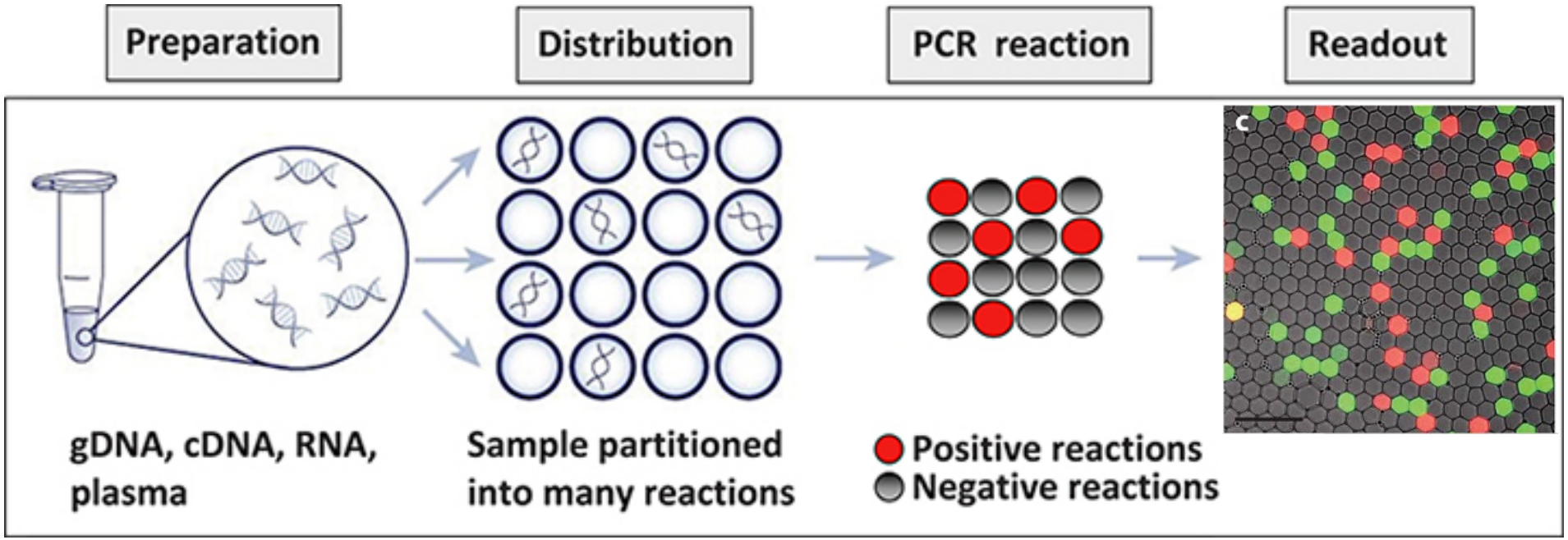
Digital PCR Seminar

Lou Ann Bierwert – ITI Center for Molecular Biology

dPCR involves performing PCR with end-point data collection in a large number of separate reaction chambers, or partitions. Results are obtained by counting the number of partitions in which the amplified target sequence is detected (regarded as positive) and the number of partitions in which there is no amplification (regarded as negative). Absolute quantification of the mean number of target sequences per partition is achieved by applying a Poisson correction to the fraction of the positive partitions. This compensates for the fact that more than one copy of template may be present in some partitions.

"Absolute quantification" used in dPCR refers to an estimate derived from the count of the proportion of positive partitions relative to the total number of partitions and their known volume.



Unlike qPCR, in which the quantification cycle (Cq) depends on variable features such as the instrument, fluorescent reporter dye, and assay efficiency, dPCR relies on a simple count of the number of successful amplification reactions. The counting of positive partitions in an ideal dPCR is definitive and does not require a calibration curve to convert Cq to copy number; knowing the partition number and volume is sufficient.

The ability to measure extremely low concentrations of specific DNA sequences, independent of a standard curve, with high precision, in a complex background, is unique to dPCR.

dPCR was initially developed to investigate minority target measurement, for which rare variants are measured in the presence of large numbers of wild-type sequences. Detection and quantification of rare mutations can provide a useful tool in several scenarios such as the diagnosis and staging of cancer.

Digital PCR has many applications, including the detection and quantification of:

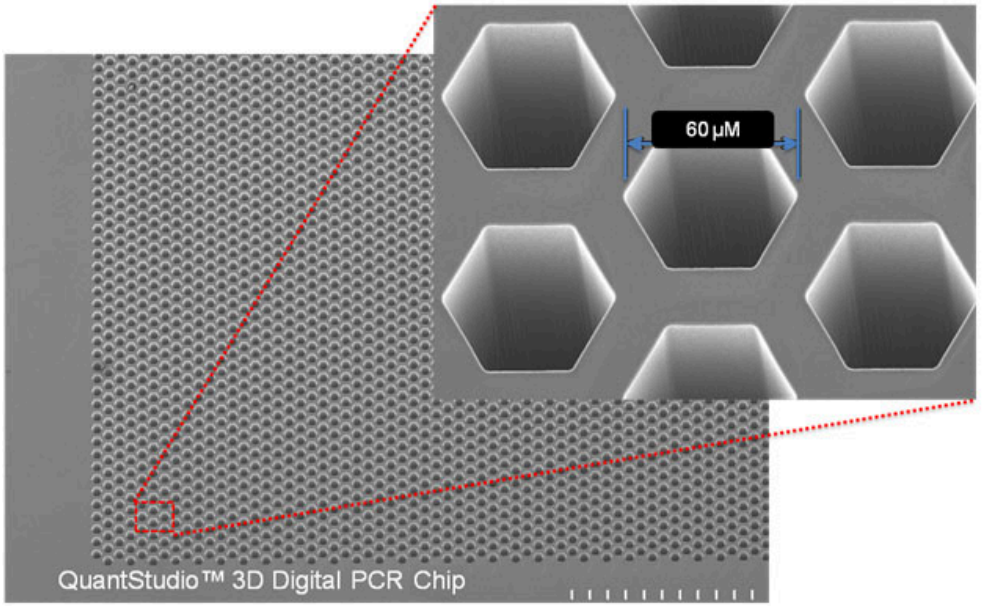
- **low-level pathogens**
- **rare genetic sequences**
- **copy number variations associated with chromosomal rearrangements or gene/chromosomal dosage**
- **relative gene expression in tissues or single cells**
- **cDNA concentrations**
- **New Digital PCR Application for Allele-Specific Copy Number Analysis**

Experimental Workflow on the QuantStudioTM 3D Digital PCR System

- Instrument reads one chip at a time
- Less than a minute to read one chip
- Factory calibrated to detect FAM™, VIC® and ROX® dyes; also compatible with SYBR Green I assays (detects SYBR on the FAM channel and can detect HEX on the VIC channel). ROX is the reference dye in MM so can only singleplex or duplex.
- Intuitive touch screen operation
- Upload results to cloud for analysis
- Instrument stores results for last ~600 chips (max)

- 20,000 reaction wells per chip
- Minimal sample loss
- One sample per chip
- Simple and consistent loading
- Sealed consumable minimizes contamination
- Each chip identified by unique 2D barcode
- Fixed reaction volume minimizes upfront sample manipulation

- Total reaction volume loaded on chip is~14.5µl
- Reaction volume is an important determinant of sensitivity.
- Partition volume is 755 pL
- Chip surface has hydrophobic coating to enable isolation of independent reactions.



•dPCR only measures the copies that **are amplifiable**.

•As such there are sequence and sample specific factors to consider prior to your experiment

Factors that affect template amplification

- ❑ Sequence damage or DNA integrity
- ❑ Assay inhibition or poor sensitivity of assay (primer design)
- ❑ Molecular dropout due to linked targets or secondary structure (RE can help, multiple primer design may be necessary)
- ❑ Chemical modifications (e.g. formalin crosslinking)
- ❑ Denaturation state (single vs double stranded)
- ❑ For RNA templates, reverse transcription efficiency

Preparing DNA Samples

- DNA Quality
  - Use an optimized DNA extraction protocol, preferably ending in water
  - salting-out procedures and crude lysates are not recommended
  - Make sure DNA extracts do not contain PCR inhibitors
  - $A_{260/230}$ and $A_{260/280}$ ratios should be between 1.7 and 1.9
    - ~2.0 for RNA
  - Make sure DNA is not degraded
    - E.g. as visualized on an agarose gel

- DNA Quantity
- Thermo recommends the following methods of quantitation:
  - nucleic acid quantitation using the Qubit® 3.0
  - Spectrophotometry (OD260) for quality ratios
- The volume of sample added to a digital PCR reaction depends on the
  - Concentration of genomic or complementary DNA (gDNA or cDNA) present in each sample
  - Number of copies of the target sequence present in the genome or total RNA of your samples.
- If target copy number is unknown, qPCR data can be used if available.
- Note-assays should be tested with regular or qPCR to verify if possible.

• Goal is to dilute the samples so that each partition will contain, on average, **~0.6 to ~1.59** copies of the target sequence.

• **Example for human gDNA templates**
• Human genomic DNA has 3.3 pg/copy of a given gene (*E. coli* 0.004 pg/copy)
• Each partition is 755 pL
• To determine an appropriate copy number per chip:

• **Low end of range**:
0.6 copies / 755 pL x 20,000 partitions = 12,000 copies total->
~795 copies/µL or 2.62 ng/µL in the dPCR reaction mix.

• **High end of range**: 1.59 copies / 755 pL x 20,000 partitions = 31,800 copies total needed->
~2105 copies/µL or 6.94 ng/µL in the dPCR master mix.
(I don't follow these numbers – easier to calculate total DNA needed – 31,800 copies * 3.3pg/copy = 104.9ng in 14.5µl so 104.5/29 half µls = 3.62 so add this to total needed = 104.9 + 3.62 = 108.5ng total in 15µl)

**Highest precision (meaning Poisson stats calculations) is achieved at 1.59 copies per partition*

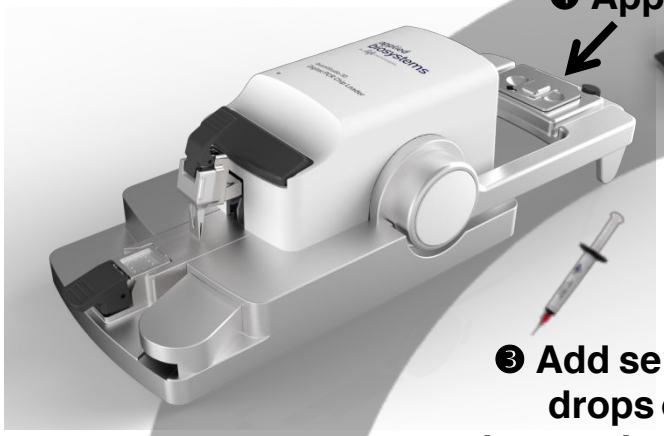
Prepare the digital PCR reactions

- **Required items**
  - TaqMan® Assay(s) ($140-170/200 rxns)
  - QuantStudio™ 3D Digital PCR Master Mix ($140/200 rxns)
  - Pipettes and tips, P10 to P1000
  - Reaction tubes
  - Molecular grade water
  - Microcentrifuge
  - Vortex
  - Gloves, marker pen, lint-free wipes
  - Chips (comes with all loading consumables) - $52/12 ($884/200)
  - $5-6 per sample

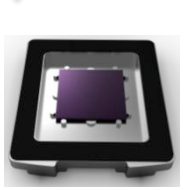
Material	Volume (µL)	Stock	Final
QuantStudio™ 3D Digital PCR Master Mix, 2X	7.5	2X	1X
TaqMan® Assay, 20X (primer/probe mix)	0.75	20X	1X
Diluted DNA	1.5	23 ng/µL*	2.3 ng/µL*
Water	5.25	-	-
Total volume (sample/1 chip)	15	-	-

* Just an example, it will depend on the application

QuantStudio™ 3D Workflow with Chip Loader



❶ Chip & rxn mix equilibrate to room temp (~15')



❷ Place chip and lid on loader & load 14.5 µL of rxn mix

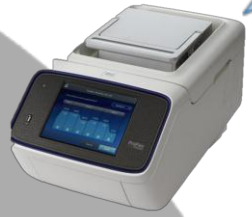
❸ Add several drops of immersion fluid after reaction is loaded

❹ Apply lid

❺ Inject immersion fluid

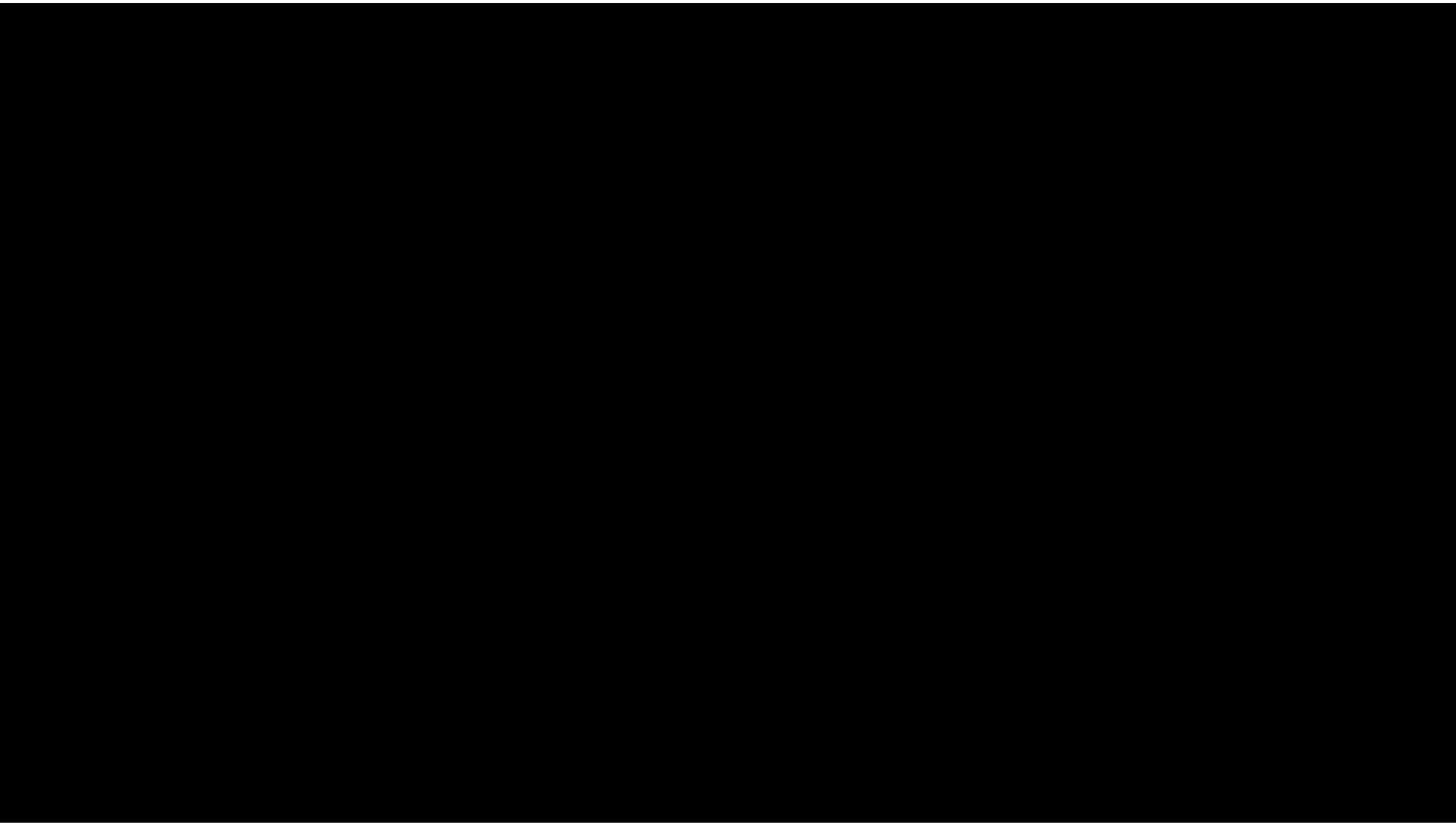
❻ Apply fold-back adhesive tape over fill port

11° Front Tilt



❼ Cycle chip on ProFlex® PCR System

❽ Read chip on QuantStudio™ 3D Digital PCR Instrument

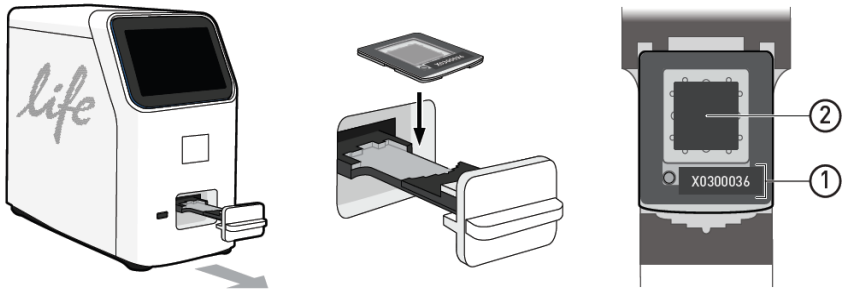


Wait for chips to reach room temperature.

•Set the destination for the imaging data

•You may, for example, use a USB memory stick to collect the run data

•Open the chip tray and load the Digital PCR 20K Chip face-up into the bay. Confirm that the Digital PCR 20K Chip is correctly aligned within the chip tray, then close it.



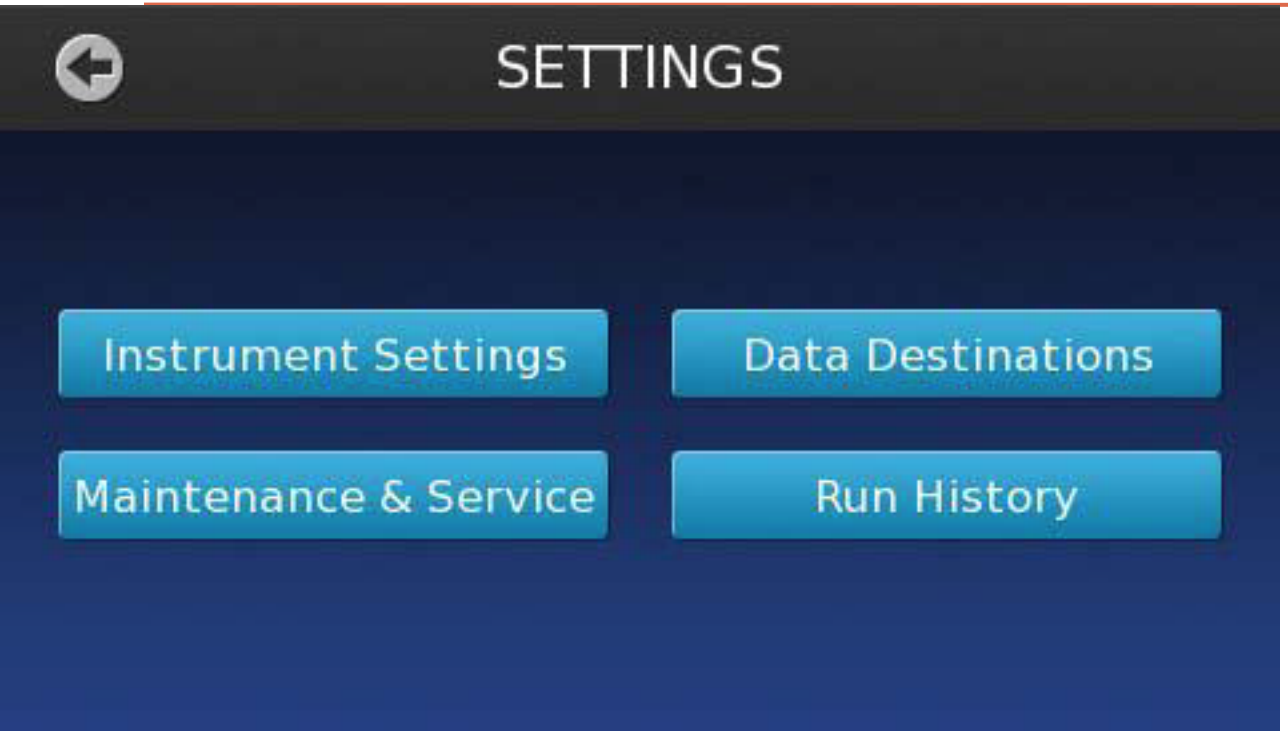
❶ Orient the chip ID and fill port toward the front of the instrument.

❷ Confirm that the chip window is clean and correctly aligned to the chip.

Enter a prefix or regret will ensue. You have about 20 seconds.

- FAM™ and VIC® data will be shown after analysis is complete
- After reviewing the results of the run, touch Done
- Further analysis using cloud- or server-based AnalysisSuite software is recommended

- You may run the imaged Digital PCR 20K Chip again **for up to 1 hour after thermal cycling.**
- If you read multiple Chips in rapid succession, touch the scroll buttons to review the results of the previously imaged chips.
- The Instrument retains a copy of the analyzed data for the imaged Chip that you can access from the Run History screen



Note: *The results will remain in the QuantStudio™ 3D Instrument cache for up to* ***~600 readings****. After ~600 chips, the instrument removes the oldest data file in the cache to store each new reading*

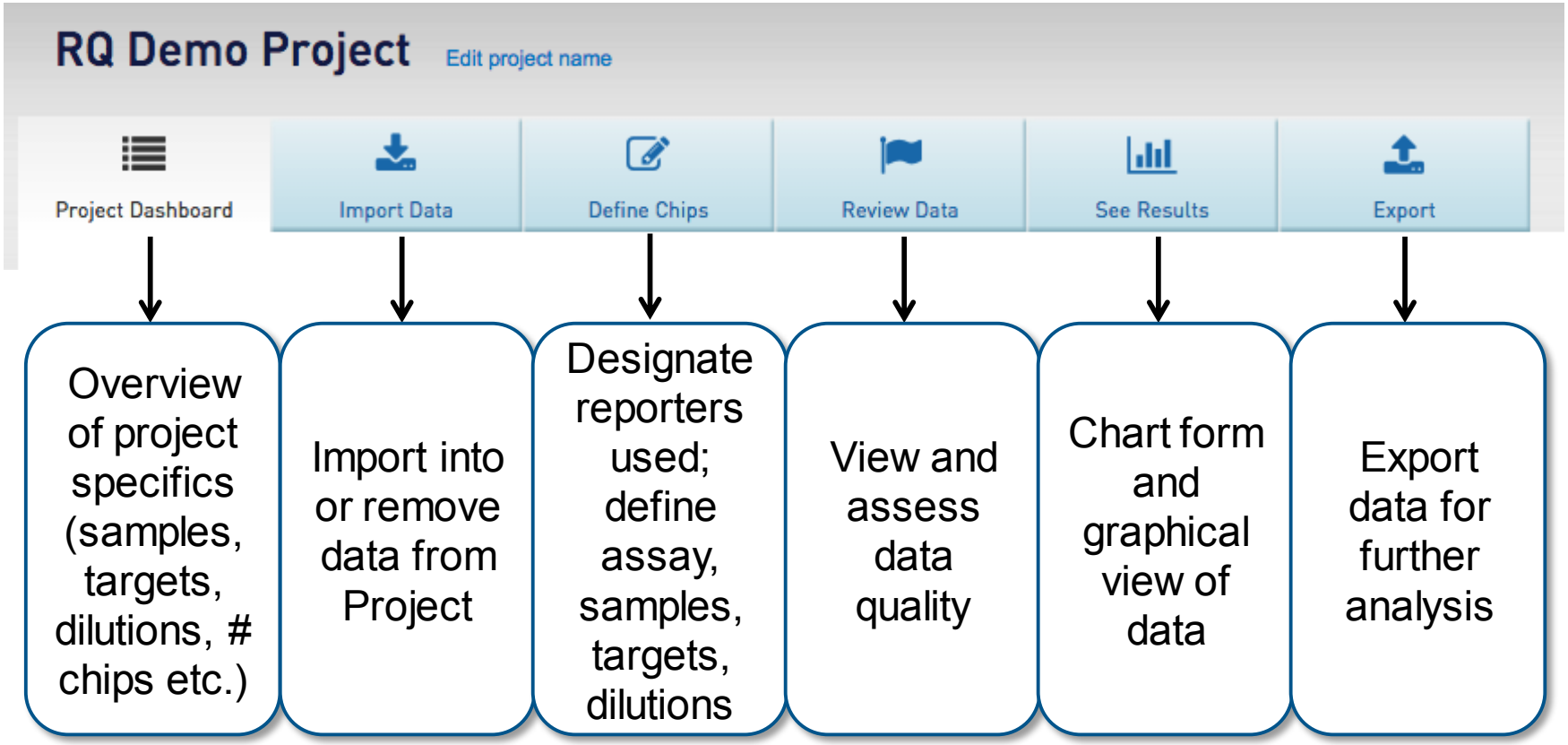
Analyze your data in AnalysisSuite in the cloud

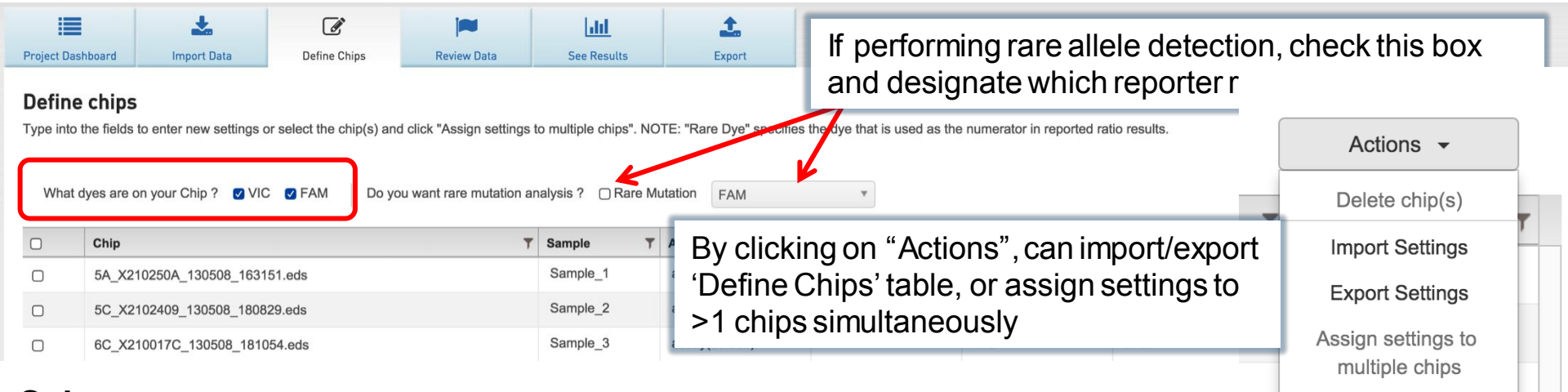
•URL: https://www.thermofisher.com/ca/en/home/life-science/pcr/digital-pcr/quantstudio-3d-digital-pcr-system.html
•Alternatively, just go to Thermofisher.com and search “AnalysisSuite”
•All users **must create a new login**, or use a pre- existing (validated) account username and password (previous Life Technologies accounts would have been transferred to Thermofisher.com and can still be used)
•Case-sensitive
•Make sure to use **Google Chrome**
- Once logged in, you are taken to this page where you can look at and add to old projects or create a new one.

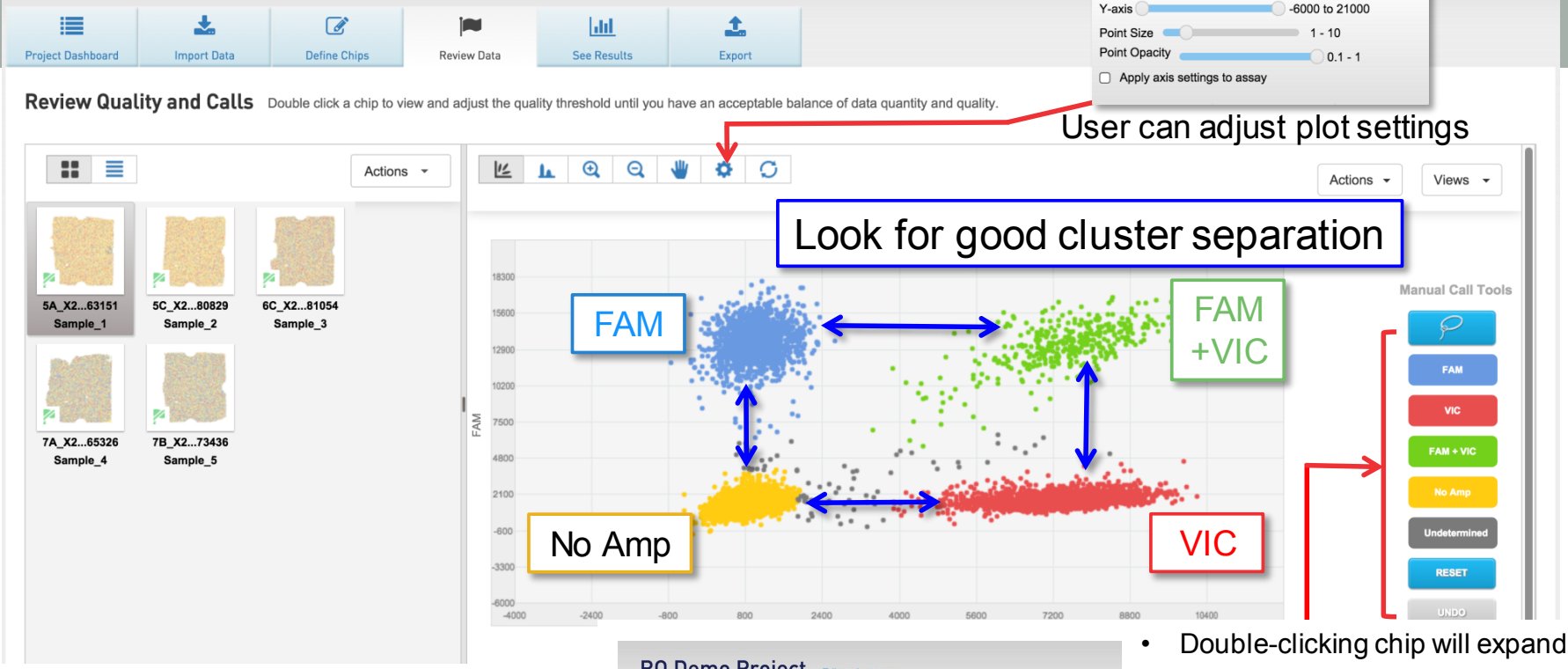
Project	# of Imported Chips	Last modified
Carlsbad Training	7	Dec 31, 2015 12:03 PM
50 100 ng 2015-12-3	3	Dec 04, 2015 02:28 PM
Cre LoxP project	2	Nov 30, 2015 01:12 PM
CMV Standard curve 3 - chip reread	7	Nov 22, 2015 01:12 AM
RQ Demo Project	5	Nov 12, 2015 03:09 PM
CNV Demo Kit	6	Nov 12, 2015 03:08 PM
SIRS samples	12	Nov 12, 2015 11:13 AM
DilutionCurve	12	Nov 12, 2015 10:48 AM
Illumina Library Quant. Demo Kit	2	Nov 06, 2015 04:39 PM

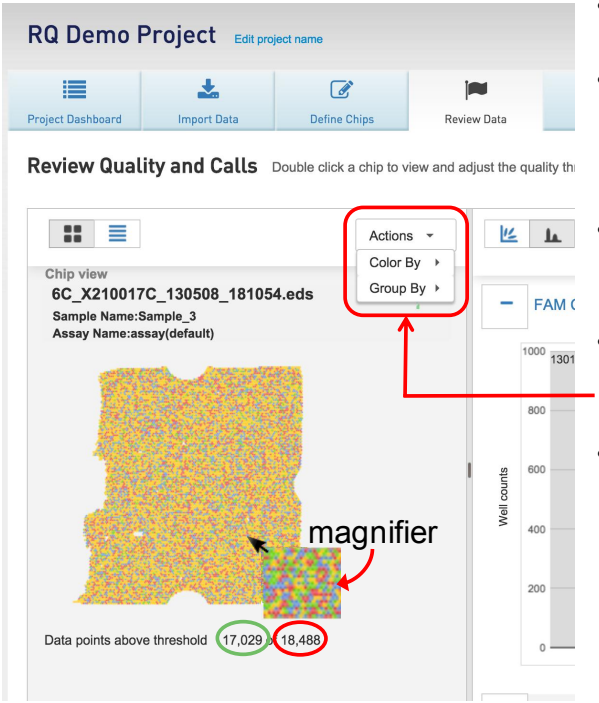
Either open an existing project, or at bottom of screen, can import a project/create a new one

Project name you created





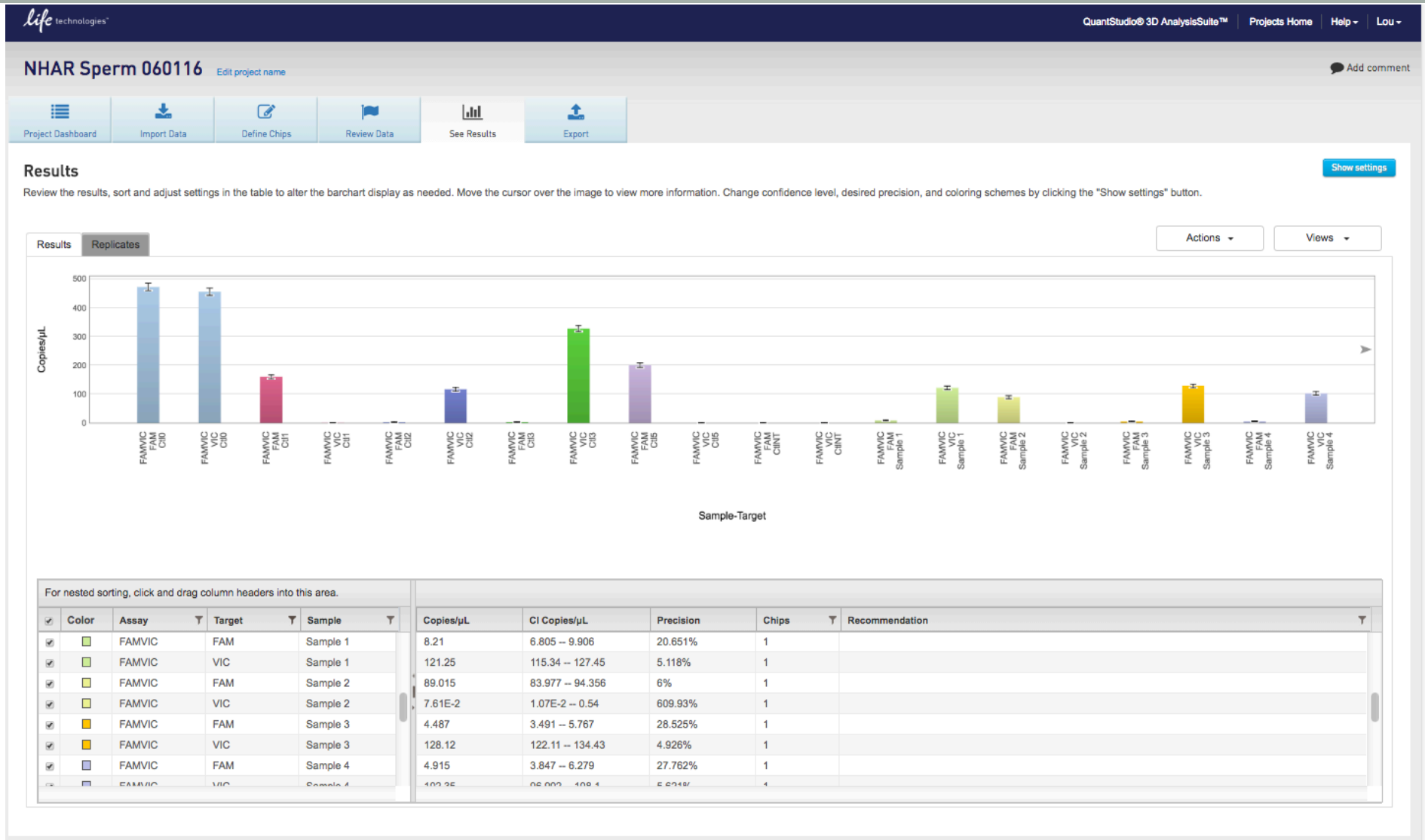




- Double-clicking chip will expand it
- Default view is to “Color by Call” (i.e. FAM +ve, VIC +ve, FAM & VIC +ve, or No Amp)
- Look for random distribution of positive partitions
- Moving cursor over chip image reveals a “magnifier”
- Clicking “Actions” reveals the ability to “Color by Quality”

17,029 — # of wells that passed the quality threshold cutoff

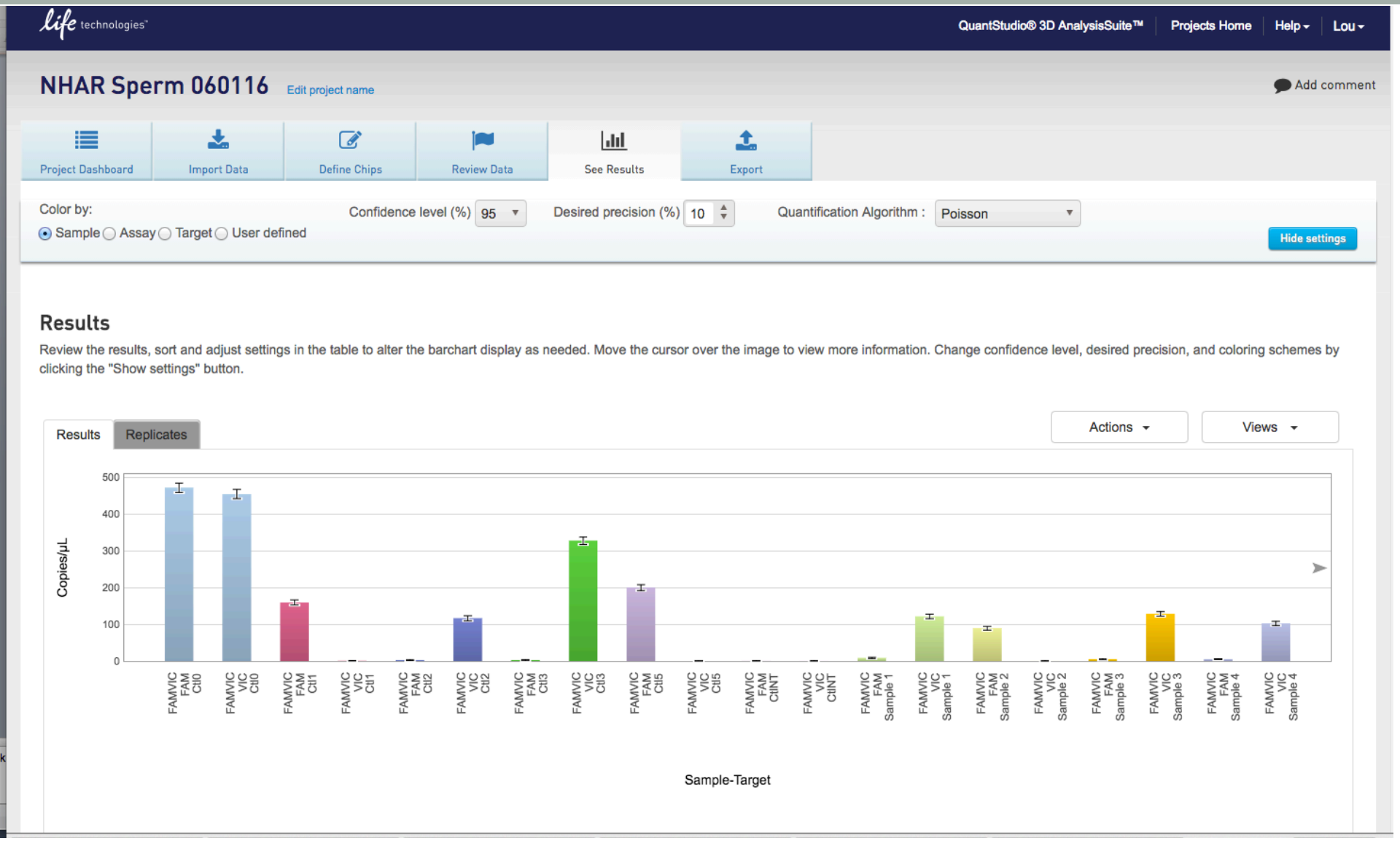
18,488 — # of wells total that were filled with dPCR reaction



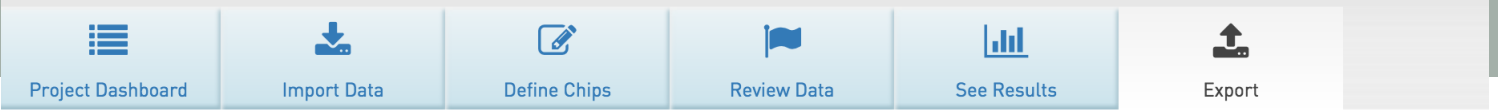
As the sample becomes more concentrated, the chance of more than 1 molecule being present within a positive partition increases. However, the distribution of molecules throughout the partitions approximates a Poisson distribution and a Poisson correction is applied.

Note how precision skyrockets with few data points. Can't be helped with rare events.

Filter can be applied to any column with a funnel icon.



Under “Show Settings”, you can change the confidence level (90, 95, or 99% - error bars grow with higher confidence desired), precision desired (wide range, 10% standard), and algorithm used (Poisson or Poisson plus).



Export

Export creates a .csv file. All data are exported in a fixed order and format regardless of the sorts and column organization in the tables below.

- Project data as CSV ← Export data as .csv, further analysis in 3rd party graphing program
- Chip data as XML ← Export well fluorescence values (for advanced users)

Export

- All data are exported in a *fixed format* that does not reflect column sorting performed in AnalysisSuite™ prior to export.

Project name	test	Assays	1
User name	lbierwer@smith.edu	Samples	1
Created by	lbierwer@smith.edu	Chips	1
Created on	Jun 03, 2016 10:48	Confidence level	95 %
Last modified on	Jul 05, 2016 15:17	Desired precision	10 %
Application version	3.0.3	Algorithm version	0.22
Project description		Algorithm type	Poisson

Rare Target Results

Assay	Sample	Target/Total	CI Target/Total	Copies/µL (VIC)	CI Copies/µL (VIC)	Precision (VIC)	Copies/µL (FAM)	CI Copies/µL (FAM)	Precision (FAM)	Chips	Recommendation
FAMVIC	Sample_1	33.631%	25.414% -- 44.457%	10.333	8.701 -- 12.271	18.756%	5.236	4.113 -- 6.664	27.285%	1	

Quantification Results

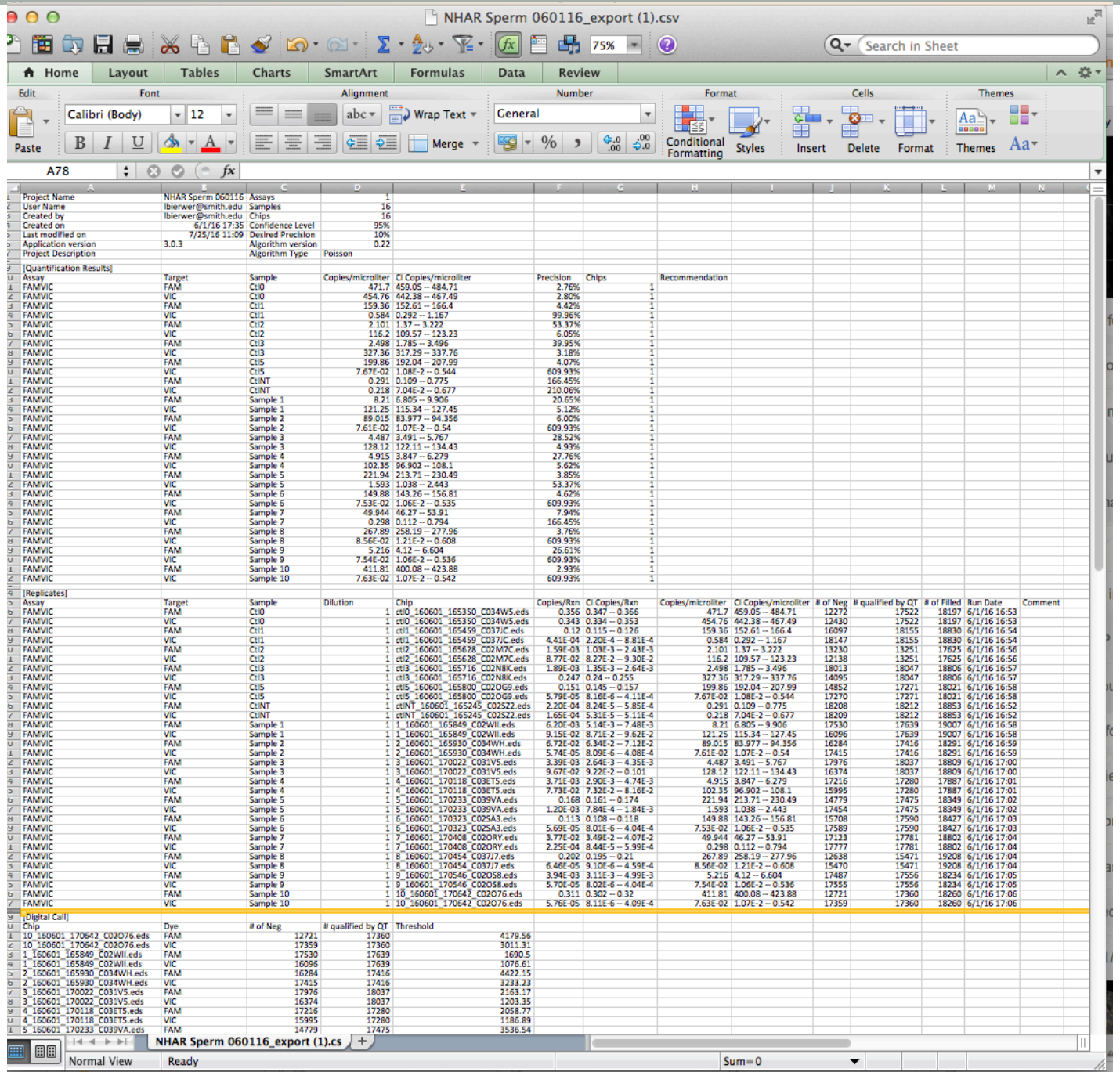
Assay	Target	Sample	Copies/µL	CI Copies/µL	Precision	Chips	Recommendation
FAMVIC	FAM	Sample_1	5.236	4.113 -- 6.664	27.285%	1	
FAMVIC	VIC	Sample_1	10.333	8.701 -- 12.271	18.756%	1	

Replicates

Assay	Sample	Dilution	Chip	Target/Total	CI Target/Total	Copies/Rxn (VIC)	CI Copies/Rxn (VIC)	Copies/Rxn (FAM)	CI Copies/Rxn (FAM)	Copies/µL (VIC)	CI Copies/µL (
FAMVIC	Sample_1	1	test_160603_104616_C02OS8.eds	33.631%	25.414% -- 44.457%	7.80E-3	6.57E-3 -- 9.26E-3	3.95E-3	3.11E-3 -- 5.03E-3	10.333	8.701 -- 12.27

Digital calls

Chip	Dye	# of Neg	# qualified by QT	Threshold
test_160603_104616_C02OS8.eds	FAM	16663	16729	1700.03
test_160603_104616_C02OS8.eds	VIC	16599	16729	2073.29



Project Name	NHAR Sperm 060116	Assays	1												
User Name	lbierwer@smith.edu	Samples	16												
Created by	lbierwer@smith.edu	Chips	16												
Created on	6/1/16 17:35	Confidence Level	95%												
Last modified on	7/25/16 11:09	Desired Precision	10%												
Application version	3.0.3	Algorithm version	0.22												
Project Description		Algorithm Type	Poisson												
[Quantification Results]															
Assay	Target	Sample	Copies/microliter	CI Copies/microliter		Precision	Chips	Recommendation							
FAMVIC	FAM	Ctl0	471.7	459.05 -- 484.71		2.76%	1								
FAMVIC	VIC	Ctl0	454.76	442.38 -- 467.49		2.80%	1								
FAMVIC	FAM	Ctl1	159.36	152.61 -- 166.4		4.42%	1								
FAMVIC	VIC	Ctl1	0.584	0.292 -- 1.167		99.96%	1								
FAMVIC	FAM	Ctl2	2.101	1.37 -- 3.222		53.37%	1								
FAMVIC	VIC	Ctl2	116.2	109.57 -- 123.23		6.05%	1								
FAMVIC	FAM	Ctl3	2.498	1.785 -- 3.496		39.95%	1								
FAMVIC	VIC	Ctl3	327.36	317.29 -- 337.76		3.18%	1								
FAMVIC	FAM	Ctl5	199.86	192.04 -- 207.99		4.07%	1								
FAMVIC	VIC	Ctl5	7.67E-02	1.08E-2 -- 0.544		609.93%	1								
FAMVIC	FAM	CtlNT	0.291	0.109 -- 0.775		166.45%	1								
FAMVIC	VIC	CtlNT	0.218	7.04E-2 -- 0.677		210.06%	1								
FAMVIC	FAM	Sample 1	8.21	6.805 -- 9.906		20.65%	1								
FAMVIC	VIC	Sample 1	121.25	115.34 -- 127.45		5.12%	1								
FAMVIC	FAM	Sample 2	89.015	83.977 -- 94.356		6.00%	1								
FAMVIC	VIC	Sample 2	7.61E-02	1.07E-2 -- 0.54		609.93%	1								
FAMVIC	FAM	Sample 3	4.487	3.491 -- 5.767		28.52%	1								
FAMVIC	VIC	Sample 3	128.12	122.11 -- 134.43		4.93%	1								
FAMVIC	FAM	Sample 4	4.915	3.847 -- 6.279		27.76%	1								
FAMVIC	VIC	Sample 4	102.35	96.902 -- 108.1		5.62%	1								
FAMVIC	FAM	Sample 5	221.94	213.71 -- 230.49		3.85%	1								
FAMVIC	VIC	Sample 5	1.593	1.038 -- 2.443		53.37%	1								
FAMVIC	FAM	Sample 6	149.88	143.26 -- 156.81		4.62%	1								
FAMVIC	VIC	Sample 6	7.53E-02	1.06E-2 -- 0.535		609.93%	1								
FAMVIC	FAM	Sample 7	49.944	46.27 -- 53.91		7.94%	1								
FAMVIC	VIC	Sample 7	0.298	0.112 -- 0.794		166.45%	1								
FAMVIC	FAM	Sample 8	267.89	258.19 -- 277.96		3.76%	1								
FAMVIC	VIC	Sample 8	8.56E-02	1.21E-2 -- 0.608		609.93%	1								
FAMVIC	FAM	Sample 9	5.216	4.12 -- 6.604		26.61%	1								
FAMVIC	VIC	Sample 9	7.54E-02	1.06E-2 -- 0.536		609.93%	1								
FAMVIC	FAM	Sample 10	411.81	400.08 -- 423.88		2.93%	1								
FAMVIC	VIC	Sample 10	7.63E-02	1.07E-2 -- 0.542		609.93%	1								
[Replicates]															
Assay	Target	Sample	Dilution	Chip		Copies/Rxn	CI Copies/Rxn	Copies/microliter	CI Copies/microliter	# of Neg	# qualified by QT	# of Filled	Run Date	Comment	
FAMVIC	FAM	Ctl0	1	ctl0_160601_165350_C034W5.eds		0.356	0.347 -- 0.366	471.7	459.05 -- 484.71	12272	17522	18197	6/1/16 16:53		
FAMVIC	VIC	Ctl0	1	ctl0_160601_165350_C034W5.eds		0.343	0.334 -- 0.353	454.76	442.38 -- 467.49	12430	17522	18197	6/1/16 16:53		
FAMVIC	FAM	Ctl1	1	ctl1_160601_165459_C037JC.eds		0.12	0.115 -- 0.126	159.36	152.61 -- 166.4	16097	18155	18830	6/1/16 16:54		
FAMVIC	VIC	Ctl1	1	ctl1_160601_165459_C037JC.eds		4.41E-04	2.20E-4 -- 8.81E-4	0.584	0.292 -- 1.167	18147	18155	18830	6/1/16 16:54		
FAMVIC	FAM	Ctl2	1	ctl2_160601_165628_C02M7C.eds		1.59E-03	1.03E-3 -- 2.43E-3	2.101	1.37 -- 3.222	13230	13251	17625	6/1/16 16:56		
FAMVIC	VIC	Ctl2	1	ctl2_160601_165628_C02M7C.eds		8.77E-02	8.27E-2 -- 9.30E-2	116.2	109.57 -- 123.23	12138	13251	17625	6/1/16 16:56		
FAMVIC	FAM	Ctl3	1	ctl3_160601_165716_C02N8K.eds		1.89E-03	1.35E-3 -- 2.64E-3	2.498	1.785 -- 3.496	18047	18013	18806	6/1/16 16:57		
FAMVIC	VIC	Ctl3	1	ctl3_160601_165716_C02N8K.eds		0.247	0.24 -- 0.255	327.36	317.29 -- 337.76	14095	18047	18806	6/1/16 16:57		
FAMVIC	FAM	Ctl5	1	ctl5_160601_165800_C02OG9.eds		0.151	0.145 -- 0.157	199.86	192.04 -- 207.99	14852	17271	18021	6/1/16 16:58		
FAMVIC	VIC	Ctl5	1	ctl5_160601_165800_C02OG9.eds		5.79E-05	8.16E-6 -- 4.11E-4	7.67E-02	1.08E-2 -- 0.544	17270	17271	18021	6/1/16 16:58		
FAMVIC	FAM	CtlNT	1	ctlNT_160601_165245_C02SZ2.eds		2.20E-04	8.24E-5 -- 5.85E-4	0.291	0.109 -- 0.775	18208	18212	18853	6/1/16 16:52		
FAMVIC	VIC	CtlNT	1	ctlNT_160601_165245_C02SZ2.eds		1.65E-04	5.31E-5 -- 5.11E-4	0.218	7.04E-2 -- 0.677	18209	18212	18853	6/1/16 16:52		
FAMVIC	FAM	Sample 1	1	1_160601_165849_C02WII.eds		6.20E-03	5.14E-3 -- 7.48E-3	8.21	6.805 -- 9.906	17530	17639	19007	6/1/16 16:58		
FAMVIC	VIC	Sample 1	1	1_160601_165849_C02WII.eds		9.15E-02	8.71E-2 -- 9.62E-2	121.25	115.34 -- 127.45	16096	17639	19007	6/1/16 16:58		
FAMVIC	FAM	Sample 2	1	2_160601_165930_C034WH.eds		6.72E-02	6.34E-2 -- 7.12E-2	89.015	83.977 -- 94.356	16284	17416	18291	6/1/16 16:59		
FAMVIC	VIC	Sample 2	1	2_160601_165930_C034WH.eds		5.74E-05	8.09E-6 -- 4.08E-4	7.61E-02	1.07E-2 -- 0.54	17415	17416	18291	6/1/16 16:59		
FAMVIC	FAM	Sample 3	1	3_160601_170022_C031V5.eds		3.39E-03	2.64E-3 -- 4.35E-3	4.487	3.491 -- 5.767	17976	18037	18809	6/1/16 17:00		
FAMVIC	VIC	Sample 3	1	3_160601_170022_C031V5.eds		9.67E-02	9.22E-2 -- 0.101	128.12	122.11 -- 134.43	16374	18037	18809	6/1/16 17:00		
FAMVIC	FAM	Sample 4	1	4_160601_170118_C03ET5.eds		3.71E-03	2.90E-3 -- 4.74E-3	4.915	3.847 -- 6.279	17216	17280	17887	6/1/16 17:01		
FAMVIC	VIC	Sample 4	1	4_160601_170118_C03ET5.eds		7.73E-02	7.32E-2 -- 8.16E-2	102.35	96.902 -- 108.1	15995	17280	17887	6/1/16 17:01		
FAMVIC	FAM	Sample 5	1	5_160601_170233_C039VA.eds		0.168	0.161 -- 0.174	221.94	213.71 -- 230.49	14779	17475	18349	6/1/16 17:02		
FAMVIC	VIC	Sample 5	1	5_160601_170233_C039VA.eds		1.20E-03	7.84E-4 -- 1.84E-3	1.593	1.038 -- 2.443	17454	17475	18349	6/1/16 17:02		
FAMVIC	FAM	Sample 6	1	6_160601_170323_C02SA3.eds		0.113	0.108 -- 0.118	149.88	143.26 -- 156.81	15708	17590	18427	6/1/16 17:03		
FAMVIC	VIC	Sample 6	1	6_160601_170323_C02SA3.eds		5.69E-05	8.01E-6 -- 4.04E-4	7.53E-02	1.06E-2 -- 0.535	17589	17590	18427	6/1/16 17:03		
FAMVIC	FAM	Sample 7	1	7_160601_170408_C02ORY.eds		3.77E-02	3.49E-2 -- 4.07E-2	49.944	46.27 -- 53.91	17123	17781	18802	6/1/16 17:04		
FAMVIC	VIC	Sample 7	1	7_160601_170408_C02ORY.eds		2.25E-04	8.44E-5 -- 5.99E-4	0.298	0.112 -- 0.794	17777	17781	18802	6/1/16 17:04		
FAMVIC	FAM	Sample 8	1	8_160601_170454_C037J7.eds		0.202	0.195 -- 0.21	267.89	258.19 -- 277.96	12638	15471	19208	6/1/16 17:04		
FAMVIC	VIC	Sample 8	1	8_160601_170454_C037J7.eds		6.46E-05	9.10E-6 -- 4.59E-4	8.56E-02	1.21E-2 -- 0.608	15470	15471	19208	6/1/16 17:04		
FAMVIC	FAM	Sample 9	1	9_160601_170546_C02OS8.eds		3.94E-03	3.11E-3 -- 4.99E-3	5.216	4.12 -- 6.604	17487	17556	18234	6/1/16 17:05		
FAMVIC	VIC	Sample 9	1	9_160601_170546_C02OS8.eds		5.70E-05	8.02E-6 -- 4.04E-4	7.54E-02	1.06E-2 -- 0.536	17555	17556	18234	6/1/16 17:05		
FAMVIC	FAM	Sample 10	1	10_160601_170642_C02O76.eds		0.311	0.302 -- 0.32	411.81	400.08 -- 423.88	12721	17360	18260	6/1/16 17:06		
FAMVIC	VIC	Sample 10	1	10_160601_170642_C02O76.eds		5.76E-05	8.11E-6 -- 4.09E-4	7.63E-02	1.07E-2 -- 0.542	17359	17360	18260	6/1/16 17:06		
[Digital Call]															
Chip	Dye	# of Neg	# qualified by QT	Threshold											
10_160601_170642_C02O76.eds	FAM	12721	17360	4179.56											
10_160601_170642_C02O76.eds	VIC	17359	17360	3011.31											
1_160601_165849_C02WII.eds	FAM	17530	17639	1690.5											
1_160601_165849_C02WII.eds	VIC	16096	17639	1076.61											
2_160601_165930_C034WH.eds	FAM	16284	17416	4422.15											
2_160601_165930_C034WH.eds	VIC	17415	17416	3233.23											
3_160601_170022_C031V5.eds	FAM	17976	18037	2163.17											
3_160601_170022_C031V5.eds	VIC	16374	18037	1203.35											
4_160601_170118_C03ET5.eds	FAM	17216	17280	2058.77											
4_160601_170118_C03ET5.eds	VIC	15995	17280	1186.89											
5_160601_170233_C039VA.eds	FAM	14779	17475	3536.54											