewing port.
3. Once cells appear in well A, stop the flow and place plate in incubator for 24 hrs. This allows cells to attach. Add fresh media into well O and/or A before placing in incubator, ~500 µL per well.
4. Check under microscope for cell attachment. Once cells are attached, remove old media w/o touching lower reservoir.



5. Add fresh media into wells (amount per well varies depending on flow profile)
6. Apply desired flow profile, place plate in incubator. Conditioning time: 48 hours
7. Remove and SAVE all spent media from flow condition. Label spent media conical tube.

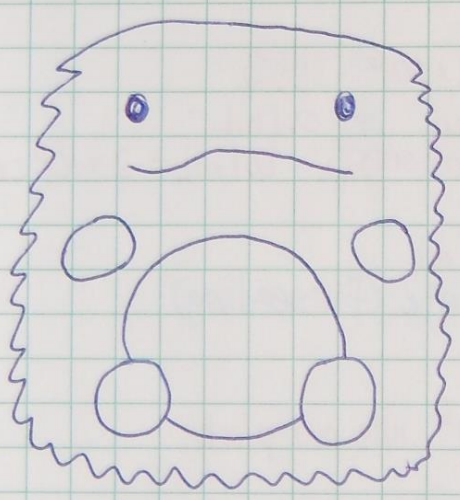
Continued on Page

Read and Understood By

Signed _____ Date _____ Signed _____ Date 9/30/2018

8. Extract RNA from cells in plate using QIAGEN or TRIzol.

Culturing SMC's with EC's spent media
Cell type: Porcine aortic Smooth muscle cells (SMC)
1. Seed SMC's in 6cm dish at 0.8×10^6 cells/mL for 24 hours. This allows cell attachment.
2. Once cells have attached, aspirate old media and add 50% fresh 50% spent media from EC's (1.5mL & 1.5mL)
3. Culture for 48 hours.
4. Remove (& save?) spent media. Extract RNA w/ TRIzol from SMCs for qPCR.



Alternatively:
Seed SMC's in 6cm dish @ 0.8×10^6 cells with 50% fresh DMEM + 50% Spent media from EC's. Culture for 48 hours, extract RNA for qPCR.

$$OSI = \frac{1}{2}\left(1 - \frac{\left|\int_0^T \tau_w \, dt\right|}{\int_0^T |\tau_w| \, dt}\right)$$

τ_w: wall shear stress
T: duration cycle
t: time

Continued on Page

Read and Understood By

Signed | Date | Signed | 9/30/2018 Date

ACTIVITY LOG

9/24/2018: Obtained porcine aortic endothelial cells (PAEC) from MSH, P8
9/27/2018: PAEC passage, P8→P9
Obtained porcine aortic smooth muscle cells (PASMC) from MSH, p5
9/29/2018: PAEC passage, P9→P10
PASMC passage, P5→P6
10/1/2018: PAEC media change, spent media saved (Static)
PASMC media change
Coated gelatin in Bioflux 24-well plate
EC cell count: 1574,000 cells/mL (live)
Seeded EC's in Bioflux 24-well plate (8 channels)

Media Used:

PAEC

Cat# P211-500
Lot: 29908
Porcine Endothelial Cell Growth Medium
CELL APPLICATIONS INC

PASMC

Lot 1989243
DMEM (1X)
+4.5 g/L D-glucose, L-glutamine
- Sodium pyruvate
gibco

EC Cell Count:
10 µL Trypan blue } 20 µL in total
10 µL EC's in media }
↓
Each count = 10 µL on counting slide

LIVE CELLS
Count 1: 9.44×10^5 cells/mL
Count 2: 6.30×10^5 cells/mL

Live: $\frac{2 \times 9.44 \times 10^5 + 2 \times 6.30 \times 10^5}{2} = \frac{1888000 + 1260000}{2} = 1,574,000$ cells/mL

C_1: Seeding concentration (min)
V_1: Seeding volume (minimum)
C_2: cell count concentration
V_2: Volume needed per channel for seeding

$C_1 V_1 = C_2 V_2$

2×10^6 cells/mL · 0.1 mL = 1574000 cells/mL · V_2

$V_2 = \frac{2 \times 10^6 \times 0.1}{1574000} = 0.127$ mL OR 127 µL per channel

1. EC's were seeded at 127 µL per channel at 16:47 on Oct. 1, 2018, P9.
2. Flow applied to fill microfluidic channels with cells
3. Stopped flow, added 100 µL of fresh media into all wells.
4. Placed plate in 37°C incubator 5% CO_2 for 24 hours.

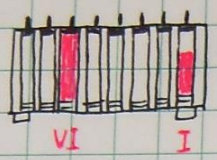
Continued on Page

Read and Understood By

Signed Date Signed 10/1/2018 Date

10/2/2018: PAEC passage, P10 → P11, static spent EC media saved.
PASMC passage, P6 → P7
2 Hrs @ 10 dynes/cm² Bioflux 24-well plate w/ P9 PAEC's:
10 dynes/cm² flow applied at 17:27.
Flow stopped at 19:31 because of temporary reservoir overflow
Rinsed tubings w/media using isopropanol
Replaced three filters behind controller

temporary reservoir



10/3/2018: Removed & tossed static media from all wells in Bioflux plate.
Added 1 mL of fresh media in column O's (8 wells total)
Applied 5 dynes/cm² steady flow at 10:11 am.
Note: Lowered shear stress because well O's were emptied too quickly!

11:01 am paused flow, well O's were empty
Transferred media in A & B columns back to O column.
6 Hrs @ 5 dynes/cm²
11:07 am 5 dynes/cm² flow resumed.
11:45 am paused flow, transferred media as before
11:50 am Flow resumed @ 5 dynes/cm²
12:50 pm paused flow, transferred media as before
1:00 pm Flow resumed @ 5 dynes/cm²
2:00 pm paused flow, transferred media
2:15 pm Flow resumed @ 5 dynes/cm²
3:00 pm Paused flow, transferred media. Bubbles accumulated in wells
3:35 pm Flow resumed @ 5 dynes/cm²
4:45 pm Stopped flow, media crawled up temporary reservoir
Removed all media, saved spent media in conical.
Pipetted fresh media (100 µL) into each well (all 24 wells)
5:08 pm Placed plate in static condition (no flow)

10/4/2018: 9:20 am Removed static media
Pipetted 3mL fresh media into column O wells
7 Hrs @ 2 dynes/cm²
9:41 am Started run @ 2 dynes/cm² steady state, lower shear, longer run
4:04 pm Removed and saved spent media
Pipetted fresh media (100 µL) into each well
4:45 pm Placed plate in static condition

10/5/2018: 11:30 am Removed static media, saved for later use
Pipetted 1.5 mL fresh media + 1.5 mL spent media @ 2 dynes/cm² into well O's.

Read and Understood By

Signed Date

Signed 10/2/2018 Date

6 Hrs @ 2 dyne/cm²
Applied flow @ 2 dynes/cm²
6:07pm Stopped flow, collected spent media
Placed in static culture
PASMC media change (p7). PAEC media change (p11)

10/6/2018: Removed static media, pipetted flow media into well O's.

6 Hrs @ 2 dynes/cm²
12:25pm Started flow @ 2 dynes/cm² steady flow
PAEC dish culture media change (p11), static spent media saved for later use
4:51pm Paused flow, moved media in A & B columns into O column
5:00pm Resumed flow @ 2 dynes/cm² (steady flow)
6:45 pm Stopped flow, saved spent media. Pipetted previously saved spent media into wells for static culture in Bioflux

Culturing PASMC with PAEC's spent media:

1. Trypsinized two 10-cm plates of PASMC
2. Cell count: Resuspended pellet in 910 µL of fresh DMEM after centrifuge

Count 1 → 5 µL + 5 µL Trypan blue
Raw reading viable = 2.63×10^6 cells/mL
non-viable = 2.72×10^6 cells/mL

Count 2 → 5 µL + 5 µL Trypan blue
Raw reading viable = 2.55×10^6 cells/mL
non-viable = 2.57×10^6 cells/mL

Average raw reading: $\frac{(2.63 + 2.55)}{2} = 2.59 \times 10^6$ cells/mL

Total cell concentration: $2 \times 2.59 \times 10^6$ cells/mL $= 5.18 \times 10^6$ cells/mL

$C_1V_1 = C_2V_2$

$C_2 = \frac{C_1V_1}{V_2} = \frac{(0.8 \times 10^6 \text{ cells/mL})(1.0 \text{ mL})}{5.18 \times 10^6 \text{ cells/mL}} = 0.154 \text{ mL} = 154 \text{ µL}$

C_2 = Seeding density
V_1 = Seeding volume
C_1 = counted cell density
V_2 = Volume needed

Used 140 µL of suspended cells to seed in a 6-cm culture dish.

154 µL suspension
1 mL fresh DMEM
1154 µL spent media from EC
6-cm PASMC P8 w/static spent media from EC

154 µL suspension
1 mL fresh DMEM
1154 µL spent media from EC
6-cm PASMC P8 w/Flow spent media from EC, steady flow @ 2-5 dynes/cm²

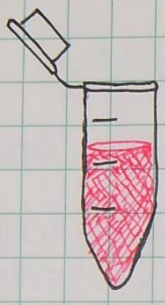
Ratio DMEM : Spent EC media = 1 : 1

PASMC spent media culture start time: 10/6/2018 17:05

Signed _____ Date _____ Signed _____ Date 10/5/2018

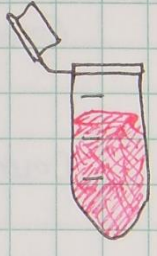
10/7/2018: Removed static media, pipetted flow media into wells.
5.75 hr @ 2 dynes/cm² — Applied flow @ 2 dynes/cm² at 1:00pm
6:45pm stopped flow, collected spent flow media.
Placed plate back in static.

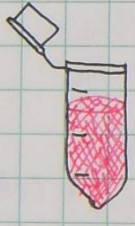
10/8/2018: 12:25pm Started flow @ 2 dynes/cm²
6.5 Hr @ 2 dynes/cm² — 19:00 Stopped flow. Pipetted TRIzol in wells, ran steady flow for 3 minutes at 10 dynes/cm². Stored TRIzol w/ EC RNA in tube in -80°C
PASMC Spent media culture end time: after 48 hours
17:05 Stored TRIzol w/PASMC RNA in tubes in -80°C
Passaged PAEC P11 → P12
Passaged PASMC P7 → P8



PASMC
Static Spent media Culture
0.8 mL TRIzol

PASMC
Steady flow Spent media culture
0.8mL TRIzol





PAEC
Average steady flow ≈ 0.8 dynes/cm²
0.8 mL TRIzol

*For purpose of convenience will use peak shear stresses of 1 dyne/cm² for 0.25 and 0.5 OSI's.

2 hrs @ 10 dynes/cm²
6 hrs @ 5 dynes/cm²
7 hrs @ 2 dynes/cm²
6 hrs @ 2 dynes/cm²
6 hrs @ 2 dynes/cm²
5.75 hrs @ 2 dynes/cm²
6.5 hrs @ 2 dynes/cm²
104 hrs @ 0 dynes/cm²

2 hrs @ 10 dynes/cm² → 20
6 hrs @ 5 dynes/cm² → 30
31.25 hrs @ 2 dynes/cm² → 62.5
104 hrs @ 0 dynes/cm² → 0
143.25 hrs — 112.5 dynes/cm²

$\frac{112.5}{143.25} = 0.785$ dynes/cm² (average) #

10/10/2018: PAEC media change
PASMC media change

Continued on Page

Read and Understood By

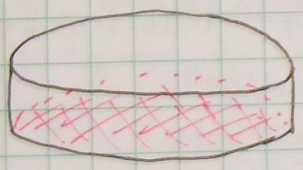
Signed — Date — Signed — 10/7/2018 Date

10/11/2018: Trypsinized two 10-cm plates of SMC (P8)
Resuspended in 1010 µL
Cell count Raw Reading: (10 µL trypan blue + 10 µL (20 µL) suspension)
Total: 1.87×10^6 cells/mL
Live: 1.80×10^6 cells/mL } count 1, 10 µL (5 µL trypan blue + 5 µL cells)
Total: 1.66×10^6 cells/mL
Live: 1.55×10^6 cells/mL } count 2, 10 µL "

Average raw reading: $\frac{1.80 + 1.55}{2} = 1.675 \times 10^6$ cells/mL

Total cell concentration = $2 \times 1.675 \times 10^6$ cells/mL
Cell concentration = 3.35×10^6 cells/mL

Want 0.8×10^6 cells seeded in 6-cm plates
$C_1 V_1 = C_2 V_2$
$0.8 \times 10^6 = 3.35 \times 10^6 V_2$
$V_2 = \frac{0.8}{3.35} = 0.2388$ mL = 238.8 µL ≈ 240 µL in fresh DMEM

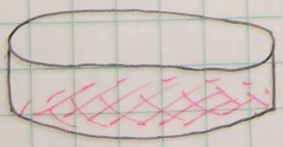

6cm plate


6cm plate

Plate 1: Static Spent media from EC

Plate 2: steady flow spent media from EC

applies to all four plates!
240 µL fresh DMEM w/ cells + 1260 µL fresh DMEM } total = 3 mL @ 50% fresh DMEM
240 µL Spent EC media + 1260 µL Spent EC media } 50% spent EC media


6 cm plate


6 cm plate

Plate 3: static spent media from EC

Plate 4: steady flow spent media from EC

Start conditioning time: 18:00
Conditioning time: 48 hrs (ends on 10/13 @ 18:00)
Continued on Page

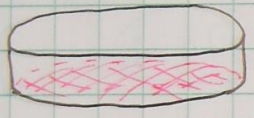
10/12/2018 : Passaged PAEC P12→P13
Passaged PASMC P8→P9

1. Trypsinized 1 10-cm plate @ P8 of PASMC for 48 hr static culture
Cell count of PASMC:
Live 1: 9.65×10^5 cells/mL
Live 2: 9.07×10^5 cells/mL
> 10 µL media w/cells + 10 µL trypan blue

$\frac{9.65+9.07}{2} = 9.36 \times 10^5$ (average raw count)

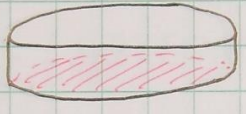
Actual cell concentration = $2 \times 9.36 \times 10^5 = 1.872 \times 10^6$ cells/mL
Want: 0.8×10^6 cells in each 6-cm plate
$0.8 \times 10^6 = 1.872 \times 10^6$ cells/mL $\times V_2$
$V_2 = \frac{0.8}{1.872} = 0.427$ mL = 427 µL

Used 430 µL


6cm plate

Static spent media from EC

430 µL DMEM w/cells
1070 µL fresh DMEM
1500 µL static spent media
} = 3mL


6cm plate

Steady flow spent media from EC

430 µL DMEM w/cells
1070 µL fresh DMEM
1500 µL steady flow spent media
} = 3mL

Start time: 19:23
Conditioning time: 48 hrs (ends on 10/14 @ 19:23)

2. Seeded PAEC in Bioflux plate
Cell count of PAEC: (from 6 6-cm plates)
Live 1: 1.36×10^6 cells/mL
Live 2: 1.20×10^6 cells/mL
> 10 µL media w/cells + 10 µL trypan blue

$\frac{1.36+1.20}{2} = 1.28 \times 10^6$ cells/mL (average raw count)

Actual cell concentration = $2 \times 1.28 \times 10^6$ cells/mL = 2.56×10^6 cells/mL
Want: 2×10^6 cells/mL $\times$ 0.1 mL per channel
$C_1V_1 = C_2V_2$
$(2 \times 10^6)(0.1) = (2.56 \times 10^6)V_2$

Continued on Page

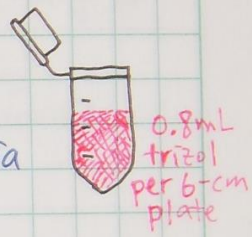
$V_2 = \frac{2 \times 0.1}{2.56} = 0.078125 = 78.125\,\mu L$ (Used 80 µL per channel)

Aliquotted $8 \times 80\,\mu L = 640\,\mu L$ of cell suspended in media to seed in 8 Bioflux channels. Plated the rest across five 6-cm culture dish, approximately 3.4816×10^6 cells in total.

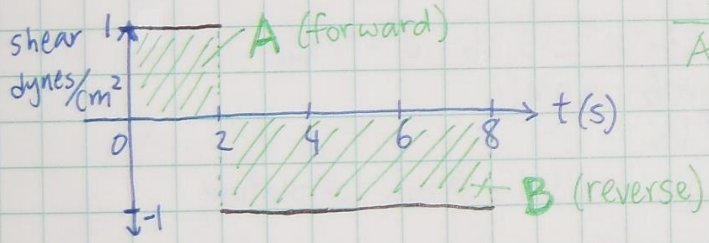
Ran Bioflux @ 1-10 dynes/cm² to make sure all channels have cells. Added 100 µL of fresh EC media for static culture to allow cells to attach in microfluidic channels. Cells in Bioflux plate will remain in static for 24 hours before flow experiments.

Time seeded: 21:49
Static conditioning time: 24 hours (ends on 10/13 @ 21:49)

10/13/2018: 18:00 End of 48 hr PASMC static culture
Transferred samples into TRIzol and placed in -80.
Total of 4 6-cm plates: Two Static EC spent media
Two steady flow EC spent media



Started 0.25 OSI flow profile



$\frac{A}{A+B} = 0.25 = OSI$

duration (s)	direction & shear (dynes/cm²)	
2	1 forward	] 8 sec
6	1 reverse	

Each full iteration is 8 seconds.
Need 21,600 iterations for a conditioning time of 48 hours.

48 hours = 172,800 seconds
$\frac{172{,}800 \text{ seconds}}{8 \text{ seconds/iteration}} = 21{,}600$ iterations

Bioflux 200 → Edit Autorun → Protocol Setup → entered duration and shear stress values → Sequence Setup → select the previously entered protocol, enable all 8 channels, enter 21600 under "step iterations", select DMEM+ for fluid type, save.

PAEC 0.25 OSI Run Start time: 10/13 @ 21:50 (ends on 10/15 @ 21:50)
Starting volume per well/column
TeamViewer: 1212510503
PW: 94dg4b

Continued on Page

Columns	A	B	O
	0.5 mL	0 mL	2.5 mL

A → O forward
O → A & B reverse

Read and Understood By

Signed _____ Date _____ Signed _____ Date 10/12/2018

10/14/2018: 16:00 paused flow (PAEC's) in Bioflux
moved media from columns A & B to 0
16:07 resumed flow
20:00 placed SMCs in TRIzol
coated new 6-cm plates w/gelatin
21:00 paused flow in Bioflux. Redistributed media to leave flow overnight. unpaused flow at 21:07.

10/15/2018: 10:00 paused Bioflux, redistributed media
10:07 resumed flow
PASMC passage P9 → P10, passaged 2 plates.
PAEC passaged two plates P13 → 14
21:50 Stopped 0.25 OSI flow, saved all spent media from all wells.
Ran TRIzol through channels to collect PAEC cell RNA
(100 µL per channel, total of 800 µL)

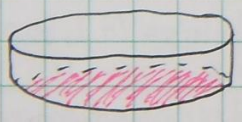
PASMC cell count:
Live 1: 3.09×10^5 cells/mL
Live 2: 2.17×10^5 cells/mL
} 10 µL in media + 10 µL Trypan blue

$$\text{Avg count} = \frac{3.09 + 2.17}{2} = 2.63 \times 10^5 \text{ cells/mL}$$

Actual count = $2 \times 2.63 \times 10^5 = 5.26 \times 10^5$ cells/mL
Want: 0.8×10^6 cells in 6-cm plate
$0.8 \times 10^6 = 5.26 \times 10^5 V_2$

$$V_2 = \frac{8}{5.26} = 1.53 \text{ mL}$$

Used 1.5 mL for plating



1.5 mL cells in suspension + 1.5 mL spent media from 0.25 OSI PAEC

Plated time: 22:20 on 10/15 (ends on 10/17 @ 22:20)

10/17/2018: 22:20 placed PASMC in TRIzol and -80

10/18/2018: Passaged PASMC P10 → P11
PAEC media change

10/21/2018: PAEC media change
TRIzol RNA Extraction on PASMC: static
steady flow
0.25 OSI

RT-PCR

Continued on Page

RNA Quantification:

Static (S)	Flow (F)	OSI@25 (0.25)
667.8 ng/µL	567.3 ng/µL	271.7 ng/µL
701.3 ng/µL	575.8 ng/µL	267.6 ng/µL
674.0 ng/µL	561.6 ng/µL	267.3 ng/µL
681.03 ng/µL	568.23 ng/µL	268.87 ng/µL AVERAGES

Need 100 ng of RNA per well for qPCR

S

$100\ ng = 681.03\ ng/\mu L \times V_S$

$V_S = \frac{100}{681.03} = 0.1468 \approx 0.15\ \mu L$ per well

F

$100\ ng = 568.23\ ng/\mu L \times V_F$

$V_F = \frac{100}{568.23} = 0.17599 \approx 0.18\ \mu L$ per well

0.25

$100\ ng = 268.87\ ng/\mu L \times V_{0.25}$

$V_{0.25} = \frac{100}{268.87} = 0.3719 \approx 0.38\ \mu L$ per well

Three RNA samples: S, F, 0.25
Four primer pairs per sample
GAPDH, PECAM1, α-SMA, VEGF — targets
Three replicates for each primer pair

Set-Up 48-well Plate

	1	2	3	4	5	6
A	S GAPDH	S GAPDH	S GAPDH	S α-SMA	S α-SMA	S α-SMA
B	S PECAM1	S PECAM1	S PECAM1	S VEGF	S VEGF	S VEGF
C	F GAPDH	F GAPDH	F GAPDH	F α-SMA	F α-SMA	F α-SMA
D	F PECAM1	F PECAM1	F PECAM1	F VEGF	F VEGF	F VEGF
E	0.25 GAPDH	0.25 GAPDH	0.25 GAPDH	0.25 α-SMA	0.25 α-SMA	0.25 α-SMA
F	0.25 PECAM1	0.25 PECAM1	0.25 PECAM1	0.25 VEGF	0.25 VEGF	0.25 VEGF

36 wells in total
Use RNA to C_T one-step kit

Cat# 4391178
Power SYBR™ Green RNA-to-C_T™ 1-Step Kit
Applied Biosystems
ThermoFisher SCIENTIFIC

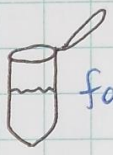
Continued on Page

Read and Understood By

Signed — Date — Signed — 10/21/2018 Date

MASTER MIXES:

1. RNA-to-C_T RT Master Mix
(Page 13 in protocol online) - For 10 µL reactions
Volume needed per sample (well):
5.0 µL SYBR green mix
0.08 µL RT Enzyme
36 + 1 extra
5.0 µL × 37 samples = 185 µL SYBR Green Mix
0.08 µL × 37 samples = 2.96 µL RT Enzyme

2. Primers
(Page 13 in protocol) - For 10 µL reactions, use 100-200 nM of each primer, but extra primers are unbound and don't fluoresce, we will use 1 µL of the 10 µM tube for both FWD & REV.
3 replicates × 3 samples = 9 primer pairs
9 + 1 extra
1 µL FP × 10 wells = 10 µL FP
1 µL RP × 10 wells = 10 µL RP
} 20 µL in  for each primer type

3. RNA Samples
12 + 1 extra
S
0.15 µL × 13 wells = 1.95 µL of S

S Reaction Volume per well:
5.0 µL SYBR Green
0.08 µL RT Enzyme
1 µL FP
1 µL RP
7.08 µL

→ 7.08 µL + 0.15 µL of S RNA
= 7.23 µL
Add DEPC water to fill up to 10 µL
DEPC H_2O = 10 − 7.23 = 2.77 µL/well

2.77 µL × 13 wells = 36.01 µL DEPC H_2O

S Tube:
1.95 µL of S + 36.01 µL DEPC H_2O ⇒ RNA + DEPC = 37.96 µL

Read and Understood By

Signed — Date — Signed — 10/21/2018 Date

F

0.18 µL × 13 wells = 2.34 µL of F
7.08 µL + 0.18 µL of F RNA = 7.26 µL
DEPC H_2O = 10 − 7.26 = 2.74 µL/well
2.74 µL × 13 wells = 35.62 µL DEPC H_2O

F Tube:
2.34 µL of F + 35.62 µL DEPC H_2O ⇒ RNA + DEPC 37.96 µL

0.25

0.38 µL × 13 wells = 4.94 µL of 0.25
7.08 µL + 0.38 of 0.25 RNA = 7.46 µL
DEPC H_2O = 10 − 7.46 = 2.54 µL/well
2.54 µL × 13 wells = 33.02 µL DEPC H_2O

0.25 Tube:
4.94 µL of 0.25 + 33.02 µL DEPC H_2O ⇒ RNA + DEPC 37.96 µL

Place Volume of Master Mixes per well:
5.08 µL of RT Master Mix
2 µL of FP + RP Primer Mix
2.92 µL of RNA + DEPC mix
] Pipette into wells start w/ largest volume

Prepare:
1 tube for each pair of primers ⇒ 4 tubes
1 tube for RT Master Mix ⇒ 1 tube
1 tube for each RNA Sample ⇒ 3 tubes
] 8 tubes total

TUBE #	Content	
1	S RNA + DEPC	1.95 + 36.01
2	F RNA + DEPC	2.34 + 35.62
3	0.25 RNA + DEPC	4.94 + 33.02
4	RT Master Mix	2.96 RT Enzyme + 185 SYBR Green
5	PECAM1	10F + 10R
6	VEGF	10F + 10R
7	α-SMA	10F + 10R
8	GAPDH	10F + 10R

Continued on Page

Read and Understood By

Signed _____ Date _____ Signed _____ 10/21/2018 Date


Experiment Menu
Setup
Run
Analysis
Amplification Plot
Gene Expression
Multicomponent Plot
Melt Curve
Raw Data Plot
QC Summary
Multiple Plots View
Experiment: Smooth muscle cell DH
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
Amplification Plot
ΔRn
Cycle
A B C D E F
Options
Target: All
Threshold:
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 GAPDH Ct: 18.91 GAPDH Ct: 21.05 GAPDH Ct: 28.21 ALPHA... Ct: 26.33 ALPHA... Ct: 26.36 ALPHA... Ct: 27.32
B PECA... Ct: 27.98 PECA... Ct: 33 PECA... Ct: 32.63 VEGF Ct: 28.39 VEGF Ct: 28.74
C GAPDH Ct: 29.96 GAPDH Ct: 30.12 GAPDH Ct: 29.34 ALPHA... Ct: 27.9 ALPHA... Ct: 24.58 ALPHA... Ct: 26.67
D PECA... Ct: 33.39 PECA... Ct: 33.24 PECA... Ct: 33.9 VEGF Ct: 28.56 VEGF Ct: 29.9 VEGF Ct: 29.16
E GAPDH Ct: 30.5 GAPDH Ct: 30.61 GAPDH Ct: 30.42 ALPHA... Ct: 29.76 ALPHA... Ct: 29.59 ALPHA... Ct: 29.74
F PECA... Ct: 28.1 PECA... Ct: 29.87 PECA... Ct: 31.85 VEGF Ct: 25.2 VEGF Ct: 25.84 VEGF Ct: 24.64
Wells: 33 Unknown 3 Negative Control 12 Empty
Analysis Summary: Total Wells in Plate: 48 Wells Set Up: 36 Wells Omitted Manually: 0 Wells Flagged: 23 Wells Omitted by Analysis: 0 Samples Used: 3 Targets Used:
Smooth muscle cell DH.eds
start
Local Instrument : connected.

10/24/2018: PASMC media change
PAEC media change
Coated Bioflux w/gelatin

10/25/2018: PAEC raw cell count (10 µL = 5 µL trypan blue + 5 µL cell suspension)
Count 1: 5.86×10^5 cells/mL
Count 2: 5.92×10^5 cells/mL > AVG = 5.89×10^5
Actual cell concentration = $2 \times 5.89 \times 10^5 = 1.18 \times 10^6$ cells/mL
Want (for seeding): 2×10^6 cells/mL × 0.1 mL per channel
$C_1V_1 = C_2V_2$
2×10^6 cells/mL × 0.1 mL = 1.18×10^6 cells/mL × V_2

$$V_2 = \frac{2 \times 10^5}{1.18 \times 10^6} = \frac{2}{11.8} = 0.169 \text{ mL} = \underline{\underline{169 \mu L}} \text{ per channel}$$

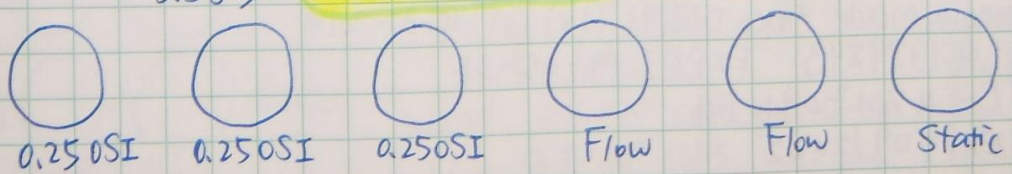
Seeded PAEC's in Bioflux @ 169 µL per channel across 6 channels
(There were enough cells for only 6 channels)
Time seeded for attachment: 16:42 on 10/25/2018

10/26/2018: PAEC media change
PAEC seeded on 10/25 in Bioflux plate did not attach. Aspirated all cells and media, ran PBS through all wells and channels.
Re-coated channels w/gelatin overnight.

10/27/2018: Passaged PAEC P14 → P15
Passaged two plates → 4 plates of PASMC P11 → P12
Conditioned two 10-cm plates of PASMC in 0.25 OSI × 3 6-cm plates
PASMC cell count:
Steady Flow × 2 6-cm plates
Static × 1 6-cm plates
Live 1: 3.44×10^6 cells/mL
Live 2: 1.94×10^6 cells/mL } AVG = 2.69×10^6 cells/mL
ACTUAL = $2 \times 2.69 \times 10^6$ cells/mL = $\underline{5.38 \times 10^6 \text{ cells/mL}}$

Want: 0.8×10^6 cells in 6-cm plate
$0.8 \times 10^6 = 5.38 \times 10^6$ cells/mL × V_2

$$V_2 = \frac{0.8 \times 10^6}{5.38 \times 10^6} = 0.1487 \text{ mL} \approx 150 \mu L$$



Six 6-cm plates

50% DMEM
50% Spent media
> Start 10/27 @ 17:45
End 10/29 @ 17:45

Continued on Page

Read and Understood By

Signed _____ Date _____ Signed _____ Date 10/24/2018

10/29/2018: PASMC passage P12→P13
PAEC media change
17:45 collected conditioned SMC in TRIzol
3 0.25 OSI samples + 2 steady Flow samples + 1 static sample

10/31/2018: PASMC media change
PAEC media change

11/2/2018:

PASMC media change
PAEC media change

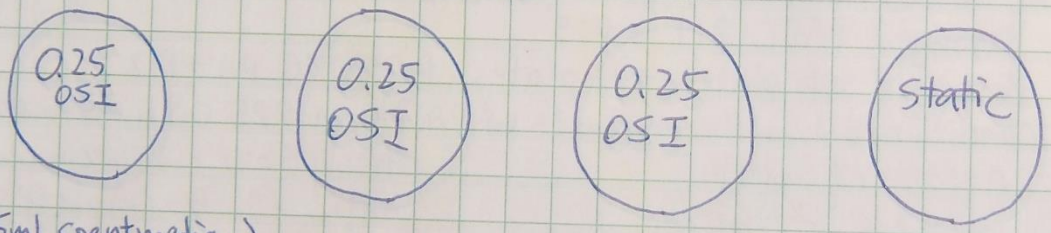
11/5/2018: PAEC media change
PASMC spent media conditioning

Cell count: 4.38×10^6 cells/mL
3.36×10^6 cells/mL
> 10 µL cells in media + 10 µL trypan blue

Average count = 3.87×10^6 cells/mL
Actual = 7.74×10^6 cells/mL
Want: 0.8×10^6 cells in 6-cm plate

$$0.8 \times 10^6 \text{ cells} = 7.74 \times 10^6 \text{ cells/mL} \times V_2$$

$$V_2 = \frac{0.8 \times 10^6 \text{ cells}}{7.74 \times 10^6 \text{ cells/mL}} = 0.103 \text{ mL} = 103 \text{ µL}$$



1.5mL spent media
1.5mL DMEM
} for all 4 plates

Start time: 4:20pm on 11/5 (Mon)
End time: 4:20pm on 11/7 (Wed)

Obtained one 6-cm PASMC from Mohammad. P3
New media made: gibco DMEM (1x) on 10-31-2018
Lot # 1989243 Exp: 2019-05-31
FBS Lot # 2017091OFG
Exp: 09/2022

Continued on Page

Read and Understood By

Signed
Date
Signed
10/29/2018
Date

11/7/2018: Transfer conditioned PASMC into TRIzol & -80°C. Used 0.8mL of TRIzol per 6-cm plate.
changed media PAEC & PASMC (P3)
Passaged PASMC (P13-P14)

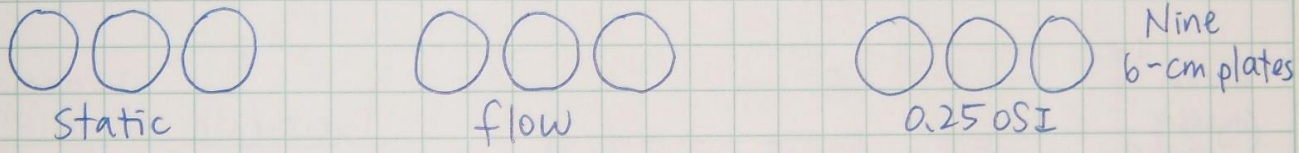
11/9/2018: changed media PAEC & PASMC (P14)
Passaged PASMC (P3 → P4)

11/12/2018: PASMC media change P4 & P14
PAEC media change

11/14/2018: PAEC passage (P15 → P16)
PASMC passage (P4 → P5)
PASMC (P14) spent media culture
Cell counts: (live)
count 1: 5.74×10^6 cells/mL
count 2: 4.44×10^6 cells/mL
AVG $> 5.09 \times 10^6$ cells/mL
Actual $2 \times 5.09 \times 10^6$ cells/mL $= 10.18 \times 10^6$ cells/mL
Want: 0.8×10^6 cells in 6-cm plate

$$V_2 = \frac{0.8 \times 10^6 \text{ cells}}{10.18 \times 10^6 \text{ cells/mL}} = 0.08\text{mL} \approx 80\mu\text{L}$$

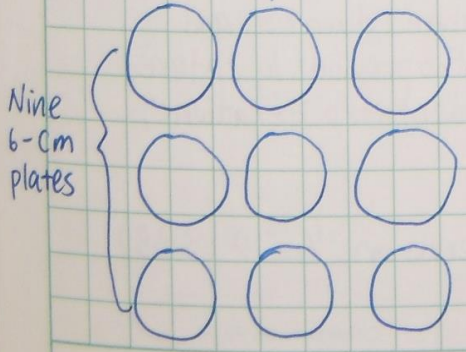
Used 100µL per 6-cm plate



Conditioning Start time: 11/14 @ 5pm
End time: 11/16 @ 5pm

11/16/2018: Placed conditioned media PASMC samples in TRIzol in -80.

11/17/2018: PAEC media change
PASMC passage P5 → P6 (one plate → 2 plates)
Spent media culture (P5) start time: 11/17 5pm
End: 11/19 5pm



Cell Counts:
4.43×10^6 cells/mL
3.79×10^6 cells/mL
AVG $4.11 \times 10^6 \Rightarrow 2 \times 4.11 \times 10^6$ cells/mL $= 8.22 \times 10^6$ cells/mL
Want: 0.8×10^6 cells per 6-cm plate

$$V_2 = \frac{0.8 \times 10^6 \text{ cells}}{8.22 \times 10^6 \text{ cells/mL}} = 0.097\text{mL} \approx 100\mu\text{L}$$

used 100uL to seed.

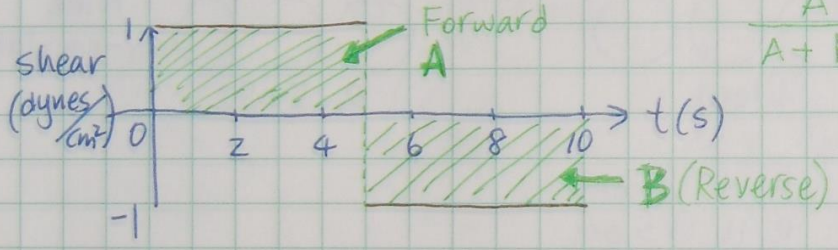
Continued on Page

Read and Understood By

Signed _____ Date _____ Signed _____ Date 11/7/2018

11/19/2018: PASMC (P6) media change
Collected PASMC that was in spent media culture (P5)
Placed PASMC in Trizol @ -80°C
Coated Bioflux plate w/gelatin
PAEC (P16) seeding in Bioflux channels:
Live count 1: 1.33×10^6 cells/mL
Live count 2: 1.06×10^6 cells/mL
$\rightarrow 1.195 \times 10^6$ cells/mL AVG
Actual = $2 \times 1.195 \times 10^6 = 2.39 \times 10^6$ cells/mL
$C_1V_1 = C_2V_2$
2×10^6 cells/mL $\times$ 0.1 mL = 2.39×10^6 cells/mL $\times V_2$
$V_2 = \frac{2 \times 10^5}{2.39 \times 10^6} = 0.0837$ mL ≈ 85 µL
Seeded 85 µL of EC per channel.
Seeding time: 6 pm, will remain in static for 24 Hrs for cell attachment.
Passaged the remaining 2 plates of PAEC P16 → P17

11/20/2018: 6:38 pm Started 0.5 OSI on the PAEC's
0.5 OSI Flow Profile



$\frac{A}{A+B} = 0.50$ OSI

Duration(s)	Direction & Shear (dynes/cm²)	
5	1 (forward)	] 10sec
5	1 (reverse)	

Each full iteration is 10 seconds
Need 17,280 iterations for a conditioning time of 48 hours.

48 hrs = 172,800 seconds
$\frac{172{,}800 \text{ seconds}}{10 \text{ seconds/iteration}} = 17{,}280$ iterations

Bioflux 200 → Edit AutoRun → Protocol Setup → Entered duration and shear stress values → Sequence Setup → Select the previously entered protocol, enable all 8 channels, enter 17,280 under "Step iterations", select DMEM+ for fluid type, save.

PAEC 0.5 OSI Run Start time: 11/20 @ 18:38 (ends on 11/22 @ 18:38)
Starting Volume per well/column

Columns	A	B	C
	2 mL	1 mL	1 mL

A → C forward
C → A+B Reverse

Continued on Page

Read and Understood By

Signed Date

Signed 11/19/2018 Date

11/21/2018: 8:30am moved 600μL of media from column B to A
moved 600μL of media from column B to O
8:40am Resumed flow
PAEC media change (P17)
PASMC Passage (P6 → P7)
22:30 paused flow, redistributed media from B to A & O
22:40 Resumed flow (0.5 OSI)

11/22/2018: 19:00 stopped flow, collected EC's in Trizol and placed in -80
Pipetted spent media into 50mL conical and placed in freezer

11/25/2018: PAEC, place all 4 6-cm plates in 2.0mL of TRIzol (static EC RNA)
PASMC: placed one 10-cm plate (without conditioned media) in TRIzol
The rest (3 plates) were used for 0.5 OSI conditioned media
Culture. Live count 1: 1.68×10^6 cells/mL
count 2: 1.60×10^6 cells/mL
AVG 1.64×10^6 cells/mL

ACTUAL = $2 \times 1.64 \times 10^6 = 3.28 \times 10^6$ cells/mL

Want 0.8×10^6 cells in each 6-cm plate

0.8×10^6 cells $= 3.28 \times 10^6 \times V_2$

$$V_2 = \frac{0.8 \times 10^6}{3.28 \times 10^6} = 0.244 \text{ mL} \approx 250 \mu L$$

Ten plates of 0.5 OSI PASMC were cultured
Start time: 11/25 @ 16:00
End time: 11/27 @ 16:00

ALL RNA GROUPS

CELL TYPE	Porcine Aortic (PASMC) Smooth muscle cells	Porcine aortic (PAEC) Endothelial Cells
GROUP	static, no spent media static PAEC spent media steady flow PAEC spent media 0.25 OSI 0.50 OSI	static, no flow steady flow @ 1 dyne/cm² 0.25 OSI, peaks @ ±1 dyne/cm² 0.50 OSI, peaks @ ±1 dyne/cm²

*Note: # of samples of PASMC ~10 per group (10 repeats)
# of samples of PAEC = 1 per group

Continued on Page 25

Read and Understood By

Signed _____ Date _____ Signed _____ Date 11/21/2018

11/27/2018: Collected conditioned PASMC (in 0.5 OSI spent media) into TRIzol.
Placed all 10 samples in -80 °C.

Bioreactor Tissue Samples

← from Vivitro, 7 days of flow exposure

Sample 1: Bioreactor flow Sample (535 pulsatile flow)
Sample 2: static Sample
Cell type of samples: Human Bone Marrow Stem Cells

11/27/2018 Extracted RNA from Sample 1 & Sample 2 (protocol on pg. 78)
Quantified RNA

	Sample 1 Bioreactor Flow (B)	Sample 2 static (S)
RNA Quantitation	208.2 ng/μL	44.4 ng/μL

11/28/2018 RNA to C_T qPCR, need 20ng ~ 100ng of RNA in each well

B: $40\,ng = 208.2\ ng/\mu L \times V_1$

$V_1 = \frac{40}{208.2} = 0.192\ \mu L$ of RNA per well

S: $40\,ng = 44.4\ ng/\mu L \times V_2$

$V_2 = \frac{40}{44.4} = 0.90\ \mu L$ of RNA per well

Targets (Primers) 20 wells in total: GAPDH, YARS, FZD2, MLC1F, KLF2A

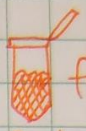
	1	2	3	4	5	6
A	S GAPDH	S YARS	S FZD2	S MLC1F	S KLF2A	
B	S	S	S	S	S	
C						
D	B	B	B	B	B	
E	B	B	B	B	B	
F						

2 replicates

1. RNA to C_T RT Master Mix (for 10 μL reactions)
Volume needed per well (from pg. 13 in protocol online):
5.0 μL SYBR Green mix
0.08 μL RT Enzyme

(20+1)
5.0 μL × 21 samples = 105 μL SYBR Green Mix
0.08 μL × 21 samples = 1.68 μL RT Enzyme

Continued on Page

2. Primers - Use 1 µL of the 10 µM tube for both FWD & REV
2 replicates × 2 samples = 4 primer pairs
1 µL FP × 5 wells = 5 µL FP (4 + 1)
1 µL RP × 5 wells = 5 µL RP
} 10 µL in  for each primer type (one tube for both FWD & REV)

3. RNA Samples (10 + 1)
B: 0.19 × 11 wells = 2.11 µL of B
S: 0.90 × 11 wells = 9.9 µL of S

Components Per Well
5.0 µL SYBR Green
0.08 µL RT Enzyme
1 µL FP
1 µL RP
7.08 µL

→ 7.08 µL + 0.19 of B = 7.27 µL
Add DEPC water to fill up to 10 µL
10 - 7.27 = 2.73 µL of DEPC water per well.
(10 + 1)
2.73 × 11 wells = 30.03 µL of DEPC water

B Tube: 2.11 µL of B + 30.03 µL of DEPC water

7.08 µL + 0.90 of S = 7.98 µL
10 - 7.98 = 2.02 µL of DEPC water per well
(10 + 1)
2.02 × 11 wells = 22.22 µL of DEPC water

S Tube: 9.9 µL of S + 22.22 µL DEPC water

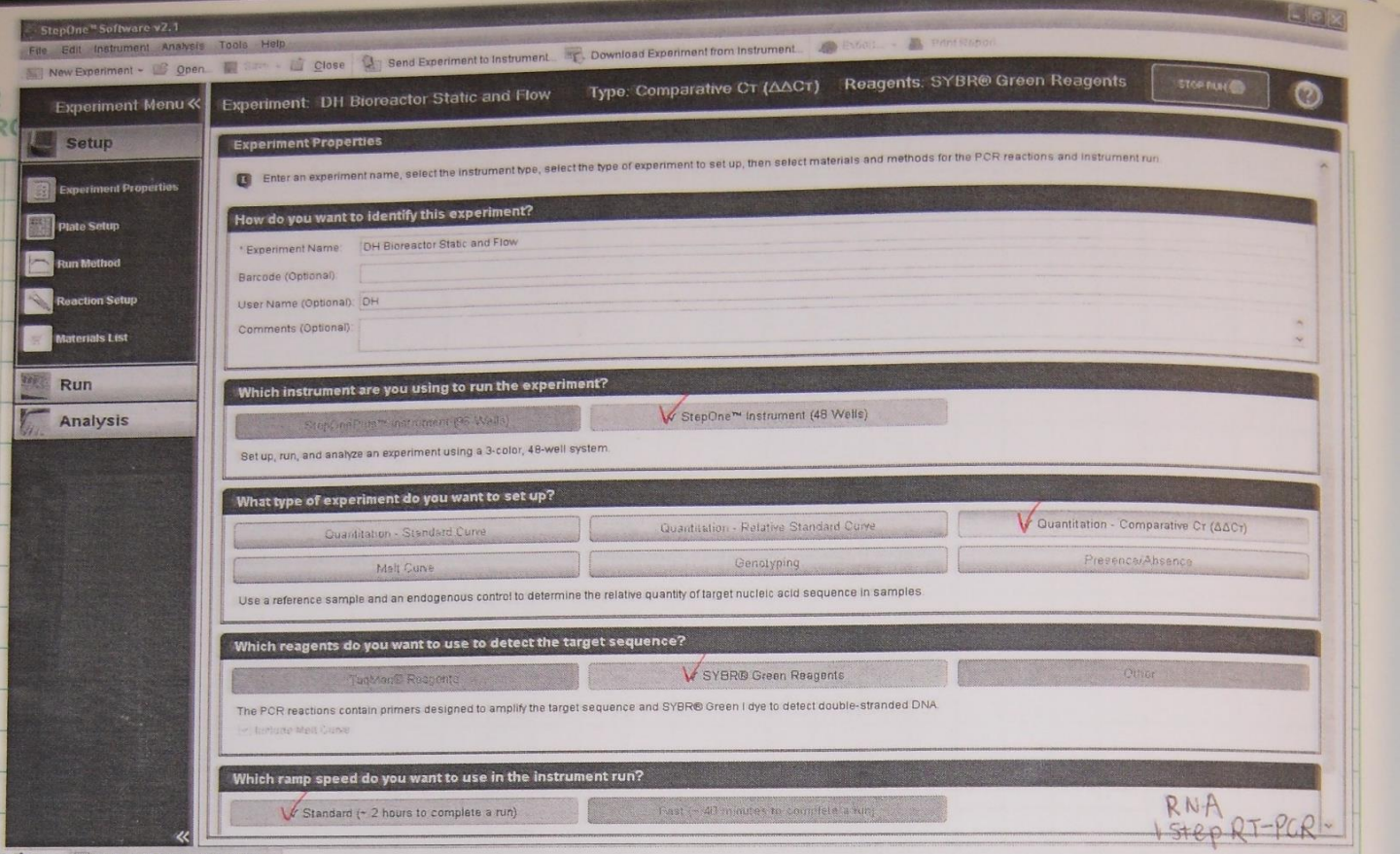
Prepare:
1 Tube for each pair of primers ⇒ 5 tubes
1 Tube for RT Master Mix ⇒ 1 tube
1 Tube for each RNA Sample ⇒ 2 Tubes (B Tube & S Tube)
} 8 tubes

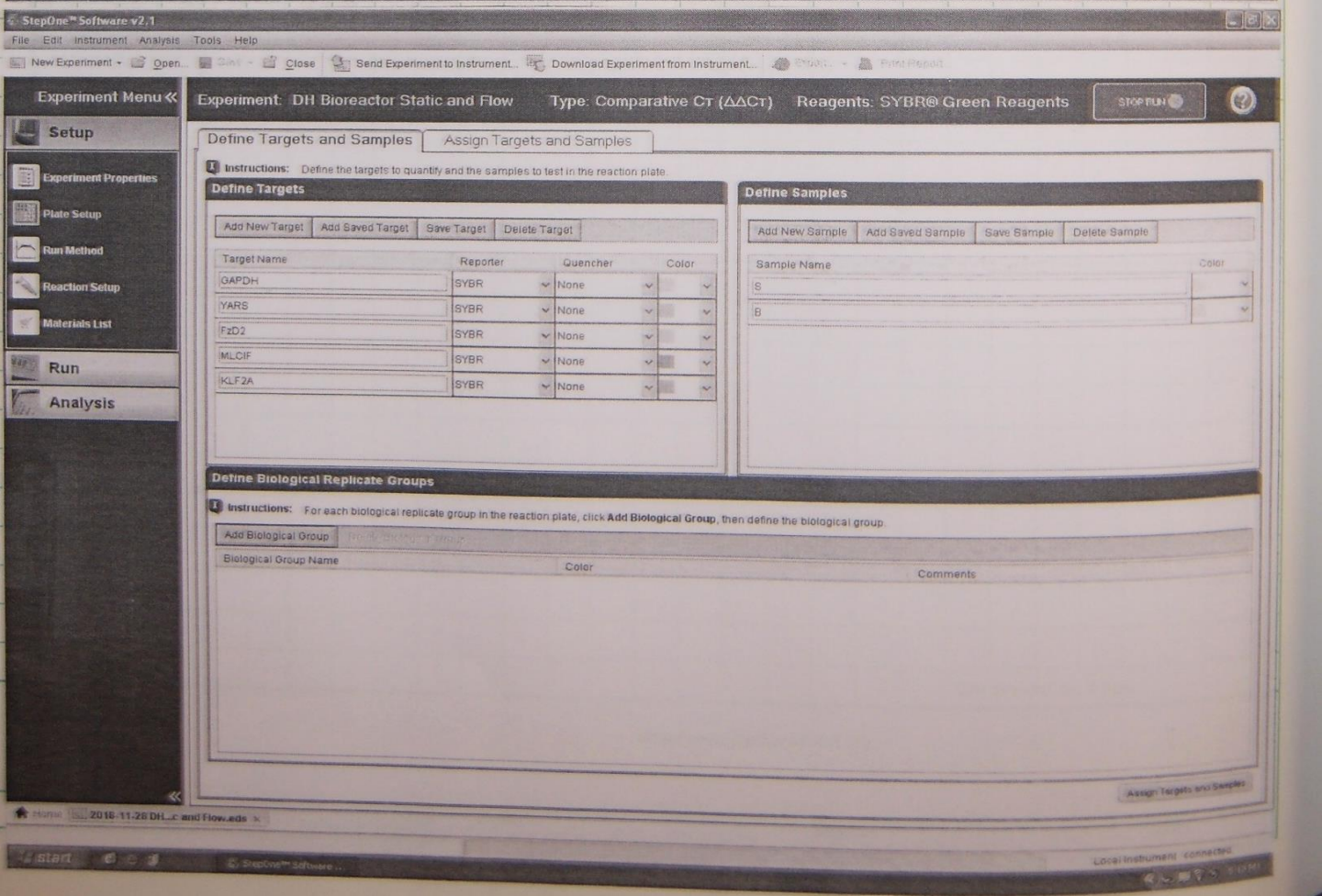
Tube#			Tube#		
1	B Tube	2.11 µL of B + 30.03 DEPC	5	YARS	5 µL FP + 5 µL RP
2	S Tube	9.9 µL of S + 22.22 µL DEPC	6	FzD2	
3	RT MM	1.68 µL RT Enzyme + 105 µL SYBR green	7	MLC1F	
4	GAPDH	5 µL FP + 5 µL RP	8	KLF2A	

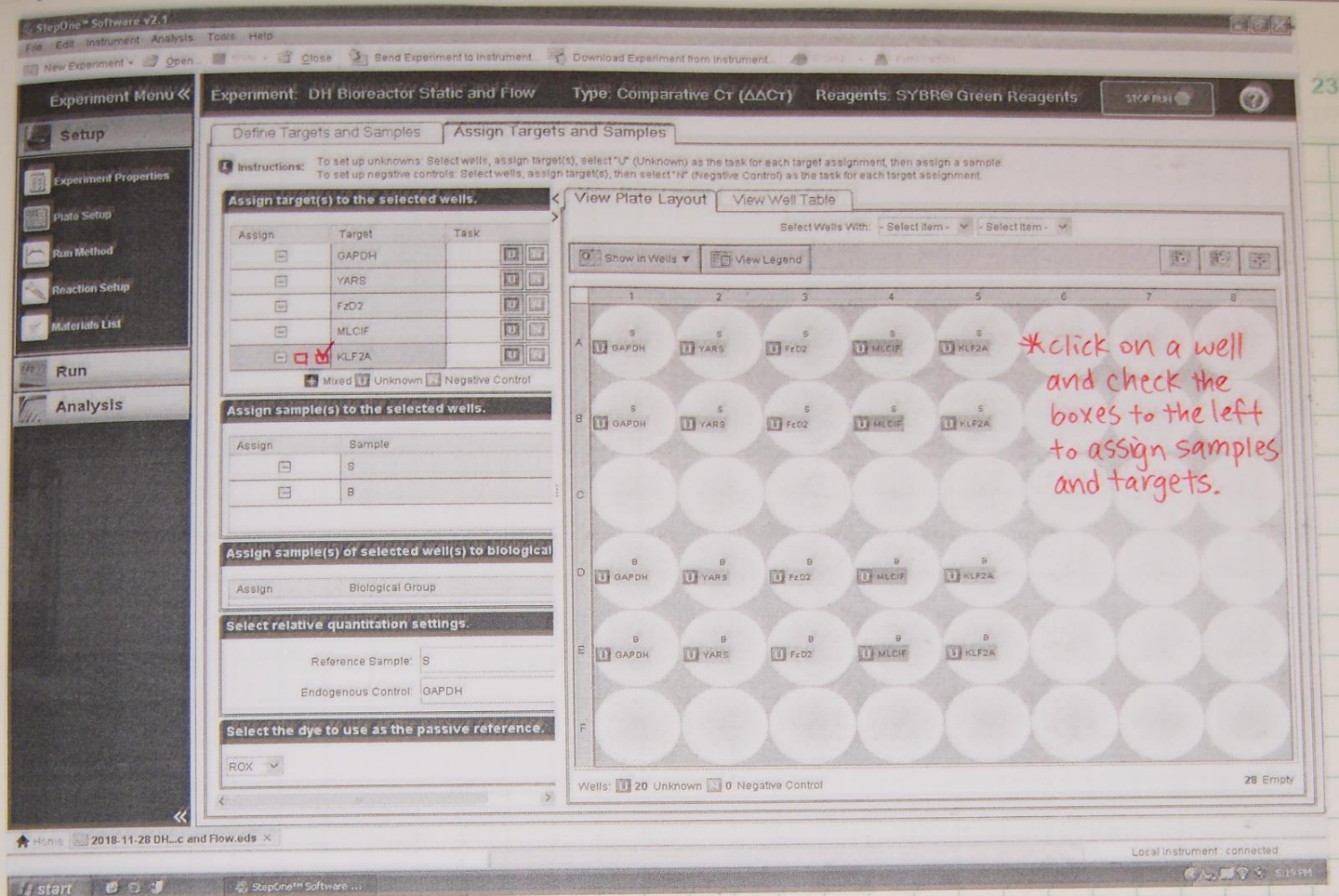
Continued on Page

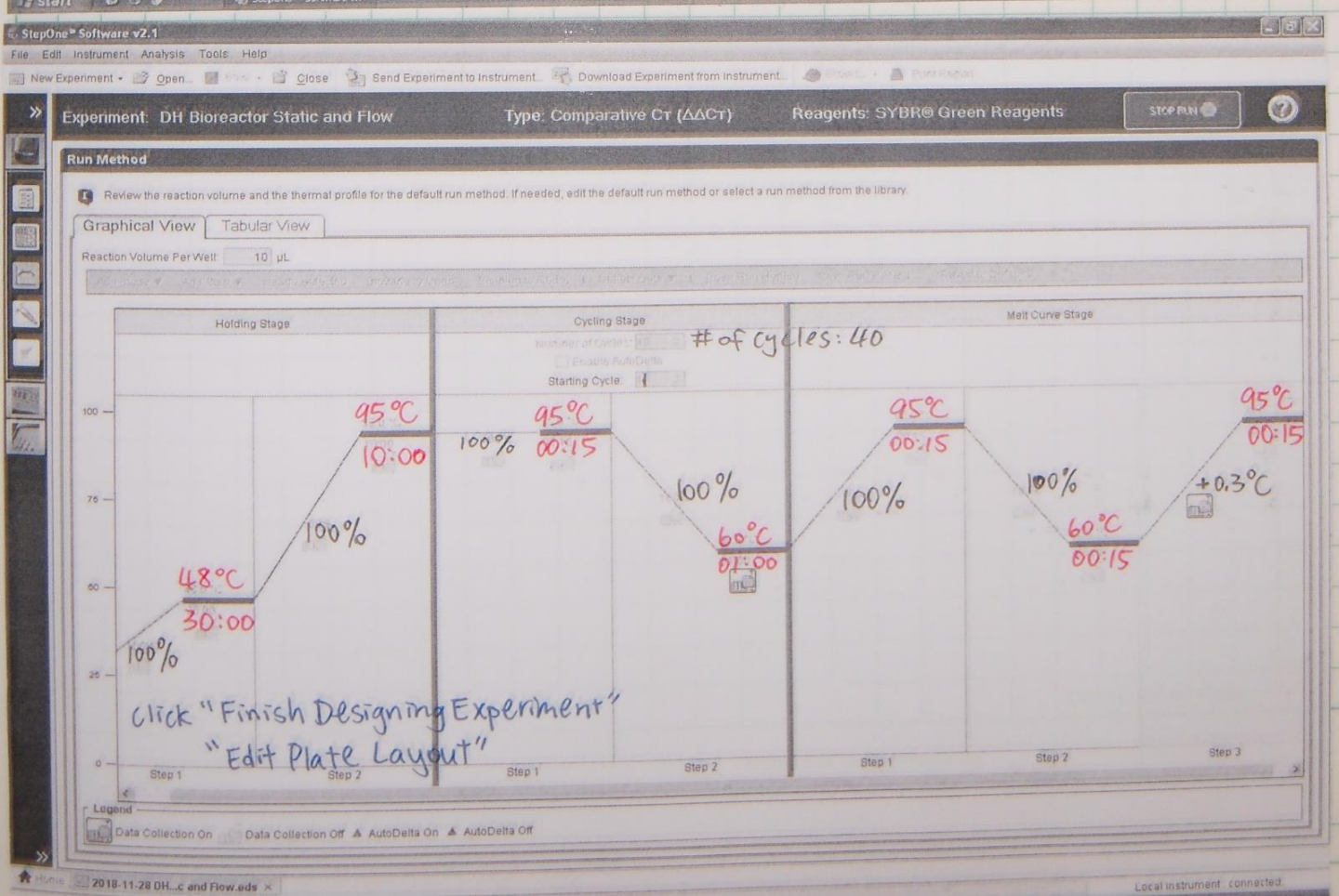
Read and Understood By

Signed _____ Date _____ Signed _____ Date 11/28/2018


StepOne™ Software v2.1
File Edit Instrument Analysis Tools Help
New Experiment Open... Close Send Experiment to Instrument... Download Experiment from Instrument...
Experiment Menu
Setup
Experiment Properties
Plate Setup
Run Method
Reaction Setup
Materials List
Run
Analysis
Experiment: DH Bioreactor Static and Flow
Type: Comparative CT (ΔΔCT)
Reagents: SYBR® Green Reagents
STOP RUN
Experiment Properties
Enter an experiment name, select the instrument type, select the type of experiment to set up, then select materials and methods for the PCR reactions and instrument run.
How do you want to identify this experiment?
* Experiment Name: DH Bioreactor Static and Flow
Barcode (Optional):
User Name (Optional): DH
Comments (Optional):
Which instrument are you using to run the experiment?
StepOnePlus™ Instrument (96 Wells)
StepOne™ Instrument (48 Wells)
Set up, run, and analyze an experiment using a 3-color, 48-well system.
What type of experiment do you want to set up?
Quantitation - Standard Curve
Quantitation - Relative Standard Curve
Quantitation - Comparative CT (ΔΔCT)
Melt Curve
Genotyping
Presence/Absence
Use a reference sample and an endogenous control to determine the relative quantity of target nucleic acid sequence in samples.
Which reagents do you want to use to detect the target sequence?
TaqMan® Reagents
SYBR® Green Reagents
Other
The PCR reactions contain primers designed to amplify the target sequence and SYBR® Green I dye to detect double-stranded DNA.
Which ramp speed do you want to use in the instrument run?
Standard (~ 2 hours to complete a run)
Fast (~ 40 minutes to complete a run)
RNA
1 Step RT-PCR
Home
2018-11-28 DH...c and Flow.eds
Local Instrument connected
start
StepOne™ Software ...


StepOne™ Software v2.1
File Edit Instrument Analysis Tools Help
New Experiment Open... Close Send Experiment to Instrument... Download Experiment from Instrument...
Experiment Menu
Setup
Experiment Properties
Plate Setup
Run Method
Reaction Setup
Materials List
Run
Analysis
Experiment: DH Bioreactor Static and Flow
Type: Comparative CT (ΔΔCT)
Reagents: SYBR® Green Reagents
STOP RUN
Define Targets and Samples
Assign Targets and Samples
Instructions: Define the targets to quantify and the samples to test in the reaction plate.
Define Targets
Add New Target
Add Saved Target
Save Target
Delete Target
Target Name
Reporter
Quencher
Color
GAPDH
SYBR
None
YARS
SYBR
None
FzD2
SYBR
None
MLCIF
SYBR
None
KLF2A
SYBR
None
Define Samples
Add New Sample
Add Saved Sample
Save Sample
Delete Sample
Sample Name
Color
S
B
Define Biological Replicate Groups
Instructions: For each biological replicate group in the reaction plate, click Add Biological Group, then define the biological group.
Add Biological Group
Biological Group Name
Color
Comments
Assign Targets and Samples
Home
2018-11-28 DH...c and Flow.eds
Local Instrument connected
start
StepOne™ Software ...


StepOne™ Software v2.1
File Edit Instrument Analysis Tools Help
New Experiment Open... Close Send Experiment to Instrument... Download Experiment from Instrument...
Experiment Menu
Setup
Experiment Properties
Plate Setup
Run Method
Reaction Setup
Materials List
Run
Analysis
Experiment: DH Bioreactor Static and Flow
Type: Comparative CT (ΔΔCT)
Reagents: SYBR® Green Reagents
STOP RUN
Define Targets and Samples
Assign Targets and Samples
Instructions: To set up unknowns: Select wells, assign target(s), select "U" (Unknown) as the task for each target assignment, then assign a sample.
To set up negative controls: Select wells, assign target(s), then select "N" (Negative Control) as the task for each target assignment.
Assign target(s) to the selected wells.
Assign
Target
Task
GAPDH
YARS
FzD2
MLCIF
KLF2A
Mixed Unknown Negative Control
Assign sample(s) to the selected wells.
Sample
S
B
Assign sample(s) of selected well(s) to biological
Biological Group
Select relative quantitation settings.
Reference Sample: S
Endogenous Control: GAPDH
Select the dye to use as the passive reference.
ROX
View Plate Layout
View Well Table
Select Wells With:
Show in Wells
View Legend
*click on a well and check the boxes to the left to assign samples and targets.
Wells: 20 Unknown 0 Negative Control
28 Empty
2018-11-28 DH...c and Flow.eds
Local instrument connected
start
StepOne™ Software ...


StepOne™ Software v2.1
File Edit Instrument Analysis Tools Help
New Experiment Open... Close Send Experiment to Instrument... Download Experiment from Instrument...
Experiment: DH Bioreactor Static and Flow
Type: Comparative CT (ΔΔCT)
Reagents: SYBR® Green Reagents
STOP RUN
Run Method
Review the reaction volume and the thermal profile for the default run method. If needed, edit the default run method or select a run method from the library.
Graphical View
Tabular View
Reaction Volume Per Well: 10 µL
Holding Stage
Cycling Stage
Melt Curve Stage
# of cycles: 40
Starting Cycle:
95°C
10:00
48°C
30:00
100%
95°C
00:15
60°C
01:00
95°C
00:15
60°C
00:15
95°C
00:15
+0.3°C
Step 1
Step 2
Step 1
Step 2
Step 1
Step 2
Step 3
Legend
Data Collection On
Data Collection Off
AutoDelta On
AutoDelta Off
Click "Finish Designing Experiment"
"Edit Plate Layout"
2018-11-28 DH...c and Flow.eds
Local instrument connected

StepOne™ Software v2.1

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

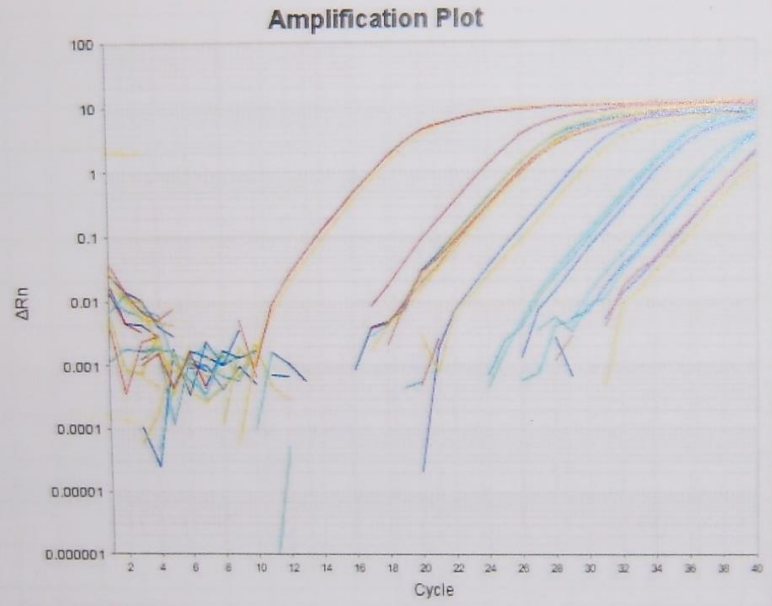
Experiment: DH Bioreactor Static and Flow Type: Comparative CT (ΔΔCT) Reagents: SYBR® Green Reagents | Analyze | Analysis Settings

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	S GAPDH CT: 17.55	1 S YARS CT: 23.23	1 S FzD2 CT: 25.94	2 S MLCIF CT: 36.34	1 S KLF2A CT: 28.74			
B	S GAPDH CT: 17.9	1 S YARS CT: 25.09	1 S FzD2 CT: 29.55	2 S MLCIF CT: 37.07	1 S KLF2A CT: 26.93			
C								
D	1 B GAPDH CT: 33.75	2 B YARS CT: 36.17	B FzD2 CT: 25.42	1 B MLCIF CT: 35.19	1 B KLF2A CT: 34.28			
E	1 B GAPDH CT: 34.56	1 B YARS CT: 36.93	B FzD2 CT: 25.37	2 B MLCIF CT: 36.47	1 B KLF2A CT: 29.73			
F								

Wells: 20 Unknown 0 Negative Control 28 Empty

Analysis Summary: Total Wells in Plate: 48 | Wells Set Up: 20 | Wells Omitted Manually: 0 | Wells Flagged: 16 | Wells Omitted by Analysis: 0 | Samples Used: 2 | Targets Used:

Home | 2018-11-28 DH...c and Flow.eds

Local Instrument : connected.

start | StepOne™ Software ... | 5:15 PM

11/30/2018: 0.5 OSI spent media conditioned PASMC RNA extraction

Bioflux Samples	RNA Concentration (ng/μL)
Static	681.03
Flow (steady)	568.23
0.25 OSI	268.87
0.5 OSI	862.40

12/2/2018: qPCR of housekeeping genes (conditioned spent media on PASMCs)

Want: 40 ng of RNA per well for qPCR

STATIC $40\,ng = 681.03\,ng/\mu L \times V_S$

$V_S = \frac{40}{681.03} = 0.059\,\mu L$

FLOW $40\,ng = 568.23\,ng/\mu L \times V_F$

$V_F = \frac{40}{568.23} = 0.070\,\mu L$

0.25 OSI $40\,ng = 268.87\,ng/\mu L \times V_{0.25}$

$V_{0.25} = \frac{40}{268.87} = 0.149\,\mu L$

0.5 OSI $40\,ng = 862.40\,ng/\mu L \times V_{0.5}$

$V_{0.5} = \frac{40}{862.40} = 0.046\,\mu L$

TARGETS (Primers): RPLP1, RPLP13A, PPIA, PGK1
SAMPLES: Static, Flow, 0.25 OSI, 0.5 OSI
# of REPLICATES: 3

} 4 targets × 4 samples × 3 reps = 48 wells

LAYOUT:

	1	2	3	4	5	6	7	8
A	Static RPLP1	static PPIA	Flow RPLP1	Flow PPIA	0.25 RPLP1	0.25 PPIA	0.5 RPLP1	0.5 PPIA
B	static RPLP1	static PPIA	Flow RPLP1	Flow PPIA	0.25 RPLP1	0.25 PPIA	0.5 RPLP1	0.5 PPIA
C	static RPLP1	static PPIA	Flow RPLP1	Flow PPIA	0.25 RPLP1	0.25 PPIA	0.5 RPLP1	0.5 PPIA
D	static RPLP13A	static PGK1	Flow RPLP13A	Flow PGK1	0.25 RPLP13A	0.25 PGK1	0.5 RPLP13A	0.5 PGK1
E	static RPLP13A	static PGK1	Flow RPLP13A	Flow PGK1	0.25 RPLP13A	0.25 PGK1	0.5 RPLP13A	0.5 PGK1
F	static RPLP13A	static PGK1	Flow RPLP13A	Flow PGK1	0.25 RPLP13A	0.25 PGK1	0.5 RPLP13A	0.5 PGK1

*ALL HOUSEKEEPING GENES

Continued on Page

Read and Understood By

Signed Date Signed 11/30/2018 Date

1. RNA to C_T RT Master Mix (for 10 µL reactions)
Volume needed per well (from pg.13 in protocol online):
5.0 µL SYBR Green Mix
0.08 µL RT Enzyme
5.0 µL × 50 samples = 250 µL SYBR Green Mix
(48+2)
0.08 µL × 50 samples = 4 µL RT Enzyme

2. Primers - Use 1 µL of the 10uM tube for both FWD & REV for dilution
3 replicates × 4 samples = 12 primer pairs
1 µL FP × 13 wells = 13 µL FP
(12+1)
1 µL RP × 13 wells = 13 µL RP
} 26 µL in  for each primer type
1 µL FWD of 10µM
1 µL REV of 10µM
+ 24 µL DEPC
* Pipette 2 µL of each primer Tube into each corresponding well
(12+1)

3. RNA Samples

Components Per Well
5.0 µL SYBR Green
0.08 µL RT Enzyme
1 µL FP
1 µL RP
7.08 µL

→ **S-tube:** 0.059 × 13 = 0.767 µL of Static
7.08 µL + 0.059 µL = 7.139 µL
10 - 7.139 = 2.861 µL of DEPC H_2O
2.861 × 13 wells = 37.193 µL of DEPC H_2O
(12+1)
S-Tube:
0.767 µL of [S] + 37.19 µL of DEPC H_2O

F-Tube: 0.070 × 13 = 0.91 µL of Flow
7.08 µL + 0.070 µL = 7.15 µL
10 - 7.15 = 2.85 µL of DEPC H_2O
2.85 × 13 wells = 37.05 µL DEPC H_2O
F-Tube:
0.91 µL of [F] + 37.05 µL DEPC H_2O

0.5-Tube: 0.046 × 13 = 0.598 µL of 0.5 OSI
7.08 µL + 0.046 µL = 7.126 µL
10 - 7.126 = 2.874 µL of DEPC H_2O
2.874 × 13 wells = 37.362 µL DEPC H_2O
0.5 Tube:
0.598 µL of [0.5 OSI] + 37.362 µL DEPC H_2O

0.25-Tube:
0.149 × 13 = 1.937 µL of 0.25 OSI
7.08 µL + 0.149 µL = 7.229 µL
10 - 7.229 = 2.771 µL of DEPC H_2O
2.771 × 13 wells = 36.02 DEPC H_2O
0.25-Tube:
1.937 µL of [0.25 OSI] + 36.02 DEPC H_2O

Continued on Page

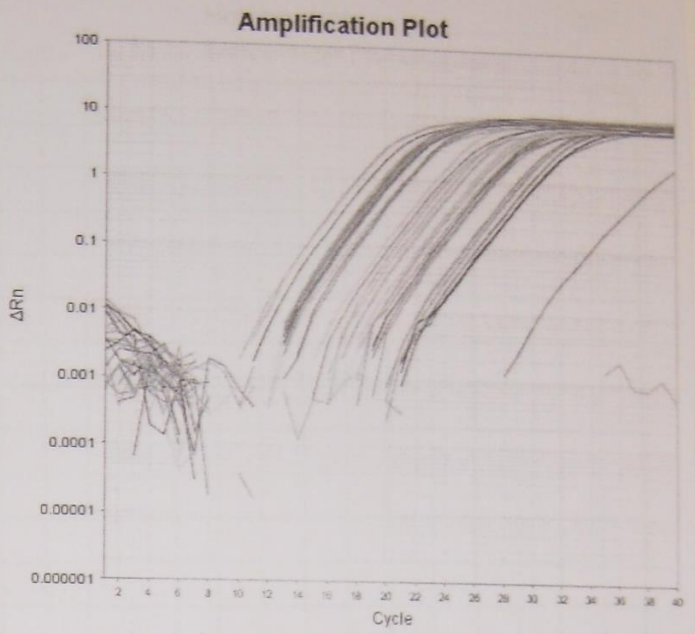
Read and Understood By

Signed — Date — Signed — 12/2/2018 Date

PROJECT Bioflux Housekeeping Genes Panel

Tube#:

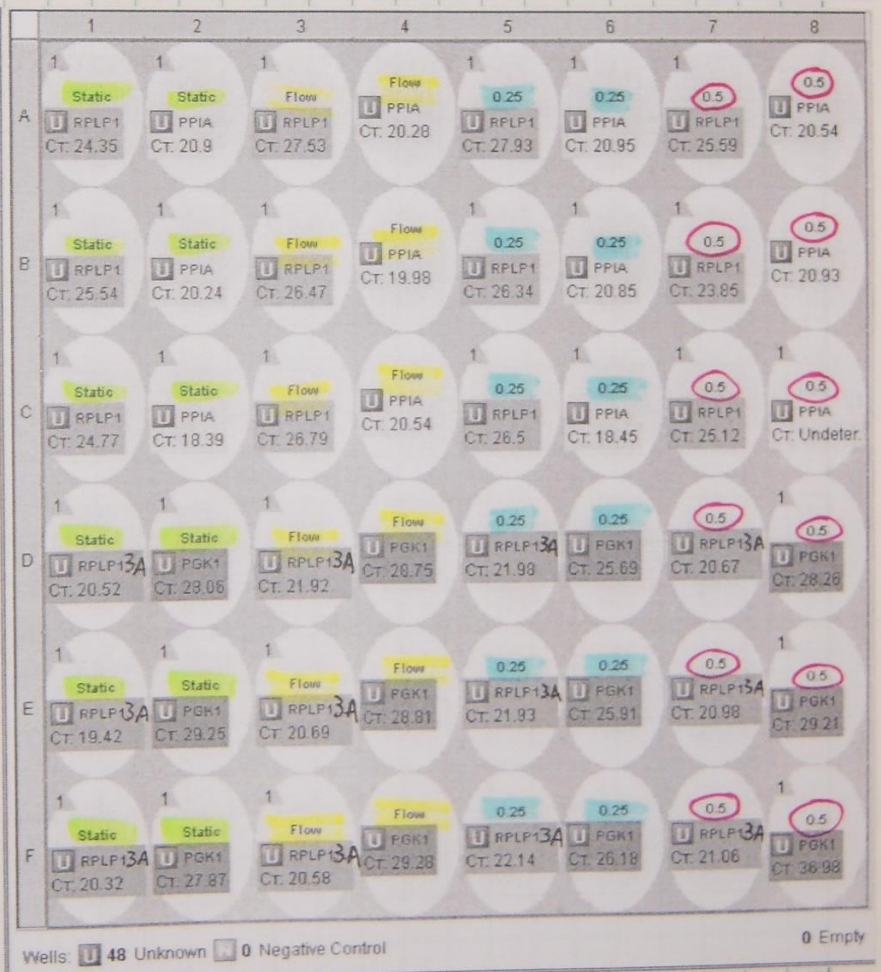
1. Static: 0.767 µL of S + 37.19 µL DEPC H_2O
2. Flow: 0.91 µL of F + 37.05 µL DEPC H_2O
3. 0.250SI: 1.937 µL of 0.250SI + 36.02 µL DEPC H_2O
4. 0.5 OSI: 0.598 µL of 0.5 OSI + 37.362 µL of DEPC H_2O
5. RT Master Mix: 4 µL RT Enzyme + 250 µL SYBR Green
6. RPLP1: 1 µL FP + 1 µL RP + 24 µL DEPC
7. PPIA:
8. RPLP13A:
9. PGK1:



C_T AVERAGES

Static	RPLP1	24.89
	PPIA	19.84
	RPLP13A	20.09
	PGK1	28.39
Flow	RPLP1	26.93
	PPIA	20.27
	RPLP13A	21.06
	PGK1	28.95
0.25 OSI	RPLP1	26.92
	PPIA	20.08
	RPLP13A	22.02
	PGK1	25.93
0.5 OSI	RPLP1	24.85
	PPIA	20.74
	RPLP13A	20.90
	PGK1	31.48 or 28.74

if did not consider outlier



Greatest Differences b/w Averages and Individual Wells

	RPLP1	PPIA	RPLP13A	PGK1
AVG.	2.08	0.9*	1.93	3.02
IND.	4.08	2.56*	2.72	3.59 or 11.29

* least observed difference

↑ includes outlier

Continued on Page

Read and Understood By

Signed

Date 12/2/2018

12/3/2018: RNA to C_T
TARGETS: PPIA, αSMA, VEGF, PECAM1 (HK: PPIA)
SAMPLES: Static, Flow, 0.25, 0.5 (4)
#REPLICATES: 3
LAYOUT:

	1	2	3	4	5	6	7	8
A	Static PPIA	Static VEGF	Flow PPIA	Flow VEGF	0.25 PPIA	0.25 VEGF	0.5 PPIA	0.5 VEGF
B	Static PPIA	Static VEGF	Flow PPIA	Flow VEGF	0.25 PPIA	0.25 VEGF	0.5 PPIA	0.5 VEGF
C	Static PPIA	Static VEGF	Flow PPIA	Flow VEGF	0.25 PPIA	0.25 VEGF	0.5 PPIA	0.5 VEGF
D	Static αSMA	Static PECAM1	Flow αSMA	Flow PECAM1	0.25 αSMA	0.25 PECAM1	0.5 αSMA	0.5 PECAM1
E	Static αSMA	Static PECAM1	Flow αSMA	Flow PECAM1	0.25 αSMA	0.25 PECAM1	0.5 αSMA	0.5 PECAM1
F	Static αSMA	Static PECAM1	Flow αSMA	Flow PECAM1	0.25 αSMA	0.25 PECAM1	0.5 αSMA	0.5 PECAM1

Tube #:

1. Static: 0.767 µL of [5] + 37.19 µL DEPC H_2O
2. Flow: 0.91 µL of [F] + 37.05 µL DEPC H_2O
3. 0.25 OSI: 1.937 µL of [0.25] + 36.02 µL DEPC H_2O
4. 0.5 OSI: 0.598 µL of [0.5] + 37.362 µL DEPC H_2O
5. RT Master Mix: 4 µL RT Enzyme + 250 µL SYBR Green
6. PPIA: 1 µL FP + 1 µL RP + 24 µL DEPC H_2O
7. α-SMA:
8. VEGF:
9. PECAM1:

Pipette Volume Per Well (µL)
2.92
5.08
2

Continued on Page

Read and Understood By

Signed | Date | Signed | Date 12/3/2018

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

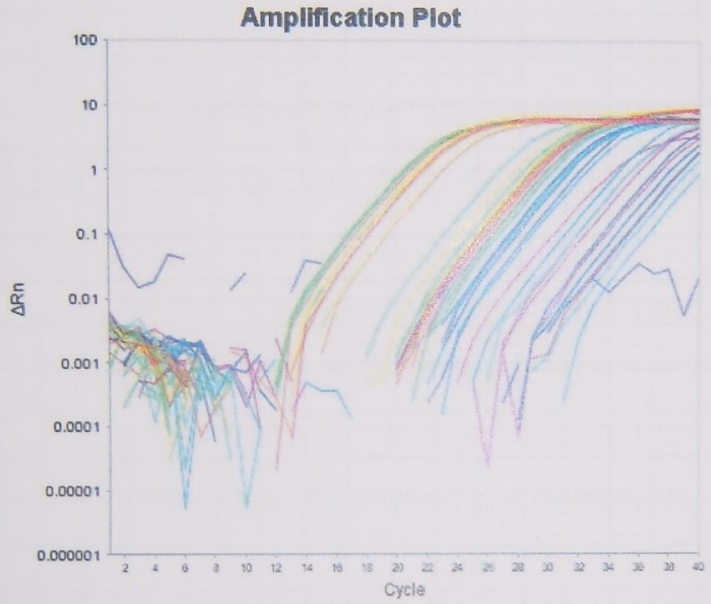
Experime... DH_Bioflux_Static_Flow_Pulsatile Ty... Comparative CT (ΔΔCT, Reagen... SYBR® Green Reagents | Analyze | Analysis Settings

Amplification Plot

Plot Settings

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

Save current settings as the default



Options

Target: All | Threshold: | Auto | Auto Baseline

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

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	1 Static PPIA CT: 21.77 (21.77)	1 Static VEGF CT: 29.7	Flow PPIA CT: 20.94 (20.94)	Flow VEGF CT: 29.21	0.25 PPIA CT: 21.87 (21.87)	0.25 VEGF CT: 29.34	0.5 PPIA CT: 23.12 (23.12)	0.5 VEGF CT: 29.55
B	1 Static PPIA CT: 21.49 (21.49)	1 Static VEGF CT: 27.99	Flow PPIA CT: 21.48 (21.48)	Flow VEGF CT: 29.5	0.25 PPIA CT: 21.92 (21.92)	0.25 VEGF CT: 29.01	0.5 PPIA CT: 23.04 (23.04)	0.5 VEGF CT: 29.07
C	1 Static PPIA CT: 20.68 (20.68)	1 Static VEGF CT: 30.45	Flow PPIA CT: 20.54 (20.54)	Flow VEGF CT: 30.11	0.25 PPIA CT: 20.66 (20.66)	0.25 VEGF CT: 28.57	0.5 PPIA CT: 21.44 (21.44)	0.5 VEGF CT: 26.92
D	1 Static alphaS... CT: 29.13	1 Static PECAM1 CT: 32.45	Flow alphaS... CT: 28.72	Flow PECAM1 CT: 36.24	0.25 alphaS... CT: 30.95	0.25 PECAM1 CT: 37.09	0.5 alphaS... CT: 32.21	0.5 PECAM1 CT: 31.66
E	1 Static alphaS... CT: 30.65	1 Static PECAM1 CT: 34.96	Flow alphaS... CT: 29.09	Flow PECAM1 CT: 35.66	0.25 alphaS... CT: 31.12	0.25 PECAM1 CT: 33.98	0.5 alphaS... CT: 31.85	2 0.5 PECAM1 CT: Undeter.
F	1 Static alphaS... CT: 29.56	1 Static PECAM1 CT: 34.32	Flow alphaS... CT: 28.85	2 Flow PECAM1 CT: 32.91	0.25 alphaS... CT: 30.7	0.25 PECAM1 CT: 32.44	0.5 alphaS... CT: 33.94	0.5 PECAM1 CT: 35.72

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: 33 | Wells Omitted by Analysis: 0 | Samples Used: 4 | Targets Used:

Home | DH_Bioflux_St...2018-12-03.eds

12/03/2018

1/8/2019: RNA Extraction - PAEC & PASMC - RNA Concentrations (ng/μL)
↑ flow conditioned in Bioflux
↑ PAEC spent media conditioned on PASMCs

	PAEC		PASMC (ng/μL)
Static	141.9	S	1001.8
Flow	56.1	F	712.96
0.25 OSI	21.65	0.25	121.43
0.50 OSI	650.1	0.5	319.40

1/10/2019: qPCR on PASMC (repeating data collection on pg. 28 for verification)
TARGETS: PPIA, αSMA, VEGF, PECAM1 (HK: PPIA)
SAMPLES: Static, Flow, 0.25 OSI, 0.5 OSI (4)
# of Replicates: 3
LAYOUT:

	1	2	3	4	5	6	7	8
A	Static PPIA	Static VEGF	Flow PPIA	Flow VEGF	0.25 PPIA	0.25 VEGF	0.5 PPIA	0.5 VEGF
B	Static PPIA	Static VEGF	Flow PPIA	Flow VEGF	0.25 PPIA	0.25 VEGF	0.5 PPIA	0.5 VEGF
C	Static PPIA	Static VEGF	Flow PPIA	Flow VEGF	0.25 PPIA	0.25 VEGF	0.5 PPIA	0.5 VEGF
D	Static αSMA	Static PECAM1	Flow αSMA	Flow PECAM1	0.25 αSMA	0.25 PECAM1	0.5 αSMA	0.5 PECAM1
E	Static αSMA	Static PECAM1	Flow αSMA	Flow PECAM1	0.25 αSMA	0.25 PECAM1	0.5 αSMA	0.5 PECAM1
F	Static αSMA	Static PECAM1	Flow αSMA	Flow PECAM1	0.25 αSMA	0.25 PECAM1	0.5 αSMA	0.5 PECAM1

4 targets × 4 samples × 3 replicates = 48 wells.

Continued on Page

1. RNA to Ct Master Mix (for 10 µL reactions)
Volume needed per well (from pg. 13 in protocol online):
5.0 µL SYBR Green Mix
0.08 µL RT Enzyme
5.0 µL × 52 samples = 260 µL of SYBR Green mix
(48+4)
0.08 µL × 52 samples = 4.16 µL of RT Enzyme

2. Primers - Use 1 µL of the 10 µM tube for both FWD & REV in total.
3 replicates × 4 samples = 12 primer pairs
Make primer tubes for 13 wells (12+1)
Each well needs 1 µL of FWD + 1 µL of REV = 2 µL
2 µL per well × 13 wells = 26 µL

1 µL of the 10 µM FWD Stock
1 µL of the 10 µM REV Stock
} + 24 µL DEPC H_2O = 26 µL

Primer Tube (26 µL each): PPIA, αSMA, VEGF, PECAM1

3. RNA Samples: (For 10 µL reactions)
Want: 40 ng of RNA per well for qPCR

Static: $40\ ng = 1001.8\ ng/\mu L \times V_{Static} \Rightarrow V_{Static} = 0.0399 \approx 0.04\ \mu L$
Flow: $40\ ng = 712.96\ ng/\mu L \times V_{Flow} \Rightarrow V_{Flow} = 0.0561\ \mu L$
0.25OSI: $40\ ng = 121.43\ ng/\mu L \times V_{0.25} \Rightarrow V_{0.25} = 0.329\ \mu L$
0.5OSI: $40\ ng = 319.40\ ng/\mu L \times V_{0.5} \Rightarrow V_{0.5} = 0.125\ \mu L$

Components Per Well (µL)

5.0 SYBR Green
0.08 RT Enzyme
FP
RP
7.08 µL

STATIC:

0.04 µL × 13 wells = 0.52 µL of [S]
(12+1)
7.08 µL + 0.04 µL = 7.12 µL
10 − 7.12 = 2.88 µL of DEPC H_2O per well
2.88 × 13 wells = 37.44 µL of DEPC H_2O

Static tube:
0.52 µL of [S] + 37.44 µL of DEPC H_2O

Continued on Page

Read and Understood By

Signed _____ Date _____ Signed _____ Date 1/10/2018

Flow: (12+1)

0.0561 µL × 13 wells = 0.7293 µL of F
7.08 µL + 0.0561 = 7.1361 µL
10 − 7.1361 = 2.8639 µL of DEPC H_2O per well
2.8639 × 13 = 37.23 µL DEPC H_2O

Flow Tube: 0.73 µL of F + 37.23 µL DEPC H_2O

0.25 OSI: (12+1)

0.329 µL × 13 wells = 4.277 µL of 0.25
7.08 µL + 0.329 µL = 7.409 µL
10 − 7.409 µL = 2.591 of DEPC H_2O per well
2.591 × 13 wells = 33.683 µL DEPC H_2O

0.25 OSI Tube: 4.28 µL of 0.25 + 33.683 µL DEPC H_2O

0.5 OSI: (12+1)

0.125 µL × 13 wells = 1.625 µL of 0.5
7.08 µL + 0.125 = 7.205 µL
10 − 7.205 = 2.795 µL DEPC H_2O per well
2.795 × 13 wells = 36.335 DEPC H_2O

0.5 OSI Tube: 1.63 µL of 0.5 + 36.34 µL DEPC H_2O

TUBE #:

1. Static RNA: 0.52 µL of 5 + 37.44 µL DEPC H_2O
2. Flow RNA: 0.73 µL of F + 37.23 µL DEPC H_2O
3. 0.25 RNA: 4.28 µL of 0.25 + 33.683 µL DEPC H_2O
4. 0.5 RNA: 1.63 µL of 0.5 + 36.34 µL DEPC H_2O
5. RT MM: 260 µL SYBR Green Mix + 4.16 µL RT Enzyme
6. PPIA: 1 µL FP + 1 µL RP + 24 µL DEPC
7. αSMA:
8. VEGF:
9. PECAM1:

Pipette Volume Per Well
2.92 µL
5.08 µL
2 µL

Continued on Page

Read and Understood By

Signed — Date — Signed — 1/10/2019 Date

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

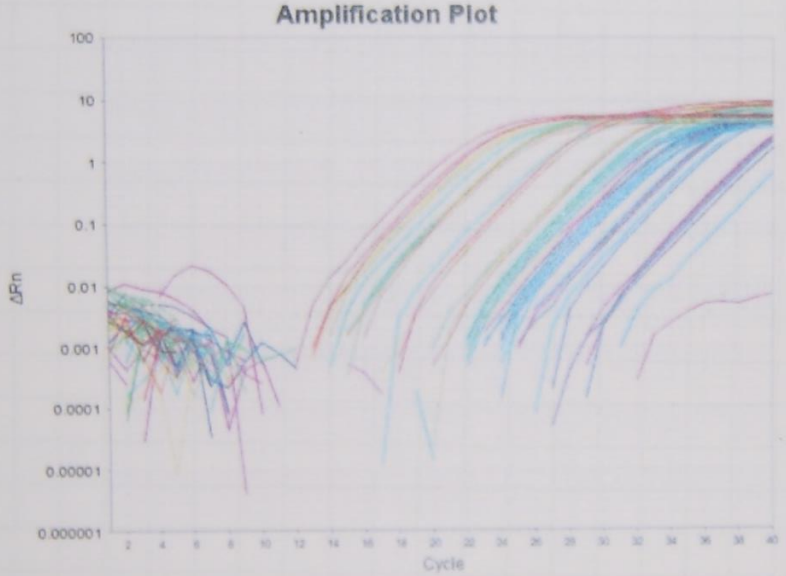
Experi... DH SMC RNA samples January 10th 20 T... Comparative CT (ΔΔC) Reage... SYBR® Green Reagent: Analyze | Analysis Settings

Amplification Plot

Plot Settings

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

Save current settings as the default



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	Static PPIA CT: 21.32	Static VEGF CT: 26.7	Flow PPIA CT: 20.21	Flow VEGF CT: 29.73	0.25 PPIA CT: 21.03	0.25 VEGF CT: 27.06	0.5 PPIA CT: 23.95	0.5 VEGF CT: 23.94
B	Static PPIA CT: 21.83	Static VEGF CT: 26.99	Flow PPIA CT: 21.71	Flow VEGF CT: 29.41	0.25 PPIA CT: 22.02	0.25 VEGF CT: 31.49	0.5 PPIA CT: 23.76	0.5 VEGF CT: 29.54
C	Static PPIA CT: 23.68	Static VEGF CT: 30.48	Flow PPIA CT: 23.63	Flow VEGF CT: 30.93	0.25 PPIA CT: 22.73	0.25 VEGF CT: 30.73	0.5 PPIA CT: 25.98	0.5 VEGF CT: 32.59
D	Static aSMA CT: 30.94	Static PECA... CT: 29.57	Flow aSMA CT: 31.32	Flow PECA... CT: 31.88	0.25 aSMA CT: 33.21	0.25 PECA... CT: 37/1	0.5 aSMA CT: 33.36	0.5 PECAM1 CT: 30.84
E	Static aSMA CT: 32.31	Static PECA... CT: 34.94	Flow aSMA CT: 32.84	Flow PECA... CT: 32.28	0.25 aSMA CT: 31.47	0.25 PECA... CT: 31.29	0.5 aSMA CT: 33.42	0.5 PECAM1 CT: 35.37
F	Static aSMA CT: 33.26	Static PECA... CT: 34.73	Flow aSMA CT: 32.49	Flow PECA... CT: 34.56	0.25 aSMA CT: Undete	0.25 PECA... CT: 31.12	0.5 aSMA CT: 32.48	0.5 PECAM1 CT: 28.53

Wells: 48 Unknown | 0 Negative Control | 0 Er

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

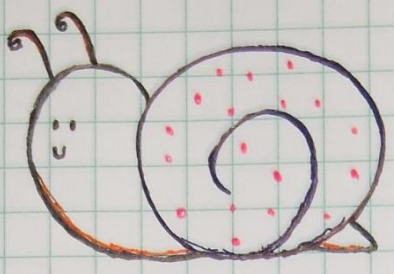
DH SMC RNA sa... 10th 2019.eds

Local Instrument: connected

Start | StepOne™ Software ...

01/10/2019

1/11/2019: qPCR on PAEC's (quantified on page 30)
TARGETS: β-actin, CD31, FzD2, YARS (HK: β-actin)
SAMPLES: static, flow, 0.25, 0.5 (Bioflux conditioned)
# of REPLICATES: 3



樹屋裡的蝸牛

作者：阿D

Want: 20ng of RNA per well for qPCR

Static $20\,ng = 141.9\ ng/\mu L \times V_{static}$
$V_{static} = 0.141\,\mu L$

FLOW $20\,ng = 56.1 \times V_{FLOW}$
$V_{FLOW} = 0.357\,\mu L$

0.25 OSI $20 = 21.65\ ng/\mu L \times V_{0.25}$
$V_{0.25} = 0.924\,\mu L$

0.5 OSI $20 = 650.1 \times V_{0.5}$
$V_{0.5} = 0.031\,\mu L$

	1	2	3	4	5	6	7	8
A	Static β-actin	static FzD2	Flow β-actin	Flow FzD2	0.25 β-actin	0.25 FzD2	0.5 β-actin	0.5 FzD2
B	static β-actin	static FzD2	Flow β-actin	Flow FzD2	0.25 β-actin	0.25 FzD2	0.5 β-actin	0.5 FzD2
C	static β-actin	static FzD2	Flow β-actin	Flow FzD2	0.25 β-actin	0.25 FzD2	0.5 β-actin	0.5 FzD2
D	static CD31	static YARS	Flow CD31	Flow YARS	0.25 CD31	0.25 YARS	0.5 CD31	0.5 YARS
E	static CD31	static YARS	Flow CD31	Flow YARS	0.25 CD31	0.25 YARS	0.5 CD31	0.5 YARS
F	static CD31	static YARS	Flow CD31	Flow YARS	0.25 CD31	0.25 YARS	0.5 CD31	0.5 YARS

Continued on Page

4 targets × 4 samples × 3 replicates = 48 wells

Read and Understood By

Signed Date Signed Date

1/11/2019

1. RNA to C_T Master Mix (for 10 µL reactions) - pg. 13 from protocol online
   5.0 µL Power SYBR Green RT-PCR Mix (2x)
   0.08 µL RT Enzyme
   5.0 µL × 54 samples = 270 µL of SYBR Green Mix
   (48+6)
   0.08 µL × 54 samples = 4.32 µL of RT Enzyme

2. Primers - Use 1 µL of the 10 µM stock for both FWD & REV
   3 replicates × 4 samples = 12 primer pairs
   Make each primer tube for 13 wells (12+1)

   1 µL FWD 10 µM stock
   1 µL REV 10 µM stock
   + 24 µL DEPC H_2O = 26 µL total
   (pipette 2 µL into each well)

   Primer tubes (26 µL each): β-actin, CD31, FZD2, YARS

3. RNA Samples (for 10 µL reactions)
   Components per well: 5.0 µL SYBR Green
   0.08 µL RT Enzyme
   1 µL FWD Primer
   1 µL REV Primer
   7.08 µL
   (12+1)

Static:
0.141 µL × 13 wells = 1.833 µL
7.08 + 0.141 = 7.221 µL (fill the rest w/ DEPC H_2O up to 10 µL)
10 µL - 7.221 µL = 2.779 µL
2.779 × 13 wells = 36.127 µL DEPC H_2O
Static Tube:
1.833 µL of [S] + 36.127 µL DEPC H_2O

Flow:
0.357 µL × 13 wells = 4.641 µL
7.08 + 0.357 = 7.437 µL
10 µL - 7.437 µL = 2.563 µL
2.563 × 13 wells = 33.319 µL DEPC H_2O
Flow Tube:
4.641 µL of [F] + 33.319 µL DEPC H_2O

Continued on Page

Signed _____ Date _____ Signed _____ Date 1/11/2019

0.25 OSI:

0.924 µL × 13 wells = 12.012 µL
7.08 + 0.924 = 8.004 µL
10 µL − 8.004 µL = 1.996 µL DEPC H_2O per well
1.996 × 13 wells = 25.948 µL DEPC H_2O
0.25 OSI Tube:
12.012 µL of [0.25] + 25.948 µL DEPC H_2O

0.5 OSI:

0.031 µL × 13 wells = 0.403 µL
7.08 + 0.031 = 7.111 µL
10 − 7.111 = 2.889 µL
2.889 × 13 wells = 37.557 µL DEPC H_2O
0.5 OSI Tube:
0.403 µL of [0.5] + 37.557 µL DEPC H_2O

TUBE #:

Pipette Volume Per Well
2.92 µL

1. 1.833 µL of [S] + 36.127 µL DEPC H_2O
2. 4.641 µL of [F] + 33.319 µL DEPC H_2O
3. 12.012 µL of [0.25] + 25.948 µL DEPC H_2O
4. 0.403 µL of [0.5] + 37.557 µL DEPC H_2O
5. 270 µL of SYBR Green Mix + 4.32 µL of RT Enzyme — 5.08 µL
6. β-Actin: 1 µL FWD Primer + 1 µL REV Primer + 24 µL DEPC H_2O — 2 µL
7. CD31:
8. FzD2:
9. YARS:

Continued on Page

Read and Understood By

Signed — Date — Signed — 1/11/2019 Date

StepOne™ Software v2.1

File Edit Instrument Analysis Tools Help

New Experiment Open... 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

Experime... Bioflux PAEC qPCR 2019-01-11 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

Amplification Plot

ΔRn

Cycle

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	Static 21.99	Static Undete.	Flow 20.96	Flow Undete.	24.4	Undete.	24.22	Undete.
B	Static 20.37	Static Undete.	Flow 20.86	Flow Undete.	25.11	Undete.	24.35	Undete.
C	Static 23.91	Static Undete.	Flow 22.47	Flow Undete.	25.2	Undete.	25.16	Undete.
D	Static 28.76	Static Undete.	Flow 26.91	Flow Undete.	28.23	Undete.	30.96	Undete.
E	Static 29.08	Static Undete.	Flow 26.95	Flow Undete.	29.07	Undete.	31.95	Undete.
F	Static 28.97	Static Undete.	Flow 27.77	Flow Undete.	29.15	Undete.	29.99	Undete.

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: 39 Wells Omitted by Analysis: 0 Samples Used: 4 Targets Used: 4

Home Bioflux PAEC ...2019-01-11.eds

Local Instrument : connected

start StepOne™ Software ...

1/11/2019

Signed Date Signed Date

1/12/2019: Total RNA extraction - PASMC & Quantification (ng/μL)

	Static	Flow	0.25	0.5
	93.5	52.2	45.0	225.9
	84.1	50.6	48.5	289.6
AVGs	88.8	51.4	46.75	257.75

Want: 40 ng per well for qPCR

Static

$40\,ng = 88.8\ ng/\mu L \times V_{static}$

$V_{static} = \frac{40}{88.8} = 0.450\ \mu L$

Flow

$40\,ng = 51.4\ ng/\mu L \times V_{Flow}$

$V_{Flow} = \frac{40}{51.4} = 0.778\ \mu L$

0.25 OSI

$40\,ng = 46.75\ ng/\mu L \times V_{0.25}$

$V_{0.25} = \frac{40}{46.75} = 0.856\ \mu L$

0.5 OSI

$40\,ng = 257.75\ ng/\mu L \times V_{0.5}$

$V_{0.5} = \frac{40}{257.75} = 0.155\ \mu L$

TARGETS: PPIA, αSMA, Col1a1, ALP
SAMPLES: Static, flow, 0.25, 0.5
# of replicates: 3
4 targets × 4 samples × 3 replicates = 48 wells

	1	2	3	4	5	6	7	8
A	Static PPIA	Static Col1a1	Flow PPIA	Flow Col1a1	0.25 PPIA	0.25 Col1a1	0.5 PPIA	0.5 Col1a1
B	Static PPIA	Static Col1a1	Flow PPIA	Flow Col1a1	0.25 PPIA	0.25 Col1a1	0.5 PPIA	0.5 Col1a1
C	Static PPIA	Static Col1a1	Flow PPIA	Flow Col1a1	0.25 PPIA	0.25 Col1a1	0.5 PPIA	0.5 Col1a1
D	Static αSMA	Static ALP	Flow αSMA	Flow ALP	0.25 αSMA	0.25 ALP	0.5 αSMA	0.5 ALP
E	Static αSMA	Static ALP	Flow αSMA	Flow ALP	0.25 αSMA	0.25 ALP	0.5 αSMA	0.5 ALP
F	Static αSMA	Static ALP	Flow αSMA	Flow ALP	0.25 αSMA	0.25 ALP	0.5 αSMA	0.5 ALP

1. RNA to C_T RT MM (for 10 μL reactions) - p.13 from protocol online
   5.0 μL Power SYBR Green RT-PCR Mix (2x)
   0.08 μL RT Enzyme → (48+6)
   5.0 μL × 54 samples = 270 μL of SYBR Green
   0.08 μL × 54 samples = 4.32 μL RT Enzyme

Continued on Page

Read and Understood By

Signed | Date | Signed | 1/12/2019 Date

2. Primers - Use 1uL of the 10uM stock for both FWD & REV
3 replicates × 4 samples = 12 primer pairs

1uL FWD 10uM stock
1uL REV 10uM stock
+ 24uL DEPC H_2O = 26uL total

Make primer tubes @26uL each for: PPIA, αSMA, col1a1, ALP

3. RNA Samples (for 10uL reaction)
Components per well: 5.0 uL SYBR Green
0.08 uL RT Enzyme
1 uL FWD primer
1 uL REV primer
7.08 uL

Static:
0.450 uL × 13 wells = 5.85 uL
7.08 + 0.450 = 7.53 uL (fill the rest w/ DEPC H_2O up to 10 uL)
10 - 7.53 = 2.47 uL
2.47 × 13 wells = 32.11 uL DEPC H_2O
Static Tube:
5.85 uL [S] + 32.11 uL DEPC H_2O

Flow:
0.778 uL × 13 wells = 10.114 uL
7.08 + 0.778 = 7.858 uL
10 - 7.858 = 2.142 uL
2.142 × 13 wells = 27.846 uL DEPC H_2O
Flow Tube:
10.114 uL [F] + 27.846 uL DEPC H_2O

0.25:
0.856 uL × 13 wells = 11.128 uL
7.08 + 0.856 = 7.936 uL
10 - 7.936 = 2.064 uL
2.064 × 13 wells = 26.832 uL DEPC H_2O
0.25 Tube:
11.128 uL of [0.25] + 26.832 uL DEPC H_2O

Continued on Page

Read and Understood By

Signed _____ Date _____ Signed _____ Date 1/13/2019

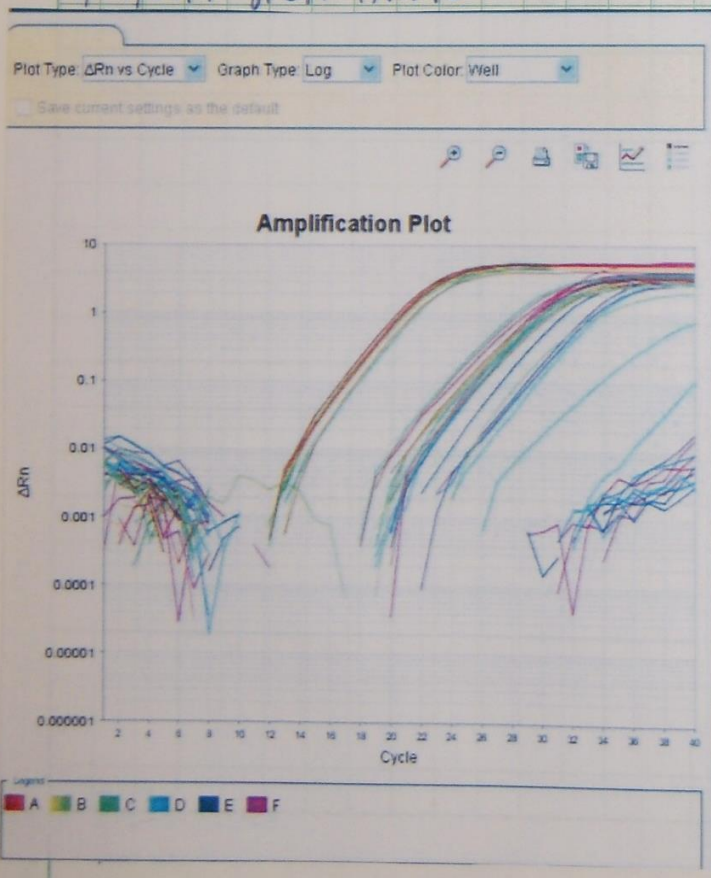
0.50: 0.155 µL × 13 wells = 2.015 µL
7.08 + 0.155 = 7.235 µL
10 − 7.235 = 2.765 µL
2.765 × 13 wells = 35.945 µL DEPC H_2O
0.5 Tube:
2.015 µL of [0.5] + 35.945 µL DEPC H_2O

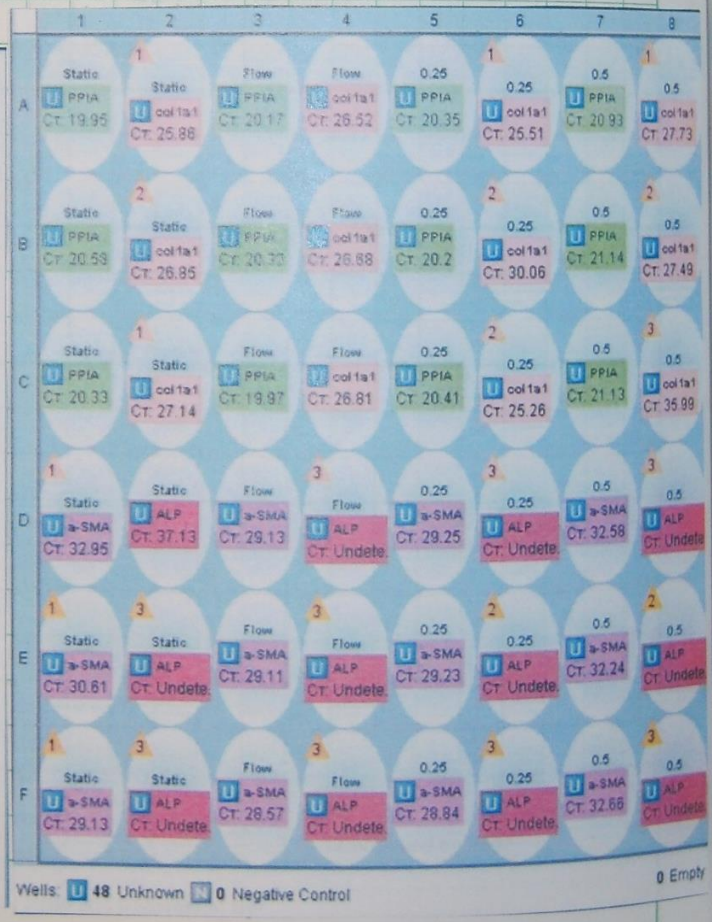
Tube #:

1. 5.85 µL of [5] + 32.11 µL DEPC H_2O
2. 10.114 µL of [F] + 27.846 µL DEPC H_2O
3. 11.128 µL of [0.25] + 26.832 µL DEPC H_2O
4. 2.015 µL of [0.5] + 35.945 µL DEPC H_2O
5. 270 µL SYBR Green Mix + 4.32 µL RT Enzyme
6. PPIA: 1 µL FWD + 1 µL REV + 24 µL DEPC H_2O
7. αSMA:
8. Col1a1:
9. ALP:

Pipette Volume Per Well (uL)
2.92
↓
5.08
2
↓

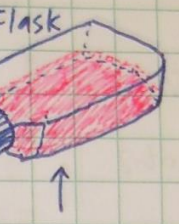
1/14/2019: qPCR - PASMC





Signed | Date | Signed | Date 1/13/2019

1/23/2019: Plated human primary aortic endothelial cells P3 in one T75 flask
Catalog No. H-6052
Human Primary Aortic Endothelial Cells - P3
0.5×10^6 cells/Vial
Date Cryopreserved 03-05-2018
Lot # 021514 F14/17 Store at -180°C
FOR RESEARCH USE ONLY
http://www.cellbiologics.com

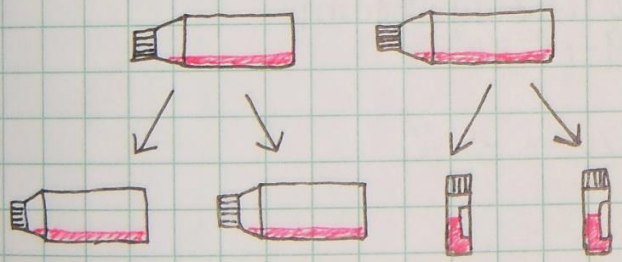


1/24/2019: Changed Human Aortic EC (HAEC) media
Gelatin-Base Coating Solution
Catalog #6950 Lot # 1218
EXP: 12/2019 Store at 4 °C
⟸ Flask was coated w/gelatin provided by Cell Biologics

1/26/2019: HAEC passage P3 → P4
1/28/2019: HAEC media change

Media: CELL APPLICATIONS, INC
Cat No. 211-500 LOT: 35601
Endothelial Cell Growth Medium
EXP 07/19

1/29/2019: HAEC passage P4 → P5
froze 2 vials @ P5



P4
P5

Freezing Media gibco
EXP 2019-09-30
Recovery™ Cell Culture
Freezing Medium Contains DMSO
Cat# 12648-010
Lot# 2004391

2/1/2019: HAEC media change
2/3/2019: HAEC passage P5 → P6
2 flasks → 4 flasks
2/6/2019: HAEC media change
2/8/2019: HAEC media change
2/11/2019: HAEC passage P6 → P7
froze 4 vials @ P7

4 Flasks in P6 → 4 flasks → P7 { 3 T75, 2 T25
4 Flasks in P6 → 4 vials @ P7

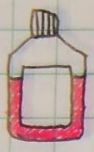
Continued on Page

Read and Understood By

Signed _____ Date _____ Signed _____ Date 1/23/2019

2/12/2019: Received 1 flask of Human Umbilical Artery Smooth Muscle Cells (proliferating in flask) ~ 500,000 cells in P2
HUASMC-p 70mL] Used PromoCell protocol online for culturing
lot# 424Z027] proliferating flasks
SMooth Muscle Cell Growth Medium

PromoCell Cat# C-22062



Smooth muscle cell Growth Medium 2
500 mL
PromoCell Cat# C-22062B
Lot# 443M297

Supplement Mix / Smooth Muscle Cell Growth Medium 2
PromoCell Cat# C-39267
Lot #440MØ10

Added C-39267 to C-22062B + 1% (5mL) P/S
Passaged HUASMC P2 → P3, P3 is split into 3 T25 flasks.

2/14/2019: HAEC media change

2/16/2019: HAEC media change
Froze 2 T25 flasks of HUASMC in Liquid N_2
Promocell will send the correct cell type (Human Aortic Smooth muscle cells) on Monday 2/18.
→ gibco Recovery™ - Cell Culture Freezing Medium
contains DMSO EXP 2019-09-30
Cat No. 12648-010 50 mL
Lot No. 1969968 Store -20 to -5 °C

2/19/2019: Passaged HAEC P7 → P8
2 T25 → 4 T25
3 T75 → 1 T75 + 6 T25

5 µL media w/cells + 5 µL trypan blue
Raw readings:
Counted cells in this group: 6.35×10^5 cells/mL
6.35×10^5 cells/mL
2.28×10^5 cells/mL
4.12×10^5 cells/mL
AVG reading 4.775×10^5 cells/mL

Actual: $2 \times 4.775 \times 10^5$ cells/mL
$= 9.55 \times 10^5$ cells/mL
* Had 3 mL of media w/cells
$3 \times 9.55 \times 10^5 \approx 2.9 \times 10^6$ cells in these flasks

Continued on Page

Read and Understood By

Signed | Date | Signed | Date
2/12/2019

2/20/2019: Received 1 T25 flask of Human Aortic Smooth Muscle Cells (proliferating in flask) ~ 500,000 cells in P2
HAoSMC-p
Cat No: C-12532
Lot No: 437Z012.2
] used Promocell protocol online for culturing proliferating flasks
changed media, capped flask w/ CO_2 ventilated caps.

2/22/2019: HASMC changed media (1 T25 flask)
HAEC changed media (2 T75, 10 T25 flasks)

Made media to run experiment (regular DMEM)

	DMEM	10% FBS	1% P/S
Lot#	04918008	C16036	1989509
Cat#	10-013-CV	S11950	15070-063
EXP	08/2019	03/2021	2019-05-30
Brand	Corning	Atlanta Biologicals	Fisher Scientific

2/25/2019: HASMC passaged P2 → P3
Added 1% P/S to Endothelial Cell Growth Medium
HAEC changed media
Plated 1.6 million HAEC's in a 6-cm dish for Static Samples
Used the regular DMEM experiment media.
Conditioning time: 48 hours. Start: 2/25 @ 12:30pm
Cell Count: 8.14×10^5 cells/mL, 9.77×10^5 cells/mL } AVG 8.955×10^5 cells/mL

Actual: $2 \times 8.955 \times 10^5 = 1.79 \times 10^6$ cells/mL

$C_1 V_1 = C_2 V_2$

$1.6 \times 10^6 = 1.79 \times 10^6 \text{ cells/mL} \; V_2$

$$V_2 = \frac{1.6 \times 10^6}{1.79 \times 10^6} = 0.893 \text{ mL} \approx 0.9 \text{ mL}$$

2/27/2019: Aspirated and saved spent media from HAEC static group @ 1pm
Centrifuged the spent media to remove debris
Collected HAEC static group RNA using TRIzol.
Stored RNA in TRIzol & spent media in -80°C.

2/28/2019: HASMC changed media
HAEC changed media

3/3/2019: HAEC changed media
HASMC passage P3 → P4

Continued on Page

Read and Understood By

Signed _____ Date _____

Signed _____ Date 2/20/2019

3/6/2019: Passaged HASMC P4 → P5
1 T75 → 2 T75
1 T75 → 2 vials stored in liquid N_2
HAEC changed media

3/9/2019: HASMC media change
HAEC redistributed all flasks to 7 flasks (T25)
cell count: 4.62×10^5 cells/mL in 1 mL
cell count: 6.56×10^5 cells/mL in 1 mL
Total cell count: 1.118×10^6 cells

3/11/2019: Added 10% FBS to Endothelial Cell Growth Media to enhance growth rate
CORNING Fetal Bovine Serum 500mL
REF 35-015-CV
LOT# 35015156
EXP DATE 04/2022

3/12/2019: Plated a previously stored HAEC in P5 from liquid N_2 to T25.
Changed Media for the rest of the HAECs.
Passaged HASMC P5 → P6.

3/15/2019: HAECs changed media
HAEC from P5 in liquid N_2 did not attach. Cells are dead. May be due to tilted lid on Fri. Feb. 8th, tank temperature rose after liq. N_2 evaporated.
Seeded 2 6-cm plates of HASMC w/ 50% static spent EC media and 50% fresh media (DMEM). Seeding density 0.8×10^6 cells per plate.
HASMC Raw Count:
Count 1: 8.79×10^5 cells/mL
Count 2: 7.05×10^5 cells/mL
} 1.58×10^6 cells/mL

$C_1V_1 = C_2V_2$ Want 0.8×10^6 cells
$0.8 \times 10^6 = 1.58 \times 10^6 V_2$

$$V_2 = \frac{0.8 \times 10^6}{1.58 \times 10^6} = 0.506 \text{ mL}$$

Use 505 uL for seeding

HASMC in static EC media — 6-cm plate
HASMC in static EC media — 6-cm plate

1.5 mL spent EC grow media in static
1.5 mL DMEM, fresh.

Start: 3/15/2019 @ 14:45
End: 3/17/2019 @ 14:45

Continued on Page

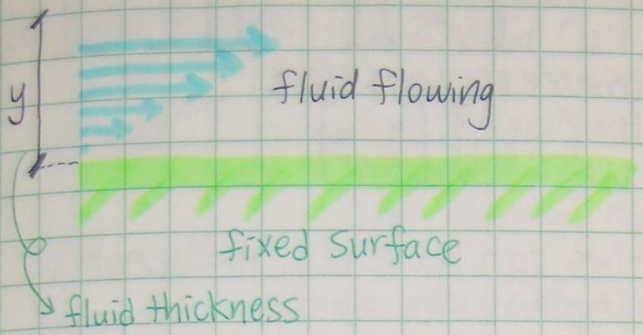
Passaged HASMC P6 → P7

Read and Understood By

Signed — Date — Signed — 3/6/2019 Date

Dynamic Viscosity aka Absolute Viscosity

Resistance to a layer of fluid when it moves over another layer of fluid



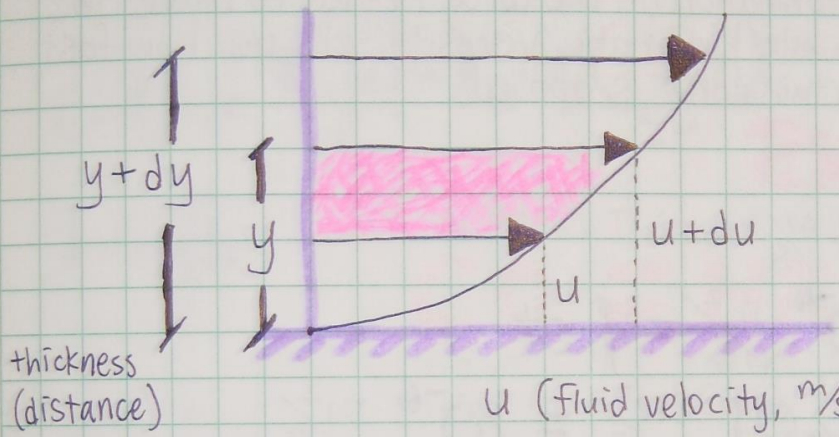
u_{max} = maximum velocity

$u_0 = 0$ no velocity @ surface

Shear Stress:

$$\tau = \mu \frac{du}{dy}$$

$$\mu = \frac{\tau}{\frac{du}{dy}} \qquad \frac{du}{dy} = \dot{\gamma} \text{ shear rate}$$



τ: shear stress in fluid. Units: $[N/m^2]$, $[dynes/cm^2]$

μ: dynamic viscosity. Units: $\left[\frac{N \cdot s}{m^2}\right]$, $[Pa \cdot s]$, $\left[\frac{kg}{m \cdot s}\right]$, [poise], [cp]

du: unit velocity. Units: $[m/s]$, $[cm/s]$

dy: unit distance between layers. Units: [m], [cm]

$\dot{\gamma}$: $\frac{du}{dy}$ = shear rate. Units: $[s^{-1}]$

CONVERSIONS

$1 \frac{N \cdot s}{m^2} = 1 \, Pa \cdot s = 1 \frac{kg}{m \cdot s}$

$1 \text{ poise} = 0.1 \frac{N \cdot s}{m^2} = 0.1 \, Pa \cdot s = 0.1 \frac{kg}{m \cdot s}$

$1 \text{ cp} = 0.01 \text{ poise} = 0.001 \, Pa \cdot s$

$1 \text{ dyne} = 10^{-5} \text{ N}$

$1 \text{ cm} = 0.01 \text{ m}$

$1 \frac{dyne}{cm^2} = 0.1 \frac{N}{m^2}$

Continued on Page

Read and Understood By

Signed

Date

Signed

3/9/2019

Date

Kinematic Viscosity

Ratio of dynamic viscosity, μ, to density, ρ.

$$\nu = \frac{\mu}{\rho}$$

ν: kinematic viscosity. Units: $[m^2/s]$, $[m \cdot m/s]$, $[Pa \cdot s / kg/m^3]$, $[st]$

μ: dynamic viscosity. Units: see previous page

ρ: density of fluid. Units: $[kg/m^3]$, $[g/cm^3]$

Two fluids with the same dynamic viscosities can have very different kinematic viscosities depending on density and vice versa. Dynamic viscosity gives you information on the force needed to make the fluid flow at a certain rate, while kinematic viscosity tells you how fast the fluid is moving when a certain force is applied.

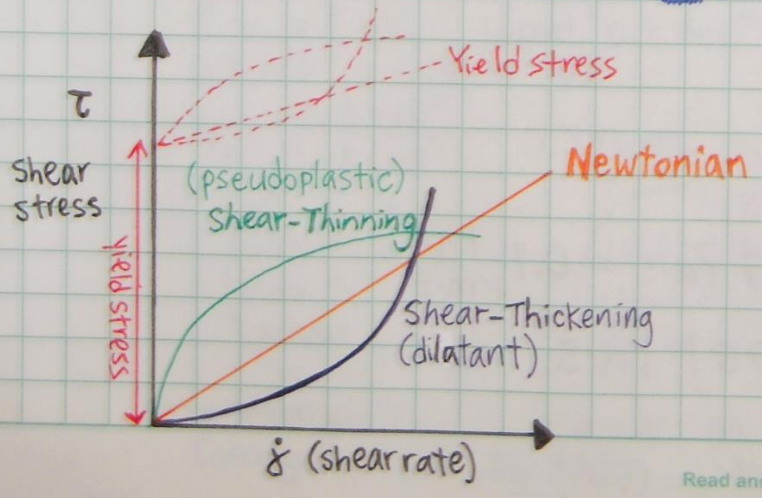
CONVERSIONS

$1\ Pa \cdot s / (kg/m^3) = 1\ m^2/s = 1\ m \cdot m/s$

$1\ \text{stoke} = 1\ cm^2/s = 10^{-4}\ m^2/s = 1\ st$

$1\ cst = 0.01\ st = 0.01\ cm^2/s = 10^{-6}\ m^2/s$

SHEAR STRESS VS. SHEAR RATE



Newtonian: linear
Shear-Thinning: logarithmic
Shear-Thickening: exponential
Yield Stress fluid: can be Newtonian or non-Newtonian. Fluid does not begin to flow until the yield stress is reached.

Continued on Page

Shear rate at inner wall of a Newtonian fluid flowing within a pipe:

$\dot{\gamma} = \frac{8v}{d}$

$\dot{\gamma}$ = shear rate, in s^{-1}
v = linear fluid velocity
d = pipe inner diameter

$v = \frac{Q}{A}$ Volumetric flow continuity equation

Q = Volumetric flow rate
A = Area of Cross Section

$A = \pi r^2$

$V = \frac{Q}{\pi r^2}$ Substituting into shear rate equation

$$\dot{\gamma} = \frac{8v}{d} = \frac{8\left(\frac{Q}{\pi r^2}\right)}{d} = \frac{8\left(\frac{Q}{\pi r^2}\right)}{2r}$$

$$\dot{\gamma} = \frac{4Q}{\pi r^3}$$

Assumptions
- Blood = Newtonian fluid
- Blood vessel = rigid pipe, straight
- Laminar flow ~~(L >> 2R)~~
- Fully developed flow (L >> 2R)
- Incompressible fluid
- Steady-state continuum
- No slip w/ boundary layer

CARDIAC OUTPUT: Amount of blood pumped by the heart per minute.

CO = HR × SV

Cardiac output | Heart Rate | Stroke Volume

Nominal Conditions:

CO = 70 bpm × 70 mL/beat = 4900 mL/min ≈ 5 Liters/min = 5000 cm^3/min

Proximal Ascending Aorta Diameters

	Men	Women
Mean (mm)	29.1 ± 4.3	27.4 ± 3.4

Source: Vriz, Olga et al. "Normal Values of Aortic Root Dimensions in Healthy Adults" (2014).

AVG: 28.25 mm (diameter)
14.125 mm (radius)

Plug into shear rate equation:

$$\dot{\gamma} = \frac{4Q}{\pi r^3} = \frac{4 \cdot 5000\ cm^3/min \times 1\ min/60s}{\pi \cdot (1.4125)\ cm^3} = 37.65\ s^{-1}$$

Source: Viscosity Measurement of Whole Blood. Anton Parr Application Report C72IA025EN-A.

Calculating shear stress on wall:

$\tau_{wall} = \dot{\gamma}\, \mu_{blood}$

μ_{blood}: dynamic viscosity of blood at 37°C
$\mu_{blood} = 2.7801\ mPa \cdot s = 2.78 \times 10^{-3}\ Pa \cdot s = 2.78 \times 10^{-3}\ kg/m \cdot s$

$\tau_{wall} = 37.65\ s^{-1} * 2.78 \times 10^{-3}\ kg/m \cdot s = 0.105\ kg/ms^2 = 0.105\ N/m^2$

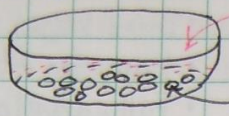
$\tau_{wall} = 0.105\ N/m^2 * \frac{1\ dyne}{10^{-5}\ N} * \frac{1\ m^2}{10^4\ cm^2} = 1.05\ dynes/cm^2$

Continued on Page

Read and Understood By

Signed | Date | Signed | 3/10/2019 Date

3/17/2019: 15:00 Removed static EC media from HASMC
Lysed HASMC w/TRIzol after washing w/PBS
Stored Media & TRIzol w/HASMC in -80 °C.



3/18/2019: HASMC media change
HAEC media change

3/20/2019: HAEC media change

3/21/2019: HASMC changed media, seeded two 6-cm plates of HASMC in static conditioned EC media.

Hemocytometer cell count:

87	58	43
~~71~~	~~71~~	~~47~~
71	61	50

← Each square is 100 nL
← AVG = 62.25

$62.1 \frac{\text{cells}}{100\,\text{nL}} \times \frac{10^6\,\text{nL}}{\text{mL}} = 622{,}500 \frac{\text{cells}}{\text{mL}}$

Want: 0.8×10^6 cells in one 6-cm plate for seeding

$0.8 \times 10^6 = 622{,}500 \frac{\text{cells}}{\text{mL}} \times V$

$V = \frac{80}{62.25} = 1.285\ \text{mL}$

HASMC static EC + DMEM

HASMC static EC + DMEM

1.5 fresh DMEM + 1.5 static EC spent media
start time: 3/21/2019 @ 16:30
End time: 3/23/2019 @ 16:30

3/23/2019: HAEC media change
Collected HASMC in static conditioned media
Stored HASMC in TRIzol in -80 °C

3/25/2019: HASMC media change

3/26/2019: HAEC cell count

9	6	3
~~8~~	~~7~~	~~4~~
11	17	12

+

19	13	13
~~17~~	~~13~~	~~5~~
12	11	9

AVG 6.5 + 13.25 = 19.75 ≈ 20

$20 \times 10^4 \frac{\text{cells}}{\text{mL}} \times 4\,\text{mL} = 800{,}000$ cells
Each channel needs 2×10^5 cells
Seeded 4 channels in Bioflux plate
changed HAEC media in the remaining flasks w/cells.

3/27/2019: Unfroze 2 vials of HAEC P7 from Liq. N_2, cultured them in 2 T25's
Bioflux steady flow (0 osi) @ 1 $\frac{\text{dyne}}{\text{cm}^2}$
Start time: 5:41 pm

Continued on Page

Read and Understood By

Signed Date Signed Date

3/17/2019

3/28/2019: HASMC media change
10:15am paused Bioflux flow to redistribute media from O→A wells.
10:20am Resumed flow
HAEC (unfrozen from yesterday) media change

3/29/2019: 5:41pm Stopped Bioflux flow
Collected conditioned media from wells (← centrifuged spent media), washed cells with PBS,
Collected RNA w/TRIzol and stored RNA in −80°C. (↳ and spent media)
HAEC media change

3/31/2019: New DMEM media

	DMEM	FBS (10%)	P/S (1%)
Lot#	04918008	C16036	1989509
Brand	Corning	Atlanta Biologicals	Fisher Scientific
EXP	08/2019	03/2021	05/2019

HASMC Hemocytometer Cell Count

52	122
32	80

AVG: 71.5
Cell concentration: 715,000 cells/mL

Want 800,000 cells in 6-cm dish

$800{,}000 = 715{,}000 \text{ cells/mL} \times V_2$

$V_2 = \frac{800{,}000}{715{,}000} = 1.11$ mL of cell suspension

HASMC in steady flow conditioned media from EC

(HASMC steady flow EC+DMEM) (HASMC steady flow EC+DMEM) + (HASMC steady flow EC+DMEM) (HASMC steady flow EC+DMEM)

3/31/2019
6:21pm

passaged the rest of HASMCs p7→p8

Start: 3/31/2019
5:41pm
HAEC changed media

4/1/2019: HAEC changed media
HASMC changed media

4/2/2019: Collected HASMC in TRIzol, placed TRIzol w/RNA in −80°C

4/4/2019: HAEC media change
HASMC media change

4/6/2019: seeded the old HBMSC's (P7) in Bioflux plates.
These cells were originally purchased for the Bioreactor experiments. However, Bioreactor experiments have now switched to the Rooster Bio cells. Hence the old HBMSC's will be used for Bioflux experiments.

Read and understood by

3/28/2019

Signed Date Signed Date

4/6/2019: (con'd) Want 200,000 cells per channel, coated w/gelatin

33	34
57	39

count w/ hemocytometer
AVG: 40.75 $\Rightarrow \times 10^4$
Cell concentration: 407,500 cells/mL, 2mL total
# of cells: 815,000 cells.
Seeded 500 µL in each channel. Start: 16:12
Placed in incubator for attachment.

4/7/2019: Start steady flow on HBMSC @ 1 dyne/cm², 6:28pm
HASMC changed media
HAEC passaged P8 → P9

4/8/2019: 8:50am paused flow, redistributed media from O to A wells
8:52am resumed flow @ 1 dyne/cm²
7:50pm paused flow, redistributed media from O to A wells
7:52pm resumed flow @ 1 dyne/cm²

4/9/2019: HAEC changed media
HASMC changed media (P8)
9:15am paused Bioflux flow, redistributed media from O → A wells
9:17am resumed flow @ 1 dyne/cm²
6:28pm Terminated HBMSC steady flow experiment. Collected RNA sample ×1 and stored in TRIzol in -80°C.
Counted HBMSC's for next round of seeding

78	28
33	52

AVG:
47.75×10^4 cells/mL
$47.75 \times 2 \times 10^4 = 955{,}000$ cells in total
Want: 200,000 cells per channel
$200{,}000 = 47.75 \times 10^4$ cells/mL $\times V_2$

$$V_2 = \frac{200{,}000}{477500} = 0.419 \text{ mL}$$

Seeded 4 channels @ 420 µL each.
Time: 6:45pm

4/10/2019: HBMSC 0.25 OSI start 6:53pm (same profile as page 9)
HAEC 8 flasks decided to undergo apoptosis :(
HASMC also underwent apoptosis (more sad face)

4/11/2019: 9:00am paused flow, redistributed media
9:05am resumed flow
6:00pm paused flow, redistributed media
6:05pm resumed flow.

4/12/2019: 10:00am paused flow, redistributed media
10:05am resumed flow

Continued on Page

Read and Understood By

Signed | Date | Signed | 4/6/2019 Date

6:50pm Stopped flow, stored RNA in TRIZOL in -80°C.
HAEC changed media

4/15/2019: HAEC media change

4/16/2019: Plated human primary aortic endothelial cells P3 in one T75 flask

Catalog No. H-6052
Human Primary Aortic Endothelial Cells-P3
0.5×10^6 cells/vial
Date cryopreserved 03-05-2018
Lot# 021514F14/17 Store at -180°C
FOR RESEARCH USE ONLY
http://www.cellbiologics.com

Primary Cells
Store at -180 °C
FOR RESEARCH USE ONLY
http://www.cellbiologics.com
Catalog No. H-6052
Human Primary Aortic Endothelial Cells-P3 0.5×10^6 cells/vial
Date Cryopreserved 03-05-2018
Lot# 021514F14/17 Store at -180 °C
FOR RESEARCH USE ONLY
http://www.cellbiologics.com

慈悲沒有敵人
智慧不起煩惱

4/17/2019: HAEC P3 media change
HAEC P9 media change

4/18/2019: Seeded HBMSC's in 4 channels Bioflux

Cell count

47	59
25	44

AVG: 43.75 → 437,500 cells/mL
With 2mL of cell suspension, total # of cells is $2 \times 43.75 \times 10^4 = 875{,}000$ cells.

Want 200,000 cells per channel.
5:45pm Seeded 4 channels @ 500uL each.

4/19/2019: HBMSC 0.50 OSI start 5:45pm (same profile as pg.18)
HAEC P3 → P4 passage
HAEC P9 media change

4/20/2019: 11:30 am paused flow, redistributed media, resumed flow @ 11:35am
7:00pm paused flow, redistributed media, resumed flow @ 7:05pm

4/21/2019: 5:45pm HBMSC 0.50 OSI stopped flow, collected RNA in TRIzol, stored in -80°C. HAEC passaged P4 → P5 (4 T75 flasks). P9 media change. (HAEC) → 4 T25's

Current cells in incubator:
4 T25's : HAEC in P9
4 T75's : HAEC in P5
1 T75 : HBMSC in P7

Extracted RNA from previously conditioned PASMC's, three samples each:
Static 1, Static 2, Static 3
Flow 1, Flow 2, Flow 3 ← steady flow } total of 12 Eppendorf tubes
0.25 OSI 1, 0.25 OSI 2, 0.25 OSI 3
0.50 OSI 1, 0.50 OSI 2, 0.50 OSI 3

Continued on Page

Read and Understood By

4/12/2019
Date

Signed Date Signed Date

PROJECT aortic SMC Porcine RNA Extraction & qPCR

Notebook No.

Continued from Page

4/21/2019: (con'd) Porcine aortic smooth muscle cell RNA extraction
Cells were cultured in conditioned endothelial cell media. Porcine aortic endothelial cells were directly exposed to four groups of shear profiles: static, steady flow (OSI=0), 0.25 OSI, 0.50 OSI
n=3 for each groups were extracted. RNA quantitation shown below:

Group SAMPLES	Concentration (ng/uL)	Average (ng/uL)	A260 (10mm)	A260/A280
Static 1	465.4	533.65	11.636	1.91
	601.9		15.048	1.92
Static 2	612.8	611.05	15.319	1.88
	609.3		15.233	1.89
Static 3	314.9	316.7	7.873	1.84
	318.5		7.962	1.84
steady Flow 1	409.1	403.25	10.228	1.85
	397.4		9.934	1.85
steady Flow 2	1042.0	943.05	26.049	1.87
	844.1		21.102	1.87
steady Flow 3	473.4	475.9	11.836	1.85
	478.4		11.960	1.86
0.25 OSI-1	287.9	280.7	7.197	2.39
	273.5		6.837	1.84
0.25 OSI-2	205.8	191.65	5.144	1.73
	177.5		4.437	1.84
0.25 OSI-3	277.6	261.05	6.940	1.83
	244.5		6.112	1.85
0.50 OSI-1	496.5	478.7	12.413	1.84
	460.9		11.522	1.84
0.50 OSI-2	575.9	499.6	14.397	1.47
	423.3		10.582	1.87
0.50 OSI-3	710.7	624.45	17.767	1.84
	538.2		13.456	1.85

TARGETS: PPIA, αSMA, Col1a1, TGFβ-1
SAMPLES: static 1 (S1), steady flow 1 (F1), 0.25 OSI-1, 0.50 OSI-1
# of Replicates: 3
4 targets × 4 samples × 3 replicates = 48 wells

Continued on Page

Read and Understood By

Signed Date Signed Date 4/21/2019

Want: 40 ng per well for qPCR

Static 1 (S1): $40\ ng = 533.65\ ng/\mu L \times V_{S1}$

$V_{S1} = \frac{40}{533.65} = 0.075\ \mu L$

Steady Flow (F1): $40 ng = 403.25\ ng/\mu L \times V_{F1}$

$V_{F1} = \frac{40}{403.25} = 0.099\ \mu L$

0.25 OSI-1: $40 ng = 280.7\ ng/\mu L \times V_{0.25-1}$

$V_{0.25-1} = \frac{40}{280.7} = 0.1425\ \mu L$

0.50 OSI-1: $40 ng = 478.7\ ng/\mu L \times V_{0.50-1}$

$V_{0.50-1} = \frac{40}{478.7} = 0.0835\ \mu L$

	1	2	3	4	5	6	7	8
A	S1 PPIA	S1 Col1a1	F1 PPIA	F1 Col1a1	0.25-1 PPIA	0.25-1 Col1a1	0.50-1 PPIA	0.50-1 Col1a1
B	S1 PPIA	S1 Col1a1	F1 PPIA	F1 Col1a1	0.25-1 PPIA	0.25-1 Col1a1	0.50-1 PPIA	0.50-1 Col1a1
C	S1 PPIA	S1 Col1a1	F1 PPIA	F1 Col1a1	0.25-1 PPIA	0.25-1 Col1a1	0.50-1 PPIA	0.50-1 Col1a1
D	S1 αSMA	S1 TGFβ1	F1 αSMA	F1 TGFβ1	0.25-1 αSMA	0.25-1 TGFβ1	0.50-1 αSMA	0.50-1 TGFβ1
E	S1 αSMA	S1 TGFβ1	F1 αSMA	F1 TGFβ1	0.25-1 αSMA	0.25-1 TGFβ1	0.50-1 αSMA	0.50-1 TGFβ1
F	S1 αSMA	S1 TGFβ1	F1 αSMA	F1 TGFβ1	0.25-1 αSMA	0.25-1 TGFβ1	0.50-1 αSMA	0.50-1 TGFβ1

1. RNA to Ct RTMM (for 10 µL reactions) - p.13 from protocol online
   - 5.0 µL Power SYBR Green RT-PCR Mix (2×)
   - 0.08 µL RT Enzyme → 48+6 extra
   - 5.0 µL × 54 samples = 270 µL SYBR Green
   - 0.08 µL × 54 samples = 4.32 µL RT Enzyme

2. Primers - Use 1 µL of the 10 µM stock for both FWD & REV
   - 3 replicates × 4 samples = 12 primer pairs
   - 1 µL FWD 10 µM stock + 25 µL DEPC H_2O = 27 µL total
   - 1 µL REV 10 µM stock
   - (pipette 2 µL of mixture per well)

Make primer tubes @ 27 µL total for PPIA
αSMA
Col1a1
TGFB1

Continued on Page

Read and Understood By

Signed _____ Date _____ Signed _____ Date 4/21/2019

3. RNA Samples (for 10 µL reaction)
Components per well: 5.0 µL SYBR Green
0.08 µL RT Enzyme
1 µL FWD Primer
1 µL REV Primer
7.08 µL

Static 1 (S1): (12+1)
0.075 µL × 13 wells = 0.975 µL
7.08 + 0.075 = 7.155 (fill the rest w/DEPC H_2O up to 10 µL total volume)
10 − 7.155 = 2.845 µL DEPC H_2O per well
2.845 µL × 13 wells = 36.985 µL DEPC H_2O
✡ 0.975 µL S1 + 36.985 µL DEPC H_2O

Steady Flow (F1):
0.099 µL × 13 wells = 1.287 µL
7.08 + 0.099 = 7.179 (fill the rest w/DEPC H_2O up to 10 µL total volume)
10 − 7.179 = 2.821 µL DEPC H_2O per well
2.821 µL × 13 wells = 36.673 µL DEPC H_2O
✡ 1.287 µL F1 + 36.673 µL DEPC H_2O

0.25 OSI-1:
0.1425 µL × 13 wells = 1.8525 µL
7.08 + 0.1425 = 7.2225 (fill the rest w/DEPC H_2O up to 10 µL total volume)
10 − 7.2225 = 2.7775 µL DEPC H_2O per well
2.7775 µL × 13 wells = 36.1075 µL DEPC H_2O
✡ 1.8525 µL 0.25-1 + 36.1075 µL DEPC H_2O

0.50 OSI-1:
0.0835 µL × 13 wells = 1.0855 µL
7.08 + 0.0835 = 7.1635 (fill volume up to 10 µL w/DEPC H_2O)
10 − 7.1635 = 2.8365 µL DEPC H_2O per well
2.8365 µL × 13 wells = 36.8745 µL DEPC H_2O
✡ 1.0855 µL 0.50-1 + 36.8745 µL DEPC H_2O

TUBE #:

		Pipette Volume Per Well (µL)
1.	0.975 µL S1 + 36.985 µL DEPC H_2O	2.92
2.	1.287 µL F1 + 36.673 µL DEPC H_2O	2.92
3.	1.8525 µL 0.25-1 + 36.1075 µL DEPC H_2O	2.92
4.	1.0855 µL 0.50-1 + 36.8745 µL DEPC H_2O	2.92
5.	270 µL SYBR Green + 4.32 µL RT Enzyme	5.08
6.	PPIA: 1 µL FWD + 1 µL REV + 25 µL DEPC H_2O	2
7.	αSMA:	2
8.	Col1a1:	2
9.	TGFβ1:	2

Continued on Page

Read and Understood By
Signed Date Signed 4/21/2019 Date

StepOne™ Software v2.1

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: DH_PASMC_04-22-2019 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

Amplification Plot

ΔRn

Cycle

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	Static-1 PPIA CT: 19.13	Static-1 col1a1 CT: 25.54	S.Flow-1 PPIA CT: 19.12	S.Flow-1 col1a1 CT: 25.67	0.25 OSI-1 PPIA CT: 19.94	0.25 OSI-1 col1a1 CT: 26.7	0.50 OSI-1 PPIA CT: 19.98	0.50 OSI-1 col1a1 CT: 27.96
B	Static-1 PPIA CT: 19.96	Static-1 col1a1 CT: 27.9	S.Flow-1 PPIA CT: 20.17	S.Flow-1 col1a1 CT: 27.37	0.25 OSI-1 PPIA CT: 20.67	0.25 OSI-1 col1a1 CT: 27.95	0.50 OSI-1 PPIA CT: 20.93	0.50 OSI-1 col1a1 CT: 28.28
C	Static-1 PPIA CT: 20.73	Static-1 col1a1 CT: 27.89	S.Flow-1 PPIA CT: 20.08	S.Flow-1 col1a1 CT: 27.62	0.25 OSI-1 PPIA CT: 20.16	0.25 OSI-1 col1a1 CT: 27.86	0.50 OSI-1 PPIA CT: 20.9	0.50 OSI-1 col1a1 CT: 27.07
D	Static-1 α-SMA CT: 28.79	Static-1 TGFB1 33.94	S.Flow-1 α-SMA CT: 28.11	S.Flow-1 TGFB1 32.9	0.25 OSI-1 α-SMA CT: 28.61	0.25 OSI-1 TGFB1 32.79	0.50 OSI-1 α-SMA CT: 30.32	0.50 OSI-1 TGFB1 37.15
E	Static-1 α-SMA CT: 29.96	Static-1 TGFB1 33.53	S.Flow-1 α-SMA CT: 28.97	S.Flow-1 TGFB1 36.62	0.25 OSI-1 α-SMA CT: 28.94	0.25 OSI-1 TGFB1 35.53	0.50 OSI-1 α-SMA CT: 30.87	0.50 OSI-1 TGFB1 32.97
F	Static-1 α-SMA CT: 29.02	Static-1 TGFB1 33.14	S.Flow-1 α-SMA CT: 29.1	S.Flow-1 TGFB1 32.85	0.25 OSI-1 α-SMA CT: 29.31	0.25 OSI-1 TGFB1 32.32	0.50 OSI-1 α-SMA CT: 30.78	0.50 OSI-1 TGFB1 36.79

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: 37 Wells Omitted by Analysis: 0 Samples Used: 4 Targets Used: 4

Home DH_PASMC_04-22-2019.eds

Local Instrument : connected.

start StepOne™ Software ...

Signed Date Signed Date 4/22/2019

55

4/22/2019: TARGETS: PPIA, αSMA, Col1a1, TGFB-1
SAMPLES: static 2 (S2), steady flow 2 (F2), 0.25 OSI-2, 0.50 OSI-2
# of Replicates: 3

Want: 40 ng per well for qPCR (RNA concentration from pg. 52)

static (S2): $40\,ng = 611.05\,\frac{ng}{\mu L} \times V_{S2}$

$V_{S2} = \frac{40}{611.05} = 0.0655\,\mu L$

Steady Flow (F2): $40\,ng = 943.05\,\frac{ng}{\mu L} \times V_{F2}$

$V_{F2} = \frac{40}{943.05} = 0.0424\,\mu L$

0.25 OSI-2: $40\,ng = 191.65\,\frac{ng}{\mu L} \times V_{0.25\text{-}2}$

$V_{0.25\text{-}2} = \frac{40}{191.65} = 0.209\,\mu L$

0.50 OSI-2: $40\,ng = 499.6\,\frac{ng}{\mu L} \times V_{0.50\text{-}2}$

$V_{0.50\text{-}2} = \frac{40}{499.6} = 0.080\,\mu L$

RNA Samples (for 10 μL reactions)

static (S2):
0.0655 μL × 13 wells = 0.8515 μL
7.08 + 0.0655 = 7.1455
10 − 7.1455 = 2.8545 μL DEPC H_2O
2.8545 × 13 wells = 37.109 μL
0.8515 μL S2 + 37.109 μL DEPC H_2O

Steady Flow (F2):
0.0424 μL × 13 wells = 0.5512 μL
7.08 + 0.0424 = 7.1224
10 − 7.1224 = 2.8776 μL DEPC H_2O
2.8776 × 13 wells = 37.409 μL
0.5512 μL F2 + 37.409 μL DEPC H_2O

0.25 OSI-2:
0.209 μL × 13 wells = 2.717 μL
7.08 + 0.209 = 7.289
10 − 7.289 = 2.711 μL DEPC H_2O
2.711 × 13 wells = 35.243 μL
2.717 μL 0.25 OSI-2 + 35.243 μL DEPC H_2O

0.50 OSI-2:
0.080 μL × 13 wells = 1.04 μL
7.08 + 0.080 = 7.16
10 − 7.16 = 2.84 μL DEPC H_2O
2.84 × 13 wells = 36.92 μL
1.04 μL 0.50 OSI-2 + 36.92 μL DEPC H_2O

TUBE #:
1. 0.8515 μL S2 + 37.109 μL DEPC H_2O
2. 0.5512 μL F2 + 37.409 μL DEPC H_2O
3. 2.717 μL 0.25-2 + 35.243 μL DEPC H_2O
4. 1.04 μL 0.50-2 + 36.92 μL DEPC H_2O
5. 270 μL SYBR Green + 4.32 μL RT Enzyme
6. PPIA: 1 μL FWD + 1 μL REV + 25 μL DEPC H_2O
7. αSMA:
8. Col1a1:
9. TGFβ1:

Pipette Volume Per Well (μL)
2.92
↓
5.08
2
↓

Continued on Page

Read and Understood By

Signed _____ Date _____ Signed _____ Date 4/22/2019

StepOne™ Software v2.1

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

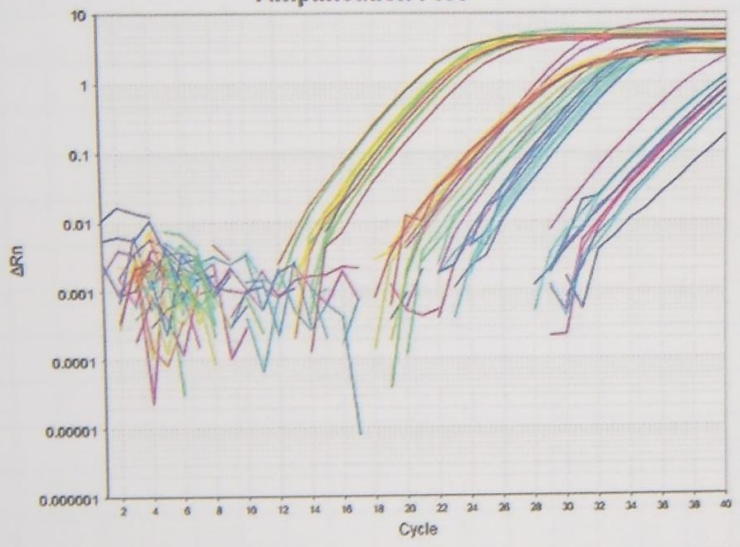
Experiment: DH PASMC 04-23-2019 Type: Comparative CT (ΔΔCT) Reagents: SYBR® Green Reagents Analyze Analysis Settings

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

View the well legend

	1	2	3	4	5	6	7	8
A	1 Static 2 U PPIA CT: 21.82	1 Static 2 U col1a1 CT: 27.92	1 S. Flow 2 U PPIA CT: 22.95	1 S. Flow 2 U col1a1 CT: 28.13	1 0.25 OSI-2 U PPIA CT: 19.98	1 0.25 OSI-2 U col1a1 CT: 28.16	1 0.50 OSI-2 U PPIA CT: 21.47	1 0.50 OSI-2 U col1a1 CT: 27.85
B	1 Static 2 U PPIA CT: 21.36	1 Static 2 U col1a1 CT: 27.93	1 S. Flow 2 U PPIA CT: 21.04	1 S. Flow 2 U col1a1 CT: 27.53	1 0.25 OSI-2 U PPIA CT: 21.1	1 0.25 OSI-2 U col1a1 CT: 28.04	1 0.50 OSI-2 U PPIA CT: 22.2	1 0.50 OSI-2 U col1a1 CT: 28.96
C	1 Static 2 U PPIA CT: 20.08	1 Static 2 U col1a1 CT: 28.26	1 S. Flow 2 U PPIA CT: 22.16	1 S. Flow 2 U col1a1 CT: 30.64	1 0.25 OSI-2 U PPIA CT: 21.48	1 0.25 OSI-2 U col1a1 CT: 29.57	1 0.50 OSI-2 U PPIA CT: 21.21	1 0.50 OSI-2 U col1a1 CT: 30.02
D	1 Static 2 U aSMA CT: 32.17	2 Static 2 U TGFB1 CT: 33.01	1 S. Flow 2 U aSMA CT: 31.95	1 S. Flow 2 U TGFB1 CT: 35.26	1 0.25 OSI-2 U aSMA CT: 31.29	2 0.25 OSI-2 U TGFB1 CT: 32.95	1 0.50 OSI-2 U aSMA CT: 32.5	1 0.50 OSI-2 U TGFB1 CT: 34.94
E	1 Static 2 U aSMA CT: 30.94	2 Static 2 U TGFB1 CT: 32.97	1 S. Flow 2 U aSMA CT: 31.63	1 S. Flow 2 U TGFB1 CT: 36.99	1 0.25 OSI-2 U aSMA CT: 31.33	2 0.25 OSI-2 U TGFB1 CT: 33.9	1 0.50 OSI-2 U aSMA CT: 32.58	2 0.50 OSI-2 U TGFB1 CT: 33.8
F	1 Static 2 U aSMA CT: 28.18	3 Static 2 U TGFB1 CT: 31.47	1 S. Flow 2 U aSMA CT: 29.93	1 S. Flow 2 U TGFB1 CT: 34.41	2 0.25 OSI-2 U aSMA CT: 27.79	1 0.25 OSI-2 U TGFB1 CT: 34.25	1 0.50 OSI-2 U aSMA CT: 31.01	2 0.50 OSI-2 U TGFB1 CT: 34.38

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: 45 Wells Omitted by Analysis: 0 Samples Used: 4 Targets Used: 4

Home DH PASMC 04-23-2019.eds

Local Instrument : connected.

start StepOne™ Software ...

4/23/2019

4/23/2019: TARGETS: PPIA, αSMA, col1a1, TGFB-1
SAMPLES: Static (S3), Steady Flow (F3), 0.25 OSI-3, 0.50 OSI-3
# of Replicates: 3

Want: 40ng per well for qPCR (concentration of RNA from pg. 52)

Static (S3): $40ng = 316.7 \frac{ng}{\mu L} \times V_{S3}$

$V_{S3} = \frac{40}{316.7} = 0.1263\ \mu L$

$0.1263\ \mu L \times 13$ wells $= 1.642\ \mu L$
$7.08 + 0.1263 = 7.2063$
$10 - 7.2063 = 2.7937\ \mu L$ DEPC H_2O
2.7937×13 wells $= 36.32\ \mu L$
1.642 µL S3 + 36.32 µL DEPC H_2O

Steady Flow (F3): $40ng = 475.9 \frac{ng}{\mu L} \times V_{F3}$

$V_{F3} = \frac{40}{475.9} = 0.08405\ \mu L$

$0.08405\ \mu L \times 13$ wells $= 1.093\ \mu L$
$7.08 + 0.08405 = 7.16405$
$10 - 7.16405 = 2.84\ \mu L$ DEPC H_2O
2.84×13 wells $= 36.87\ \mu L$
1.093 µL F3 + 36.87 µL DEPC H_2O

0.25 OSI-3: $40ng = 261.05 \frac{ng}{\mu L} \times V_{0.25\text{-}3}$

$V_{0.25\text{-}3} = \frac{40}{261.05} = 0.1532\ \mu L$

$0.1532\ \mu L \times 13$ wells $= 1.992\ \mu L$
$7.08 + 0.1532 = 7.2332\ \mu L$
$10 - 7.2332 = 2.7668\ \mu L$ DEPC H_2O
2.7668×13 wells $= 35.97\ \mu L$
1.992 µL 0.25 OSI-3 + 35.97 µL DEPC H_2O

0.50 OSI-3: $40ng = 624.45 \frac{ng}{\mu L} \times V_{0.50\text{-}3}$

$V_{0.50\text{-}3} = \frac{40}{624.45} = 0.06406\ \mu L$

$0.06406\ \mu L \times 13$ wells $= 0.8327\ \mu L$
$7.08 + 0.06406 = 7.144\ \mu L$
$10 - 7.144 = 2.856\ \mu L$ DEPC H_2O
2.856×13 wells $= 37.13\ \mu L$
0.8327 µL 0.50 OSI-3 + 37.13 µL DEPC H_2O

TUBE #:
1. 1.642 µL S3 + 36.32 µL DEPC H_2O
2. 1.093 µL F3 + 36.87 µL DEPC H_2O
3. 1.992 µL 0.25-3 + 35.97 µL DEPC H_2O
4. 0.8327 µL 0.50-3 + 37.13 µL DEPC H_2O
5. 270 µL SYBR Green + 4.32 µL RT Enzyme
6. PPIA: 1 µL FWD + 1 µL REV + 25 µL DEPC H_2O
7. αSMA:
8. col1a1:
9. TGFB1:

Pipette Volume Per Well (µL)
2.92
↓
5.08
2
↓

4/23/2019: HAEC P5 changed media

Continued on Page

Read and Understood By

Signed | Date | Signed | Date
4/23/2019

StepOne™ Software v2.1

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

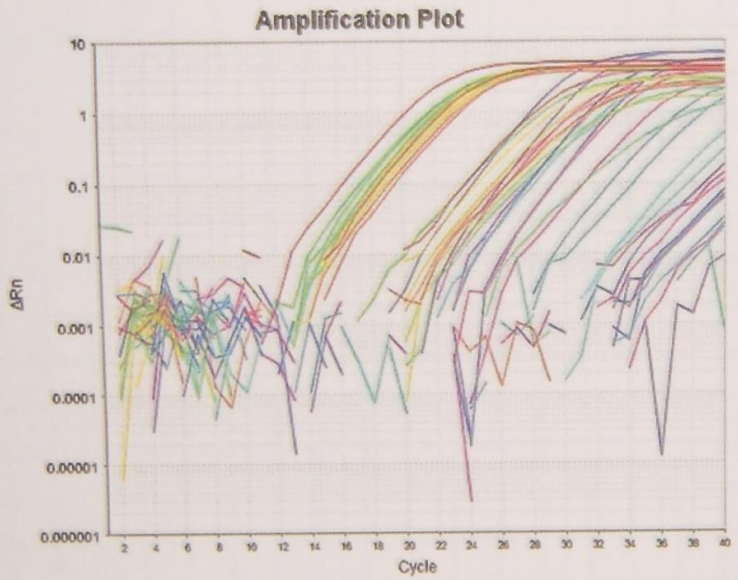
Experiment: DH PASMC 04-23-2019 run 2 Type: Comparative CT (ΔΔCT) Reagents: SYBR® Green Reagents | Analyze | Analysis Settings

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	1 Static-3 PPIA CT: 19.84	1 Static-3 col1a1 CT: 26.23	1 S. Flow-3 PPIA CT: 22.05	2 S. Flow-3 col1a1 CT: 29.13	1 0.25 OSI-3 PPIA CT: 23.14	1 0.25 OSI-3 col1a1 CT: Undete.	1 0.50 OSI-3 PPIA CT: 22.89	1 0.50 OSI-3 col1a1 CT: 28.57
B	1 Static-3 PPIA CT: 22.2	1 Static-3 col1a1 CT: 27.18	1 S. Flow-3 PPIA CT: 21.94	2 S. Flow-3 col1a1 CT: 29.34	1 0.25 OSI-3 PPIA CT: 22.56	2 0.25 OSI-3 col1a1 CT: 28.15	1 0.50 OSI-3 PPIA CT: 21.64	2 0.50 OSI-3 col1a1 CT: 28.97
C	1 Static-3 PPIA CT: 21.19	1 Static-3 col1a1 CT: 26.48	1 S. Flow-3 PPIA CT: 21.26	2 S. Flow-3 col1a1 CT: 30.51	1 0.25 OSI-3 PPIA CT: 21.95	1 0.25 OSI-3 col1a1 CT: 34.52	1 0.50 OSI-3 PPIA CT: 21.75	1 0.50 OSI-3 col1a1 CT: Undete.
D	2 Static-3 αSMA CT: 37.01	1 Static-3 TGFB1 CT: 34.07	1 S. Flow-3 αSMA CT: 28.49	1 S. Flow-3 TGFB1 CT: 38.4	1 0.25 OSI-3 αSMA CT: 34.62	1 0.25 OSI-3 TGFB1 CT: Undete.	1 0.50 OSI-3 αSMA CT: 33.25	1 0.50 OSI-3 TGFB1 CT: 37.14
E	1 Static-3 αSMA CT: 28.21	1 Static-3 TGFB1 CT: 34.96	1 S. Flow-3 αSMA CT: 24.65	1 S. Flow-3 TGFB1 CT: 37.05	1 0.25 OSI-3 αSMA CT: 29.66	2 0.25 OSI-3 TGFB1 CT: Undete.	1 0.50 OSI-3 αSMA CT: 26.43	1 0.50 OSI-3 TGFB1 CT: 36.79
F	1 Static-3 αSMA CT: 28.05	1 Static-3 TGFB1 CT: 35.84	1 S. Flow-3 αSMA CT: 26.81	1 S. Flow-3 TGFB1 CT: 38.97	1 0.25 OSI-3 αSMA CT: 31.56	0.25 OSI-3 TGFB1 CT: 37.12	1 0.50 OSI-3 αSMA CT: 29.71	1 0.50 OSI-3 TGFB1 CT: 34.25

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: 41 Wells Omitted by Analysis: 0 Samples Used: 4 Targets Used: 4

Home DH PASMC 04-23-2019 run 2.eds

Local Instrument : connected.

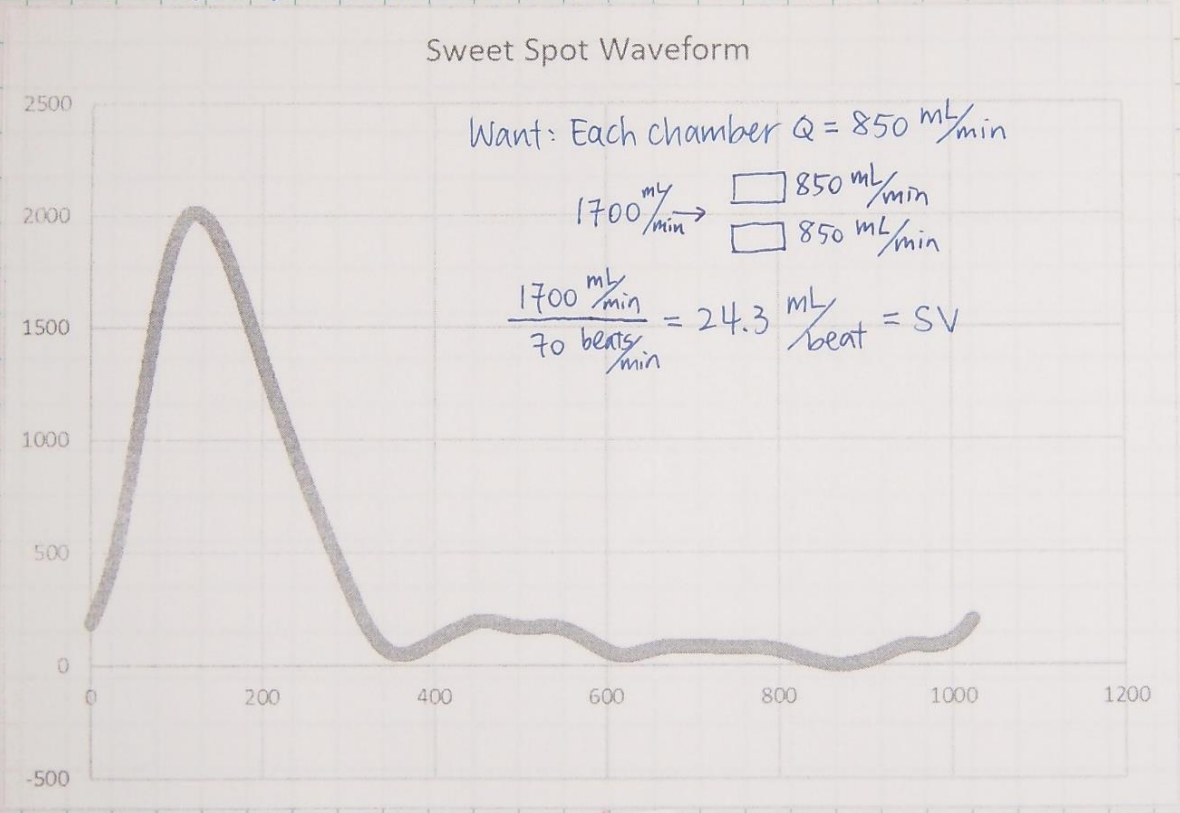
start StepOne™ Software ...

Signed Date Signed Date 4/23/2019

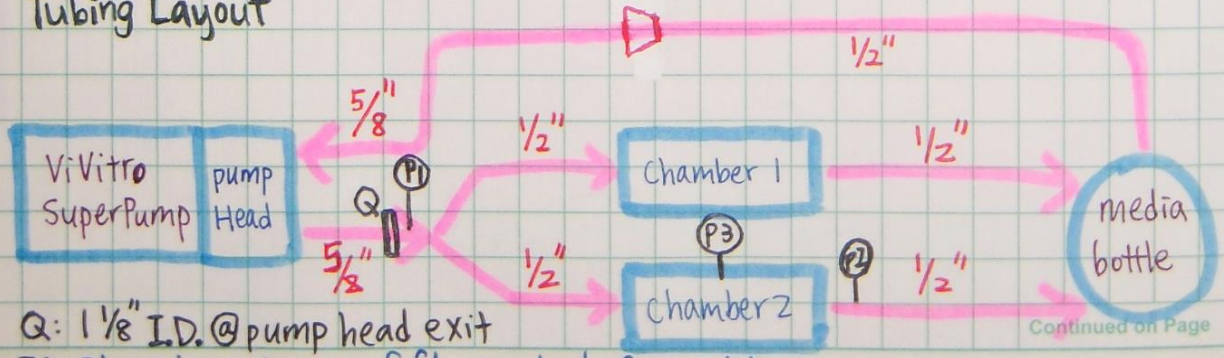
4/24/2019: Extracted HBMSC RNA, quantified below

GROUP	CONCENTRATION (ng/uL)
Static	629.4
Steady Flow	208.8
0.25 OSI	95.0
0.50 OSI	38.2

4/25/2019: HAEC passaged P5 → P6
Collected P9 HAEC in TRIzol (static group)
Vivitro calibration for the bioreactor experiment
Pressure & flow validation



Tubing Layout



Q: 1 1/8" I.D. @ pump head exit
P1: Blue: downstream of flow probe before split
P2: Red: Bioreactor outlet
P3: Yellow: Inside bioreactor chamber

Continued on Page

Read and Understood By

Signed | Date | Signed | 4/24/2019 Date

4/27/2019: HAEC cell count (4 T75 flasks)

140	148
128	139

AVG: 138.75×10^4 cells/mL
1387500 cells/mL

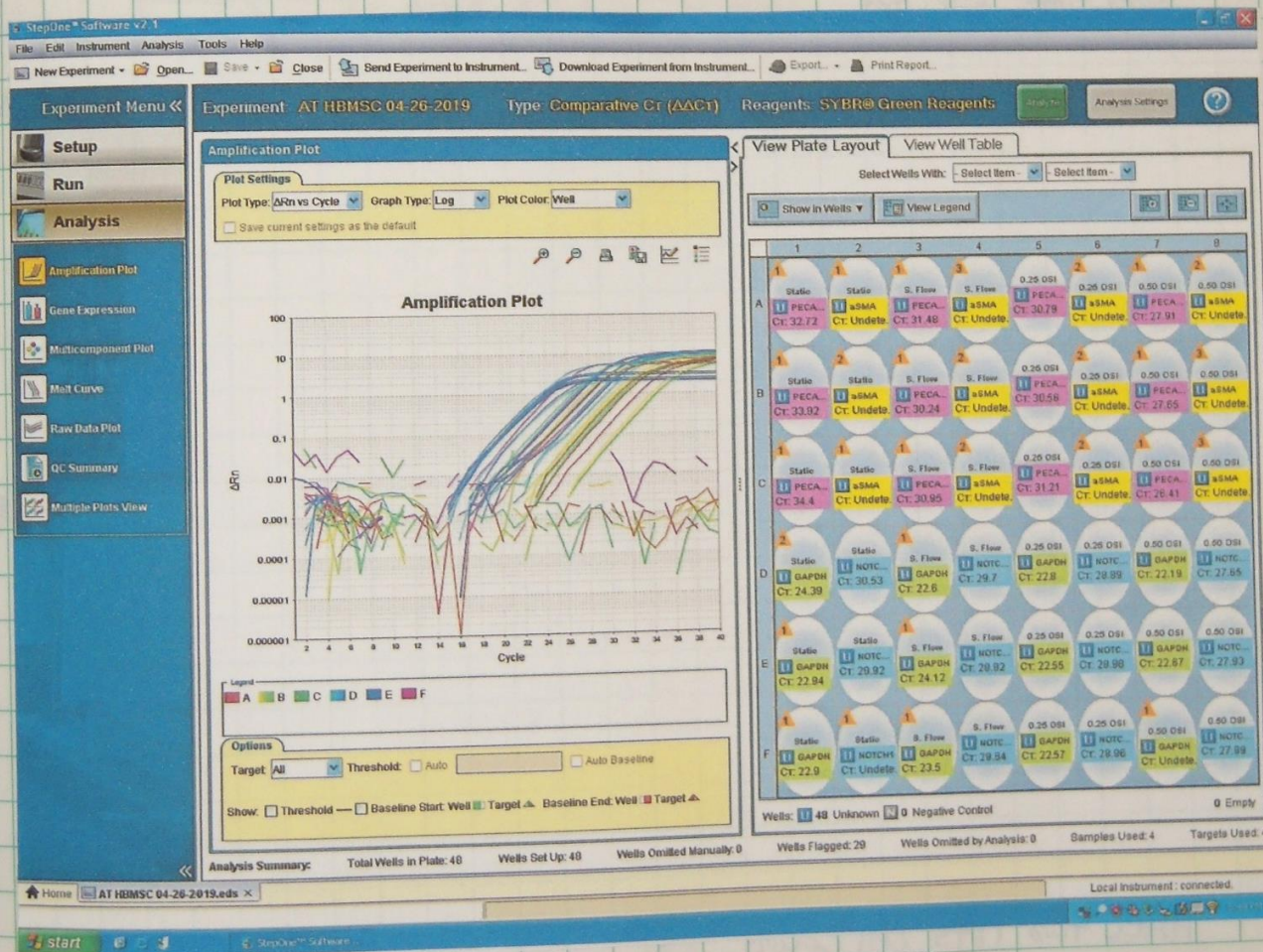
Want: 200,000 cells per channel

200,000 cells/channel = 1387500 V

$V = \frac{200,000}{1387500} = 0.144$ mL Seed 150 uL per channel

Seeded the rest in two T175's

HAEC cell passage P6 → P7 (2 T75's → 4 T75's)
Froze 2 T75's in 4 vials, placed 4 vials in -80°C for 1 hr before transferring them to liquid N_2. (P7)



HBMSC qPCR results (RNA from 04/24/2019)
20 ng of RNA in each well was used

Continued on Page

Read and Understood By

4/27/2019
Date

Signed

Date

Signed

4/28/2019: 10:00am started HAEC 0.25 OSI (2 samples)
8:30 pm paused, re-distributed media, resumed @ 8:35 pm
Started bioreactor experiment: 2 chambers
↙ HBMSC's in PGA-PLLA ×3
↘ PSIS ×3
Plan to run bioreactor for 14 days.

4/29/2019: 10:00am paused flow, redistributed wells, resumed flow
HAEC counted two flasks

81	90
110	97

AVG: 94.5×10^4 cells/mL

200,000 cells = 945000 cells/mL × V

$V = \frac{200,000}{945000} = 0.2116$ mL Seeded 220 µL per channel

Seed time: 7:45pm
HAEC changed media (P7): two T175 + two T75 flasks
Paused 0.25 OSI flow @ 8:00pm, redistributed media, resumed @ 8:05pm.
Fixed leakage in bioreactor chamber w/ putty & parafilm
Added media 200 mL (10% FBS + 2% anti-anti)
Requantified RNA samples from page 60

HBMSC Group	Concentration (ng/µL)	
Static	574.0	600.0
Steady Flow	183.1	192.4
0.25 OSI	100.0	
0.50 OSI	136.0	144.7

4/30/2019: 10:00am stopped 0.25 OSI flow, saved media. Collected 2 samples in TRIzol.
Placed media & TRIzol RNA in −80 °C
5:30pm Start 0.50 OSI
Cleaned bioreactor chamber leaks & added 200mL media

5/1/2019: 9:45am paused flow, redistributed media, resumed flow @ 9:50am
HAEC passage P7 → P8 (2 T75 → 4 T75)
HBMSC (old batch) changed media in 1 T75 flask.
Added 200mL fresh media into bioreactor (10% FBS + 2% AntiAnti)
6:15pm paused flow, redistributed media, resumed flow @ 6:20pm

5/2/2019: 7:30am paused flow, redistributed media, resumed flow @ 7:35am.
HAEC two T175 cell count

77	84
75	105

AVG: 85.25×10^4 cells/mL
Want 200,000 cells per channel
$V = \frac{200,000}{852500} = 0.234$ mL
Seeded 240 µL per channel
plated the rest in 2 T175's (P8)

Continued on Page

Signed Date Signed 4/28/2019 Date

5/2/2019: (con'd) 5:30pm Stopped 0.50 OSI, collected RNA in TRIzol, placed in -80°C.
10:00pm started steady flow (OSI = 0)

5/3/2019: 8:30am paused steady flow, redistributed media, resumed flow 8:35am
HAEC media change (P8)
Unfroze 1 vial of HASMC P5, plated in 1 T75.
8:00pm paused steady flow, redistributed media, resumed flow @ 8:05pm

5/4/2019: 9:50am paused flow, redistributed media, resumed flow @ 9:55am

Endothelial Cell Growth Medium	FBS (10%)	P/S (1%)
Cell Applications	Corning	1989509
Cat# 211-500	REF: 35-015-CV	Fisher Scientific
EXP: 2019/10	LOT: 35015156	EXP: 2019/05
	EXP: 2022/04	

Trypsinized two T175's, counted cells, seeded 8 channels

61	100
64	167

AVG: 98×10^4 cells/mL
980,000 cells/mL

$V = \frac{200{,}000}{980{,}000} = 0.204$ mL

seeded 205 µL per channel

Plated the rest in 4 T75's (P9)
HASMC P5 media change (1 T75)
9:00pm collected steady flow RNA in TRIzol, stored in -80
saved steady flow media, centrifuged & stored in -80 } 2 samples per plate.

5/5/2019: 11:00am started 0.25 OSI.
HASMC P7 changed media
9:00pm paused flow, redistributed media, resumed flow at 9:05pm

5/6/2019: 10:00am paused flow, redistributed media, resumed flow at 10:05am.
Trypsinized 2 T75's HAEC

49	93
43	111

AVG: 74×10^4 cells/mL

$V = \frac{200{,}000}{740{,}000} = 0.270$

seeded 270 µL per channel. Plated the rest in 3 T75's (P9)
Collected static HAEC media
collected RNA in TRIzol (3 samples)
Media for nitric oxide (NO) assay:
1. Fresh media
2. Static in HAEC for 48 hrs
3. steady Flow HAEC for 48 hours
4. 0.25 OSI for 48 hrs
5. 0.50 OSI for 48 hrs
8:30 pm paused flow, redistributed media, resumed flow at 8:35pm

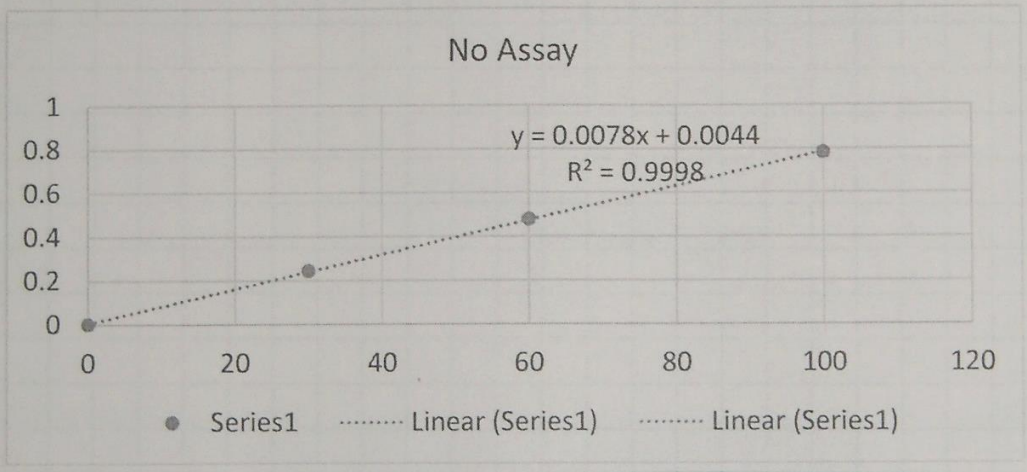
Continued on Page

Read and Understood By

Signed _____ Date _____ Signed _____ Date 5/3/2019

5/7/2019: 10:00am stopped 0.25 OSI flow
Collected 2 RNA samples in TRIzol, placed in -80
started 0.50 OSI at 10:40 am
changed HAEC & HASMC media
NO Assay on the five samples: fresh media, static, steady flow, 0.25 OSI, 0.5 OSI

	1	2	3	4	5	6	7	8
A	0.047	0.047	0.046	0.047	0.047	0.046	0.047	0.046
B	0.047	1.005	0.7	0.465	0.222	0.046	0.046	0.046
C	0.047	0.046	0.046	0.047	0.047	0.046	0.046	0.046
D	0.046	0.324	0.311	0.315	0.312	0.317	0.046	0.046
E	0.046	0.046	0.046	0.046	0.046	0.046	0.046	0.047
F	0.047	0.047	0.046	0.047	0.046	0.046	0.047	0.047
G	0.047	0.047	0.046	0.047	0.046	0.046	0.046	0.046
H	0.046	0.048	0.048	0.047	0.046	0.047	0.046	0.047



Standards	Absorbance	Concentration
A	0.783	100
B	0.478	60
C	0.243	30
D	0	0

Samples	Absorbance	Concentration(μM)	NO (mg/dl)
Media	0.324	13.07692	0.03927
Static	0.311	11.41026	0.034265
Steady flow	0.315	11.92308	0.035805
0.25 OSI	0.312	11.53846	0.03465
0.5 OSI	0.317	12.17949	0.036575

Continued on Page

10:00pm paused flow (0.5 OSI), redistributed media, resumed at 10:05pm.

Read and Understood By

5/7/2019

Signed | Date | Signed | Date

5/8/2019: 10:00am paused flow, redistributed media, resumed flow at 10:05am.
6:00pm paused flow, redistributed media, resumed flow at 6:05pm.

5/9/2019: 11:00am Stopped 0.50 OSI flow, collected RNA in TRIzol, collect spent media, stored everything in -80.
HAEC media change (P9) - 3 flasks

5/10/2019: HAEC media change (P9) - 4 flasks
HASMC passage P5 → P6

5/12/2019: HAEC cell count (3 T75's)

47	78
43	89

AVG: 64.25×10^4 cells/mL

$V = \frac{200{,}000}{642500} = 0.311$ mL. Seeded each channel with 315 uL.

Seed time: 11:40am

Placed the rest in TRIzol as static EC sample.

5/13/2019: HAEC started steady flow @ 12 noon
Conditioned HASMC in 0.25 & 0.50 OSI. Started at 1pm.
Stopped bioreactor, placed 3 PSIS & 3 PGA-PLLA in TRIzol & formalin
3 PSIS (static) in TRIzol & formalin (10%)
6:00pm paused flow, redistributed volumes, resumed flow at 6:05pm

5/14/2019: 10:00am paused flow, redistributed volumes, resumed flow at 10:05am.
Unfroze 1 vial of HASMC P5 from liq. N_2.
HAEC cell count:

58	74
66	79

AVG: 69.25×10^4 cells/mL

$V = \frac{200{,}000}{692500} = 0.2888$ mL

Seeded 290 uL per channel at 7:00pm.
Paused steady flow at 7:00pm, redistributed media, resumed at 7:10pm.

5/15/2019: 9:30am paused flow, redistributed media, resumed flow @ 9:35am
HASMC changed media. Collected 0.25 & 0.50 OSI conditioned HASMC in TRIzol in -80 at 1pm. Stopped steady flow @ 1pm. and started 0.25 OSI flow on HAEC's.
7:30pm paused flow, redistributed wells, resumed flow at 7:35pm.

5/16/2019: 10:00am paused flow, redistributed media, resumed flow at 10:05am.
Trypsinized 2 T75 HAEC flasks

40	69
57	113

AVG: 69.75×10^4 cells/mL.

$V = \frac{200{,}000}{697500} = 0.2867$ mL.

Seeded 8 channels of Bioflux at 290 uL per channel.

Continued on Page

Trypsinized 1 T75 HBMSC and conditioned in 50% 0.25 OSI + DMEM in one T25 and 50% 0.50 OSI + DMEM in another T25.
Start time: 12-noon.
Paused and redistributed media at 6:15pm (0.25 OSI)

Read and Understood by _____ 5/8/2019
Signed Date

Signed Date

5/17/2019: 10:30am paused flow, redistributed media, resumed flow at 10:35am. 1:00pm stopped 0.25 OSI flow. Collected media and EC RNA in TRIzol. Stored media & RNA in -80. Started 0.50 OSI at 2:00pm.

5/18/2019: 12:00 noon paused flow, redistributed media, resumed flow (0.50 OSI) Collected 0.25 OSI & 0.50 OSI conditioned HBMSC in TRIzol. Stored in -80.

5/19/2019: 11:00am paused flow, redistributed media, resumed flow. 5:00pm: collected 0.50 OSI HAEC in TRIzol. Collected spent media. Stored RNA & media in -80.

5/21/2019: RNA Extraction & quantification (for RNA Seq.)

TUBE #	Cell Type	Flow Group	Nanodrop (ng/μL) Concentration		AVG (ng/μL) Concentration	A260	A260/A280
S1	HAEC	Static	18.2	16.0	17.1	0.399	2.06
S2	HAEC		27.1	24.8	25.95	0.619	2.18
S3	HAEC		154.4	178.1	166.25	3.859	1.84
S4	HASMC		59.5	31.2	45.35	1.486	1.84
S5	HASMC		19.7	19.2	19.45	0.493	1.89
S6	HASMC		38.9	47.7	43.3	1.193	2.03
F1	HAEC	Steady Flow	146.7	182.0	164.35	3.668	2.09
F2	HAEC		36.6	35.4	36.0	0.915	2.12
F3	HAEC		187.5	188.0	187.75	4.700	2.14
F4	HASMC		268.1	252.8	260.45	6.321	1.86
F5	HASMC		138.0	122.2	130.1	3.054	1.89
F6	HASMC		50.0	32.6	41.25	0.814	1.75
X1	HAEC	0.25 OSI	110.0	62.3	86.15	2.749	3.40
X2	HAEC		103.9	163.4	133.65	4.086	1.79
X3	HAEC		41.9	18.4	30.15	1.046	2.00
X4	HASMC		634.8	612.1	623.45	15.304	1.88
X5	HASMC		305.2	323.1	314.15	8.077	1.89
X6	HBMSC		381.1	408.1	394.6	7.239	1.90
Y1	HAEC	0.50 OSI	503.5	385.5	444.5	6.786	1.87
Y2	HAEC		801.3	703.0	752.15	17.575	1.92
Y3	HAEC		206.8	171.6	189.2	4.291	1.86
Y4	HASMC		595.6	575.7	585.65	14.890	1.92
Y5	HASMC		545.0	469.9	507.45	13.625	1.92
Y6	HBMSC		342.0	350.6	346.3	8.764	1.87

5/22/2019: Delivered the above 24 samples to UM for RNASeq.
1501 NW 10th Ave (BRB 627)
Miami, FL 33136
William Hulme (305) 243-8718 & (305) 213-7799
Margaret Estevez (305) 778-6690

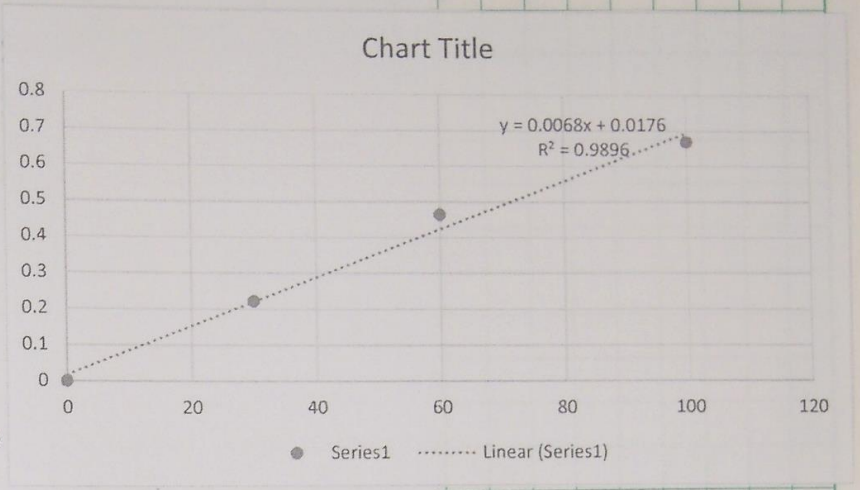
Continued on Page

Read and Understood By
Signature
Date
Signed
5/17/2019
Date

5/23/2019: No Assay: Static, Steady Flow, 0.25 OSI, 0.50 OSI media collected from 5/12 ~ 5/17. Added fresh media as control. Assay performed by MSH.

Software Version 3.00.19

Experiment File Path:
Protocol File Path:
Plate Number Plate 1
Date 5/23/2019
Time 11:35:39 AM
Reader Type: Synergy HTX
Reader Serial Number: 16092729
Reading Type Reader



Procedure Details

Plate Type 96 WELL PLATE
Eject plate on completion
Shake Linear: 0:05 (MM:SS)
Frequency: 567 cpm (3 mm)
Read Absorbance Endpoint
Full Plate
Wavelengths: 540
Read Speed: Normal, Delay: 100 msec, Measurements/Data Point: 8

Results

Actual Temperature: 20.8

	1	2	3	4	5	6	7	8
A	0.046	0.047	0.046	0.046	0.047	0.046	0.047	0.046
B	0.046	0.832	0.628	0.386	0.164	0.046	0.046	0.046
C	0.047	0.046	0.046	0.046	0.047	0.046	0.046	0.046
D	0.046	0.283	0.307	0.294	0.284	0.046	0.231	0.046
E	0.046	0.046	0.046	0.046	0.046	0.046	0.046	0.047
F	0.047	0.046	0.046	0.046	0.046	0.046	0.046	0.046
G	0.047	0.047	0.046	0.046	0.046	0.046	0.046	0.046
H	0.046	0.048	0.048	0.047	0.046	0.047	0.046	0.046

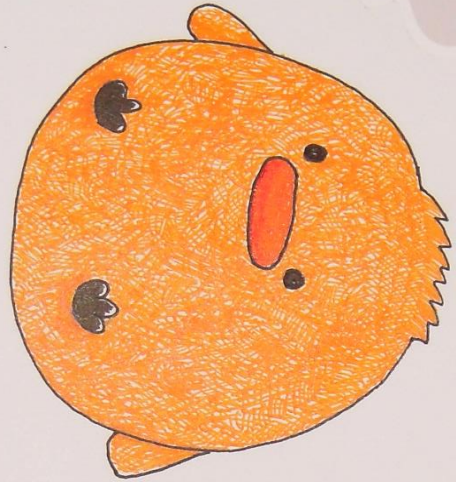
Standards	Absorbance	Concentration
A	0.668	100
B	0.464	60
C	0.222	30
D	0	0

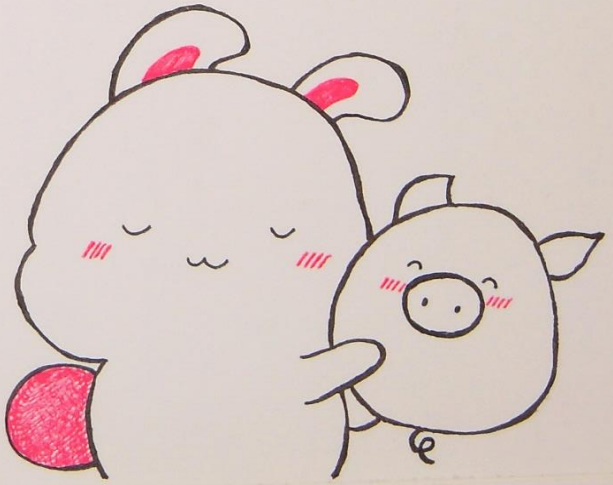
Samples	Absorbance	Concentration(μM)	NO (mg/dl)
Media	0.231	9.852941	0.029588
Static	0.284	17.64706	0.052994
Steady flow	0.294	19.11765	0.05741
0.25	0.307	21.02941	0.063151
0.5	0.283	17.5	0.052553

Continued on Page

Read and Understood By

Signed _____ Date _____ Signed _____ Date 5/23/2019

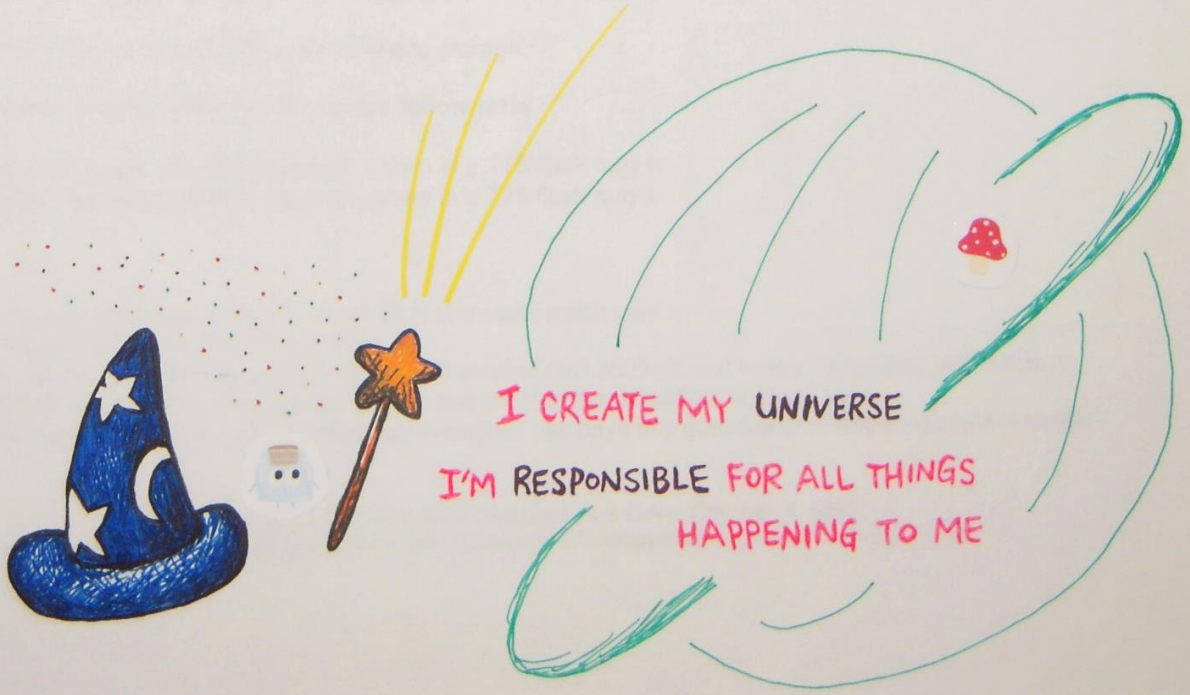




DENISE HSU


Ho[oPono]²
對不起 I'm Sorry
請原諒我 please forgive me
謝謝 Thank you
我愛你 I love you

TABLE OF CONTENTS



PROTOCOLS

Valve Endothelial Cell (VEC)
Valve Interstitial Cell (VIC)
Isolation

1. Sterile collagenase solution — no FBS OR FGRO
   10~18mg powdered Type II collagenase in 10mL of media (DMEM + 1% P/S)
   Sterilize solution with a 0.2μm syringe filter
   Place solution in conical tube (15mL or 50mL), label and store in fridge.


type II collagenase


DMEM


FBS/FGro

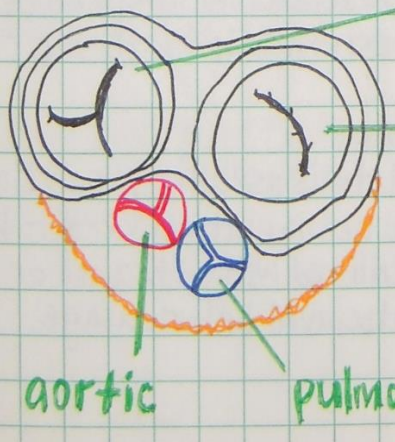
Fetal Bovine Serum
Fetalgro

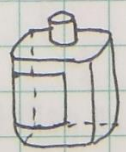

P/S (antibiotics)
penicillin/streptomycin
or anti-anti (antibiotic-antimycotic)

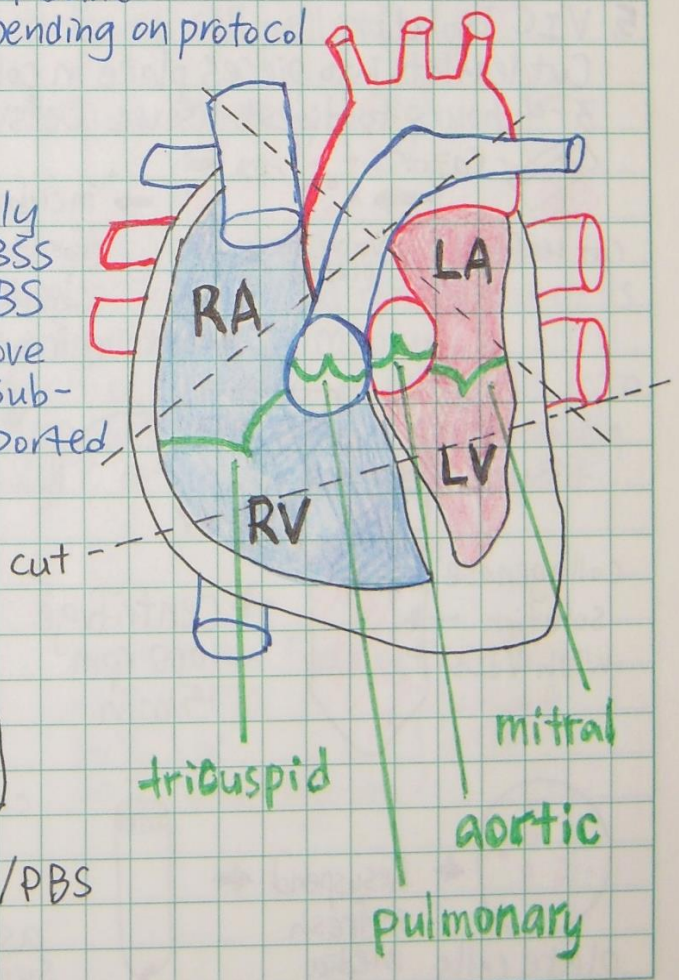
2. Cell medium (for culturing)
   Add 50mL (10%) of sterile FBS and 5mL (1%) of P/S into 500mL of sterile DMEM (Dulbecco's Modified Eagle Medium) with:
   - high glucose
   - high L-Glutamine
   - no sodium pyruvate

   ] check label on bottle can vary depending on protocol

3. Heart valve harvest
   Aortic root was excised immediately after sacrifice, washed with cold HBSS (Hank's Balanced Salt Solution) or PBS (Phosphate buffered saline) to remove blood. Aortic root with valve was submerged in cold HBSS / PBS and transported back to lab hood on ice.

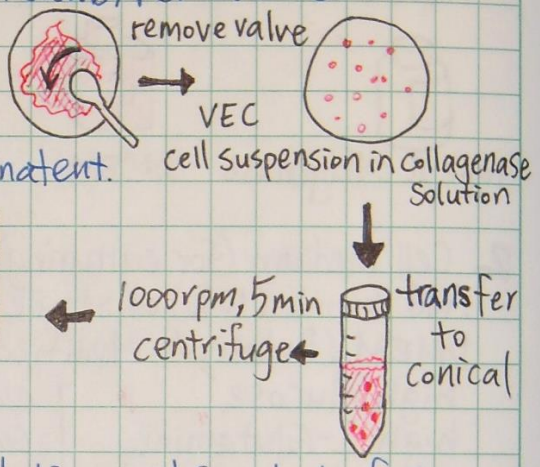



HBSS/PBS



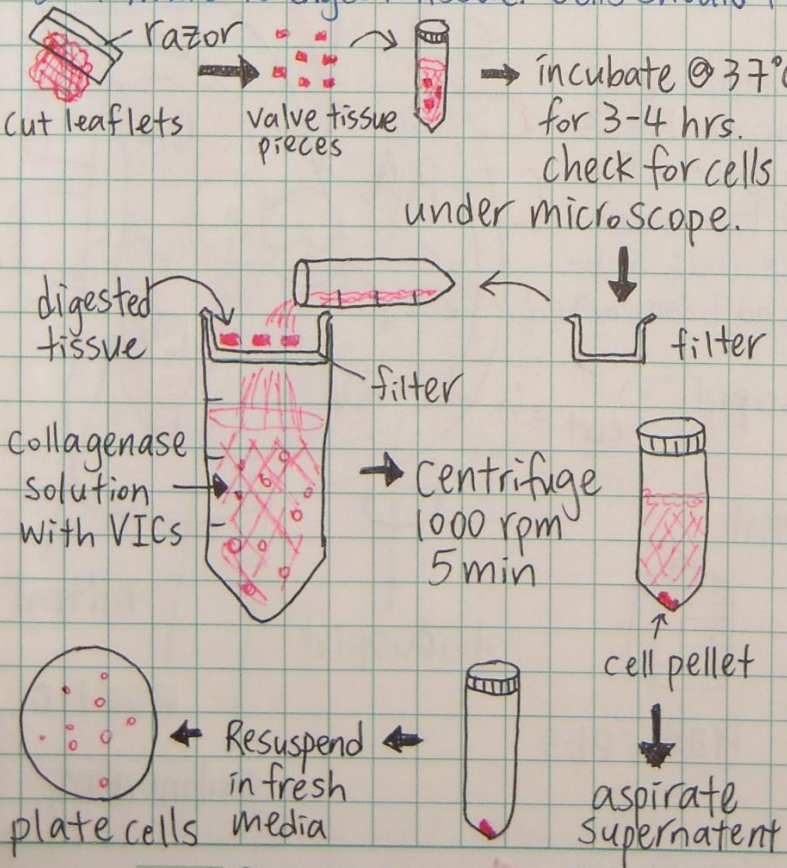
4. VEC Isolation

Cut leaflets from tissue, wash with sterile PBS. Pin leaflets down using needles. Add collagenase solution to cover leaflets. Incubate for 10 minutes at 37°C. Using a dry, sterile cotton swab, remove the endothelial cell layer with rotating motion. Collect cell suspension in collagenase solution in conical tube, centrifuge @ 1000 rpm for 5 minutes to pellet the cells. Aspirate supernatent. Resuspend cells in fresh media and plate the cells. Perform the steps above on both sides of leaflets. (fibrosa and ventricularis)



5. VIC Isolation

Cut leaflets into pieces place in collagenase solution and incubate for 3-4 hours to digest tissue. Cells should float in solution after incubation. Pippette 100 µL to check floating cells under microscope. Using a cell filter, separate digested tissue from cells and remove media (collagenase solution) with cells into new tube. Centrifuge the new tube to pellet cells. Aspirate supernatent. Resuspend cells in fresh media. Plate the cells.



* Note:
All tools used for tissue cutting and cell isolation need to be sterilized with ethanol or autoclaved before usage.

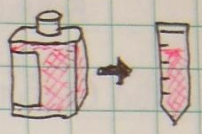
Cell Media Change, Cell Passaging, Cell Freezing

Cell Media Change

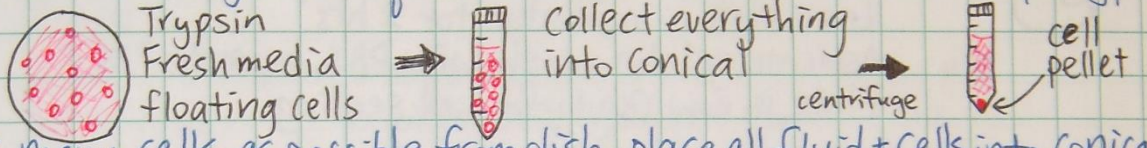
Aspirate old media, add fresh media.

Serine protease used for cell detachment → 0.05% Trypsin aliquotted

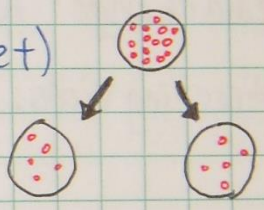
Cell Passaging (Subculture)



Aspirate old media, add 3-5 mL HBSS, aspirate HBSS.
Add 3-5 mL trypsin, incubate in trypsin for 5 minutes.
Observe under microscope to see if cells are suspended.
Once cells are floating, add equal volume of fresh media to stop trypsin activity.



Collect as many cells as possible from dish, place all fluid + cells into conical.
Centrifuge conical at 1500 rpm for 5 minutes.
Aspirate supernatent (make sure not to touch cell pellet)
Add fresh media to resuspend cells.
Plate the resuspended cells equally into two dishes.
Incubate at 37 °C, 5% CO_2.



Cell Freezing

Recovery Cell Culture Freezing Medium] contains DMSO

After centrifuging with Trypsin/Media, aspirate supernatent and resuspend cells with 1 mL of freezing medium.
Transfer freezing medium with cells into screw top vials. Store cells immediately into -80 °C or liquid nitrogen. **

↑ proper storage

DMSO: Dimethyl sulfoxide
prolonged DMSO exposure at room temperature can kill the cells. **

Classic Freezing Medium Recipe:
- High-glucose DMEM
- 10% FBS
- 10% DMSO

Cell Biologics Freezing Media Recipe: (catalog # 6916)
- 10% DMSO
- 50% FBS
- 40% Culture medium

Endothelial Cell Culture (Vascular or Valvular)

Continued on Page

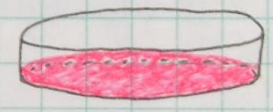
Media: Porcine Endothelial Cell Growth Medium
(Pig) Cat No. P211-500
Brand: Cell Applications, Inc.

Gelatin Coating: Gelatin Solution
2% gelatin in H_2O, Type B
Jello-like in fridge, place in room temperature to liquidify
Pipette 1 mL of gelatin into 5cm plate, cover all surfaces
Leave plate in incubator for 2 hrs
Aspirate gelatin, wash with PBS, aspirate PBS
Plate is now ready for cell seeding.

Mediachange: Aspirate old media, put 3mL new media into 5cm plate

Unfreezing vials: Use 10 cm plate, 10mL media

5cm plate for culturing

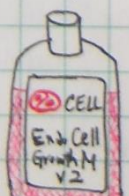


10cm plate to neutralize DMSO

Centrifuge: Use 1000 RPM for 5 minutes

Media: Human Endothelial Cell Media - Endothelial Cell Growth Medium V2
(Human) Cat No: 213-500 (EMG V2)
Enriched w/growth factors for accelerated growth and includes VEGF, FBS, EGF, bFGF, IGF, Ascorbic acid, hydrocortisone, heparin

211-500
No VEGF



213-500

Brand: Cell Applications, Inc.

CELL APPLICATIONS, INC.

Use with: Human
aortic endothelial cells
femoral artery endothelial cells
pulmonary artery endothelial cells
umbilical vein endothelial cells
valve endothelial cells

Vascular Smooth Muscle Cell Culture
Valve Interstitial Cell Culture

Media: Smooth Muscle Cell Media 2 (Ready-to-use)
(Human) Catalog Number: C-22062
Brand: PromoCell

500 mL
Basal Medium

Vial of SupplementMix

Add: 1% of P/S

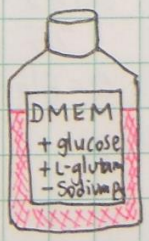
Use with:
Human umbilical artery smooth muscle cells
coronary artery smooth muscle cells
pulmonary artery smooth muscle cells
aortic smooth muscle cells
bronchial smooth muscle cells
tracheal smooth muscle cells
uterine smooth muscle cells

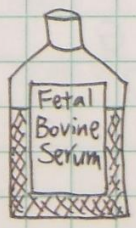
Media: Dulbecco's Modified Eagle Medium (DMEM) with
(Human or Pig) high glucose
high L-glutamine
no Sodium pyruvate
] check bottle label and protocol

Add: 10% FBS
1% P/S

Use with:
valve interstitial cells

500 mL
DMEM





10%
FBS

1%
P/S

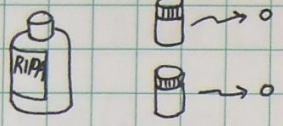
BCA Assay (Protein Quantification) - Microplate

Bicinchoninic acid Assay

Read and Understood By

Continued on Page

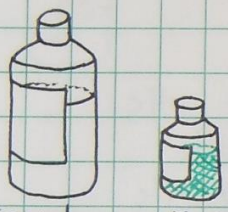
Complete RIPA Solution:



10 mL RIPA buffer
1 tablet of protease inhibitor
1 tablet of phosphatase inhibitor
] Vortex well!

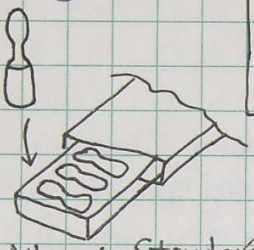
Pierce BCA Protein Assay Kit:

Thermo SCIENTIFIC

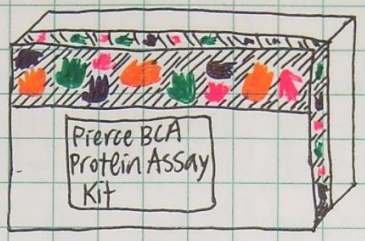


500 mL Reagent A

25 mL Reagent B



Albumin Standards @ 2 mg/mL each
Box of 10



Contents:
BCA Reagent A
BCA Reagent B
Albumin Standards

Preparation of Standards and Working Reagents (WR)

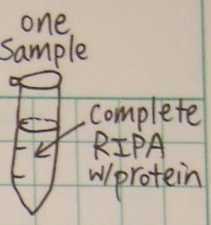
Microplate Procedure

vial	Volume of diluent (uL)	Volume & source of BSA (uL)	final BSA concentration (ug/mL)	
A	0	300 of stock	2000	
B	125	375 of stock	1500	
C	325	325 of stock	1000	
D	175	175 of B	750	
E	325	325 of C	500	9 standards
F	325	325 of E	250	
G	325	325 of F	125	
H	400	100 of G	25	
I	400	0	0 = Blank	

diluent: Complete RIPA Solution
define Samples
determine total volume of working reagents:

(# standards + # unknowns) × (# replicates) × (Volume of WR per sample) = total volume of WR required

Volume of WR per sample: 200 uL (microplate)



Example: 12 samples, find total volume of WR required
(one sample = one eppendorf tube w/RIPA solution & protein)

#standards = 9
#unknowns = 12 + 1 Note: add one extra to compensate for volume loss
#replicates = 3
Volume of WR per sample = 200 µL

Total WR required = (9+13) × 3 × 200 µL = 13200 µL = 13.2 mL

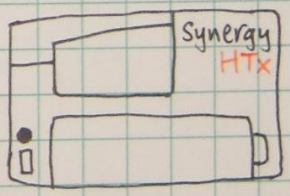
WR: 50 parts of Reagent A
1 part of Reagent B] A:B = 50:1

$\frac{13200}{51} = 259$ (Reagent B) 13200 − 259 = 12941 (Reagent A)

WR: **12.941** mL of Reagent A
0.259 mL of Reagent B] mix well

1. Pipette 25 µL of each standard or unknown sample replicate into a microplate well.
2. Add 200 µL of WR to each well, mix thoroughly on shaker for 30 seconds.
3. Cover plate and incubate at 37°C for 30 minutes.
4. Cool plate to RT, measure absorbance at 562 nm on plate reader

Using the Plate Reader



1. Place plate into Synergy HTX multimode reader
2. On the computer, open Gen5 3.00
3. New


Icon on desktop

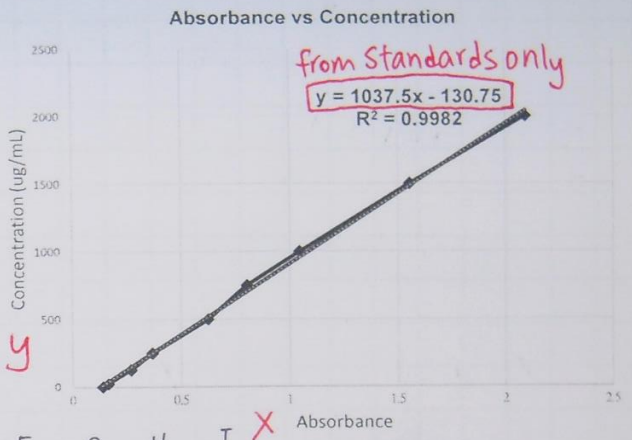
↓
Shake linear for 30 seconds
↓
Shake orbital for 30 seconds
↓
Read @ 562 nm
↓
Save, export to excel

Software Version 3.00.19

Experiment File Path: C:\Users\Public\Documents\Experiments\BCA Assay 09.17.2018 cell gradient 12 sampes.xpt
Protocol File Path:
Plate Number Plate 1
Date 9/17/2018
Time 11:26:57 AM
Reader Type: Synergy HTX
Reader Serial Numbe Unknown
Reading Type Reader

Procedure Details

Plate Type 96 WELL PLATE
Eject plate on completion
Shake Orbital: 0:30 (MM:SS)
Frequency: 282 cpm (3 mm)
Shake Linear: 0:30 (MM:SS)
Frequency: 567 cpm (3 mm)
Read Absorbance Endpoint
Full Plate
Wavelengths: 562
Read Speed: Normal, Delay: 100 msec, Measurements/Data Po



Results

Actual Temperature: 19.7

Standards: A B C D E F G H I

	1	2	3	4	5	6	7	8	9	10	11	12	
A	2.145	1.573	1.061	0.817	0.658	0.375	0.275	0.163	0.135	0.047	0.047	0.046	562
B	2.105	1.571	1.062	0.793	0.618	0.369	0.272	0.158	0.136	0.045	0.046	0.046	562
C	2.03	1.529	1.017	0.802	0.6	0.356	0.257	0.168	0.14	0.061	0.061	0.059	562
D	0.06	0.059	0.058	0.056	0.055	0.054	0.053	0.052	0.052	0.053	0.055	0.058	562
E	0.303	0.341	0.22	0.256	0.204	0.25	0.216	0.24	0.194	0.226	0.249	0.193	562
F	0.325	0.358	0.245	0.279	0.224	0.265	0.225	0.238	0.198	0.217	0.246	0.186	562
G	0.332	0.365	0.241	0.271	0.244	0.261	0.243	0.26	0.206	0.232	0.256	0.198	562
H	0.057	0.061	0.063	0.063	0.067	0.068	0.069	0.071	0.072	0.075	0.077	0.081	562

Averages of three

Samples: 1 2 3 4 5 6 7 8 9 10 11 12

Graph this
Get equation (linear relationship)

Standards	Absorbance	Concentration (ug/mL)
A	2.093333333	2000
B	1.557666667	1500
C	1.046666667	1000
D	0.804	750
E	0.625333333	500
F	0.366666667	250
G	0.268	125
H	0.163	25
I	0.137	0

obtained from plate reader

Averages of three

Concentration = 1037.5(absorbance) - 130.75

Samples	Absorbance (avg)	Concentration (ug/mL)	Volume of protein (uL)	Volume of RIPA (uL)
1	0.32	201.25	10.255	19.745
2	0.354666667	237.22	8.7004	21.3
3	0.235333333	113.41	18.199	11.801
4	0.268666667	147.99	13.946	16.054
5	0.224	101.65	20.304	9.6963
6	0.258666667	137.62	14.997	15.003
7	0.228	105.8	19.507	10.493
8	0.246	124.48	16.581	13.419
9	0.199333333	76.058	27.135	2.8646
10	0.225	102.69	20.099	9.9014
11	0.250333333	128.97	16.003 (*)	13.997
12	0.192333333	68.796	30	0

Choose the lowest concentration, set volume of protein at 30 uL and volume of RIPA at 0
Find the ratio of the selected lowest concentration to the next sample

$\frac{68.796}{128.97} \times 30 = 16.003$

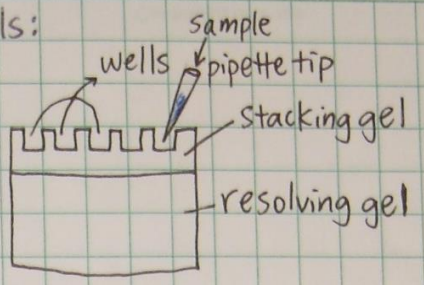
Complete

Volume of protein + Volume of RIPA = 30 uL

Signed Date Signed Date Read and Understood By

Western Blot

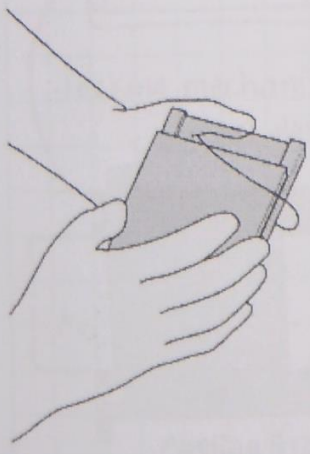
Ingredients for making TWO gels:



STACKING GEL	
H_2O	6.1 mL
30% Acrylamide/Bisacrylamide	1.3 mL
Tris-HCl, 0.5M, pH 6.8	2.5 mL
SDS, 10%	100 µL
TEMED	10 µL
10% APS (ammonium persulphate)	100 µL

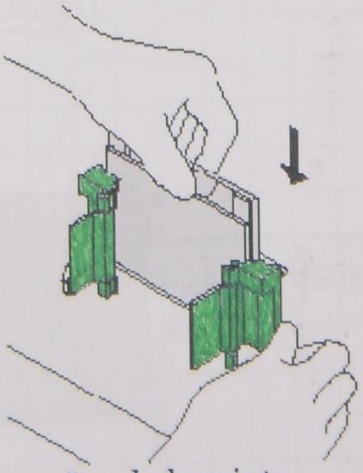
RESOLVING GEL	10%	12%
H_2O	4.1 mL	5.03 mL
30% Acrylamide/Bisacrylamide	3.3 mL	6 mL
Tris-HCl, 1.5M, pH 8.8	2.5 mL	3.75 mL
SDS, 10%	100 µL	150 µL
TEMED	10 µL	7.5 µL
10% APS (ammonium persulphate)	32 µL	75 µL

1.



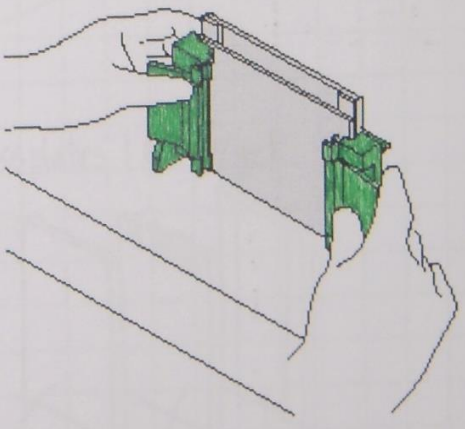
Place glass together

2.



Load glass into casting frame

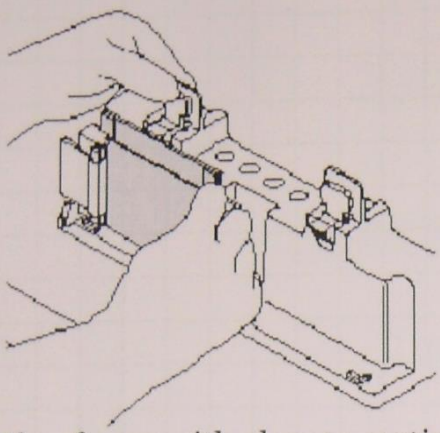
3.



Lock casting frame by pushing pressure cam to the "lock" position

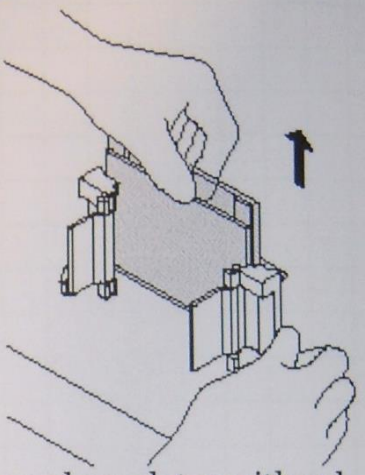
9/20/2018

4.



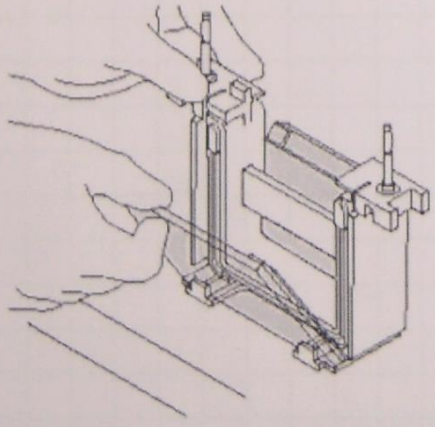
Secure casting frame with glass to casting stand. Each casting stand fits up to two frames. Pour resolving gel between glass plates in each frame up to gel line. Pour a thin layer (200µL) of isopropanol to remove bubbles. Let resolving gel set (20-30 minutes). Remove isopropanol by inverting frame. Pour stacking gel until overflowing. Place gel comb into stacking gel liquid. Let stacking gel set.

5.



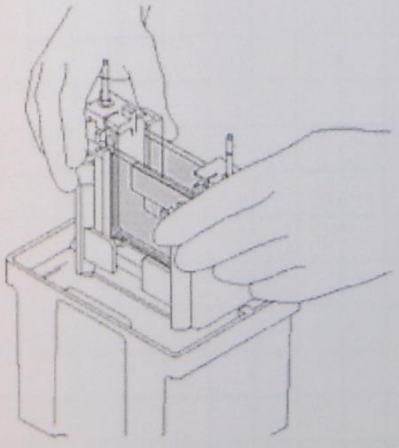
Remove glass plates with gel from casting frame. Do not remove gel comb. Gel in glass plates with comb can be wrapped in soaked paper towels and stored in zip-locked plastic bags in fridge for few days. If gel shrinks, toss the shrunken gel and remake a new one for WB.

6.



Secure glass plates and gel to gasket and electrode assembly.

7.

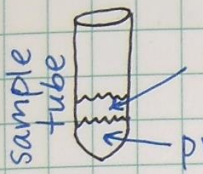


Place electrode assembly in tank. Pour 1x running buffer into electrode assembly, overflow buffer to the designated gel line.

Continued on Page

Loading samples into gel:

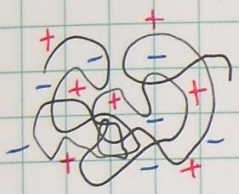
8. Using results from BCA assay, volume of protein is normalized for all samples, and volume of protein + volume of complete RIPA = 30 µL



10 µL of SDS-sample buffer (4X, reducing, blue color)

protein + complete RIPA = 30 µL

] Total = 40 µL



native protein

Heat + reductant + SDS

denatured protein with bound SDS

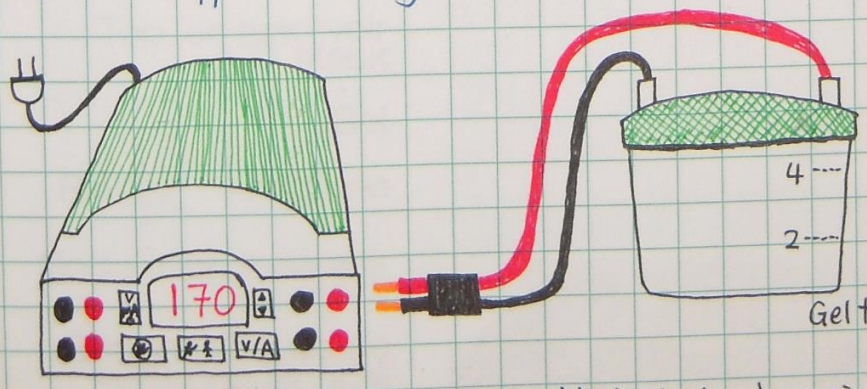
9. Place the 40 µL tubes on hot plate at 100°C for 5 minutes (denature)

10. Centrifuge the tubes

11. Remove gel comb. Pipette 4 µL precision plus protein LADDER into first well

12. Pipette 20 µL of each sample in a well. Each well should be loaded with 20 µL of a sample. Place lid on gel tank

13. Connect gel tank to power, set voltage at 170 V and press RUN. Chamber should start bubbling. Turn power off when bands reach end of glass at bottom. (approximately 30 min - 1 hr)

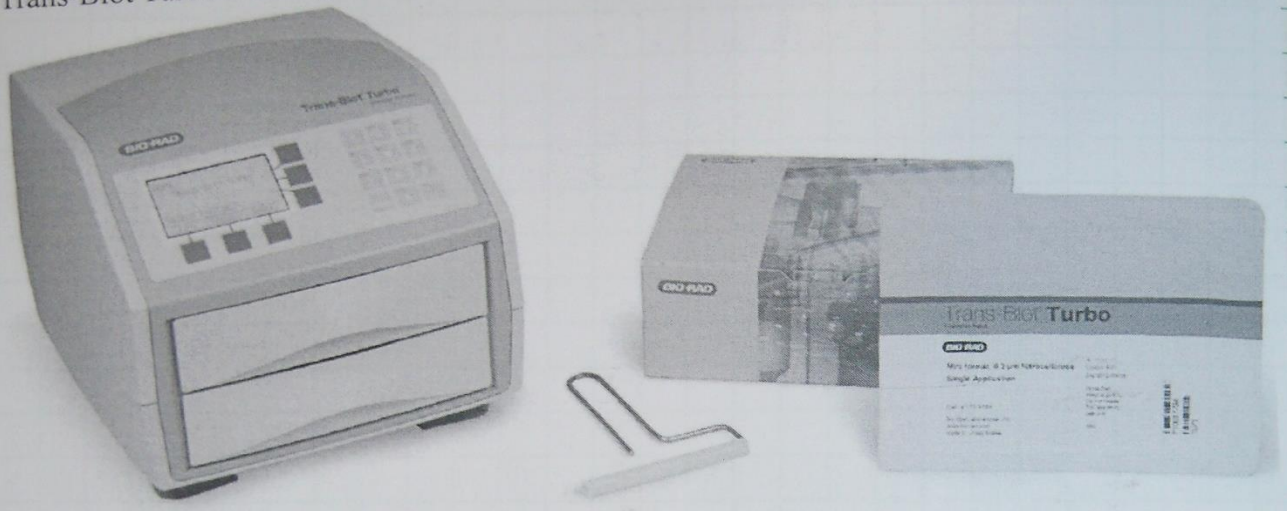


Bio-Rad PowerPac Basic Mini Electrophoresis system

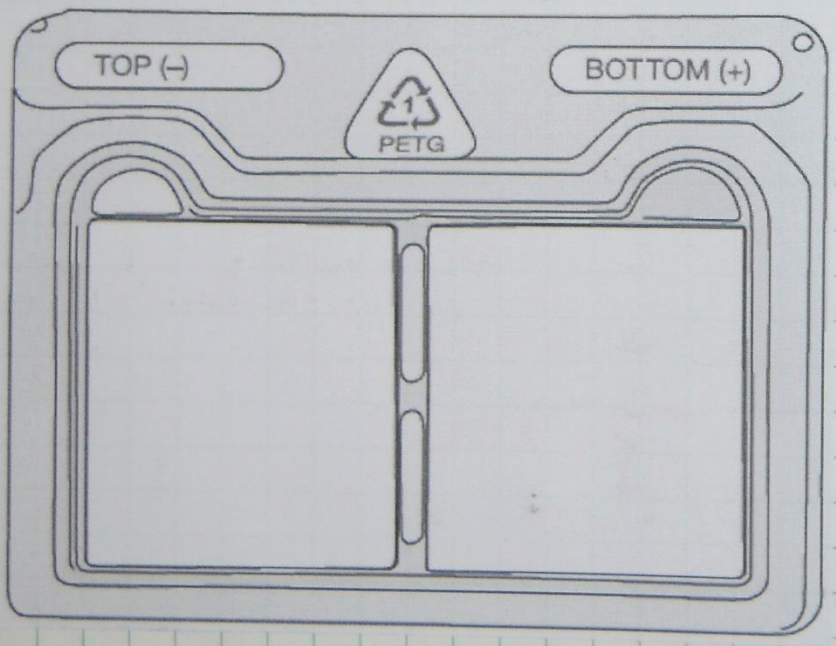
Transferring gel to membrane:

14. Membrane: Trans-Blot Turbo Mini Format, 0.2 um Nitrocellulose

Trans-Blot Turbo Blotting System

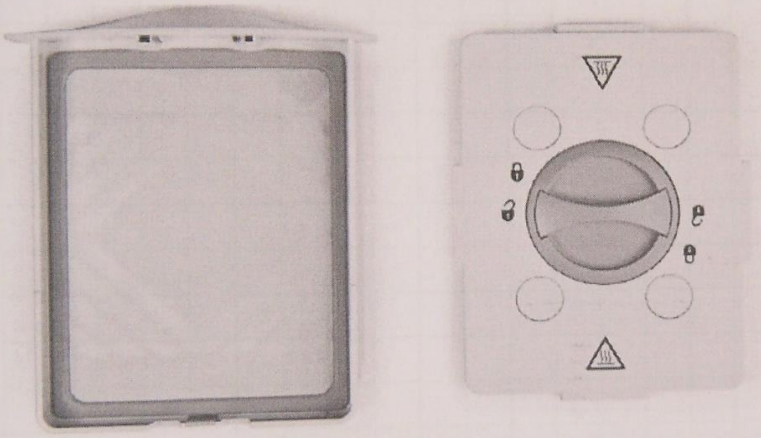


Mini Transfer Pack (Single Mini Gel) Setup

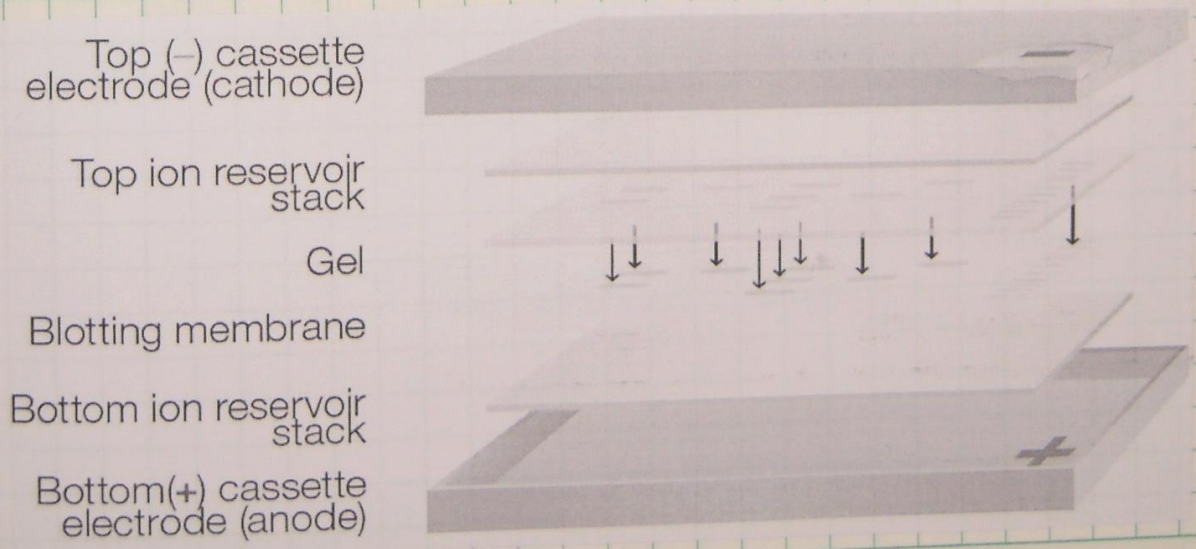


Trans-Blot Turbo Instrument

Cassette Base (left) and Lid (right)

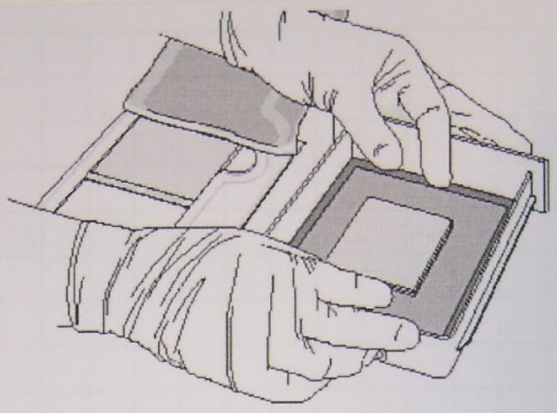


Lid with cathode (-) on the underside, base with anode (+)

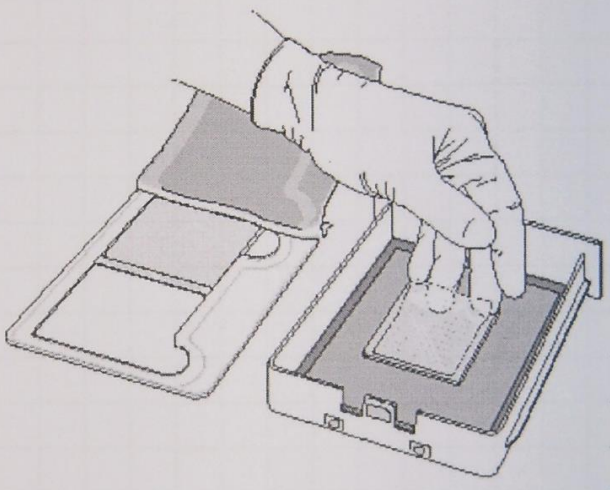


Read and Understood By

a. Lay the ion reservoir stack with the membrane (anode stack) in the center of the cassette base. Ensure that the stack is not overlapping the green rubber molding in the base.

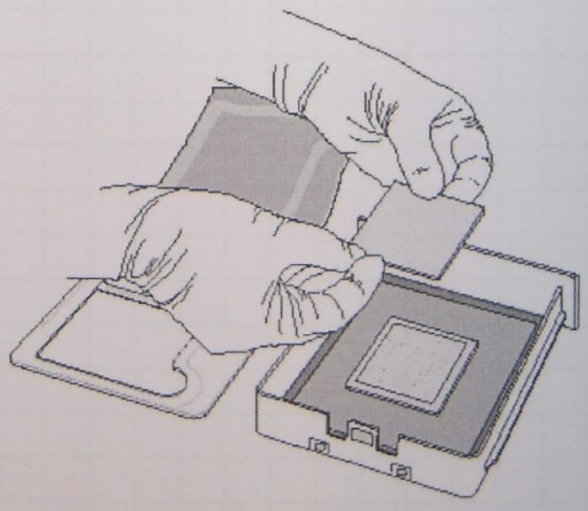


b. Carefully align the gel on the membrane. If necessary, gently use the blot roller to remove air bubbles between the gel and membrane. If transferring two mini gels, place them on the membrane so that the feet of the gels are facing toward each other.



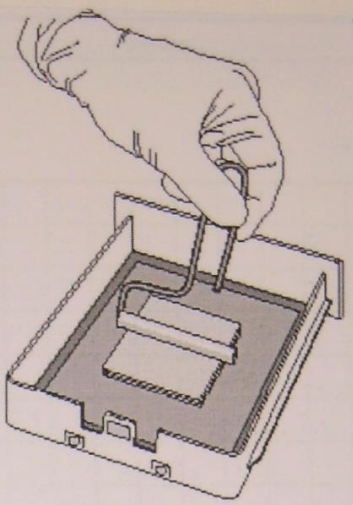
b.

c. Gently place the second ion reservoir stack (cathode stack) on the gel.

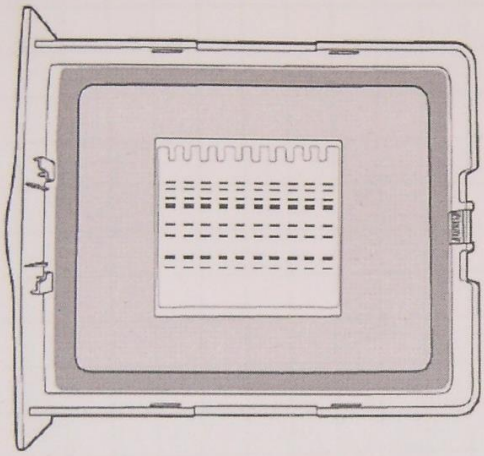


c.

d. Use the blot roller to remove any air bubbles in the assembled transfer pack and provide consistent contact between the layers.



d.



15. Make a sandwich from bottom to top: cellulose bottom, gel, cellulose top. Follow steps a~d on left page.

16. Place sandwich in Trans-Blot Turbo Instrument and bake for 7 minutes.

sample membrane

↓

09-21-2018, 10% gel, vimentin

12 samples, cell density gradient

17. Prepare blocking buffer: 10mL total
TBS-Tween-20 (1x)
Casein Blocking Buffer (1x)] mix

Dilute Casein (10x) in 1x TBS


Casein


TBS

9 mL H_2O + 1 mL TBS 10X
= 10 mL of 1X TBS
Remove 1mL from 10mL of (1X) TBS
Add 1 mL of Casein (10x) to 9mL (1X) TBS

3.3 Transfer Using the Turbo Protocol

The Turbo button immediately accesses the MIXED MW program. It is designed for efficient transfer of a wide variety of proteins over a broad range of molecular weights. The same parameters can also be accessed in the Bio-Rad preprogrammed protocols under LIST > BIO-RAD > MIXED MW.

1. Turn on the Trans-Blot Turbo system using the switch located on the right side of the instrument. After an initial Boot screen, the system will proceed to the Home screen.
2. On the Home screen press the Navigation button that corresponds with Turbo (Figure 12).

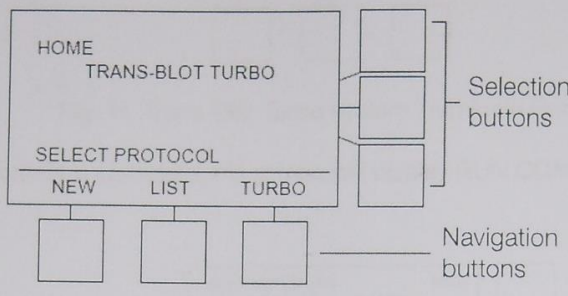


Fig. 12. Home Screen of the Trans-Blot Turbo system.

3. After selecting Turbo, there will be a selection screen for the number, size, and type of gel to be transferred. Use the corresponding selection button to choose the option for the combination of gels in the run. The option varies the current for the run (1.3 A for a single mini format gel, 2.5 A for a single midi or 2 mini format gels). Mini PROTEAN® TGX™ gels can be transferred using the "1 Mini Gel" protocol or the "1 Mini PROTEAN TGX" protocol, which transfers a single Mini PROTEAN TGX gel in 3 min.
4. Press the Navigation button that corresponds to A:RUN for the cassette in the upper bay or B:RUN for the cassette in the lower bay (Figure 13). A beep will sound to signal the start of the transfer for the chosen cassette. If you are running both cassettes, press the button to begin one transfer (either A:RUN or B:RUN), and then press the other button at any time during the run to immediately start the second cassette. Both cassettes must use the same protocol.

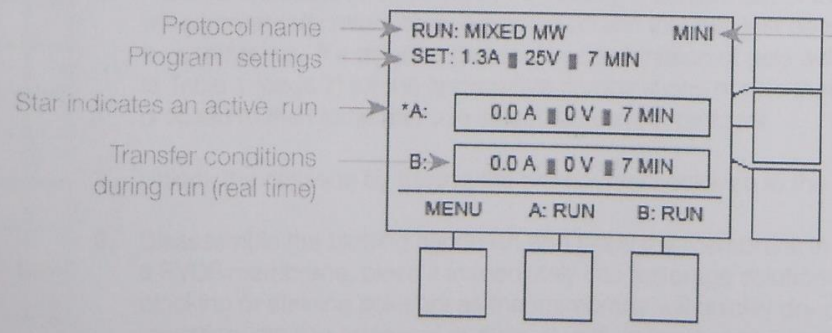


Fig. 13. Transfer Turbo Run screen.

5. The protocol will run automatically. The screen will display the conditions of the transfer and the progress of the run.
6. Turn off the Trans-Blot Turbo system with the power switch if it is no longer required.

Note: A run can be paused by pressing the corresponding A: STOP or B: STOP Navigation button during the run. The user has a choice to continue from the time point of the pause, restart the run from the beginning, or terminate the run (Figure 14.)

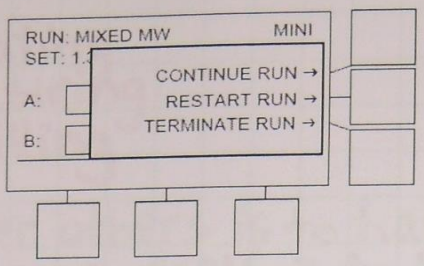


Fig. 14. Trans-Blot Turbo system Terminate Run screen.

When the transfer protocol is complete, the screen will display RUN COMPLETE, and an alarm will be heard (Figure 15).

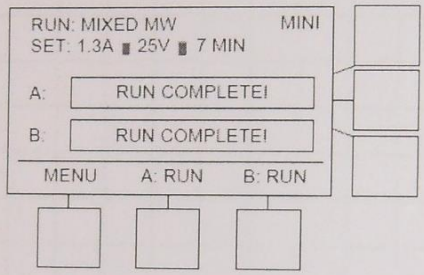


Fig. 15. Trans-Blot Turbo Run Complete screen.

3.3.a. Disassembling and Removing the Membrane

Note: Use caution when removing a cassette from the unit after a transfer run. The cassette may be warm.

1. Remove the cassette from the bay by pulling it straight out of the instrument. The LCD menu screen will automatically return to the protocol screen that has just been completed. The system is ready for another run. If a different protocol or combination of gels will be blotted in the next run, refer to Table 1 (page 7) for the appropriate combinations of gels that can be used in a single run, and proceed to the Home menu to select the correct protocol.
2. Unlock the cassette by turning the dial counterclockwise to the Unlock position.
3. Disassemble the blotting sandwich and place the membrane in a suitable container. If you are using a PVDF membrane, place it immediately into a storage solution (for example, deionized water or blocking or staining solution) as the membrane will quickly dry out. If a PVDF membrane requires rewetting, dip it in methanol or ethanol until uniformly opaque, then wash with deionized water.
4. Discard the ion reservoir stacks after one use; do not attempt to reuse them.
5. Empty residual liquid from the cassette. If no additional transfer will be performed immediately, rinse the base and lid of the cassette with deionized water and dry them with a paper towel.
6. Turn off the Trans-Blot Turbo system with the power switch if it is no longer required.

18. Remove gel from membrane w/ tweezer
19. Place membrane in box, pour blocking solution (10 mL) over it.
20. Place membrane in box on shaker for 1.5 hr w/ blocking buffer (blocking sol'n)

ANTIBODY PREPARATION

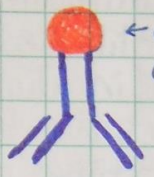
PRIMARY

pAb: polyclonal antibody
mAb: monoclonal antibody

Rabbit to CD31 (ab28364) pAb
1:500

Chicken to Vimentin (ab24525) pAb
1:10,000

SECONDARY

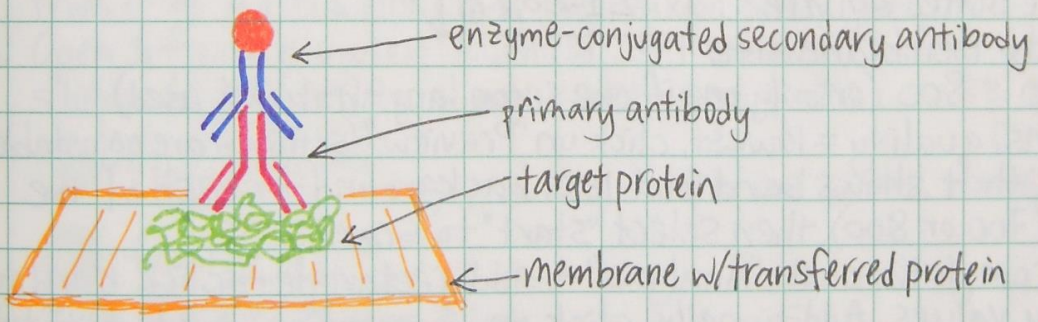


← colorimetric OR chemiluminescent

LI-COR IRDye 680 RD
Goat anti-Rabbit 1:20,000

LI-COR IRDye 800 CW
Donkey anti-chicken 1:20,000

* For volume ratios, check protocol online.



21. Dilute antibodies according to protocol ratio. Use the LI-COR Odyssey blocking buffer (TBS) to dilute all antibodies.

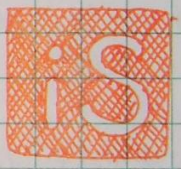
22. Pour used blocking buffer to waste.

23. Pour 10mL primary antibody on membrane, place membrane on shaker. This can be done at room temperature for ONE hour, or place shaker in fridge overnight. (this allows primary antibodies to bind to proteins)

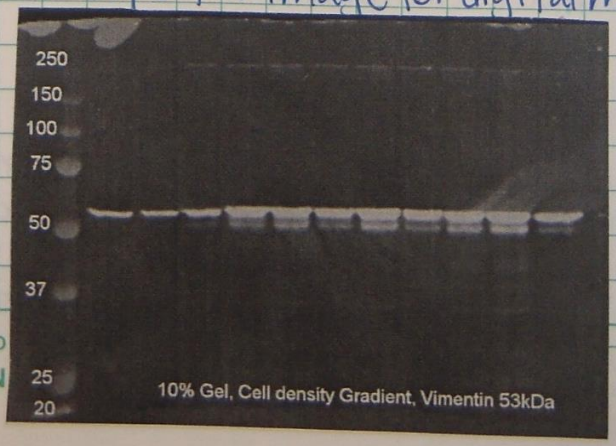
24. Make 1x washing solution by diluting 10x TBS-Tween-20 to 1x with Milli-Q.
25. After antibodies are bound, collect the unused antibodies back into conical. Antibodies can be REUSED! They're expensive, don't waste them!
26. Wash the membrane with 10mL washing solution for 15-20 minutes each. Place the wash on shaker. Toss the washed solution.
27. Pour 10 mL secondary antibody on membrane, place on shaker for 1 hr.
28. Collect secondary antibodies back into conical.
    Note: Secondary antibodies have fluorescent tag and are sensitive to light. Wrap conical in aluminum foil to keep them in the dark.
    All antibodies are REUSABLE!
29. Wash membrane 3 times on shaker, 15-20 minutes each.

Immunofluorescence

30. Place the membrane faced down in Li-COR machine, use roller to remove bubbles.
31. Turn on Li-COR machine, go to computer and select Li-COR Tech profile in Image Studio
32. Adjust the blue dotted square accordingly to the membrane size

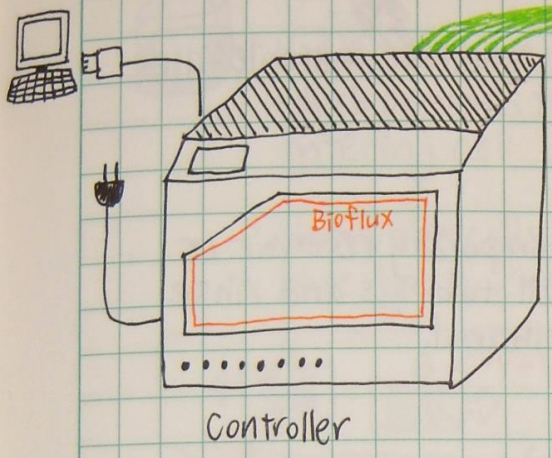


33. Check both 700 & 800 (or only one if one secondary antibody's used)
34. μ = 169 microns, quality = lowest, click on "Preview" (settings are adjustable)
35. Pick an image that shows band but less background for each of the wavelengths (700 or 800), then select "start" to scan
36. After scan is complete, click "Analysis" → add rectangle → click band to show intensity values. Additionally, click on "Annotation" → add text to add remarks.
37. To export: click on iS → Export → image for digital media
    Save as TIFF or jpg.

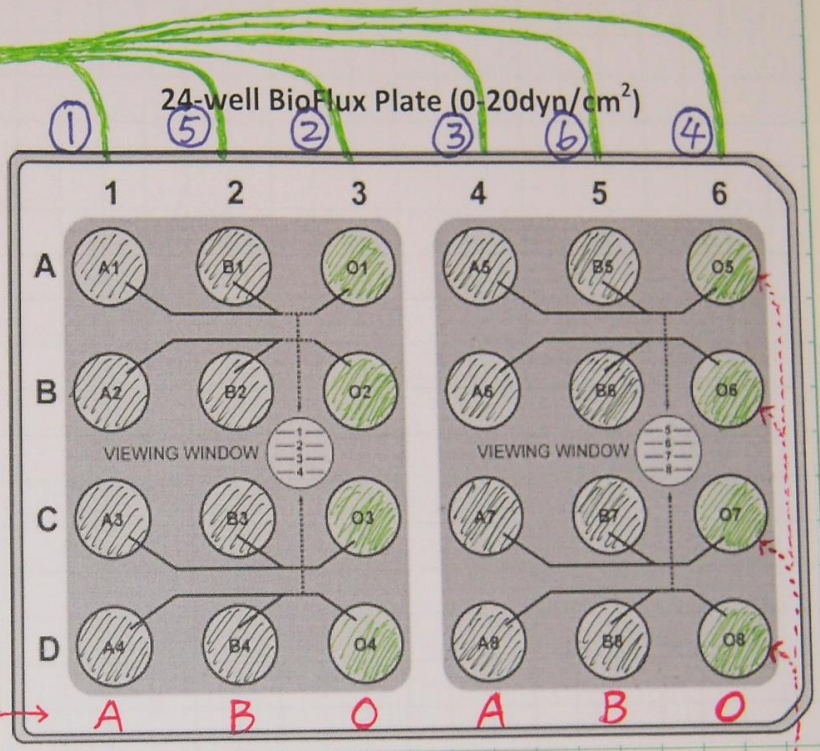


Bioflux System

BIOFLUX PLATE FLUIDIC LAYOUTS



Controller



Direction of flow:

A → O
B → O
O → A & B

} Columns

1. To prime channels, pipette fluid into well O's. Apply pneumatic pressure from O to A & B wells using the Fluxion Software.
2. Open Software, under "Manual", set steady shear (dyne/cm²) to a value between 0-20.

Fluxion (icon)

BioFlux 200

flow FROM

flow TO

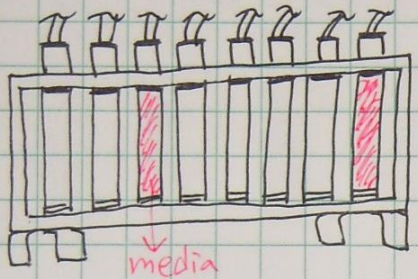
Click on wells to establish flow

3. Click STOP to stop flow
4. Click "Edit AutoRun" to introduce flow profile at various time points/duration
5. Click "View well Volumes" to set up initial well volume. When flow is "ON", use this to view instantaneous volumes in each well.
6. Images/Videos can be taken w/CCD on the viewing window through a microscope.

NOTES: Do not overflow wells
Do not let well run dry
Use "reverse" flow to introduce pulsatile flow profiles

Cleaning the Bioflux System:
Use isopropanol to clean tubes/temporary reservoir
Replace filters behind controller

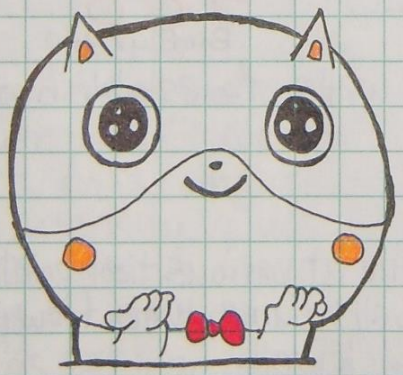

sterile filter



Temporary reservoir

If media crawls up temporary reservoir or tubings, disconnect all tubings and rinse with isopropanol or ethanol.

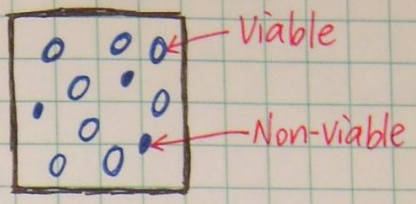
Run air through tubing to speed dry.
Leave air dry over night (or longer if necessary)



Cell Counting w/ Trypan Blue

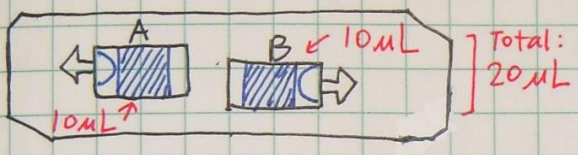
Continued on Page

Trypan Blue 0.4 or 0.5% Solution



Viable cells have intact cell membrane, which prevents trypan blue dye from entering cell.

Non-viable cells have broken cell membrane, which allows trypan blue dye to enter and stain the cell blue.



BioRad Counting Slides TC 20

1. Trypsinize cells to detach from plate. Add equal volume of media.
2. Pipette cells in trypsin + media into conical. Centrifuge conical (Refer to cell passaging protocol)
3. Resuspend cells in 1000 µL (1 mL) + 10 µL = 1010 µL micropipette (or 810 µL), as long as there's 10 µL extra to the known/easily calculated volume).
4. Remove 10 µL of resuspended cells, mix with 10 µL of trypan blue.

Trypan Mix  10 µL media + suspended cells; 10 µL trypan blue — 20 µL in total

5. Load 10 µL of Trypan mix into counting slide window A. Place side A into automated cell counter machine.
6. Repeat Step 5 with window B.
7. Average live cell count from windows A & B.
8. ~~Multiply the average from step 7 to obtain actual cell concentration in cells/mL. This is because only the 10 µL out of the 20 µL trypan mix had cells, the other 10 µL trypan blue was a cell-less dye.~~
9. Use to find desired seeding volume.

$$C_1 V_1 = C_2 V_2$$

C_1: minimum seeding concentration in cells/mL
V_1: minimum seeding volume
C_2: Counted seeding concentration in cells/mL
V_2: Volume needed for seeding

Update 3/21/2019:
Instrument accounts for 1:1 dilution of trypan blue to cell suspension.

Protocol for TC20 Automated Cell Counter can be found: http://www.bio-rad.com/webroot/web/pdf/lsr/literature/100244 23.pdf

Useful information for various sizes of cell culture dishes and flasks

There are various sizes of dishes and flasks used for cell culture. Some useful numbers such as surface area and volumes of dissociation solutions are given below for various size culture vessels.

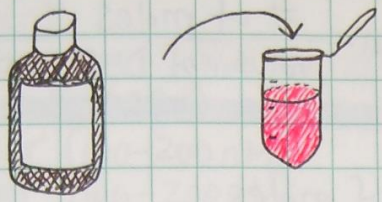
	Surface area (cm^2)	Seeding density	Cells at confluency[1]	Versene (mL of 0.05% EDTA)	Trypsin (mL of 0.05% trypsin, 0.53 mM EDTA)	Growth medium (mL)
Dishes						
35mm	9	0.3×10^6	1.2×10^6	1	1	2
60mm	28.2	0.8×10^6	3.2×10^6	3	2	3
100mm	78.5	2.2×10^6	8.8×10^6	5	3	10
150mm	176.7	5.0×10^6	20.0×10^6	10	8	20
Culture plates						
6-well	9	0.3×10^6	1.2×10^6	2	2	3–5
12-well	4	0.1×10^6	0.4×10^6	1	1	1–2
24-well	2	0.05×10^6	0.2×10^6	0.5	0.5	0.5–1.0
Flasks						
T-25	25	0.7×10^6	2.8×10^6	3	3	3–5
T-75	75	2.1×10^6	8.4×10^6	5	5	8–15
T-160	162	4.6×10^6	18.4×10^6	10	10	15–30

https://www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/cell-culture-useful-numbers.html

TRIzoL RNA Extraction

1. Thaw samples in TRIzol, incubate @ RT for 5 minutes

2. Add 200 µL of chloroform into TRIzol tubes, Shake, incubate @ RT for 2-3 min.

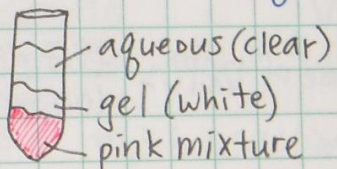


chloroform causes phase separation:
protein - organic phase
DNA - interface
RNA - aqueous phase

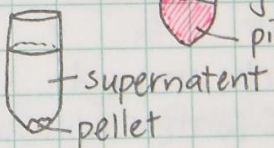
3. Transfer all liquid to Quantabio 5 PRIME Phase lock Gel Heavy 2 mL - 200
Cat # 2302830
Lot # 55708964

} one Quantabio tube per sample

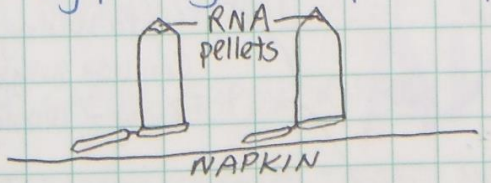
4. Centrifuge Quantabio tubes for 15 minutes @ 15000 g, 4 °C
5. Transfer aqueous phase to new Eppendorf tube. RNA is in the aqueous soln.
6. Add 500 µL isopropanol to the new tube.
isopropanol precipitates RNA by removing salts.



7. Incubate @ RT for 5 minutes
8. Centrifuge for 10 minutes @ 15000 g, 4 °C



9. Remove supernatent
10. Wash pellet w/ 75% ethanol (2mL)
11. Vortex
12. Centrifuge for 10 min. @ 15000 g, 4 °C
13. Remove supernatent
14. Dry RNA by placing tube open, top down, on a napkin



15. Resuspend pellet in 15-20 µL of "DEPC-treated Nuclease Free water for RNA Work"
↑
also diluant for primers

BP561-1
Water (for RNA work)
DEPC-Treated and Nuclease-free autoclave
Fisher Bioreagents

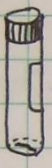
qPCR: RNA to C_T

Read and Understood By → See 拾壹 for Primer Design Guide

PRIMERS

Continued on Page

PECAM1
Sus scrofa (species)
FWD: 30.8 nMole



PECAM1
Sus scrofa
REV: 29.7 nMole

1. Making 100 μM stocks
Eg. PECAM1

$$\text{Concentration} = \frac{\#\text{ of moles}}{\text{volume of DEPC water}}$$

$$\text{Volume of DEPC water} = \frac{\#\text{ of moles}}{100\ \mu M}$$

FWD:

$$\text{Volume of DEPC} = \frac{30.8 \times 10^{-9}\ mol}{100 \times 10^{-6}\ M} = 308 \times 10^{-6}\ L = 308\ \mu L$$

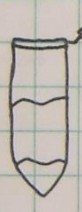
add DEPC → PECAM1 FWD

REV:

$$\text{Volume of DEPC} = \frac{29.7 \times 10^{-9}\ mol}{100 \times 10^{-6}\ M} = 297 \times 10^{-6}\ L = 297\ \mu L$$

add DEPC → PECAM1 REV

Once 100 μM stocks are made, dilute from 100 μM to 10 μM in new tube by adding DEPC water (diluant).



90 μL of DEPC water
+
10 μL of the 100 μM stock
] mix

QUANTIFY RNA

2. Measure RNA w/ Nanodrop
Measure 3 times, take the average
Values are usually expressed in ng/μL

Note:
Up to 100 ng of RNA
Can use anywhere between
20ng ~ 100ng

EQUATION:
n = concentration × volume
n = CV

3. Need 100ng of RNA in each well for qPCR
eg. measured avg value = 681.03 ng/μL

$$100\ ng = 681.03\ ng/\mu L \times V$$

$$V = \frac{100}{681.03} = 0.1468 \approx 0.15\ \mu L \text{ of RNA per well}$$

Kit: Cat# 4391178
Power SYBR™ Green
RNA-to-C_T™ 1-Step Kit
Applied Biosystems
Thermo Fisher
SCIENTIFIC

MASTER MIXES

4. (From pg. 13 in online protocol)

Component	Volume for One Reaction 10 µL	20 µL	50 µL
POWER SYBR Green RT-PCR Mix (2x)	5.0 µL	10.0 µL	25.0 µL
RT Enzyme Mix (125x)	0.08 µL	0.16 µL	0.4 µL
FWD primer (100-200 nM)	VARIABLE (SEE CALCULATION)		
REV primer (100-200 nM)			
RNA Sample (100 ng)			
DEPC H_2O	to 10	to 20	to 50
TOTAL VOLUME	10 µL	20 µL	50 µL

Determine: # of sample RNA types eg. Static, flow, 0.25 OSI ⇒ 3 samples
# of Replicates eg. 3 replicates per sample
# of primer pairs eg. GAPDH, PECAM1, VEGF, α-SMA ⇒ 4 targets

Total # of Reactions = samples × replicates × targets
= 3 × 3 × 4 = 36 reaction wells.

Set up the 48-well plate:

	1	2	3	4	5	6
A	S GAPDH	S GAPDH	S GAPDH	S α-SMA	S α-SMA	S α-SMA
B	S PECAM1	S PECAM1	S PECAM1	S VEGF	S VEGF	S VEGF
C	F GAPDH	F GAPDH	F GAPDH	F α-SMA	F α-SMA	F α-SMA
D	F PECAM1	F PECAM1	F PECAM1	F VEGF	F VEGF	F VEGF
E	0.25 GAPDH	0.25 GAPDH	0.25 GAPDH	0.25 α-SMA	0.25 α-SMA	0.25 α-SMA
F	0.25 PECAM1	0.25 PECAM1	0.25 PECAM1	0.25 VEGF	0.25 VEGF	0.25 VEGF

Samples: Static (S)
Flow (F)
0.25 OSI (0.25)

Primers: GAPDH
PECAM1
VEGF
α-SMA

* Layout software on Step-One Software V2.1 and turn on the Applied Biosystem Step-One RT PCR machine before pipetting
see page 22-23 for software setup

See page 11-13 for MM calculations

FOLD CHANGE

Continued on Page

5. C_T, ΔC_T, and $\Delta\Delta C_T$

Raw C_T values needed: Housekeeping gene ⌐ control conditions, experimental conditions

Gene being tested ⌐ control conditions, experimental conditions

Sample Data from pg. 29
Raw C_T Values

	PPIA	VEGF	αSMA	PECAM1
Static	21.77	29.7	29.13	32.45
	21.49	27.99	30.65	34.96
	20.68	30.45	29.56	34.32
AVERAGE	21.31		29.78	
Flow	20.94	29.21	28.72	36.24
	21.48	29.50	29.09	35.66
	20.54	30.11	28.85	32.91
AVERAGE	20.99		28.89	
0.25 OSI	21.87	29.34	30.95	37.09
	21.92	29.01	31.12	33.98
	20.66	28.57	30.70	32.44
AVERAGE	21.48		30.92	
0.5 OSI	23.12	29.55	32.21	31.66
	23.04	29.07	31.85	-
	21.44	26.92	33.94	35.72
AVERAGE	22.53		32.67	

Example:

SAMPLE	GENE	
	C_T αSMA (target)	C_T PPIA (reference)
Control (Static)	29.78	21.31
Test (Flow)	28.89	20.99

Continued on page

$\Delta\Delta C_T$ Method (Livak)

$\Delta\Delta C_T$

1. Normalize C_T of the target gene to the C_T of the reference gene.

ΔC_T (control) = C_T (target, control) − C_T (reference, control)
= 29.78 − 21.31 = 8.47

ΔC_T (test) = C_T (target, test) − C_T (reference, test)
= 28.89 − 20.99 = 7.9

2. Normalize ΔC_T of the test sample to the ΔC_T of the control

$\Delta\Delta C_T = \Delta C_T$ (test) − ΔC_T (control)
= 7.9 − 8.47 = −0.57

3. Calculate the fold difference in expression

Normalized Expression Ratio = $2^{-\Delta\Delta C_T}$

$= 2^{-(-0.57)} = 1.48$ #

* Flow samples express αSMA at 1.48 # fold higher level than static samples.

ΔC_T Method

ΔC_T

1. Normalize C_T of the target gene for each sample:

$2^{(C_T(PPIA) - C_T(\alpha SMA))}$ = Relative expression

Control: $2^{(21.31-29.78)} = 2^{-8.47} = 0.00282$

Test: $2^{(20.99-28.89)} = 2^{-7.9} = 0.00419$

2. Expression of αSMA relative to PPIA in both control & test samples.

Control expression = $\frac{\text{control}}{\text{control}} = \frac{0.00282}{0.00282} = 1$

Test expression = $\frac{\text{test}}{\text{control}} = \frac{0.00419}{0.00282} = 1.486$ #

Notice the similarity in $\Delta\Delta C_T$ method

* Flow samples express αSMA at a 1.486 fold higher level than static samples.

- Livak method is useful only when reaction efficiencies of target and reference genes are similar. If reaction efficiencies are different, an alternative formula must be used to determine relative expression of the target gene in different samples.

Continued from Page

Pfaffl Method

$$\text{Ratio} = \frac{(E_{target})^{[\Delta C_T, \text{target}(\text{control}-\text{test})]}}{(E_{reference})^{[\Delta C_T, \text{reference}(\text{control}-\text{test})]}}$$

E_{target} & $E_{reference}$ are reaction efficiencies of target and reference genes, respectively.

If reaction efficiencies are 100%, Pfaffl simplifies to $\Delta\Delta C_T$:

$$\text{Ratio} = \frac{2^{[\Delta C_T, \text{target}(\text{control}-\text{test})]}}{2^{[\Delta C_T, \text{reference}(\text{control}-\text{test})]}}$$

$$= 2^{-[(C_T, \text{target}(\text{test})) - (C_T, \text{target}(\text{control}))] - [(C_T, \text{ref}(\text{test})) - (C_T, \text{ref}(\text{control}))]}$$

$$= 2^{-\Delta\Delta C_T}$$

Primer Design Guide

NCBI GenBank: National Center for Biotechnology Information
https://www.ncbi.nlm.nih.gov/genbank
Top search bar, type in species and gene of interest
eg. Sus scrofa alpha smooth muscle actin
Click **Search**

Select from list, click for more info
Copy NCBI ID#, under "Locus", eg. NM_001164650

Go to IDT PrimerQuest® Tool (Integrated DNA Technologies)
https://www.idtdna.com/site/account/login?returnurl=Primerquest/Home/Index
Click "Download Sequence(s) using Genbank or Accession ID" ← login required
Enter NCBI ID# (See Hints to the right)
Click **Get Sequence**
Click **PCR 2 Primers**

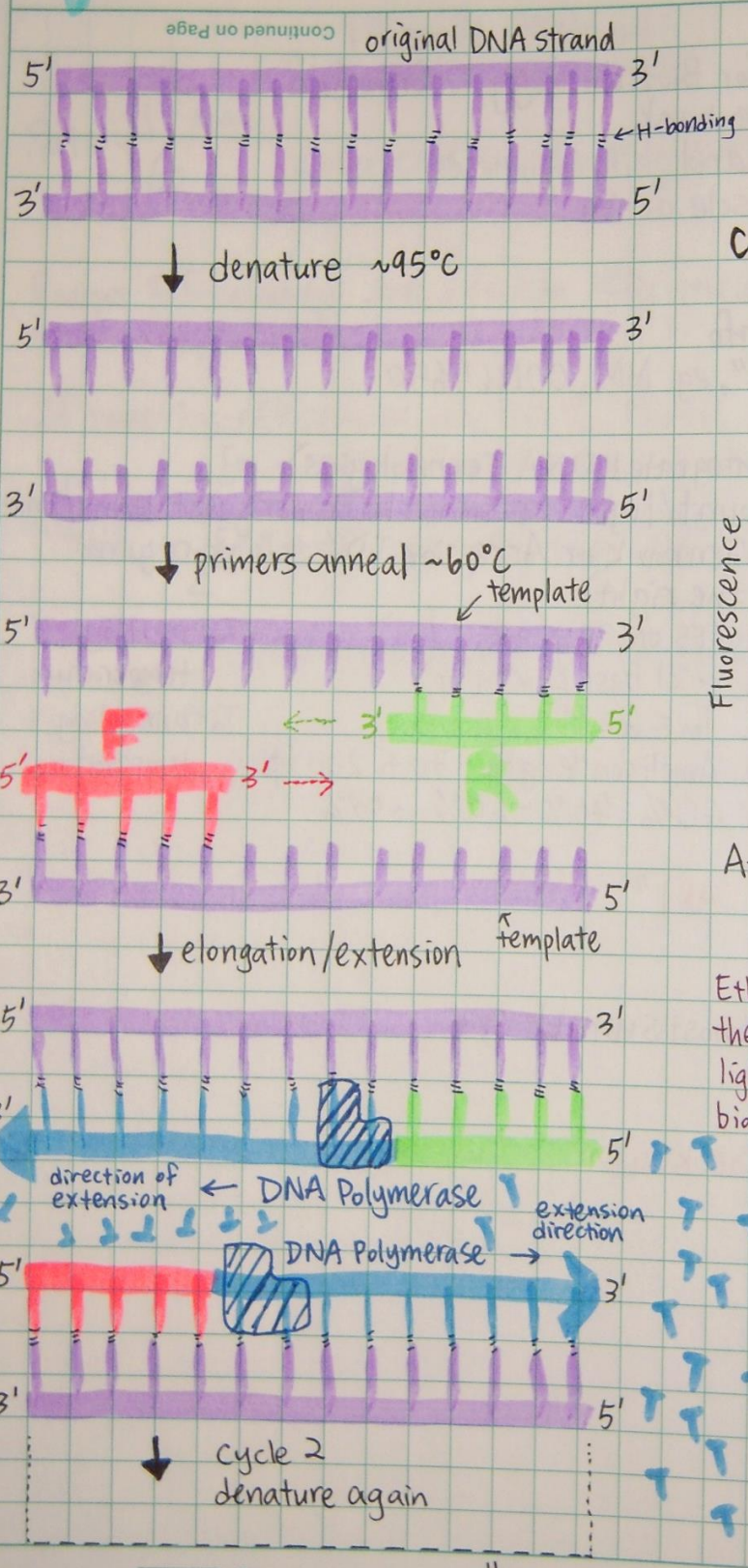
* NOTES ON PRIMERS:
1. ~21 base pairs (bp)
2. $T_m \approx 62°C$
3. Amplicon length ~70 to 200 bp
4. GC%: 40%-60%, ~50%

T_m: melting temperature
Ta: annealing temperature

Click "ADD SELECTED ASSAYS TO CART"
Forward & Reverse
Oligos in Tubes
Select the correct/chosen/most suitable set
CONTINUE
ADD TO CART
E-MAIL CART/QUOTE or Check Out

qPCR Mechanisms

quantitative Polymerase Chain Reaction

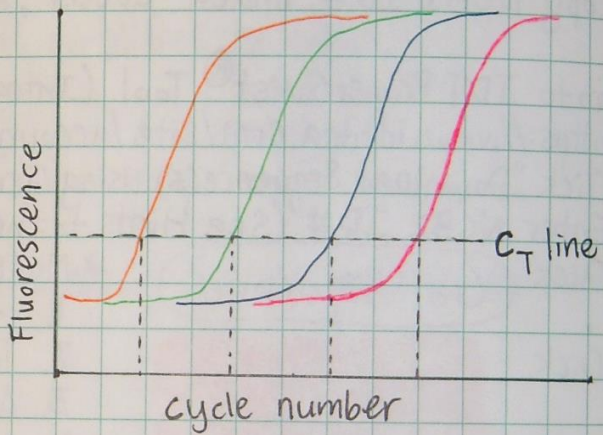


PCR results in 2^n copies of DNA, where n is the # of cycles.

$MgCl_2$: buffer

BSA: Bovine serum albumin (PCR enhancer)

C_T: cycle threshold, # of cycles required for fluorescent signal to cross the threshold



A_{260}/A_{280}: ratio of absorbance at 260 and 280 nm is a function of purity.

Ethidium bromide / ROX / SYBR Green: these bind to DNA & fluoresce under some light or UV... or... bind w/species specific biotinylated oligonucleotide probe

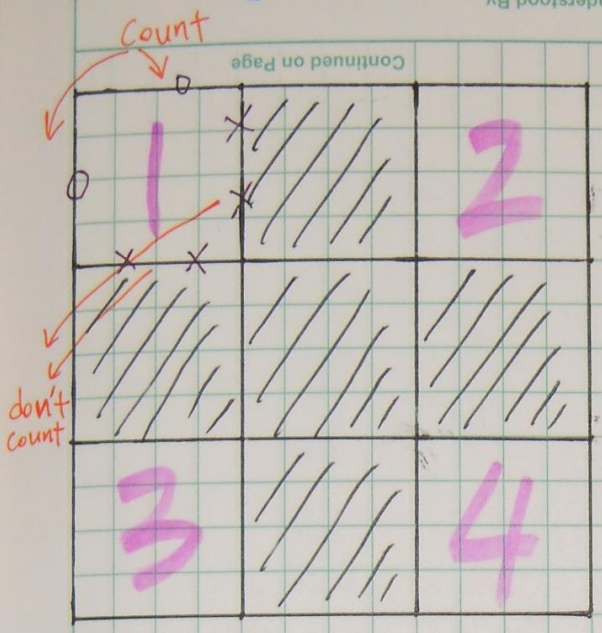
dNTPs: deoxynucleotide, or deoxyribonucleotide triphosphates

ΔC_T: case control w/housekeeping gene

$\Delta\Delta C_T$: same, but for good amplification efficiencies

Amplicon/PCR product

Hemocytometer Cell Counting



Four quadrants consisting of 16 little squares.
Each quadrant is 100 nL

- Count cells in 1, 2, 3, and 4
- Pay attention to edges (choose two edges for count if cell is on the edge line)
- Average the four quadrants
- Multiply by 10,000 (10^4) to get cells/mL (if not diluted w/media or trypan blue)

1. Clean hemocytometer and coverslip w/alcohol. Moisten the coverslip w/water to affix coverslip to hemocytometer
2. Pipette 10 uL of cell suspension under the coverslip. Allow cell suspension to be drawn out by capillary action.
3. Observe all four quadrants under the microscope. Use a hand tally counter to count live cells.
4. Average the four quadrants.
5. Multiply the average by 10^4 to get cells/mL.

Note: If there are too many cells clumped together, dilute the suspension w/ fresh media or Trypan blue. Factor the dilution ratio into calculation.

QuantiChrom™ Nitric Oxide Assay Kit (D2NO-100)

Quantitative Colorimetric Determination of Nitric Oxide at 540 nm

DESCRIPTION

Nitric oxide (NO) is a reactive radical that plays an important role in many key physiological functions. NO, an oxidation product of arginine by nitric oxide synthase, is involved in host defense and development, activation of regulatory proteins and direct covalent interaction with functional biomolecules.

Simple, direct and automation-ready procedures for measuring NO are becoming popular in Research and Drug Discovery. Since NO is oxidized to nitrite and nitrate, it is common practice to quantitate total NO_2^-/NO_3^- as a measure for NO level. BioAssay Systems' QuantiChrom™ Nitric Oxide Assay Kit is designed to accurately measure NO production following reduction of nitrate to nitrite using improved Griess method. The procedure is simple and the time required for sample pretreatment and assay is reduced to as short as 30 min.

KEY FEATURES

Sensitive and accurate. Detection range 0.6 - 200 µM in 96-well plate.

Rapid and reliable. Using an optimized VCl_3 reagent, the time required for reduction of NO_3^- to NO_2^- is 10 min at 60°C.

Simple and high-throughput. The procedure involves mixing sample with three reagents, incubation for 10 min at 60°C and reading the optical density. Can be readily automated to measure thousands of samples per day.

APPLICATIONS

Direct Assays: NO in plasma, serum, urine, tissue/cells and foods.

Drug Discovery/Pharmacology: effects of drugs on NO metabolism.

KIT CONTENTS (100 tests in 96-well plates)

Reagent A:	12 mL	**Reagent B**:	500 µL	**Reagent C**:	12 mL
NaOH:	1 mL	**$ZnSO_4$**:	1 mL	**Standard**:	1 mL

Storage conditions. The kit is shipped at room temperature. Store the Standard at -20°C and all other reagents at -20 to 4°C. Shelf life of six months after receipt.

Precautions: reagents are for research use only. Please refer to Material Safety Data Sheet for detailed information.

PROCEDURES

Prior to assay, equilibrate all components to room temperature. If precipitates are present in Reagent B, warm at 37°C until redissolved (~10-15 min).

Sample treatment: tissue or cell samples are homogenized in 1 × PBS (pH 7.4). Centrifuge at 14,000 rpm at 4°C. Use supernatant for NO assay.

Samples that need deproteination include serum, plasma, whole blood, cell culture media containing FBS, tissue or cell lysates. Urine and saliva do not need deproteination.

Deproteination. Mix 150 µL sample with 8 µL $ZnSO_4$ in 1.5-mL tubes. Vortex and then add 8 µL NaOH, votex again and centrifuge 10 min at 14,000 rpm. Transfer 100 µL of the clear supernatant to a clean tube. *Note: If samples need to be deproteinated, 150 µL of each standard should be prepared and also treated with $ZnSO_4$ and NaOH to eliminate the need for a dilution factor.*

Procedure using 96-well plate:

1. *Standards.* Prepare 500 µL 100 µM Premix by mixing 50 µL 1.0 mM Standard and 450 µL distilled water. Dilute standards in 1.5 mL centrifuge tubes as described in the Table.

No	Premix + H_2O	Nitrite (µM)
1	250 µL + 0 µL	100
2	150 µL + 100 µL	60
3	75 µL + 175 µL	30
4	0 µL + 250 µL	0

2. *Reaction.* Add 100 µL of each sample to separate, labeled eppendorf tubes. (We recommend that samples be measured in at least duplicate). Immediately prior to starting the reaction, prepare enough Working Reagent (WR) for all samples and standards by mixing per reaction tube: 100 µL Reagent A, 4 µL Reagent B and 100 µL Reagent C. Add 200 µL of the WR to each sample and standard tube and incubate for 10 min at 60°C. (Alternatively, the reaction can be run at 37°C for 60 min or RT for 150 min.) *Note:* If precipitates are present in Reagent B, warm at 37°C until redissolved (~10-15 min).
3. *Measurement.* Briefly centrifuge the reaction tubes to pellet any condensation and transfer 250 µL of each reaction to separate wells in a 96 well plate. Read OD at 500-570 nm (peak 540 nm).

Procedure using Cuvette:

Prepare standards and samples as described for the 96-well procedure except quadruple (4×) the volumes. After the reaction, transfer 1 mL to a cuvette. Measure OD_{540nm} in the cuvette.

CALCULATION

Subtract blank OD (Std 4) from the standard OD values and plot the OD against standard concentrations. Determine the slope using linear regression fitting. The NO concentration of Sample is calculated as

$$[\text{Nitric Oxide}] = \frac{OD_{SAMPLE} - OD_{BLANK}}{\text{Slope}} \quad (\mu M)$$

OD_{SAMPLE} and OD_{BLANK} are optical density values of the sample and water, respectively.

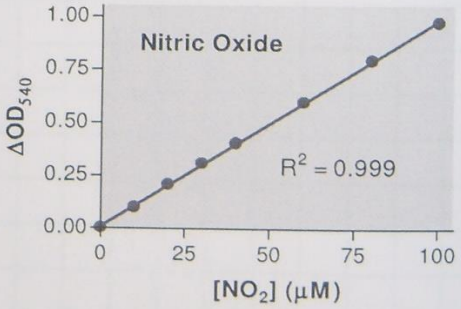
Conversions: 1 mg/dL NO equals 333 µM, 0.001% or 10 ppm.

MATERIALS REQUIRED, BUT NOT PROVIDED

Pipetting devices, eppendorf tubes, eppendorf centrifuge, clear, flat bottomed 96 well plates or cuvettes, plate reader or spectrophotometer and heat block or hot water bath (optional).

GENERAL CONSIDERATIONS

Antioxidants and nucleophiles (e.g. β-mercaptoethanol, glutathione, dithiothreitol and cysteine) may interfere with this assay. Avoid using these compounds during sample preparation.



Standard Curve in 96-well plate assay

LITERATURE

1. Bolander Jr, F. F. (2005). The compartmentalization of prolactin signaling in the mouse mammary gland. Mol. Cell. Endocrinol 245:105–110.
2. Bulau, P. et al. (2007). Analysis of methylarginine metabolism in the cardiovascular system identifies the lung as a major source of ADMA. Am J Physiol Lung Cell Mol Physiol 292: L18-L24.
3. Hasegawa, K. et al (2007). Role of asymmetric dimethylarginine in vascular injury in transgenic mice overexpressing dimethylarginie dimethylaminohydrolase. Circ Res. 101(2):e2-10.

 · 3191 Corporate Place, Hayward, CA 94545, USA · Website: www.bioassaysys.com
Tel: 510-782-9988, Fax: 510-782-1588 · Email: order@bioassaysys.com, info@bioassaysys.com

Power Analysis : Sample Size Determination

H_0: Null hypothesis
H_A: Alternative hypothesis

	Reject H_0	Do Not Reject H_0
H_0 true	Type I	Correct
H_0 false	Correct	Type II

Type I Error: Reject H_0 when it is true
Type II Error: Fail to reject H_0 when it is false
α: Type I Error probability
β: Type II Error probability
Power $= 1 - \beta$

One Population Sample Size Determination

$H_0: \mu = \mu_0$, $H_A: \mu \neq \mu_0$ } two-sided test $\quad n = \dfrac{[\sigma(Z_{\alpha/2} + Z_\beta)]^2}{(\mu - \mu_0)^2}$

$H_0: \mu = \mu_0$, $H_A: \mu > \mu_0$ } one-sided test $\quad n = \dfrac{[\sigma(Z_\alpha + Z_\beta)]^2}{(\mu - \mu_0)^2}$

σ: population std. dev.
σ^2: population variance

if $\alpha = 0.05$
$\beta = 0.20$

Two Population Sample Size Determination

$H_0: \mu_1 - \mu_2 = 0$, $H_A: \mu_1 - \mu_2 \neq 0$ } two-sided test $\quad n = \dfrac{2[\sigma(Z_{\alpha/2} + Z_\beta)]^2}{(\mu_1 - \mu_2)^2}$

$H_0: \mu_1 - \mu_2 = 0$, $H_A: \mu_1 - \mu_2 > 0$ } one-sided test $\quad n = \dfrac{2[\sigma(Z_\alpha + Z_\beta)]^2}{(\mu_1 - \mu_2)^2}$

In R:
$Z_{0.025} = \text{qnorm}(0.975) = 1.96$
$Z_{0.20} = \text{qnorm}(0.80) = 0.84$

Eg. How many samples are required for treatment and control groups? (Treatment + control, two population sample size determination)

$$n = \frac{2\sigma^2(Z_{\alpha/2} + Z_\beta)^2}{d^2}$$

where σ^2: population variance
α: Type I Error probability
β: Type II Error probability
d: difference the examiner would like to detect

If $\alpha = 0.05 \Rightarrow$ 95% confidence
$\beta = 0.20 \Rightarrow$ Power is 80%

$\Delta = \dfrac{|\mu_1 - \mu_2|}{\sigma}$, standardized difference

$n = \dfrac{2(Z_\alpha + Z_\beta)^2}{\Delta^2}$ (one-sided test)