Cluster Generation

Module 2: Overview

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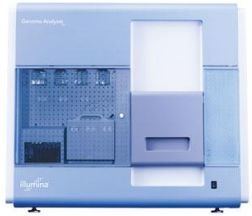
Sequencing Workflow

Sample Preparation

Cluster Generation



Sequencing

Data Analysis

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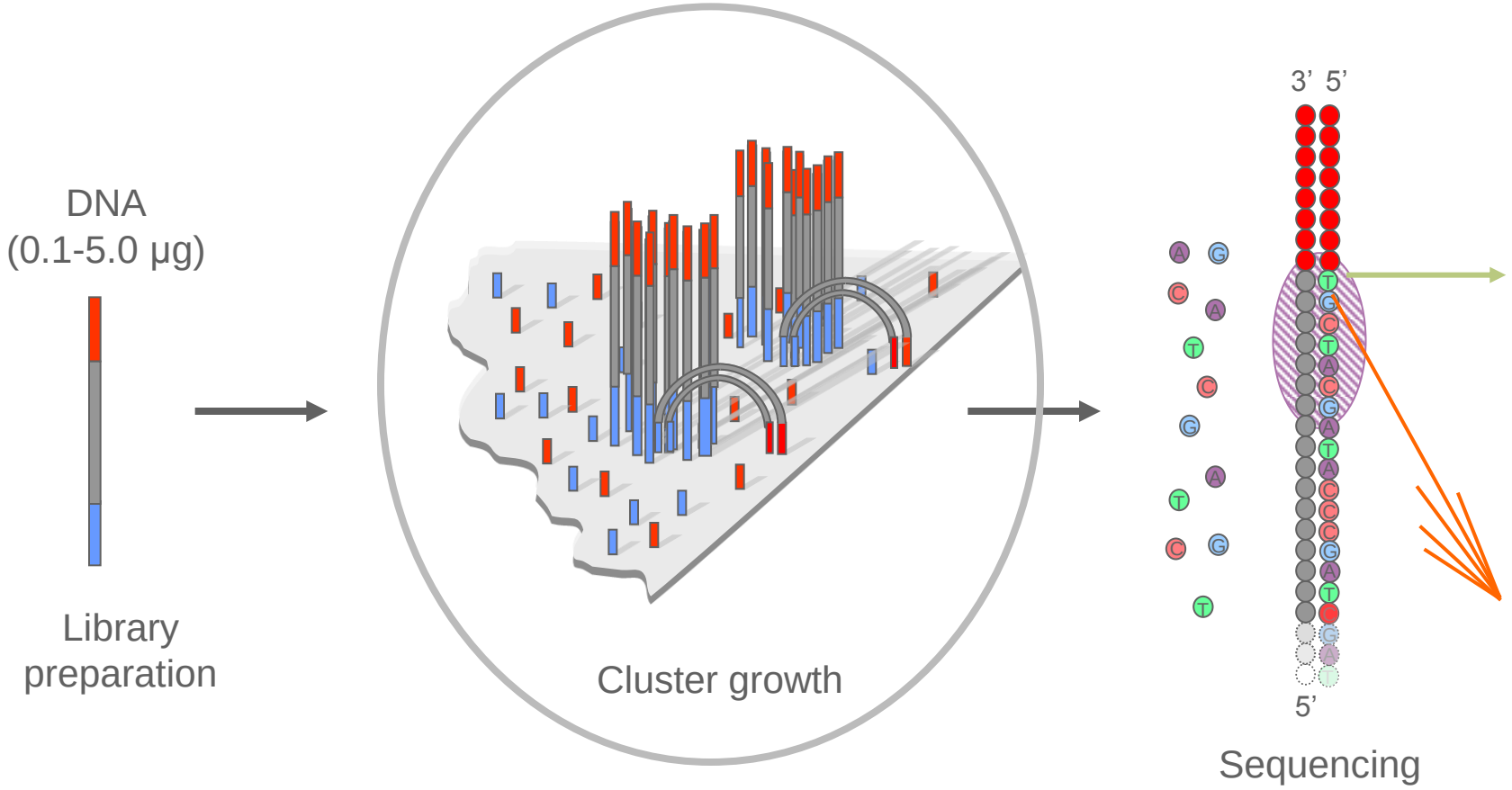
Cluster Generation



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Cluster Generation

Cluster Generation

- Cluster Generation turns libraries into clonal clusters on a flow cell
- All library preparation protocols result in dsDNA
  - Each sample is treated identically
  - Samples are denatured and hybridized to flow cell
  - Captured sample DNA is used as template for second strand synthesis
  - Second strand is amplified into a clonal cluster
  - The cluster is linearized and active sites are blocked
  - Added sequencing primer provides a site for sequencing by synthesis
- Cluster Station/cBot delivers fluidics and controls temperature

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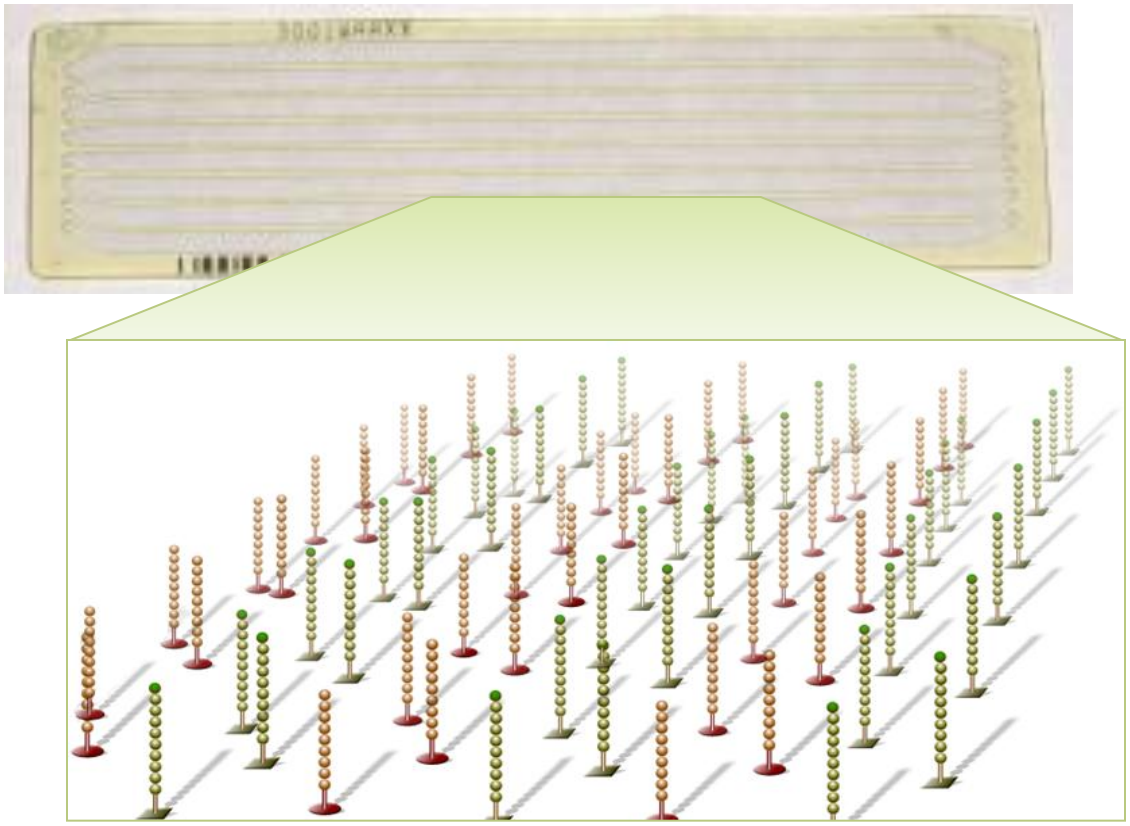
Flow Cell



Surface of flow cell coated with a lawn of oligo pairs

- Simple workflow
- Clusters are generated in a contained environment (no need for clean rooms)
- Sequencing performed on the clusters on the flow cell

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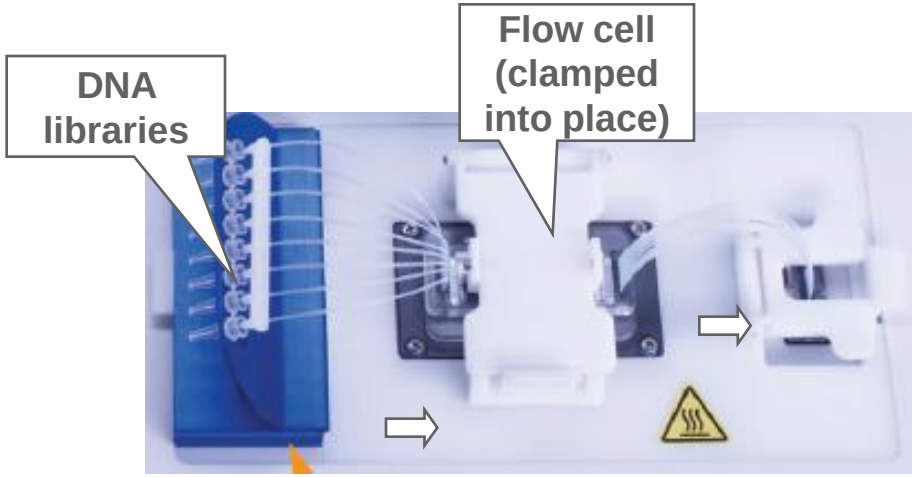
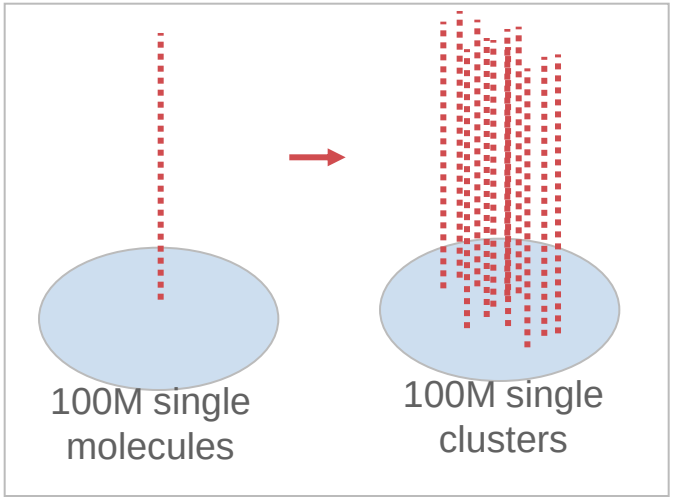
Cluster Station/cBot

- Aspirates DNA samples into flow cell
- Automates the formation of amplified clonal clusters from the DNA single molecules





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Cluster Generation Workflow

Prepare Reagents

↓

Open and Run a Recipe

↓

Follow Recipe Prompts to Load Flow Cell

↓

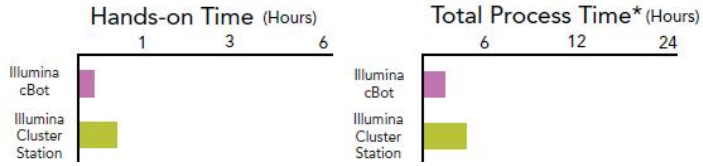
Follow Recipe Prompts to Load Reagents

↓

Cluster Generation Steps
Hybridization, Amplification, Linearization, Blocking, Primer Hybridization

↓

Prepare to Sequence Flow Cell



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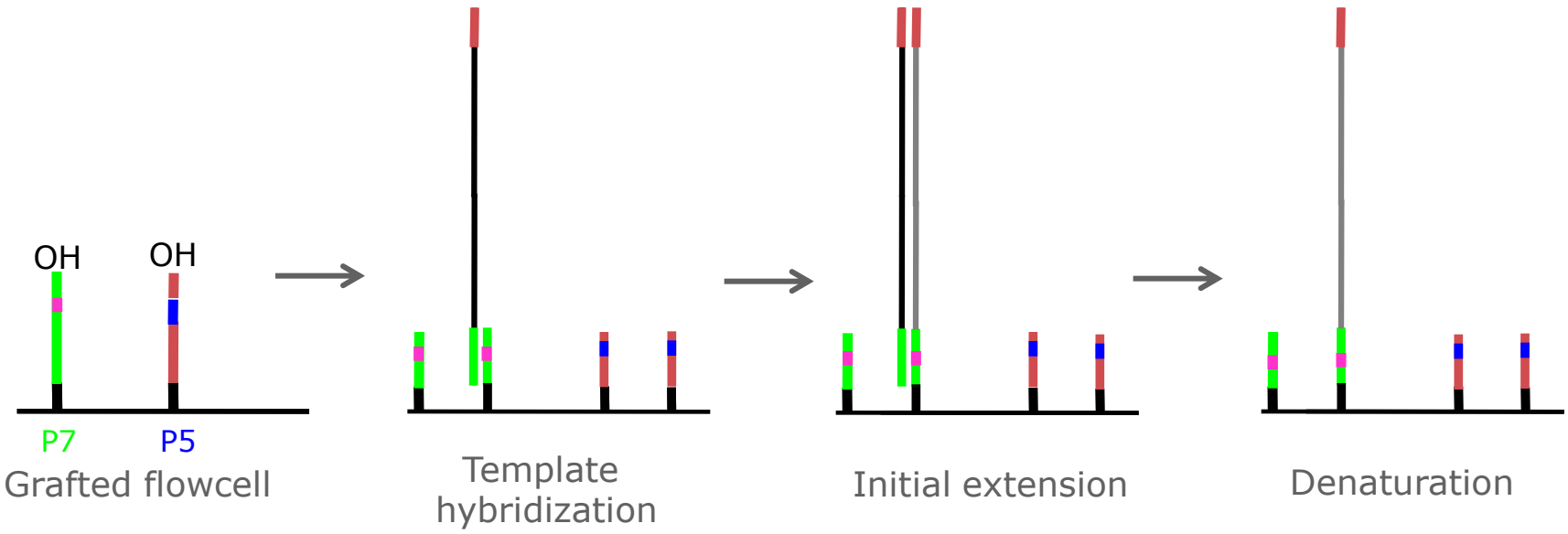
Cluster Generation (v4 Chemistry)

Hybridize Fragment & Extend



Hyb Manifold	Denatured Library	Amp Premix AMP1	NaOH Wash
Strip Tubes	Temp Ramp: 96-40°C	Phusion HFE 90 sec	Wash Buffer HT2
Hyb Buffer HT1	Wash Buffer HT2	Temp Ramp: 20°C	

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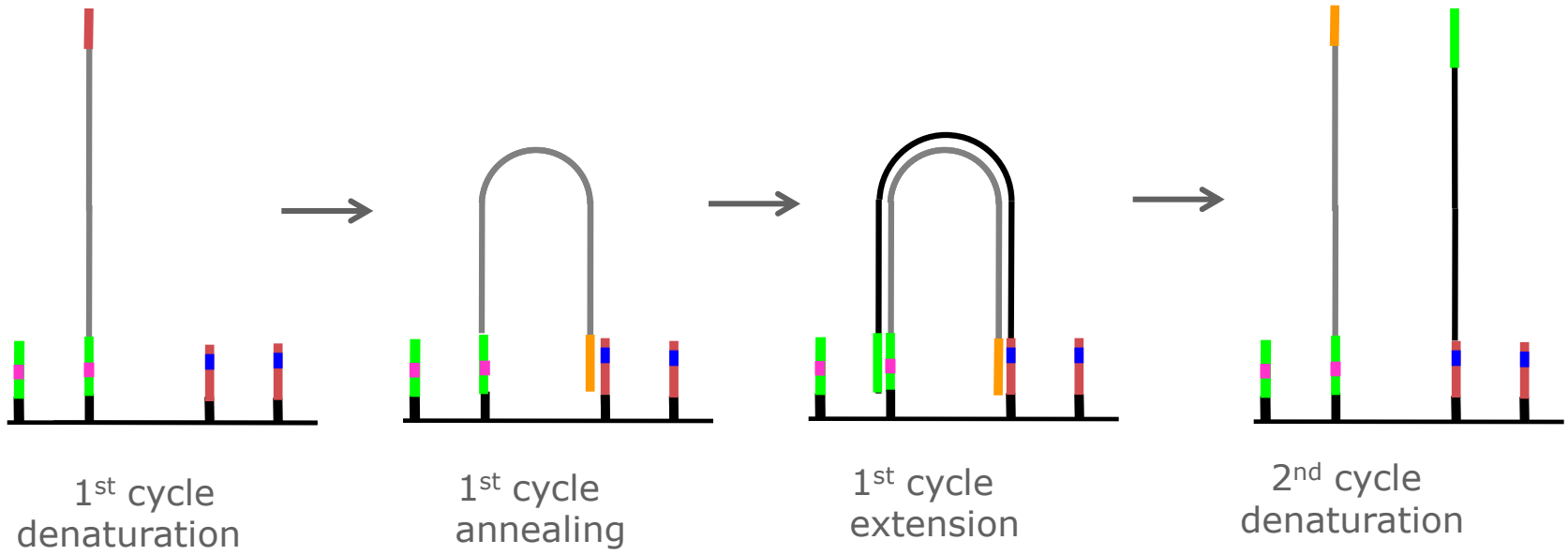
Cluster Generation

Bridge Amplification



AMP Manifold

Ramp to 60°C

Formamide AT1

Amp Premix APM1

Amp Mix AMX1

Contains BST pol & nucleotides

Formamide AT1

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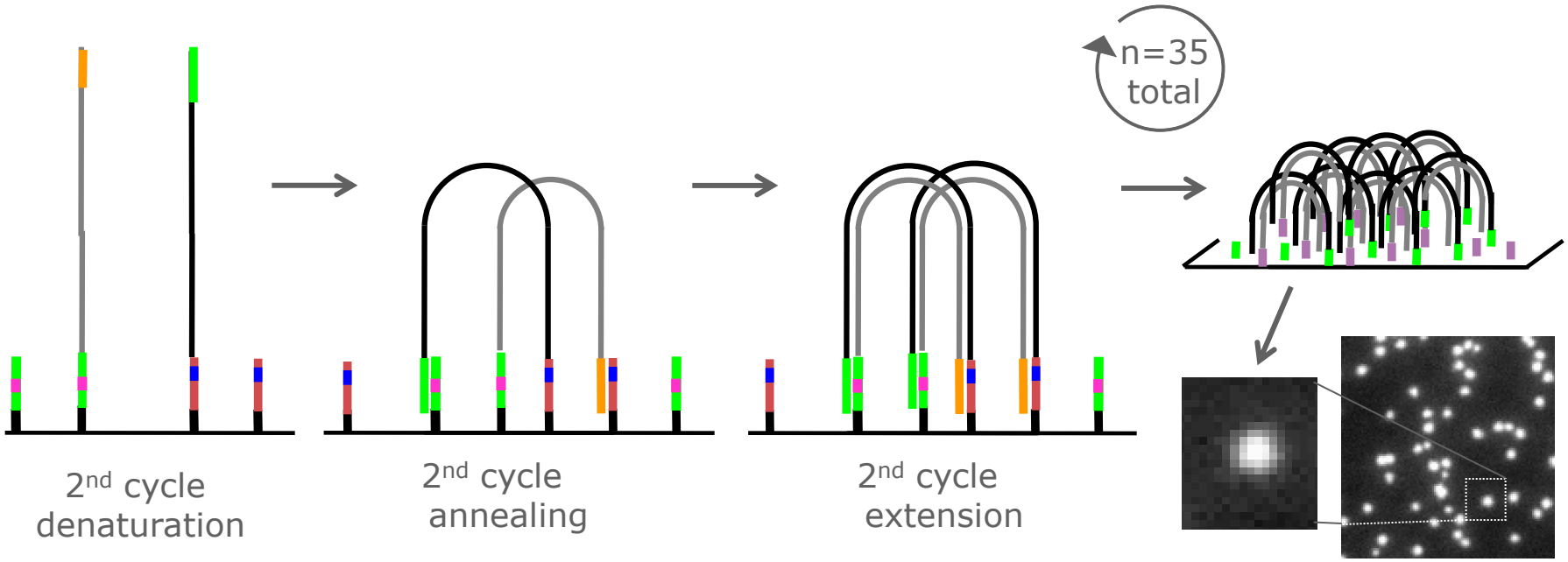
Cluster Generation

Bridge Amplification



Formamide AT1

Amp Premix APM1

Amp Mix AMX1

Contains BST pol & nucleotides

35 Cycles

Wash Buffer HT2

Hyb Buffer HT1

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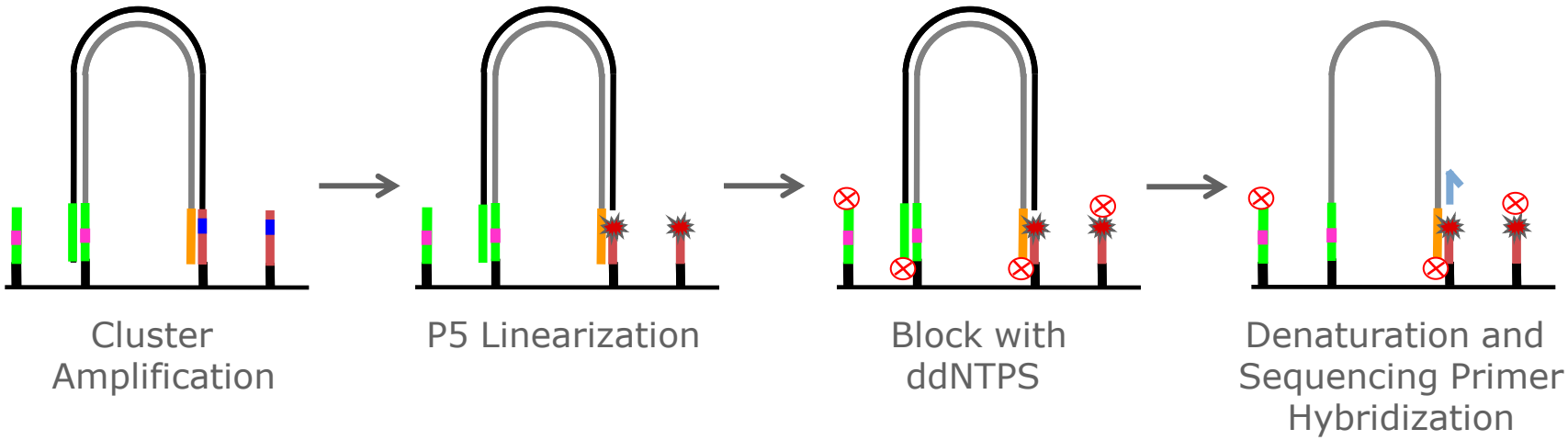
Cluster Generation

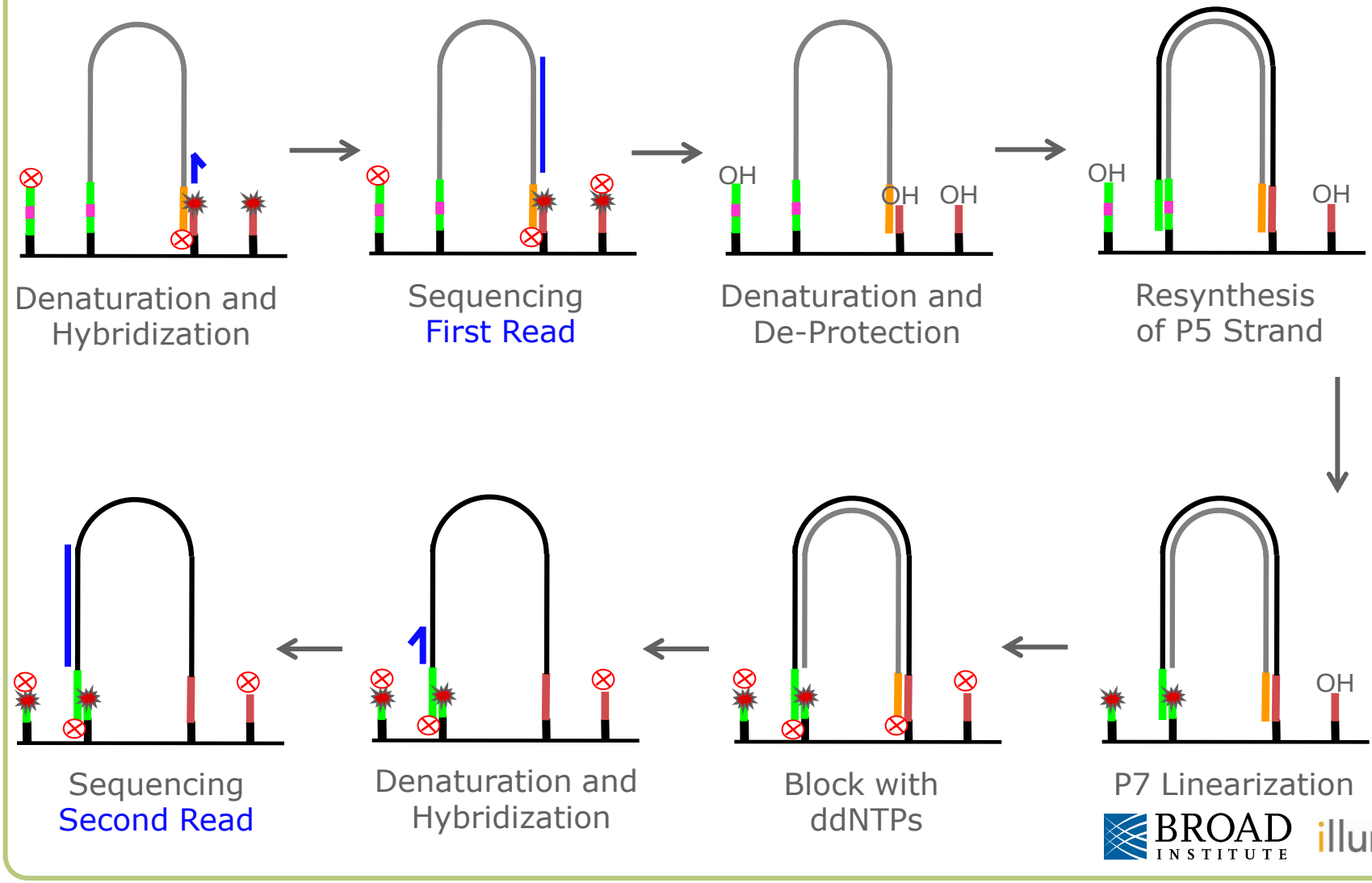
Linearization, Blocking & Sequencing Primer Hybridization



AMP Manifold	PE Linearization LMX1	Blocking Mix BMX	0.1N NaOH
Hyb Buffer HT1	Ramp 37.9°C, 30 min	38°C, 30 min	Seq. Primer
Temp 20°C	Temp Ramp: 20°C	60°C, 15 min	60°C, 5 min
	Wash Buffer HT2	20°C, HT2, HT1 Washes	20°C, HT2, HT1 Washes

Cluster Generation: Read 2 Turnaround

Resynthesis of the second strand



Best Practices

Cluster Generation

- Store reagents as recommended
  - 9 month shelf life from date of manufacture
  - Track lot numbers and barcodes
- Prepare all reagents fresh before use
  - Mix reagents well
- Use proper recipes and remove unnecessary recipes
- Check fluidics delivery both visually and by weight of reagent delivered
- Ensure that Cluster Station is washed regularly

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Cluster Generation

Best Practices from The Broad Institute

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Cluster Generation

QPCR

Cluster Amplification

SYBR QC

Linearization & Blocking / Primer Annealing (LB/PA)

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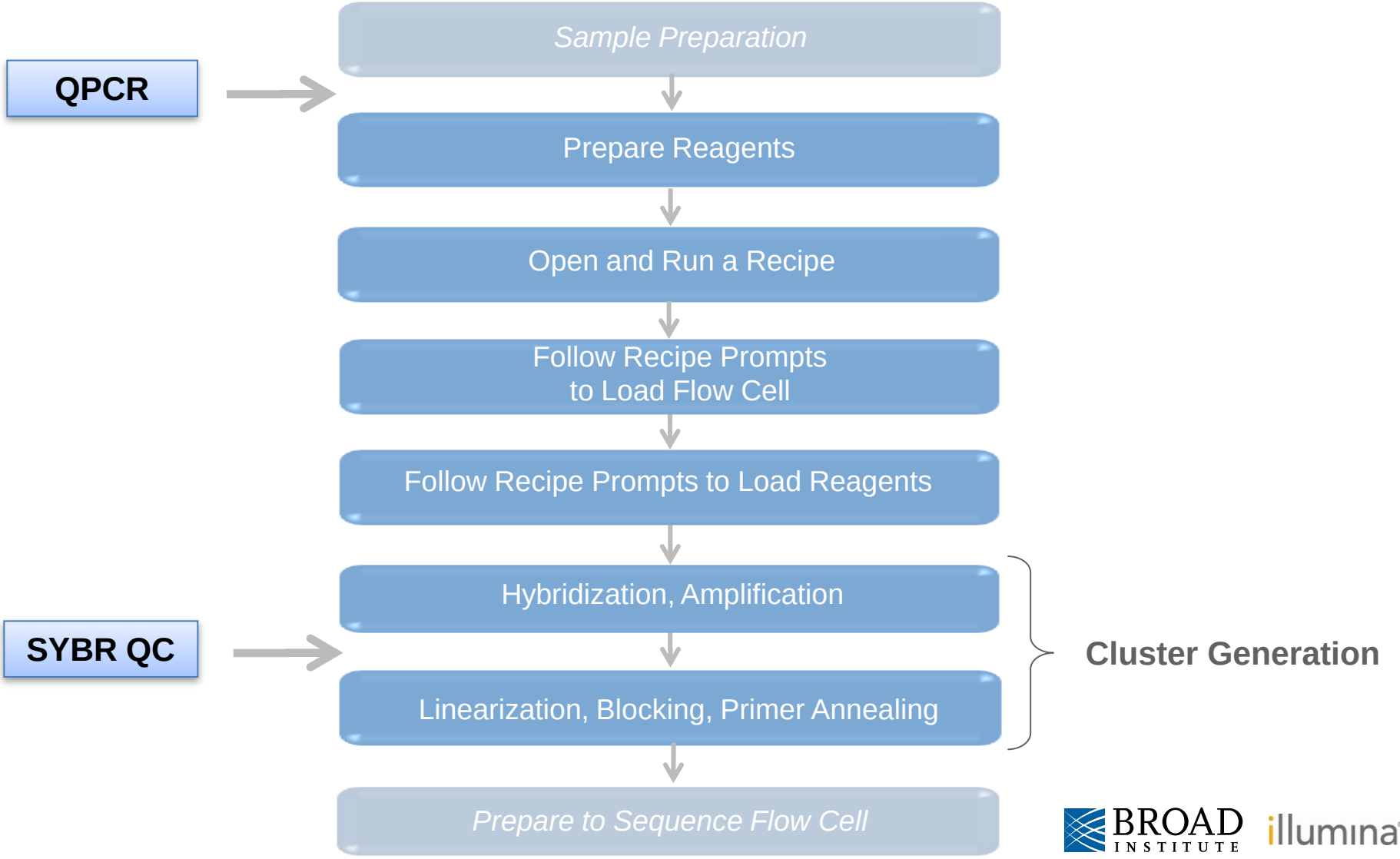
Cluster Generation Workflow -- Broad

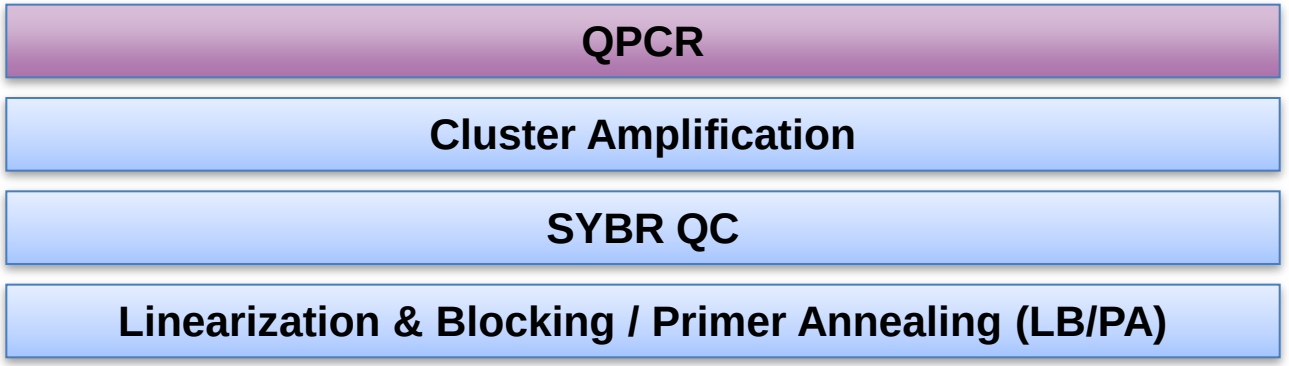


Cluster Generation Overview

- qPCR: Obtaining optimal cluster density
- Addition of Process Controls
- SYBR QC: Ensure successful amplification before continuing
- Lab: Various process details can be monitored and standardized

Quantitative PCR (qPCR)

- **GOAL:** Quantitate amplifiable library fragments in order to obtain optimal cluster density without performing a titration flow cell.



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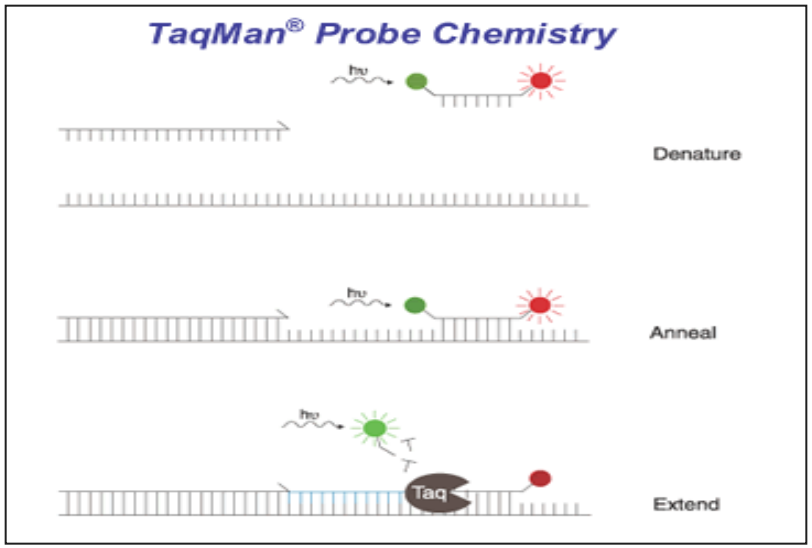
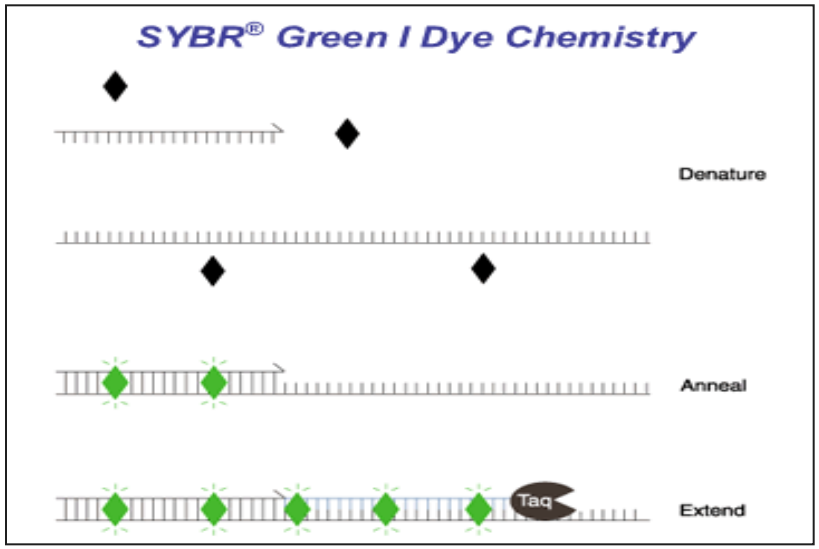
Quantitative PCR (qPCR) Overview

Quantitative Real-Time Polymerase Chain Reaction

- Fluorescence is detected and measured at each cycle
  - Increases proportional to PCR product
- Library's fluorescence is used to determine its concentration





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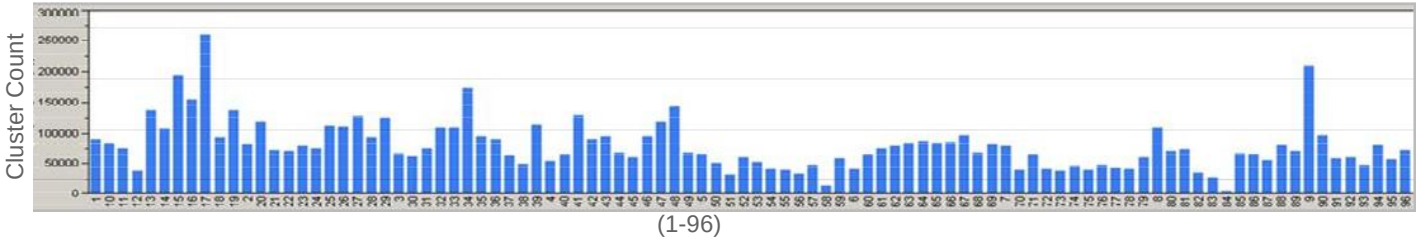
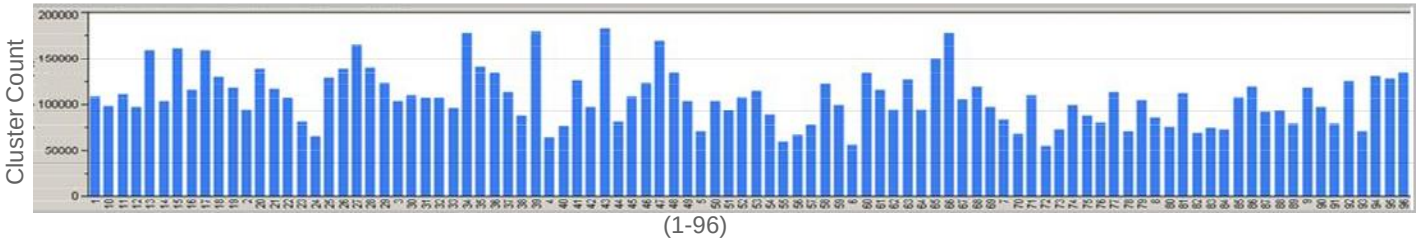
Quantitative PCR (qPCR) Accuracy

- 96 barcoded (indexed) libraries were quantified, normalized, and pooled. qPCR produced more accurate quantitation results, leading to more consistent cluster counts among the libraries.

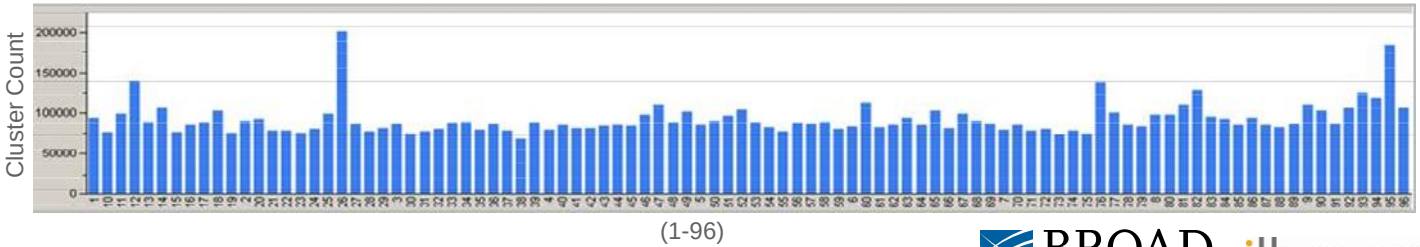
Quant Method:
None (volume)
Fold Difference: 60X
% CV: 50.8%



Quant Method:
Pico
Fold Difference: 3.3X
% CV: 27.4%



Quant Method:
Automated qPCR
Fold Difference: 3.0X
% CV: 21.4%



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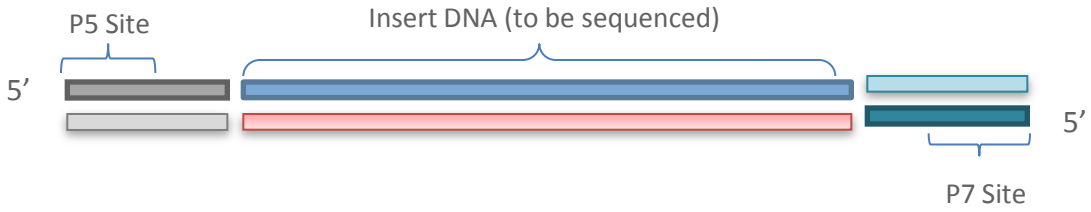
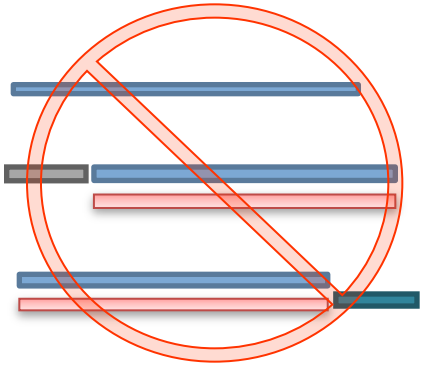
Quantitative PCR (qPCR)

- qPCR is designed to quantify only cluster-forming fragments in the sample

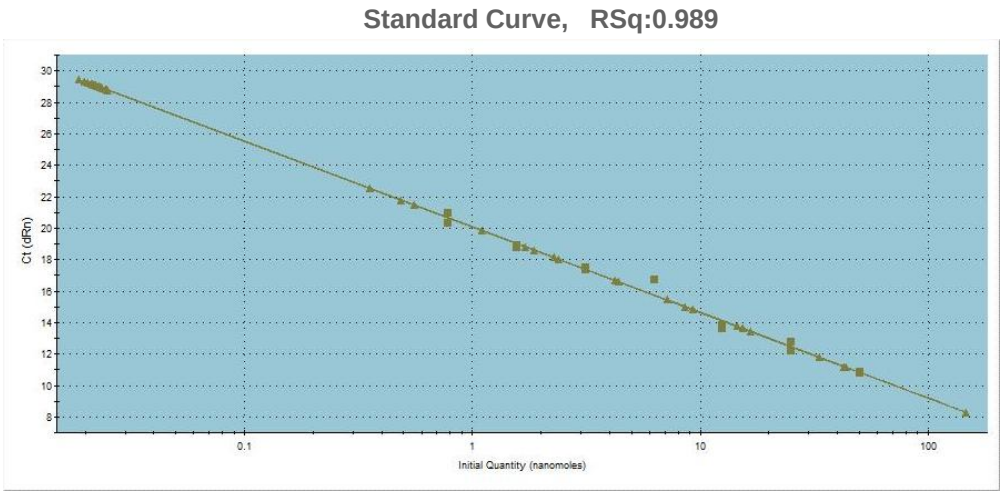




  - Uses P5 & P7 primers to mimic amplification on the flow cell
  - Primers only amplify library fragments with proper adapters

Quantitative PCR (qPCR) Standards

- Using high-quality standards is critical
- Standards' fragment size is critical
  - Ideally, a different set of standards would be used for each library size
  - Library concentration is calculated using size ratio of a monotemplate standard to library* *(*size determined by Agilent assay)*
- We have had success using two types of standards
  - a) Monotemplate (from KAPA)
  - b) Well-characterized genomic library with predictable cluster-forming behavior (PhiX from Illumina)



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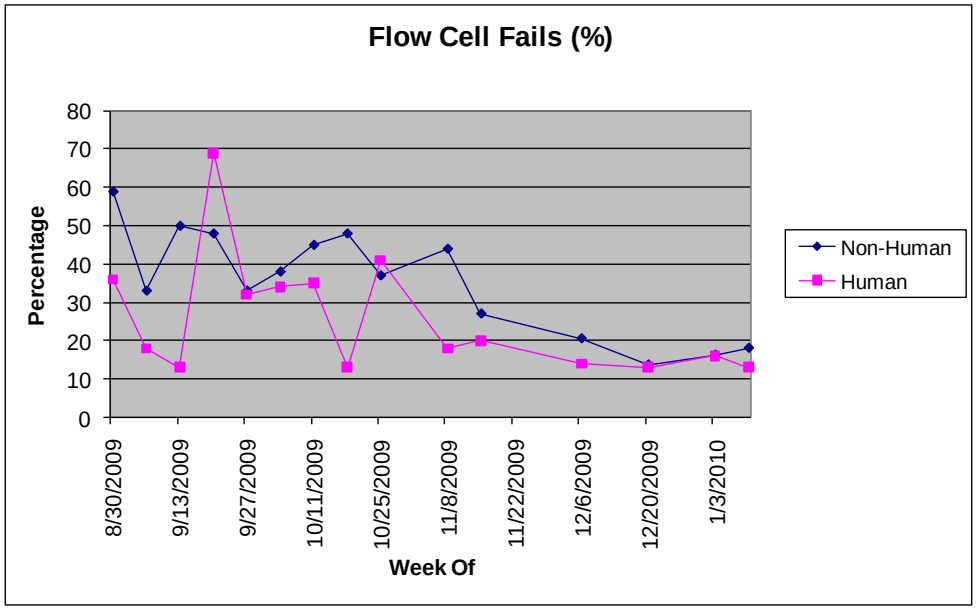
KAPA Library Quantification

- qPCR-based assay designed specifically for Illumina libraries
- 452bp monotemplate standards
- Multiple different samples 384 well plate = 96 Samples (triplicate)
  - Quants are adjusted to library size: (**452**/average library size)*Quant)
- Reduce flow cell rework rate due to high or low densities
  - (failed flow cell has 3 or more failed lanes)



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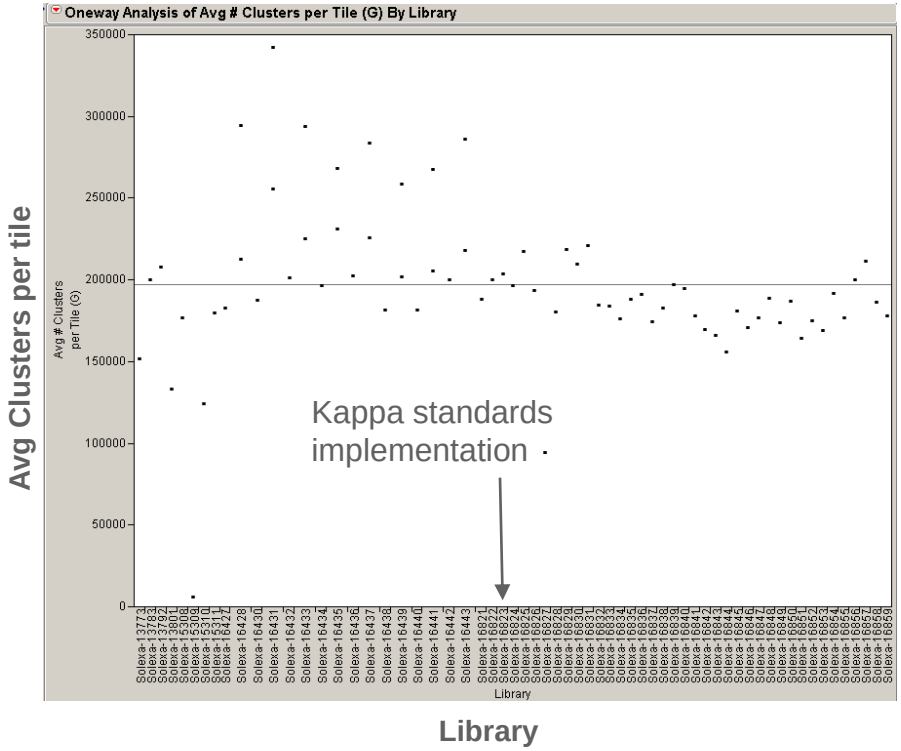
KAPA Library Quantification

- Reduction in Variabilty
  - KAPA standard qPCR vs. in-house standard qPCR
- Low Yielding Library
  - Pico unable to detect DNA
  - KAPA quant: .3nM
    - Successfully sequenced



Quantitative PCR (qPCR) Standards

- Commercial standards work well at large scale (pre-QCd)
- In-house standards work well at small scale if:
  - Standard sets should be made in large batches
  - QC: Test each new batch against the last batch
  - Ensures accuracy and reproducibility
  - Stored at -80°C

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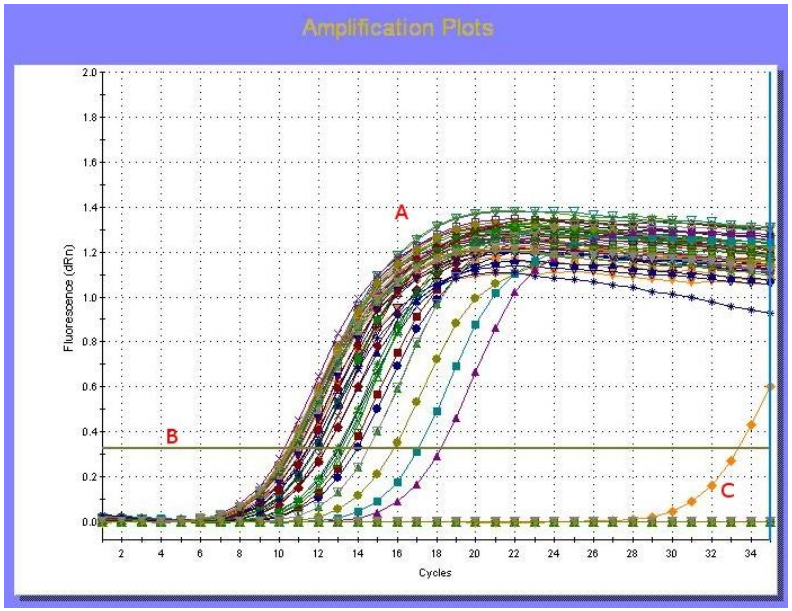
Quantitative PCR (qPCR) Specs

Enrichment Curves

- Flat for at least 4 cycles
- Should increase, then plateau

A. Enrichment Curve
B. Threshold
C. NTC (No Template Control) Enrichment Curve



Threshold

- Should be above background
- Should be below plateau
- Automatically generated

No-Template (Negative)Control

- Displays low level of enrichment, mostly due to primer dimers
- C_t (Cycle threshold) should be at least 10 cycles after template samples.

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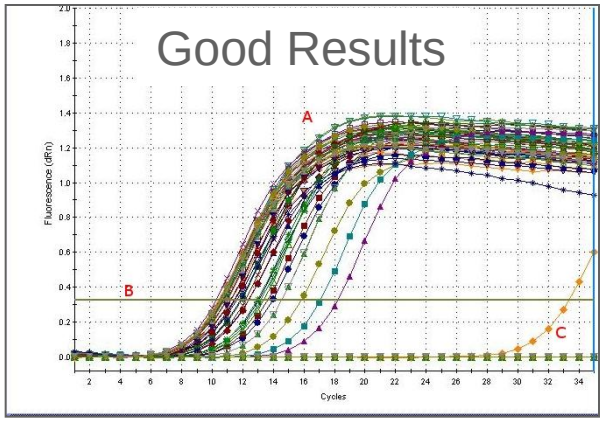
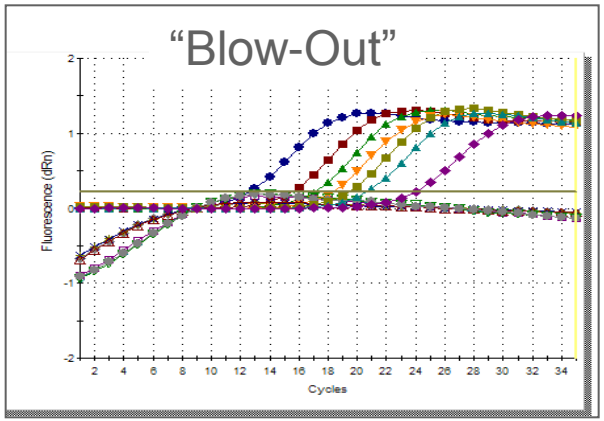
Quantitative PCR (qPCR) Sample Dilutions

- qPCR requires very low amounts of input DNA
  - "Blow-outs" occur if samples are too concentrated





- Two options for dilution of library
  - Dilute to ~10nM (based on fragment size (Agilent) and rough quant (Pico or Agilent))
  - Perform qPCR on several serial dilutions and ignore blow-outs

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Quantitative PCR (qPCR) Best Practices

- THOROUGH MIXING IS CRITICAL!
  - Sample dilutions (vortex and spin)
  - Vortex mastermix (even though it contains enzyme)
  - Place paper between plate and vortex to keep plate clean
  - Always spin plates before removing caps (to avoid cross-contamination or sample loss)
- UNIFORMITY
  - Use automated deck whenever possible
  - Designate a qPCR user to reduce operator-to-operator variability
  - Make mastermix in a large tube
  - If using a multichannel pipettor, check for uniform volume between tips
  - With any pipettor, check for (and be able to recognize) correct volume levels in tips

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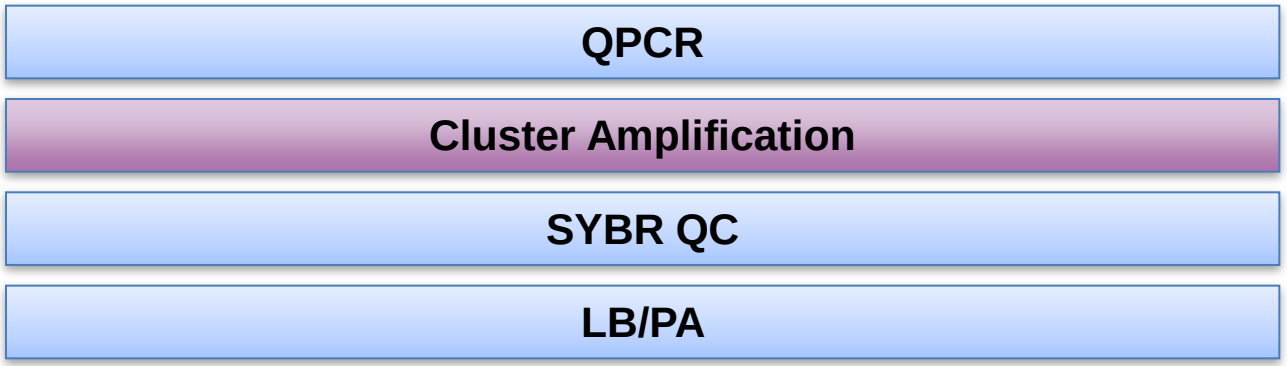
Control Samples

- **GOAL:** Detect normal and aberrant variation by including a control sample during cluster generation/amplification



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Choosing a Control Sample

- We currently use one of two types of control samples

Phi X Lane	Internal Controls (ICs)
Create a good crosstalk matrix for use in base-biased samples	Regain “real estate” by adding a small amount to each experimental lane (1/100 reads instead of 1/8 reads)
Well characterized small genome	Synthetic sequences require no error-inducing PCR enrichment
~ 50% GC	~ 50% GC
Simple data analysis	Easily identified monotemplates*
Simple workflow (treat as library)	Control in every lane

**Using Broad-designed pipeline software*

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Internal Controls (IC) Design

- Randomly-generated, unique synthetic sequences don't align to any genomes (easy to identify)
- The Internal Controls pool contains 4 monotemplate sequences:
  - Pool contains equal base composition at every cycle.
  - Easily-identified, known sequences allow accurate calculation of error rate at any cycle*.

CACGTCACGTGTACGTAGTACGTAC…
TCTAGAGTACAGTGACGAGTGACGA…
GTGCAGTGCGCACATATCACATGCT…
AGATCTCATATCGTCGCTCGTCATG…

- ICs add a level of lane-to-lane control
  - Control fragments are subject to identical chemistry, imaging, and analysis as each lane's experimental sample.

*Covered in more detail in the Working with Data Module

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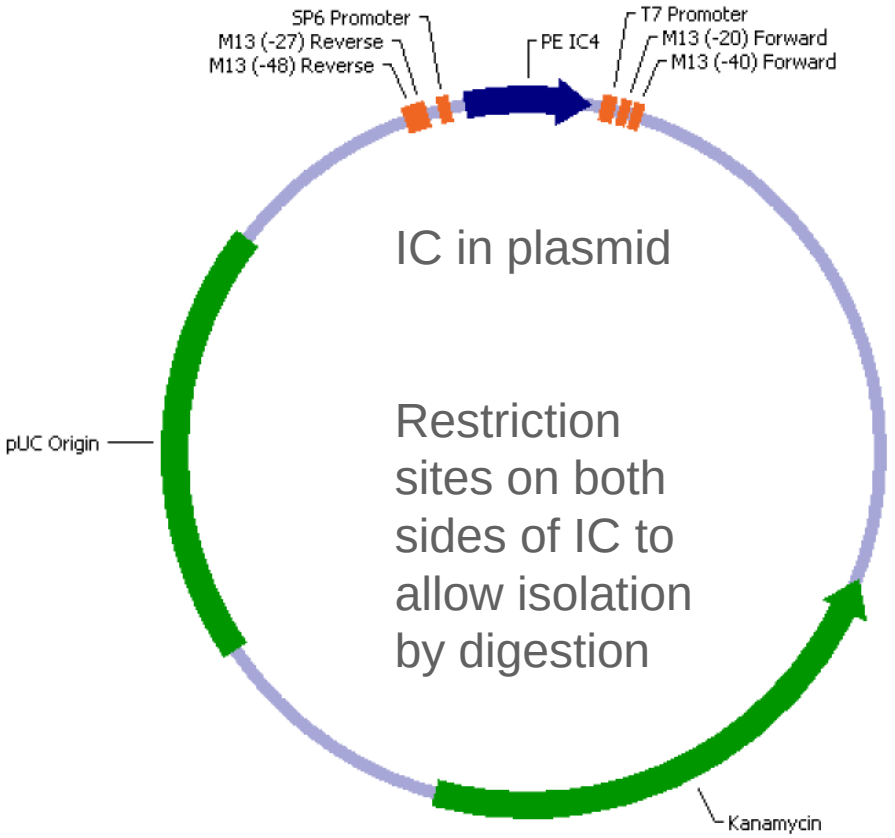
Internal Controls (IC) Preparation

- Cellular reproduction of plasmid (more accurate than PCR amplification)
- IC fragment with final adapter sequence are flanked by unique restriction sites in plasmid
- ICs with adaptors are digested/purified from plasmid
- After final purification, all 4 IC's are quantified (KAPA qPCR) and pooled equimolarly to give even representation of each base at each cycle



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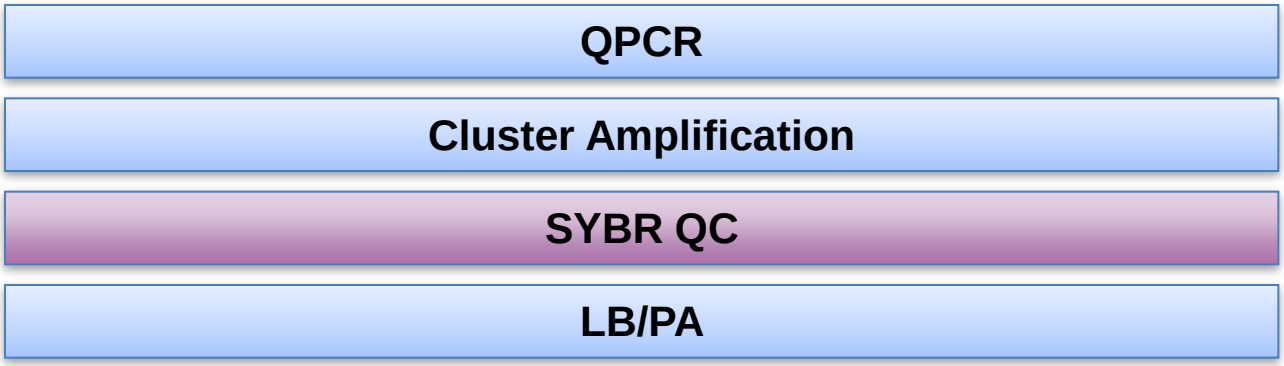
SYBR Green Cluster Generation QC

- **GOAL: Visually confirm successful cluster generation and optimal density before continuing with LBPA and sequencing**



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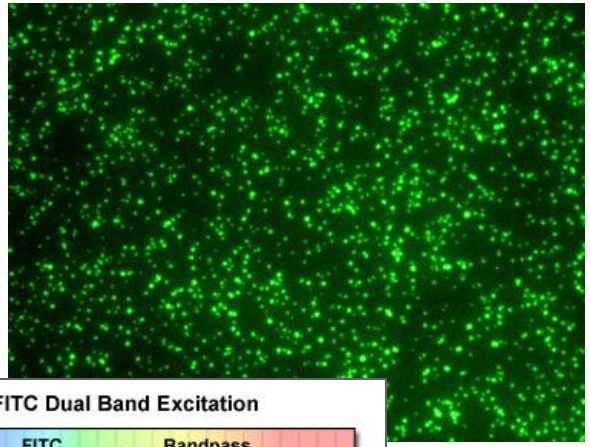
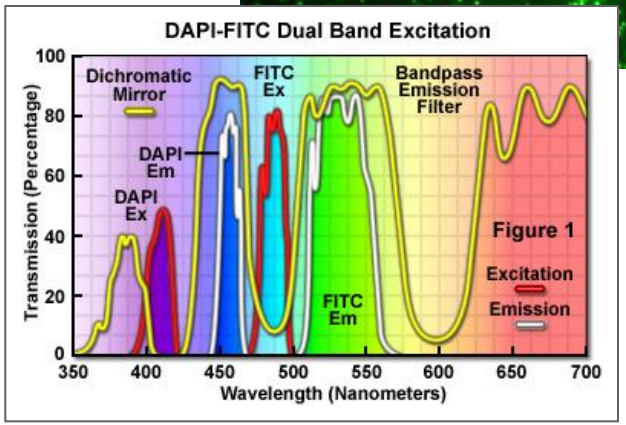
SYBR Green Cluster Generation QC

- SYBR Green dye is run through flow cell after cluster generation is complete.
  - SYBR intercalates into dsDNA
  - May be less mutagenic than ethidium bromide
- Visible clusters using standard microscope
  - Excited by Xenon bulb*
  - Fluoresces @ 500-560λ (green)
  - Visible through FITC filter
  - Clusters visible at 40X magnification
- Will see more in lab module





* Xenon bulb must be changed every 350 hours to prevent explosion risk

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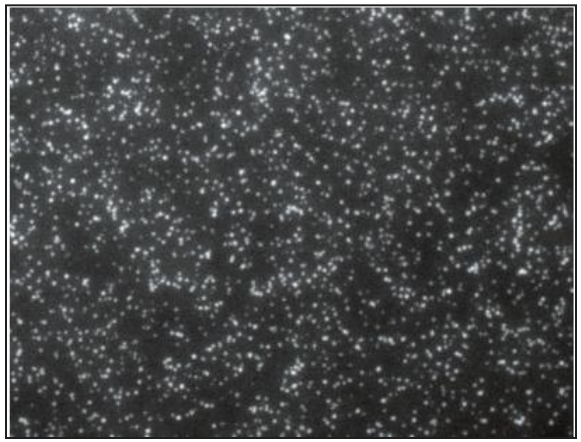
SYBR Green Cluster Generation QC



Sparse

Good

Dense

*1.6 RTA

- Visual QC of flow cells: Verify successful amplification and cluster count
  - Too Sparse: Loss of valuable real estate on flow cell
  - Too Dense: Analysis problems
- High density limits ability to accurately count clusters at SYBR step
- Afterward, flow cell can be stored for up to ~1 month

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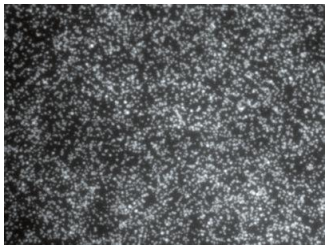
SYBR Green Cluster Generation QC

SYBR image → Estimated Cluster #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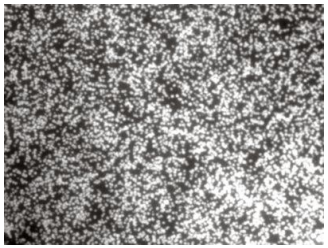
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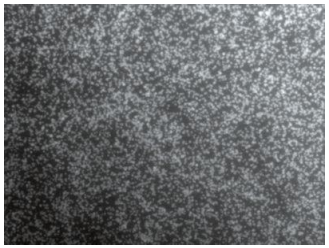
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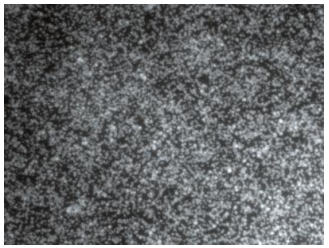
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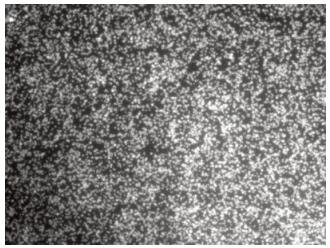
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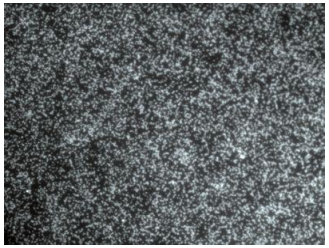
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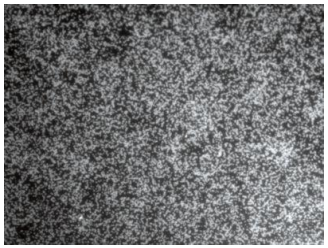
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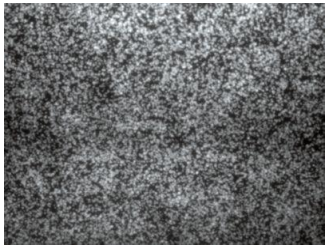
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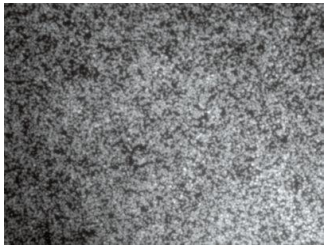
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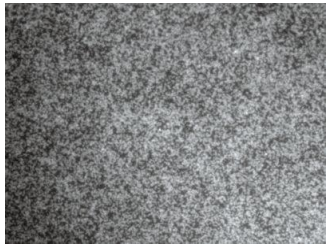
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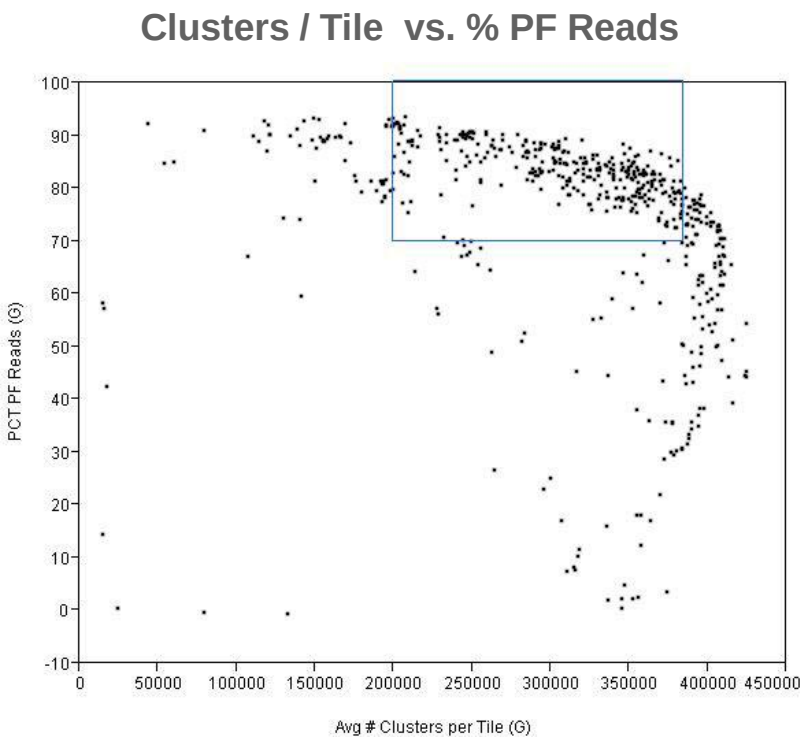
Cluster Densities (1.6 RTA)

- 1.6 RTA is able to identify many more clusters than previous pipelines
- SYBR QC images are so dense that it is difficult to differentiate between 350,000 and 400,000 clusters/tile
- QPCR accuracy, along with historical data, allow us to pinpoint optimal loading density



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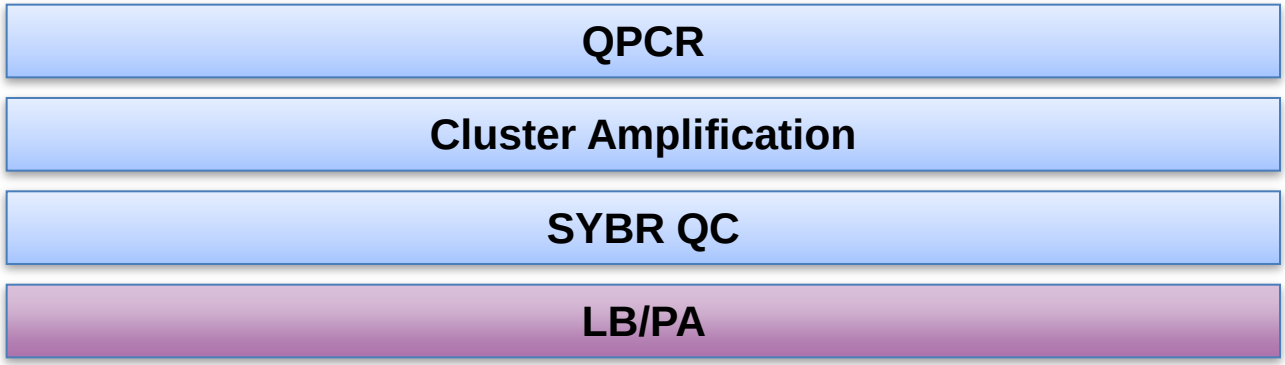
LBPA: Linearization, Blocking, Primer Annealing

- Prepare clusters for sequencing
- Use same amplification manifold as in cluster generation
  - Use wash flowcell to run water through manifold
- Ensure proper flow of reagent using Hybridization Buffer
- Immediately place on sequencer



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Cluster Generation – Summary

- QPCR
  - Using high quality standards is critical
  - Determines loading concentration for optimal cluster density
- Control Samples
  - Phi X – simple workflow and good for use on flow cells with base biased samples
  - Internal Controls – Control in every lane,
- SYBR QC
  - Determine number of clusters amplified prior to placing on sequencer

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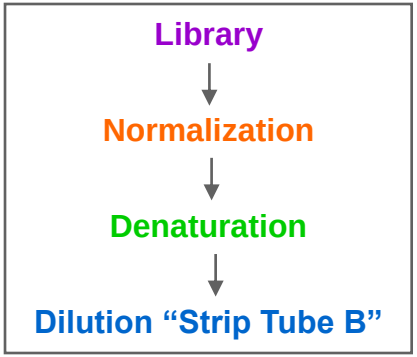
Lab Best Practices – Pipetting Variability

- Experiment: Compare variation in cluster counts between lanes containing **the same E.coli genomic DNA sample** (split off at various points in the process):



  - Replicate libraries
  - Same library, replicate Normalizations
  - Same library, same normalization, replicate denaturations
  - Same library, same normalization, same denaturation, replicate dilutions
  - Same library, same normalization, same denaturation, same dilution

- Problem confirmed: Small-volume pipetting introduces significant variation at each phase between library prep and cluster generation. Inserting variation earlier in the process increases amount of final variation.

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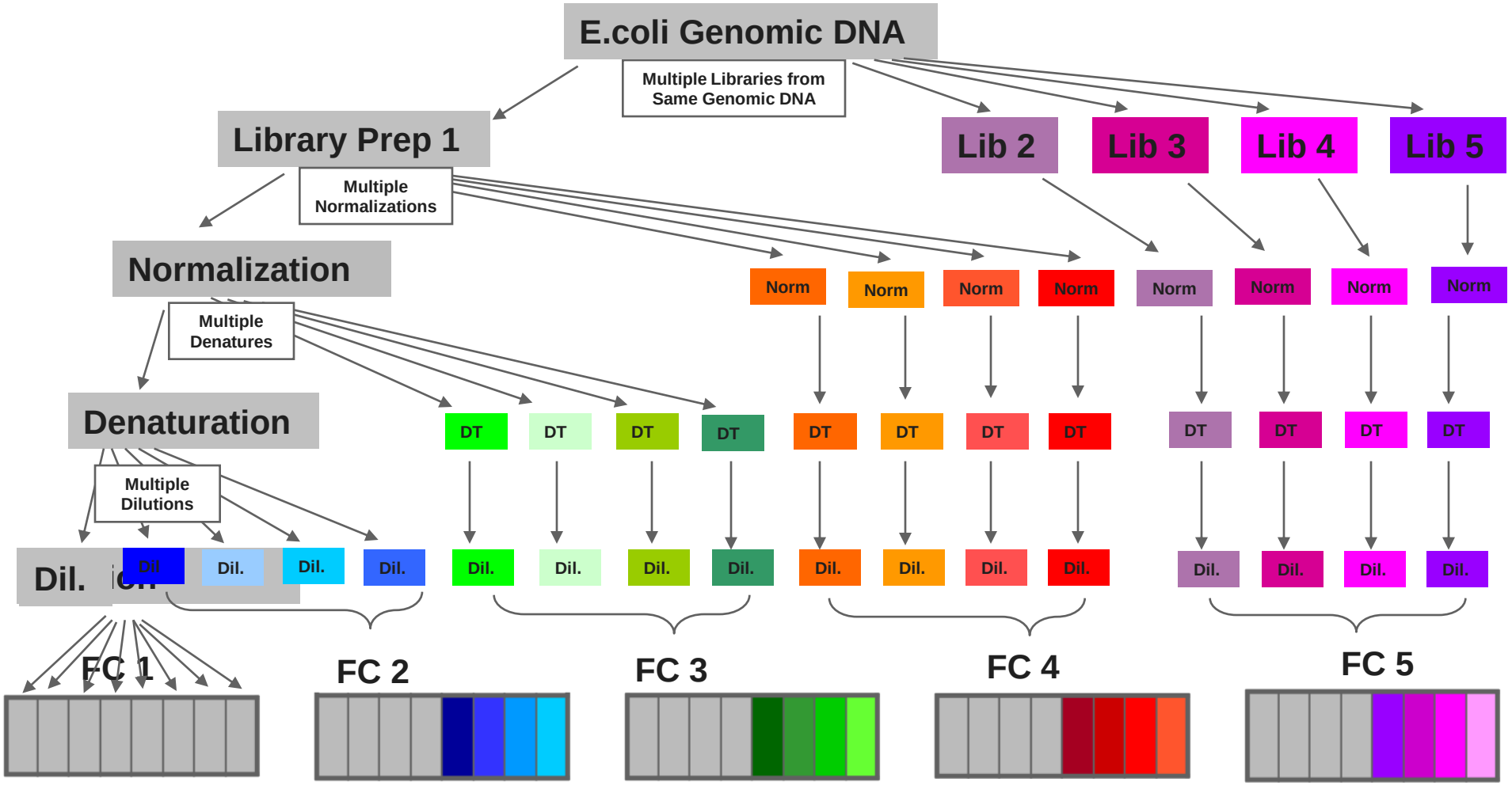
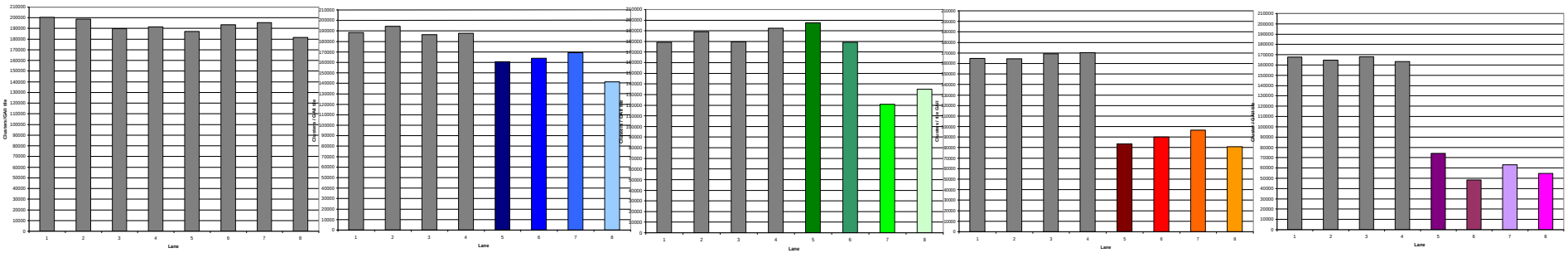
Lab Best Practices – Pipetting Variability

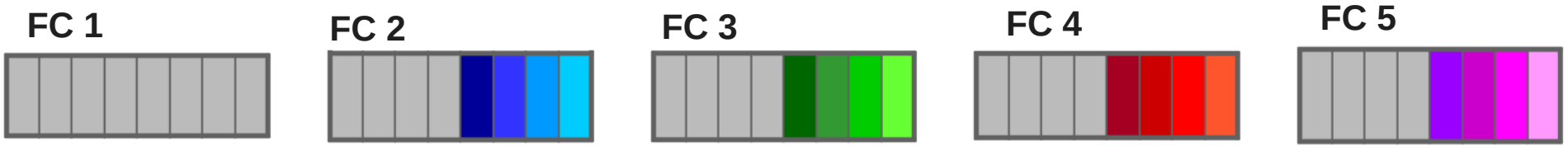


Lab Best Practices – Pipetting Variability

Same Library	Same Library	Same Library	Same Library	**REPLICATE Libraries**
Same Normalization	Same Normalization	Same Normalization	**REPLICATE Normalizations**	
Same Denaturation	Same Denaturation	**REPLICATE Denaturations**		
Same Dilution (Aliquotted into different lanes)	**REPLICATE Dilutions**			

Cluster Count By Lane





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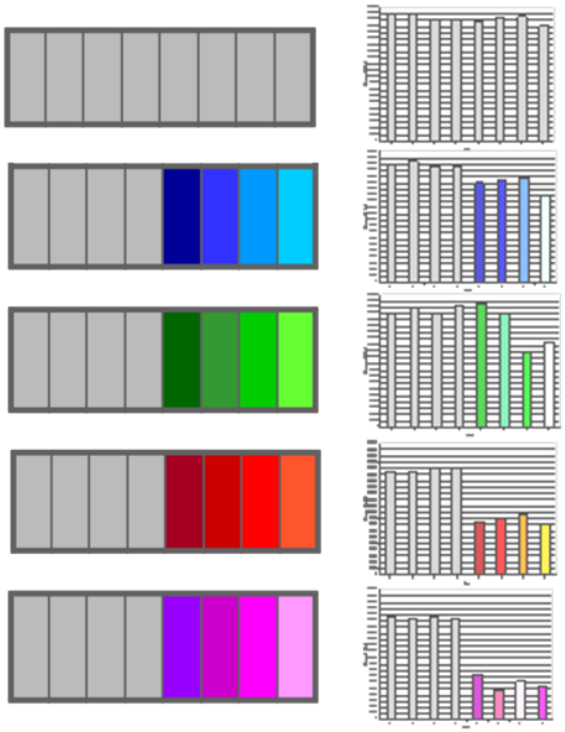
Lab Best Practices – Pipetting Variability



CV across 8 lanes: 3.2%

CV across 8 lanes: 10.5%
CV across 4 lanes: 7.7%
Split @ Dilution

CV across 8 lanes: 16.4%
CV across 4 lanes: 22.9%
Split @ Denature

CV across 8 lanes: 33.5%
CV across 4 lanes: 8.0%
Split @ Normalization

CV across 5 lanes: 50.5%
CV across 4 lanes: 18.6%
Split @ Library Prep

- Inserting variation earlier in the process between library prep and cluster generation increases final variation in cluster counts.
- **To reduce small-volume pipetting over time:**
  - **Use automated qPCR to determine optimal loading concentrations**
  - **Use automated denaturation**
  - **Store denatured libraries for re-use**

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Lab Best Practices – Cluster Station Use

- Reagent Preparation
- Flow Problems
  - Line clogs, Manifold gaskets, Watch Prime, Wiping Ports
- Cluster Station Maintenance
  - Routine Decon Wash
- Tracking – manifold re-use
- Thermal Profile
  - Peltier block failures
- Lab Bench Organization

- Reagent and waste volumes are monitored and tracked for future troubleshooting

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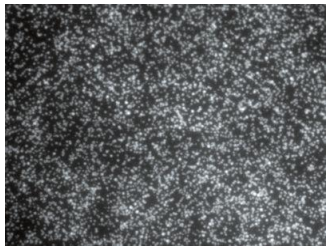
Lab Best Practices – SYBR QC

- Visualizing clusters on microscope
- SYBR image → Estimated Cluster # (Pass/Fail with trained eye)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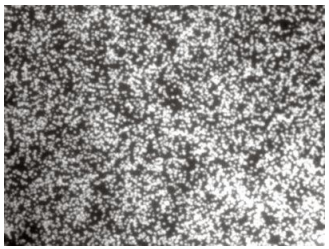
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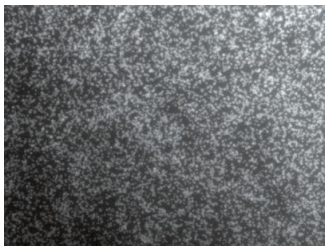
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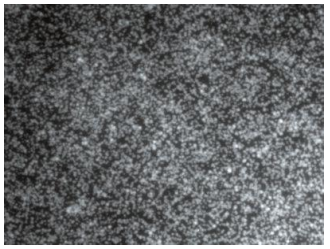
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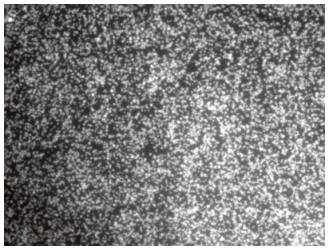
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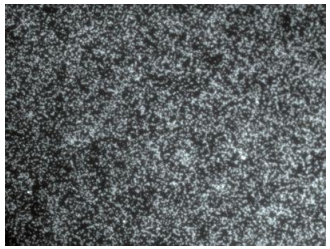
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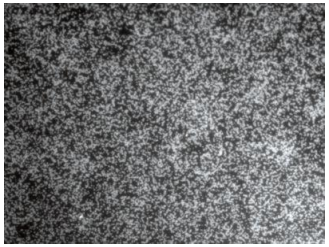
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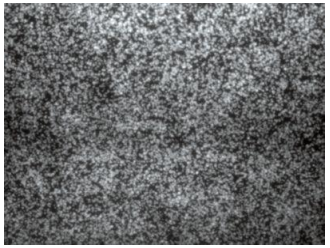
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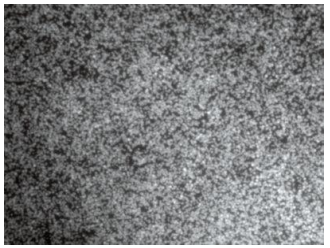
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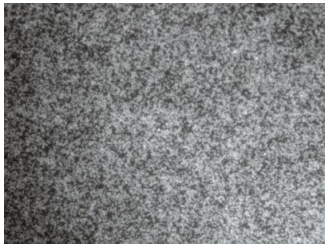
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