

CHAPOL Lineage Analysis

These first few steps are explained in detail in another protocol:

- Make CHAPOL virus
- Titer virus
- Check for helper virus

The lab already has a stock of the virus with a G-coat so use BL2 guidelines when handling the virus.

- Inject into specimen
- Harvest specimen and fix
- Whole-Mount Alkaline Phosphatase Stain:
 1. Wash in PBS
 2. Incubate at 65° C for 45 min in PBS to inactive endogenous alk. phos.
 3. Incubate at RT in Chap detection buffer - 30 min
 4. O/N at RT (in dark) in Chap detection solution
 5. Wash in TE at RT to stop stain
 6. Wash in PBS at RT
- Evaluate infection and take pictures
- Embed
- Section (since cell thickness is estimated at 10 µm, section at no less than 10 µm)
- AP stain in section:
 1. Wash in PBS at RT - 3 x 5 min
 2. Incubate at RT in Chap detection buffer - 15 min
 3. Incubate at RT (in dark) in Chap detection solution - 6 to 8 hrs
 4. Wash in TE at RT - 3 x 5 min
 5. Wash in PBS at RT - 3 x 5 min
- Antibody stain or other immunohistochemistry to visualize different cell types if necessary
- Coverslip with gelvatol or other water soluble mounting media

Before picking cells:

- Take pictures of sections with positive cells

- Print pictures

*Make sure you take and print enough pictures to pick a whole 96 well plate at one sitting.

- Pull needles (pull 100 to have extra)
- Make sure have PK buffer, PK, oligos, dNTP's, 10x PCR buffer, and Taq
- Set up room where picking cells with:

1. sterile water in a 50 ml conical
2. CLEAN 10 µl multi-channel pipet
3. CLEAN 1000 µl pipetman
4. Aersol Barrier Tips - 1000 µl
5. Tips for 10 µl multi-channel pipet
6. Reagent Reservoirs
7. 96-well plates & lids
8. Papertowels and Kimwipes
9. Mouth Pipet with adaptor to hold needles
10. Forceps
11. Black sharpie and orange or red sharpie
12. Individually wrapped sterile transfer pipets

- Make up mixture of PK Buffer and PK right before picking cells:

*need 10 µl per well with final conc of PK at 0.2 mg/ml

for 96-well plate (x 105)

21 µl PK
1029 µl PK Buffer

for half a plate (x 55)

11 µl PK
539 µl PK Buffer

- Using black sharpie number and date the 96-well plate and number each well 1 to 96
*use Marsh Bio Products plates (#N2-9078) they are clear which makes it possible to see that the cells picked have been placed in the well
- Add 10 µl of PK Buffer w/PK to each well in 96-well plate
- Remove coverslip and place a few drops of water onto the section to be picked
- Pick cells into 96-well plate (leave a few wells blank for neg. contol)
- Place a drop of mineral oil in each well
(this is to make sure there is no evaporation & it helps lower the chances of contamination)

- PK digestion:

Program - CHAP 1 (approx. 2.5 hrs)

I.	60° C	2 hrs	1 cycle
II.	85° C	20 min	1 cycle
III.	95° C	10 min	1 cycle

- PCR #1:

For 96-well plate (x 105)

For half a plate (x 55)

DNA in PK solution	10 μ l	-	-
PCR Buffer	2 μ l	210 μ l	110 μ l
Oligo #0	0.75 μ l	78.75 μ l	41.25 μ l
Oligo #5	0.75 μ l	78.75 μ l	41.25 μ l
dNTP	0.15 μ l	15.75 μ l	8.25 μ l
Taq	0.15 μ l	15.75 μ l	8.25 μ l
H ₂ O	16.2 μ l	<u>1701 μl</u>	<u>891 μl</u>
		2100 μ l	1100 μ l

*For this PCR add cocktail (**20 μ l to each well**) on top of mineral oil and let sink into the well with the DNA in PK solution. DO NOT let the tip touch anything in the well!

Program - CHAP 2 (approx. 2.5 hrs)

I.	93° C	2.5 min	1 cycle
II.	94° C	45 sec	
	70° C	2 min	30 cycles
III.	72° C	5 min	1 cycle

- PCR #2:

* Using a black sharpie number and date a new 96-well plate (Marsh Bio Products) and number each well 1 to 96

		<u>For 96-well plate</u> (x 105)	<u>For half a plate</u> (x 55)
DNA from PCR #1	1 μ l	-	-
PCR Buffer	2 μ l	210 μ l	110 μ l
Oligo #2	1 μ l	105 μ l	55 μ l
Oligo #3	1 μ l	105 μ l	55 μ l
dNTP	0.25 μ l	26.25 μ l	13.75 μ l
Taq	0.25 μ l	26.25 μ l	13.75 μ l
H ₂ O	16.2 μ l	<u>1522.5 μl</u>	<u>797.5 μl</u>
		1995 μ l	1045 μ l

- Add 19 μ l of cocktail to each well
- Add 1 μ l of DNA from PCR #1 to each well
- Add a drop of mineral oil to each well

Program - CHAP 3 (approx. 2.5 hrs)

I.	93° C	2.5 min	1 cycle
II.	94° C	45 sec	
	72° C	2 min	30 cycles
III.	72° C	5 min	1 cycle

- Run 5 μ l of PCR #2 on a 2% agarose gel in TAE - 120 volts for 1 hr & 5 min

- Take picture of gel
- Determine which wells contain amplified DNA (PCR product - 121 bp)
(see excel spreadsheet named gel key to number picture of gel correctly)
- Fill out 96-well template with positive PCR numbers (plate # . well #) ex-10.02
(see excel spreadsheet named 96-well plate grid for template)
- Once fill in an entire 96-well template with positive PCR numbers...
 1. Label a new 96-well plate (number and date) and number each well 1 to 96
(use Marsh Bio Products plates)
 2. Transfer 15 µl of appropriate PCR product (PCR #2) into its appropriately labeled well

- Purify PCR products:
 - WEAR GLOVES & use DIRTY lab bench space
 - Follow directions in QIAquick 96-well PCR Purification Kit
 - Use programmable, automatic multi-channel pipet & vacuum manifold

*Changes to protocol:

- dilute Buffer EB 1:10 in water to 1mM
- Add 200 µl water to wells during step 5 to dilute final conc of template

- Sequencing reaction:

		<u>For 96-well plate (x 105)</u>	<u>For 2 x 96-well plates</u>
Template	3 µl	-	-
Oligo #3 (0.9 pmol/µl)	3.5 µl	367.5 µl	735 µl
Water	7.5 µl	787.5 µl	1575 µl
Gen Pak	6 µl	630 µl	1260 µl
Big Dye	2 µl	<u>210 µl</u>	<u>420 µl</u>
		1995 µl	3990 µl

- Use Lab New England #T323-96SKNBC (PCR plate) with MJ Research #MSA-5001 (Micorseal "A" Film) - there is no need to number 1 to 96 since you have the template telling you what is in each well
- Add 19 µl to each well using CLEAN multi-channel pipet
- Add 3 µl of purified template using DIRTY 50 µl multi-channel pipet
- DO NOT add oil

Program - BIG DYE (approx. 2.5 hrs)

I.	96° C	10 sec	
	50° C	5 sec	
	60° C	4 min	25 cycles

- Purify sequencing products:
 - Use 96-well Gel Filtration Kit

- Use flat-bottom well plates
- All spins are done using the Qiagen Centrifuge 4-15C on Program #1 (speed - 2200, RCF - 779, Time - 2 min 30 sec)

1. Spin down column into flat bottom well plate
2. Place 96-well plate for 3700 into holder (black)
3. Label plate with name/number and date
4. Place spun down column onto plate
5. Transfer sequence reaction (~20 μ l) onto column - DO NOT TOUCH COLUMN
6. Spin samples through the column

- Dry down purified sequence sample:

Program - DRY80

I. 80° C 1 hr 1 cycle

- The BioPolymer Facility resuspends samples in water and the sequence is read by 3700
- Analyze and organize sequence and match up to cells picked

Add all sequences you retrieve to excel spreadsheet named CHAPOL SEQUENCES when working on your project to check yourself for possible contamination and for the lab to know the complexity of this batch of virus!!!! THANK YOU!

All you need to do lineage analysis

Equipment:

Microscopes

1. scope to take pictures of sections of your specimen with positive cells and visualize appropriate antibodies
2. dissecting scope to pick cells on

Multi-channel pipet & pipetman

1. CLEAN 10 µl pipetman
2. CLEAN 200 µl pipetman
3. CLEAN 1000 µl pipetman
4. CLEAN 10 µl multi-channel pipet
5. CLEAN 100 µl multi-channel pipet
6. DIRTY 20 µl pipetman
7. DIRTY 10 µl multi-channel pipet
8. DIRTY 50 µl multi-channel pipet
9. Programmable automatic pipet (Matrix Impact2 850 µl)

*The distinction here between clean and dirty is because when handling solutions for PCR's you want CLEAN pipets and once the PCR has been completed you want to transfer all PCR products using the DIRTY pipets so not to contaminate future PCR reactions. This distinction is VERY IMPORTANT!

Bench Space

You want to have two separate bench spaces 1. to do "clean" work to set up the PCR's and 2. a "dirty" work area to purify your PCR products

Sequencing Analysis

3700 DNA Analyzer by Applied BioSystems

Needle Puller

Kopf Neddle/Pipette Puller (Model 750)

Vacum Manifold

for 96-well plate

Electrophoresis

Comb with 42 wells or 50 wells

Centrifuge

Qiagen Centrifuge 4-15C

(for purifying sequencing reactions) - we use the one in the sequencing facility

96-well plates

1. For PK digestion and PCR's:
Marsh Bio Products #N2-9078 (polycarbonate PCR plate) & lid
2. For Sequencing reaction:
Lab New England #T323-96SKNBC (PCR plate)
MJ Research #MSA-5001 (Micorseal "A" Film)
3. Plate to spin purified sequencing reaction into for use in the 3700:
Marsh Bio Products #T0296PE

Other

1. Reagent Reservoir (50 ml) - Corning Costar #4870
2. Sterile Transfer Pipettes - Samco #202-1S

Kits, Reagents and Solutions:

1. QIAquick 96-well PCR Purification Kit by Qiagen (#28181) - \$550.00
2. Big Dye Termination Reaction Kit by Applied Biosystems (#4303153) - \$5500.00
3. halfBD sequencing reagent by Genpak (#HBD5000) - \$3225.00
4. 96-well Gel Filtration Kit by Edge BioSystems (#94880) - \$655.00

Chap Detection Buffer

(500 ml)

<u>Stock</u>	<u>Volume</u>	<u>Final []</u>
1M Tris-HCl, pH 9.5	50 ml	100 mM
5M NaCl	10 ml	100 mM
1M MgCl ₂	25 ml	50 mM
Water	415 ml	

Chap Detection Solution

(10 ml)

<u>Stock</u>	<u>Volume</u>	<u>Final []</u>
NBT	200 µl	1 mg/ml
BCIP	100 µl	0.1 mg/ml
Chap det. buffer	10 ml	

STOCKS:

1. NBT - 50 mg/ml in 70% DMF / 30% water
for 10 ml
7 ml DMF
3 ml water
500 mg NBT
2. BCIP (X-Phos) - 10 mg/ml in water
for 10 ml

100 mg BCIP
10 ml water

KMT (100 ml)

<u>Stock</u>	<u>Volume</u>	<u>Final []</u>
1M KCl	5 ml	50 mM
1 M MgCl ₂	225 µl	2.25 mM
1 M Tris-HCl, pH 8.0	1 ml	10 mM
Water	93.775 ml	

STOCKS:

	<u>M.W.</u>	<u>for 100 ml of 1M solution</u>
KCl	74.55	7.455 g
MgCl ₂	203.30	20.33 g
Tris-HCl, pH 8.0	157.60	15.76 g

Proteinase K buffer

(~60 ml)

<u>Stock</u>	<u>Volume</u>	<u>Final []</u>
KMT	56.42 ml	
10% Tween-20	3.1 ml	0.5%
Oligo #0 (8 pmol/µl)	620 µl	0.1 µM
Oligo #5 (8 pmol/µl)	620 µl	0.1 µM
	60.76 ml	

*make single use aliquots of 1029 µl for a 96-well plate (x105) and 539 µl for half a plate (x55)

Proteinase K Roche #745 723 (100 mg)

Stock - 10 mg/ml

Add 10 ml water to make

*Make single use aliquots of 22 µl

Primers

Stock - 8 pmol/µl

CLAP 0 TGTGGCTGCCTGCACCCCAGGAAAG

CLAP 5 GTGTGCTGTGAGCCGCCTTCAATG

CLAP 2 GCCACCACCTACAGCCCAGTGG

CLAP 3 GAGAGAGTGCCGCGGTAATGGG

*Make single use aliquots of 80 μ l for #0 & #5 and 110 μ l for #2 & #3

**For the sequencing reaction the stock for #3 is 0.9 pmol/ μ l

105 μ l Oligo #3 (8 pmol/ μ l)

828 μ l Water

933 μ l

Already Made Solutions

25 mM dNTP's - Roche #1 277 049

10x PCR Buffer - Roche #1 271 318

Taq - Roche #1 435 094

*you want to have you own stock of these to help lower the chances of contamination

Other

DNA Molecular Weight Marker VI (0.15 - 2.1 kbp) - Roche #1 062 590

Gelvatol

Solutions:

- 0.01 M KH_2PO_4 (monobasic)

M.W. - 136

$$136 \times 0.01 = 1.36 \text{ g/l}$$

$$1.36 \text{ g/l} / 5 = \mathbf{0.272 \text{ g} / 200 \text{ ml}}$$

- 0.01 M Na_2HPO_4 (dibasic)

M.W. - 141.96

$$141.96 \times 0.01 = 1.419 \text{ g/l}$$

$$1.419 \text{ g/l} / 2 = \mathbf{0.7095 \text{ g} / 500 \text{ ml}}$$

PROCEDURE:

1. Take 200 ml of 0.01 M KH_2PO_4 (= ~ pH 5.0) and add enough 0.01 M Na_2HPO_4 to bring pH up to 7.2.
2. Then take 250 ml of 0.01 M KH_2PO_4 / Na_2HPO_4 and add 2.05 g NaCl to give a 0.14 M NaCl concentration.
3. Dissolve 62.5 g Gelvatol in the 250 ml of 0.01 M KH_2PO_4 / Na_2HPO_4 / 0.14 M NaCl. Stir on magnetic stirrer in warm room for a few hours.
4. Add glycerol in an amount equal to one-half the total volume of the Gelvatol buffered saline solution and stir O/N at RT.
5. Centrifuge the Gelvatol solution at 12,000 rpm for 15 min in 30 ml Corex tubes in Beckman J2-21 centrifuge at RT to remove the undissolved particles.
6. Pipette the supernatant into small screw cap bottles. Check pH of Gelvatol solution. It should be between pH 6 and 7.

7. Store Gelvatol solution at 4° C. Screw caps on tightly to prevent evaporation. Do not leave Gelvatol uncapped for longer than necessary when working with it.

Gelvatol

Vinol Grade 205 (polyvinyl alcohol resin)

Air Products & Chemicals Inc

Allentown, PA 18105

1-800-345-3148

Other Information

Known sequence in CHAPOL: PCR product - 121 bp

(oligo # 3)

(seq to find start of 24 bp insert)

GAGAGAGTGCCGCGGTAATGGGATCTGTCTGAAGATCTCGAGTTAATTAACGCG

TAAC {24 bp insert} AAA GGCGCGCCATCGACCCCACTGGGCTGTAGGTGGTGGC

(unique seq to analyze)

(oligo # 2)

Colors of bases for sequence:

A - green

T - red

C - blue

G - black

BL2 Guidelines

For use of VSV-G pseudotyped virus

Tissue Culture Room Guidelines

1. Place sign on door notifying that BL2 work is in progress.
2. Use disposable pipettes for all work.
3. Keep a beaker of Wescodyne in hood to soak all plastic that comes into contact with virus.
4. Place a sign on incubator notifying and stating VSV experiments in use.
5. Wescodyne interior surface of hood after use.

6. Place all contaminated plastic into autoclave bag, seal within the BL2 room and autoclave.
7. Lab Coat and Gloves must be worn at all times.
8. Door to the room must remain closed.

Chick Room Guidelines

1. Place sign on door notifying that BL2 work is in progress.
2. Autoclave all waste generated when injecting.
3. Soak injection needles in Wescodyne before disposal.
4. Wipe down area after finished with Wescodyne.
5. Wipe down syringe needle on Hamilton syringe with Wescodyne after use.
6. Place sign on egg incubator for first 24 hours stating the incubator contains eggs injected with BL2 virus.
7. Lab Coat and Gloves must be worn at all times.
8. Door to the room must remain closed during injections.