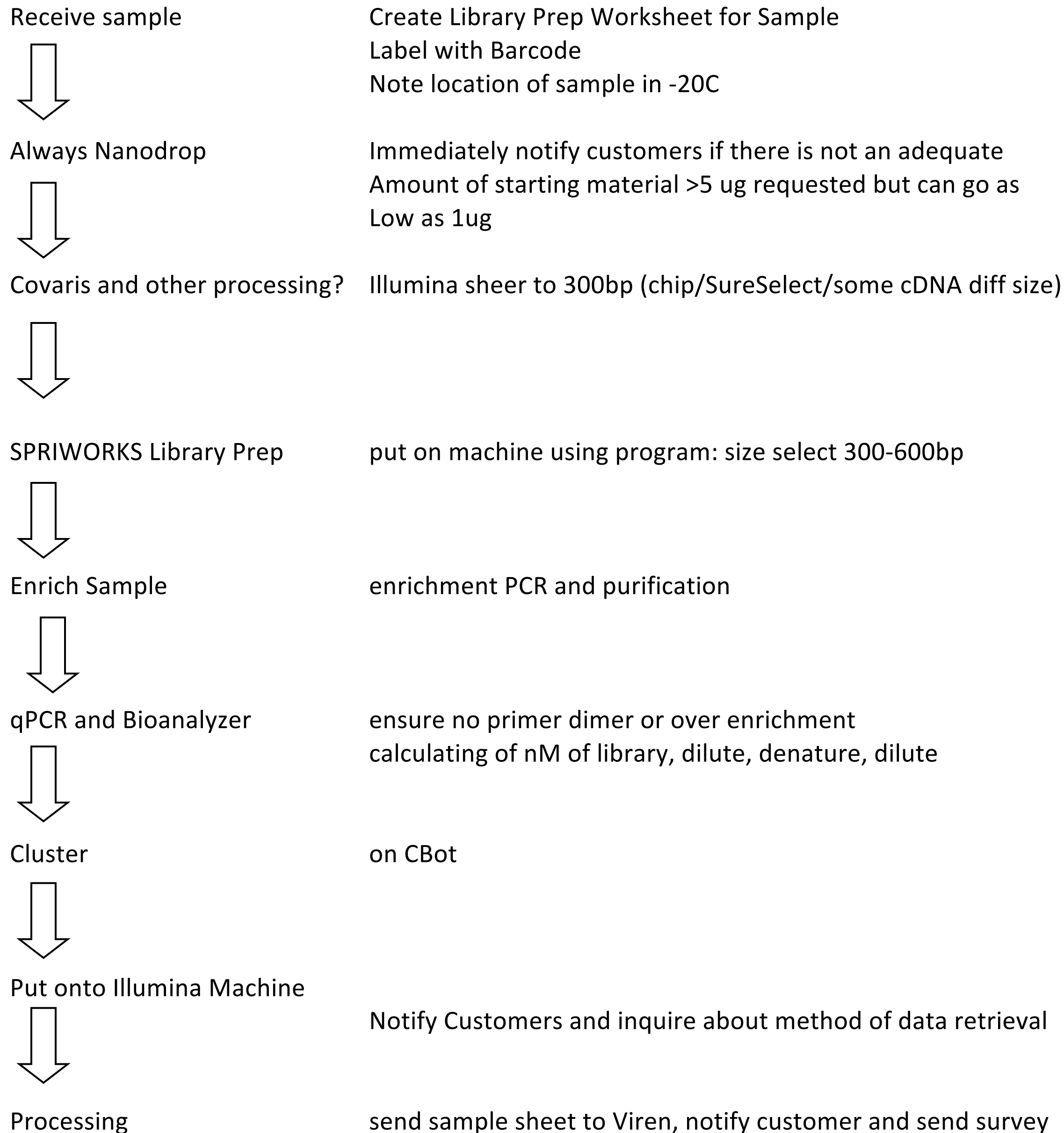
Processing Samples for Illumina HiSeq
Emory Genomics Core

Flow Chart for Illumina Samples (Receiving to Sequencing)



Receiving Samples:

- Upon Receiving samples:
  - Fill out sample sheet
    - Include date received, # received, (location box name and -20 or 4 degree), project ID/Service Agreement number, and any special notes regarding samples .

- Nanodrop sample:
  - also determine amount of volume in the tube
  - calculate how much sample you have

- Barcode sample
  - Include
    - barcode
    - PI name
    - Sample name
    - Tag: leave blank
    - step “SAMPLE”
    - concentration

Covaris:

- To sheer most samples for library prep (excluding some cDNA, and CHIP or SureSelect samples) shear to 300bp
  - Diluting sample:
    - if > 5ug then remove 5ug and dilute to 120uL
    - if <5ug use all sample (final volume 120uL)
  - Racks
    - 1-24 samples use rack #500111 with regular covaris tubes
    - 25-96 sample use rack #500143 with crimped covaris caps
  - To sheer to 300 set up computer using following parameters (configure)
    - Click all wells to be sheared (use the delete button to remove previous configuration on wells)
      - Temp: 6-7C
      - Water level: 5
      - Use intensifier
      - Duty: 10%
      - Intensity: 4
      - Cycles/burst: 200
      - Time (sec): 120
  - Hit return and do not save
  - Hit Start
  - During this time thaw red library reagents 1 per sample

SPRIWORKS Library Prep:

- Set up tubes:
  - In a 1.5mL tube rack set up three rows of 2mL screw tops (located under Spri TE)

    - Row 1: barcoded samples (the DNA will go into these)
      - Include barcode, PI, sample, tag – leave blank, step “Spri Bot”, conc –leave blank

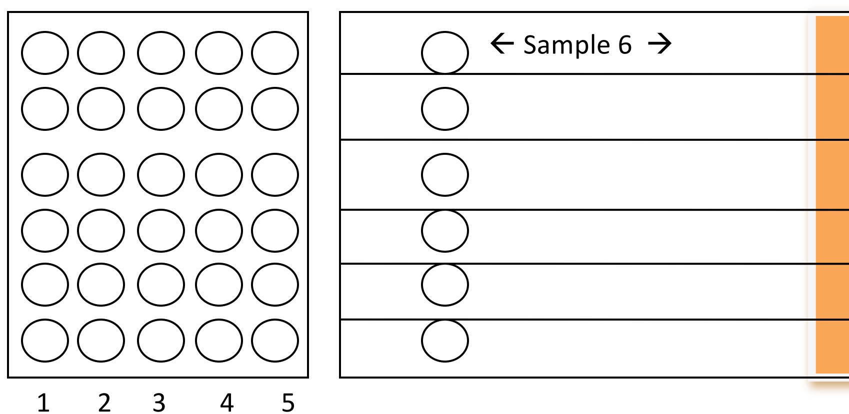
- Row 2: diluted adaptor tubes (side labeled)
  - Use index adaptor from Illumina's Multi-Sample Prep Oligo Kit cat: 1005709
  - Use following dilutions based on input DNA (see library_ calc.xls)

Ug DNA	uL Adaptor	uL H2O
5	10	0
4	9	1
3	8	2
2.5	7.5	2.5
2	7	3.0
1.5	6.5	3.5
1	5	5

- Row 3: sheered sample (side labeled)
  - 120uL of sheered sample in screw top tube

➢ In the metal rack from the SpriTE set up all 5 rows:
- Row 1: black topped tubes (A50102)
- Row 2 and 3: clear topped tubes (A50098)
- Row 4: Adaptor (remove tops)
- Row 5: barcoded library tubes (remove tops and save)

➢ In the metal reagent slide holder set up reagents with sheered sample:
- Ensure reagents are thoroughly thawed by inverting
- Orient metal reagent slide holder so that the looped bottom is closest to you
- Place reagent strip into slide holder with orange tag closest to you (near looped bottom)
- Place sheered sample into hole in reagent strip and remove and discard cap.
- Ensure that samples match from metal rack to metal reagent slide holder (see below)



❖ Loading machine

➢ Open door

➢ Place metal reagent slide holder in back with orange label facing you

- Place metal tube holder in front
- Ensure all samples are still correctly aligned
- Close door

- Starting machine:
  - Press START
  - For size selection press 1 (yes)
  - For 300-600 press 2
  - To confirm press enter (located to the left of STOP)
  - Ensure run has begun before leaving machine.

Enrichment PCR and Purification Protocol

- Enrichment PCR (to process sample after SPRIWORKS)
  - Prepare PCR reaction using appropriate volume of DNA to water and PCR index Primer for each sample (using Illumina's Multiplexing Sample Prep OligoNucleotide Kit #1005709) and cycle using 10-15 cycles

Amnt input DNA To library prep	Vol of purified library into PCR	volume of water
5ug	1uL	21uL
4ug	2uL	20uL
3ug	3uL	19uL
2ug	4uL	18uL
1ug	5uL	17uL
<1ug	10uL	12uL

BARCODE	SAMPLE	ug DNA (rounded)	uL library	uL water	index	cycles

XuL DNA + XuL water = 22uL total
1uL PCR Primer InPE 1.0
1uL PCR Primer InPE 2.0
1uL PCR Primer Index X
<u>25uL Phusion DNA Polymerase</u>
50uL total

- Amplify using the following parameters (stored on thermocycler under *Illumina* then under *ENRICHXcycles or SPRIRICHXcycles*

  1 cycle of:
  98C 30sec
  18 cycles of:
  98C 10 sec
  65C 30 sec
  72C 30 sec
  1 cycle of:
  72C 5 min
  4C hold
- During this time warm Ampure XP Beads to RT and make labels/tubes (2/sample only one with barcode).
- To make Labels:
  - Open user Bartender Security User gRA
  - Open Batchmaker from Start menu
  - Click on blue label (lower left corner)
  - Open GRA label
  - Click on FILE, Print Batch
    - Enter the ID, the Index used, and PI's name
  - Print each label. Adhere top labels to two tubes and the barcode to one of the two tubes.

- Ampure XP Purification
  - Add 1.0X Ampure Beads (50uL sample so use 50uL beads)
  - Adhere sample to beads:
    - Add Ampure Beads to tube w/o barcode
    - Add DNA to tube
    - Vortex, shake down, and let sit @ RT for 5 min
    - Put on MPC ~1min (til clear)
  - Wash
    - Remove super and discard
    - Wash 6X with 300uL 70% ethanol (let sit ~30sec after wash, do not remove from stand)
    - Allow to dry approx 2 min (tap upside down and fan)
  - Resuspend
    - Resuspend in 28uL EB
    - Leave at RT for 2 min, put on magnet ~2 min
    - transfer 26uL into tube with barcode

- Bioanalyzer:
  - Run on High Sensitivity DNA chip (1uL) – look for overenrichment and primer dimer (not good)

- KAPA Quant qPCR Protocol:
  - Add 1mL of the III GA Primer Mix to Kapa qPCR Master Mix (5mL)
  - Make 1:1000 dilution of library with 10mM Tris + 0.05% Tween.
    - Note if concentration is greater than 100nM make 1:10,000 dilution of library
  - Mark plate for samples
  - Kapa

    Cat: ________________________

    Lot: ________________________

standards neg control

	1	2	3	4	5	6	7	8	9	10	11	
A												
B												
C												
D												
E												
F												
G												
H												

- Add Reaction to Wells:

  12uL of Master Mix to each well
  4uL of water to each well
  4uL of DNA/Standard/or water to each well
  20uL total volume

- Cover plate with sealing tape, vortex 5 sec, spin @ 1000rpm for 1 min.
- Run using Kapa Quant template:
  - Login to computer using USER: admin PASS: Roche480
  - Choose EXISTING TEMPLATE
  - Choose wells:
    - Click on the ADD NEW and highlight wells to be used

- Click APPLY
- Edit Samples:
  - Click on SAMPLES
  - Choose: ABSOLUTE QUANT then from template click on NEW SUBSET 1\
- Designate samples
  - Highlight all standards and click on STANDARD, also put "PM" as quantity
  - Highlight all negative controls and click NEGATIVE CONTROL
  - Standards:
    - Highlight std 1 wells and type in STD1and click APPLY TO ALL then input standard values (do this for all standards):
      - Std 1: 20pM
      - Std2: 2pM
      - Std 3: 0.2pM
      - Std4: 0.02pM
      - Std5: 0.002pM
      - Std6: 0.0002pM
  - Highlight sample wells and type in name and click APPLY TO ALL
- Run

- Dilutions and Denaturing:
  - See qPCR template for calculating total nM of library and subsequent dilutions:
  - Note when you run out of PhiX dilute/denature/dilute in the same manner.
  - Dilute to 10nM

    (desired volume*desired nM/total nM)

    Example: (200uL*10nM)/154nM = 13uL library + 187uL EB
  - Denature

    Determine amnt library to add

    20/x=N

    Example: (20/10nm)=2uL DNA

    Detemine amnt of EB to add

    19-N = uL EB

    Example: 19-2uL = 17uL EB

    Add 2N NaOH

    1uL 2N NaOH

    Incubate for 5 min at room temperature then immediately move to next step (DNA will be at a 1nM dilution)

  - Dilute the sample for clustering 10pM-20pM

Final Concentration	10pM	12pM	15pM	18pM	20pM
1nM den. DNA	10uL	12uL	15uL	18uL	20uL
Pre-chilled HT1	990uL	988uL	985uL	982uL	980uL

CBOT

- Prepare Samples for Clustering
  - Thaw a plate of reagents
    - TruSeq Cluster Kit v2 C-Bot HS (cat: 15019751)
      - Note: if Paired end remove PE kit, is single read remove SR kit (ditto with flow cell)
    - Record Flow Cell ID: ____________________
    - Record RGT: _______________________
    - Ensure reagents are thawed and invert each several times
    - Also ensure reagents are inserted completely into plate (will click)
    - Weigh the plate _______________________G
    - Spin 1 min at 1Kg
  - Determine which samples will be multiplexed = N
  - Label a PCR strip tube 1-8
    - Add 1.2uL PhiX to lanes 1-7
    - Add 120uL PhiX to lane 8
    - For a multiplexed well, add 120/N of each sample to the well
      - Example: 3 samples for 1 lane so 120/3=40uL of each sample to the well
  - Bring to Warren Lab:
    - Samples, manifold, flow cell (at 4C either SR single read or PE), reagents

Loading CBOT

- Turn off using silver button on left then restart
  - Check waste!
  - Input USR name and hit Start
  - WASH
    - Fill to top with Wash Buffer and click reservoir filled
  - Selections:
    - Choose name
    - Choose either
      - SR_Amp_Lin_Block_Hyb_v7.0 (for single reads) OR
      - PE_Amp_Lin_Block_Hyb_v7.0 (for paired end reads)
  - Follow instructions

- Unloading Flow Cell in Warren lab
  - Check waste!
  - Will say run complete, hit OK
  - Hit complete until it disappears from screen
  - Open top
    - Undo manifold and discard
    - Discard pcr tubes
    - Remove reagent plate

  - Ensure all templates drawn equally across tubes in plate
  - Measure weight of reagent plate (~40g removed)
    Weight: ________________
  - Place in 4C for 1 day in case of rehyb

- Remove flow cell and put into buffer
  - The computer will register that the flow cell has been unloaded
  - Close lid
- Run a wash cycle
  - Fill up reservoirs with H20 until water rises just above metal pins.

HiSeq: Loading Samples

- Check Waste before beginning and empty if it's getting too full!
- Record:

  FLOW CELL ID: __________________________

  EXPERIMENT: ____

  SIDE_____________________

- Thaw reagents the night before the run! (thaw all but polymerase – see below)
    - Per run you will require a 2-3 kits which includes:
        - 1) SBS Reagent Box 1 of 2 @ 4 degrees
        - 2) SBS Reagent Box 2 of 2 @ -20 degrees (thaw the day before except polymerase)
    - 3) if multiplexing thaw TruSeq Multiplex Seq Primer Box 15017557 (in our lab) @ -20 degrees
    - Reagents for each cycle:
        - For a 50 cycle SR run thaw 1 50 cycle kit (both boxes)
        - For a 50 cycle PE run thaw 2 50 cycle kits or a 200 cycle kit and divide the reagents in 1/2
        - For a 100 cycle SR run, thaw 2 50 cycle kits or a 200 cycle kit and divide reagents in 1/2
        - For a 100 cycle PE run, thaw 4 50 cycle kits or a 200 cycle kit
- Pre Wash:
    - Choose "WASH" then "WATER"
        - Top off all bottles and PE tubes with Washing Buffer
        - Click NEXT until wash starts
    - During this time prepare Reagents:

- Preparing Reagents:
    - NOTE:
        - Cleavage Buffer***after touching cleavage buffer always change gloves!
        - Incorp Mix: if you are doing a PE run, divide the buffer in half and add ½ the amount of FFN and polymerase (store remaining Incorp mix at 4C store FFN and rest of polymerase at -20C)
        - Water: fill bottle for slot #2 with wash buffer (from accessories box)
    - Change all reagent tops with tops that have a hole from the accessories box
        - If not using immediately, store @4C

      Reagent 1 of 2 RGT: ____________________________
      Reagent 1 of 2 RGT: ____________________________
      Index Reagent RGT______________________

- Sequence:
    - Choose "SEQUENCE" then "NEW RUN"
    - First Screen:
        - FLOW CELL ID: zap the flow cell tube (use the longer barcode) should end in XX
        - EXPERIMENT: input PI's name etc.
        - CONTROL LANE: 8

    - OUTPUT FOLDER: click on COMPUTER, S:\ , HISEQ
    - Click on Advanced
        - Check KEEP INTENSITY FILE
    - NEXT
- Second Screen:
    - CYCLE: 50 or 100
    - INDEX: 0 (if no index) or 7 (if indexing) TruSeq Multiplex Seq Primer Box 15017557
    - READ: 0 (if single read) 50 or 100 if (paired end)
    - NEXT
- Third Screen
    - SBS REAGENT ID: scan reagent box
    - BOX CYCLE: leave @ 200
    - Check PRIME SBS
    - NEXT
- Fourth Screen
    - Review: ensure the following
        - Output File: S:\HiSeq
        - Keep intensity Files
        - Control lane 8
        - NEXT
- Fifth Screen:
    - Load Reagents:
        - Load Reagents 1-8
        - If Indexing: Load Reagents 9-19 keep empty if not indexing
            - #17 seq primer
            - #18 NaOH (175uL of 2N NaOH + 3325uL H2O)
            - #19 Wash buffer
        - Check PWI (25mL in P2)
        - NEXT
- Sixth Screen:
    - Vacuum engaged fluidics check
    - NEXT takes ~20min
        - During this time wash flow cell with ddH20 and wipe thoroughly
- Seventh Screen: "Priming Complete"
    - NEXT
- Eight Screen:
    - Remove flow cell
    - Wipe surface of machine with kim wipe
    - Change gaskets
    - Place flow cell onto machine (barcode to right, illumine to left) holes down
    - Engage vacuum
    - NEXT
- 9th Screen
    - PUMP
        - Ensure all cells flowing
    - NEXT

- If all cells not flowing remove flow cell, place in buffer, put @ 4C and wash machine with water.

- Close Door
  - NEXT
  - START
- To view clusters and 1st base report click on the tab at the bottom of the screen that says "Illumina HiSeq Cont"
  - Should take ~10 minutes.

- Run will finish in approximately :
  - 50 cycles ~3 days
  - 100 cycles ~ 5 days
  - 100PE ~10-11 days

- Turnaround:
  - Thaw a TruSeq PE cluster kit (the one with the 15mL tubes).
  - Make new NaOH (3,325uL water + 175uL 2N NaOH) → position #18
  - Add 15uL of 100uM Multiplexing Primer Read 2 to the Sequencing Primer Read 2 Mix →position #16
  - Load PE reagents
    - Position 10→ resynthesis mix
    - Position 11→ linearization mix
    - Position 12→ blocking mix
    - Position 13→ Amplification mix 2
    - Position 14→ AMX2 Premix
    - Position 15→ Formamide
    - Position 16→ Prepared Sequencing Primer Read 2 Mix
    - Position 17→ empty
    - Position 18→ Prepared NaOH
    - Position 19→ Wash Buffer
  - Load reagents
    - Remake a fresh Incorp mix with remaining FFN and polymerase and replace
    - Load all PE reagents
  - Follow instructions on HiSeq.

- After each run:
  - Do a Maintenance Wash:
    - On the screen choose WASH
      - First load water
      - Then load 1N NaOH wash (diluted from 10N NaOH)
      - Finally load water again
  - Reboot the computer: shut down, wait a minute, and start back up
  - Delete temp files:
    - Open computer and go into either the D drive or E drive.
    - Choose the oldest runs (listed by date) to delete – you can leave 2-3 folders in the drive. f

Note/Comments:____

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Trouble shooting: Illumina HiSeq

- Other Issues:
  - For Bubbles:
    - Fluidics Issues?
      - Blocked capillaries
        - After the maintenance Wash if capillaries are still use a syringe to force warm water through the capillaries.
        - To open the machine unscrew the bottom right screw and lift up and out to remove panel.
        - Remove vacuum on flow cell and replace with cream colored boat (red thing on left side) and groove in front. Engage vacuum.
        - Fill boat with hot water. Completely empty syringe of air and unscrew the tube entering the "NO" part of the machine and screw into syringe. Aspirate the water through the syringe checking that the water is being removed from the boat.
        - Also you can force water through the capillaries ensuring that all water is immediately soaked up in a kim wipe so that it doesn't damage the imager.
      - Faulty Solenoid – Illumina will have to come replace (we have on in our lab)
      - Faulty gasket - replace gasket to see if it solves the issue
  - No clusters/low clusters – see trouble shoot CBOT below

- Trouble shooting CBOT…OR… Rehyb
  - Immediately after clustering:
    - Before Clustering weight the reagent plate ___________
    - After clustering weight the reagent plate __________
      - The difference should be ~ 40g
    - View reagents and ensure even aspiration across tubes in a row
  - To Rehyb Flow Cell :
    - Enter UserName and click START
      - WASH
      - Prepare flow cell
        - Wash with ddH20 and wipe thoroughly
      - Input Experiment name
        - Choose "repeat hyb v 7.0" paired end v2

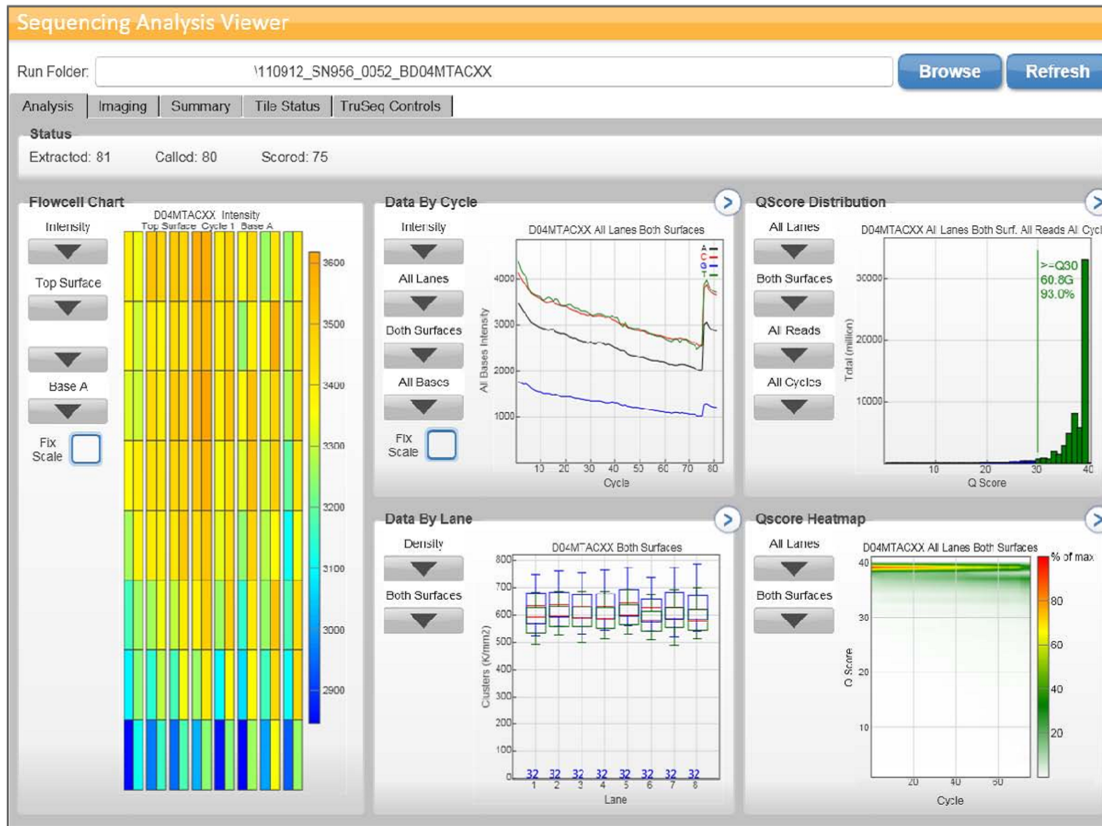
        - Choose “repeat hyb v8.0” paired end v3
            - Note computer can only “read” paired end so use paired end even for single read
            - Can only use reagents ONE day after cluster ONCE
        - Scan paired end plate
- Follow instructions
    - PRECHECK
    - START
        - Takes ~15min

Quality Control: Illumina Quality Control Specifications:

1) Q scores: at least 80% passing Q30
    a. Look at Qscores per lane (top right corner)
    b. Were all >80%? ______________________________________
2) Intensities over time
    a. Look at intensities by cycle over time, should see a slightly decreasing slope with a spike around cycle 75
    b. ____
3) Cluster densities
    a. Range:
        i. V3 HiSeq: 200K – 850K
        ii. V2 HiSeq: 200K – 425K
        iii. GAIIX: 100K – 800K
    b. Also want green and blue boxes to overlap and be tight – good distribution
    c. Blue boxes are raw cluster #s, green are cluster #'s passing filter.
    d. Cluster densities per lane :

    Lane 1____________ lane 2___________ lane 3___________ lane 4_________
    Lane 5____________ lane 6___________ lane 7___________ lane 8_________

4) Flow cell chart (fluidics issues)
    a. View intensities on both sides of the flow cell
    b. Start at cycle 1 and, choosing base T or G, scroll through all cycles
    c. Don't want to see a lane that goes blue and doesn't recover – fluidics issue (can also bleed into other lanes due to fluidics backflow)
    d. Fluidics Issues?_______________________________
5) FWHM
    a. View data by cycle (full width half mass)
    b. Want all 4 data lines (representing the bases) to be at ~3 in a horizontal line
    c. If you see spikes, drops, or step-ups that indicates a focus issue
    d. Issues?________________________________



Calculating reads = clusters (which are same as reads) as well as Gb data per flowcell and per lane:

A) To calculate cluster density passing filter per tile:

Raw clusters*** X % passing filter clusters
700K X 80% = 560K (for V3 HiSeq)
425K X 80% = 340K (for V2 HiSeq)
750K X 80% = 600K (for GAIIX)

For raw cluster number use a number within the acceptable range (min and max listed) :

V3 HiSeq: 200K – 850K
V2 HiSeq: 200K – 425K
GAIIX: 100K – 800K

B) Then calculate # tiles per lane: (note swath = column)

#tiles/swath X # swatchs/surface X # surfaces X # lanes
8 tiles/swath X 3 swatchs/surface X 2 surfaces X 1 lane = 48 (for V3 HiSeq)
8 X 2 X 2 X 1 = 32 (for V2 HiSeq)
60 X 2 X 1 X 1 = 120 (for GAIIX)

C) Then calculate the cluster density per lane

Cluster Density passing filter per tile (A) X # tiles per lane (B) X tile size
560K X 48 tiles/lane X 5.7mm = ~153 million clusters/lane (for V3 HiSeq)

425K X 32 X 5.7mm = 62 million clusters/lane (for V2 HiSeq)
750K X 120 X 0.5724mm = 41 million clusters/lane (for GAIIX)

If you want to determine how many reads or cluster per sample divide the above number (C) by the # samples per lane (ex using 2 samples per lane)
186 million/2 = ~77 million reads per sample (for V3 HiSeq)
62 million/2 = 31 million reads per sample (for V2 HiSeq)
41 million/2 = 20.5 million reads per sample (for GAIIX)

D) to determine GB per lane
Cluster density per lane (C)X cycle number
153E6 clusters/lane X 50 = 7.6 GB data per lane (for V3 HiSeq)
62E6 X 50 = 3 GB data per lane (for V2 HiSeq)
41E6 X 52 = 2 GB data per lane (GAIIX)