Genomics and Clinical Virology

Library preparation and Illumina sequencing

March 2025

Targeted-enrichment

Tuesday 4th March

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Sequencing-by-synthesis

What happens in the sequencer

Stages of Illumina sequencing

1. Sample prep:
   - add adapters and indexes
2. Clustering:
   - Isothermal amplification of each fragment molecule
   - Each single fragment produces 1000 identical copies to amplify signal
3. Sequencing:
   - Produces the ‘reads’ - output DNA sequences
   - we are doing paired-end, on the MiSeq
4. Data analysis:
   - Run analysis
   - Demultiplexing
   - Down-stream analysis

Following slides are screenshots from Illumina SBS video found at: https://emea.illumina.com/science/technology/next-generation-sequencing/sequencing-technology.html

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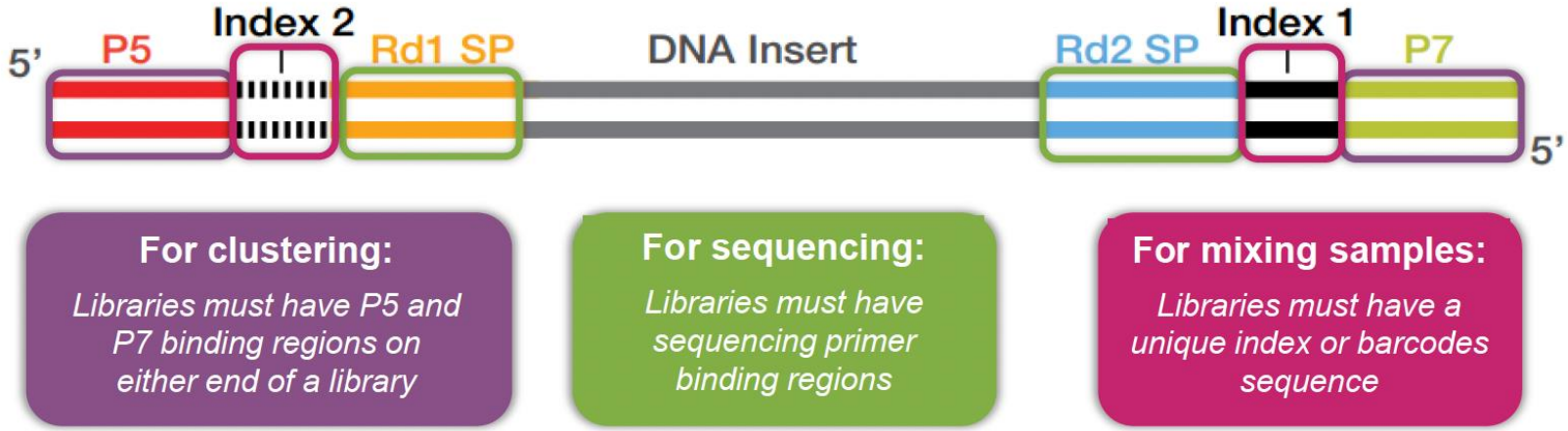

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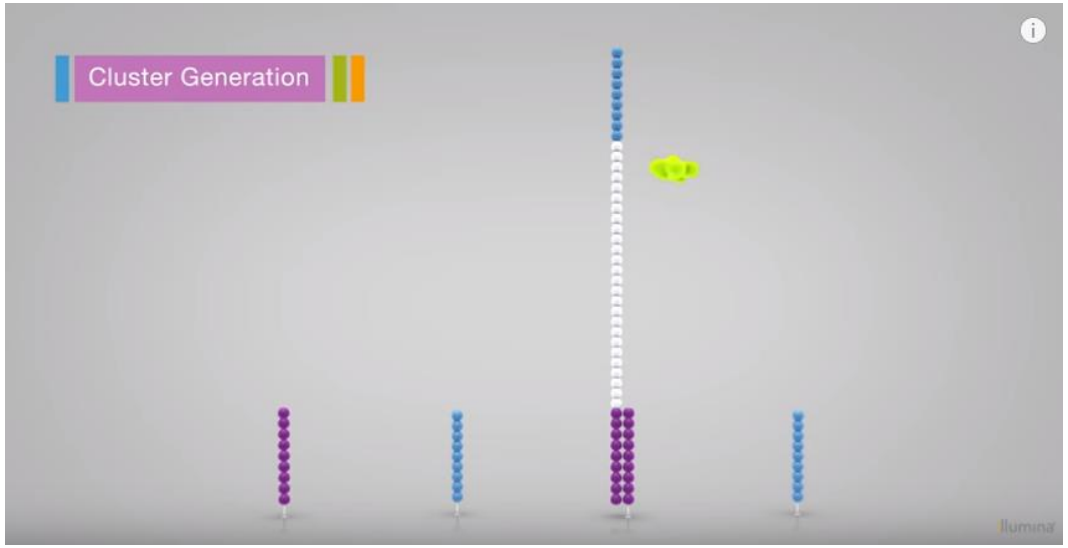
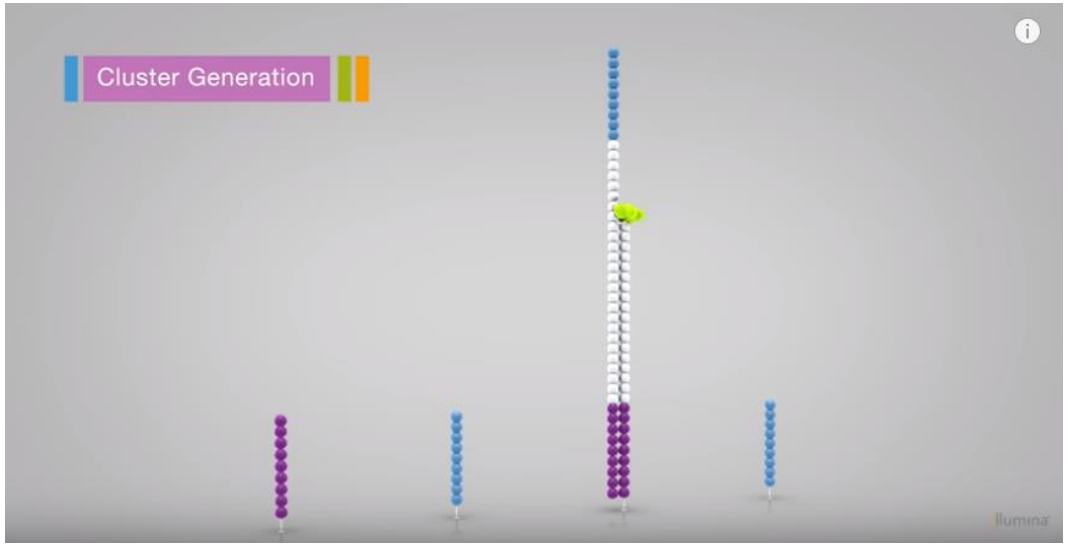
Clustering: starts with binding to the flow cell

There are two types of oligo on the flow cell which recognise the different ends of the fragment

- First, the adaptor on the fragment strand binds to a complementary oligo on flow cell

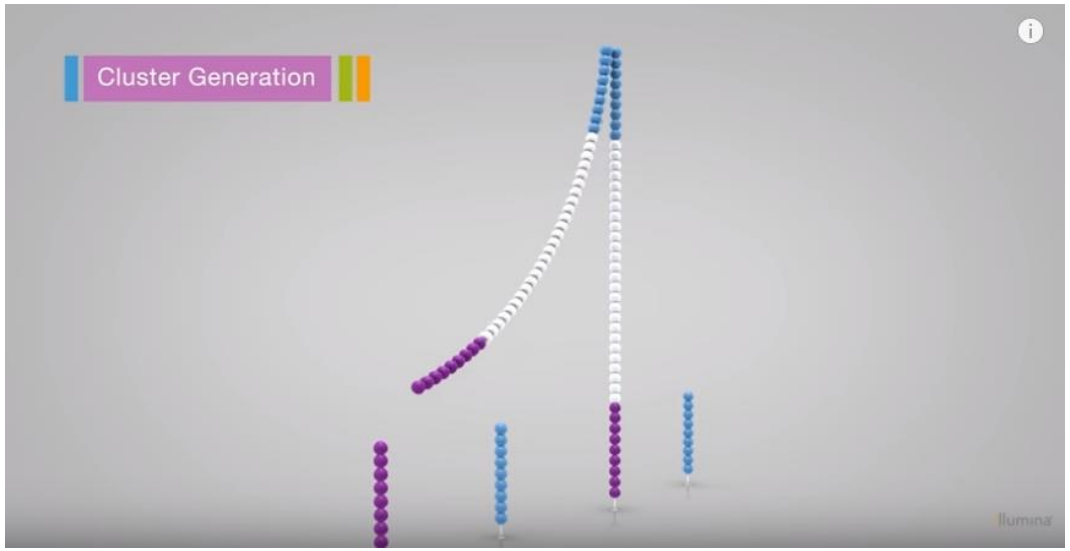
- A polymerase creates a complement of the hybridised fragment





Clustering: the initial strand is denatured

The double-stranded molecule is denatured and the original template is washed away

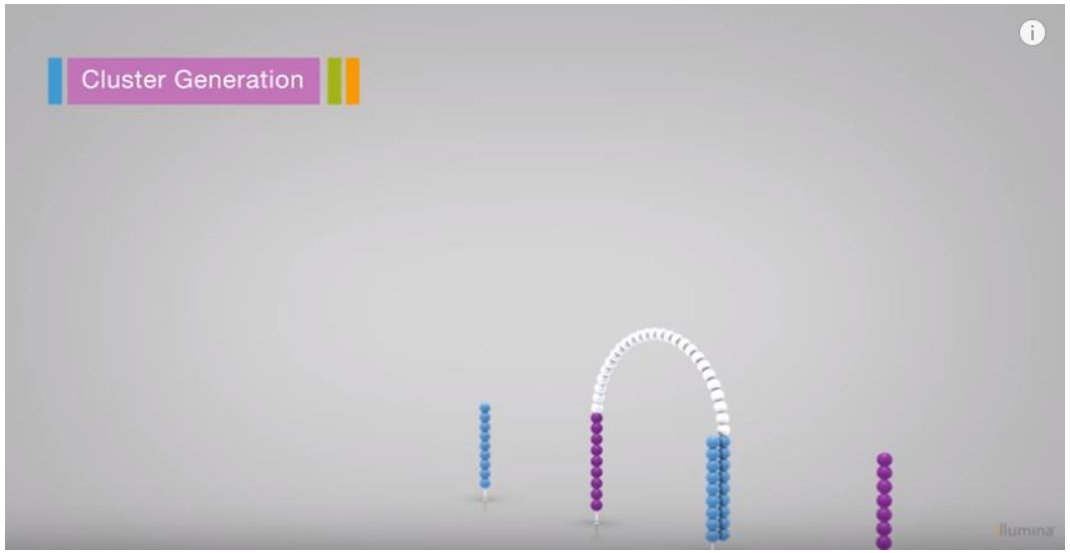


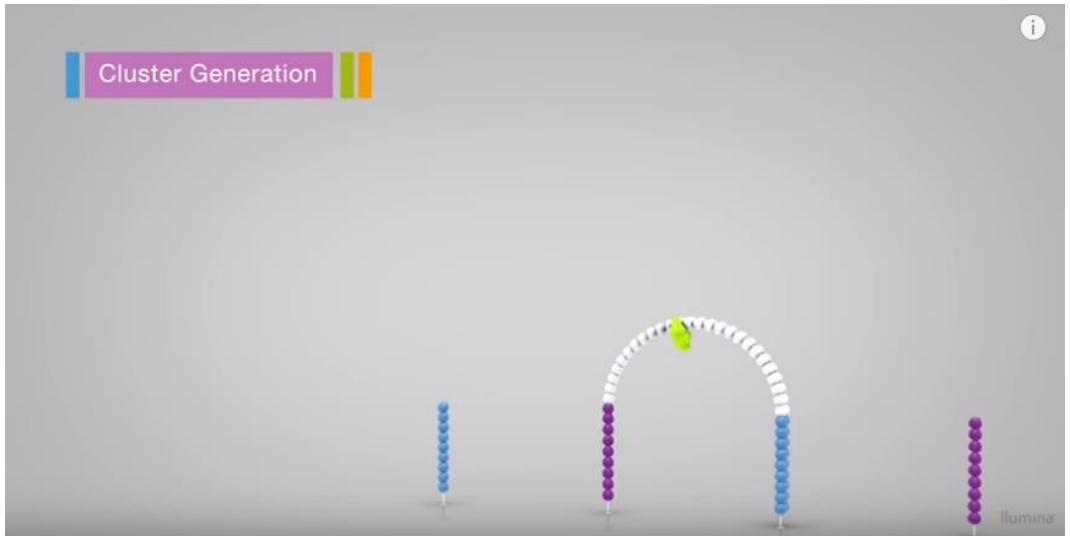
Clustering: bridge amplification

The strands are clonally amplified through bridge amplification

- The strand folds over and the adapter hybridises to the second type of oligo on the flow cell

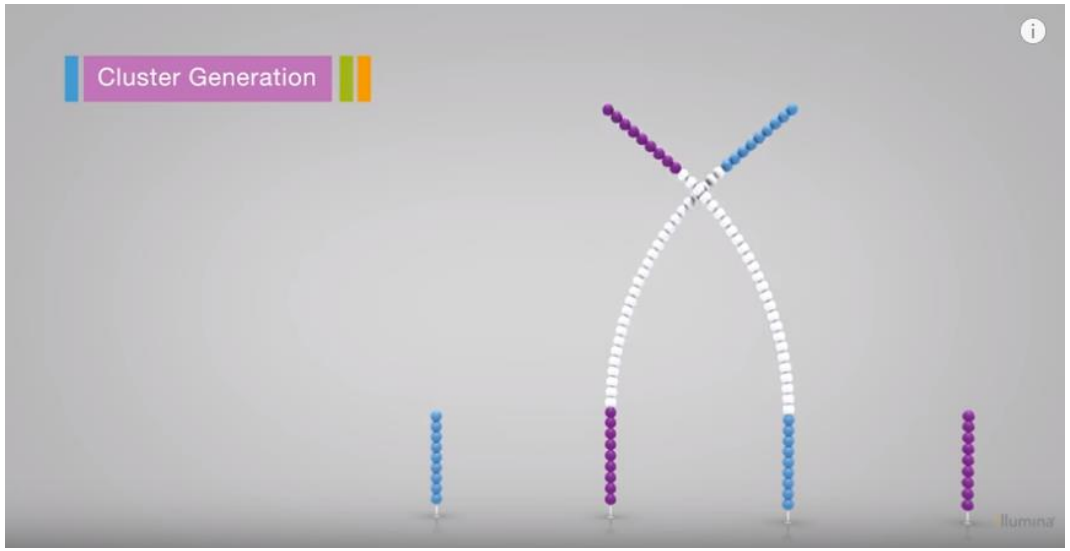
- Polymerases generate the complementary strand, forming a double-stranded bridge





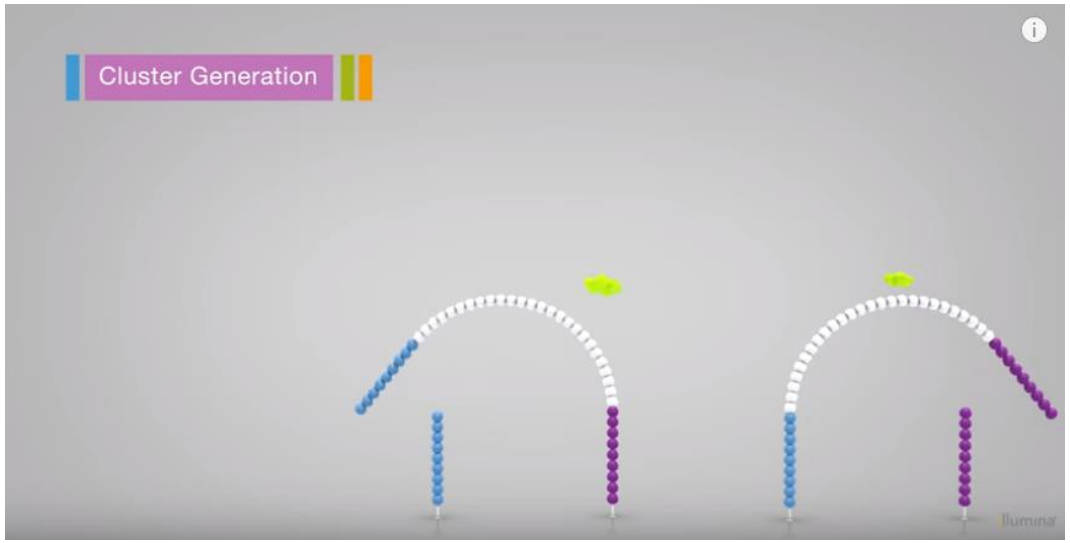
Clustering: bridge amplification

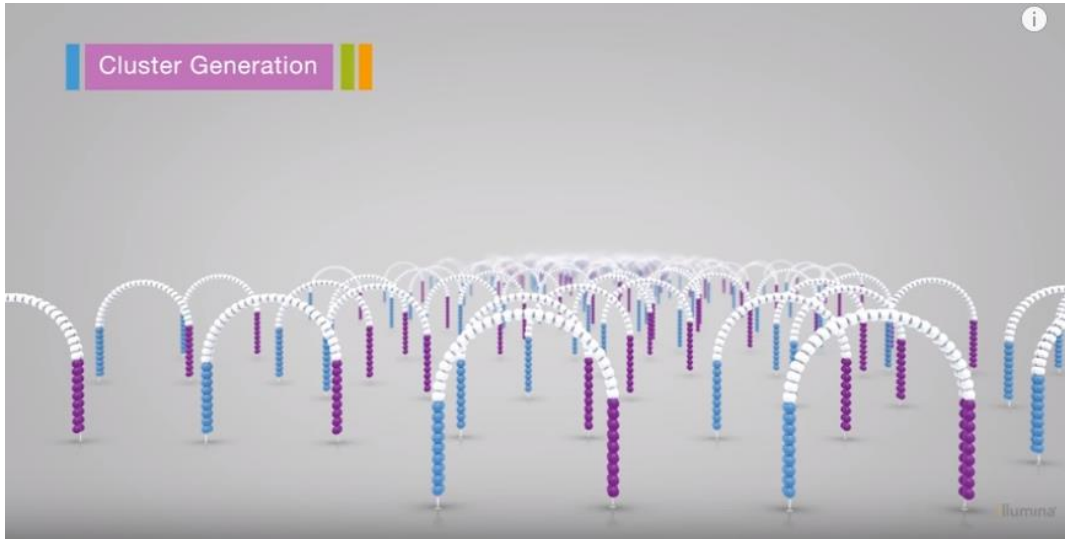
This bridge is denatured resulting in two single-stranded copies of the molecule that are tethered to the flow cell



Clustering: bridge amplification

The process is then repeated over and over, and occurs simultaneously for millions of clusters, resulting in clonal amplification of all the fragments

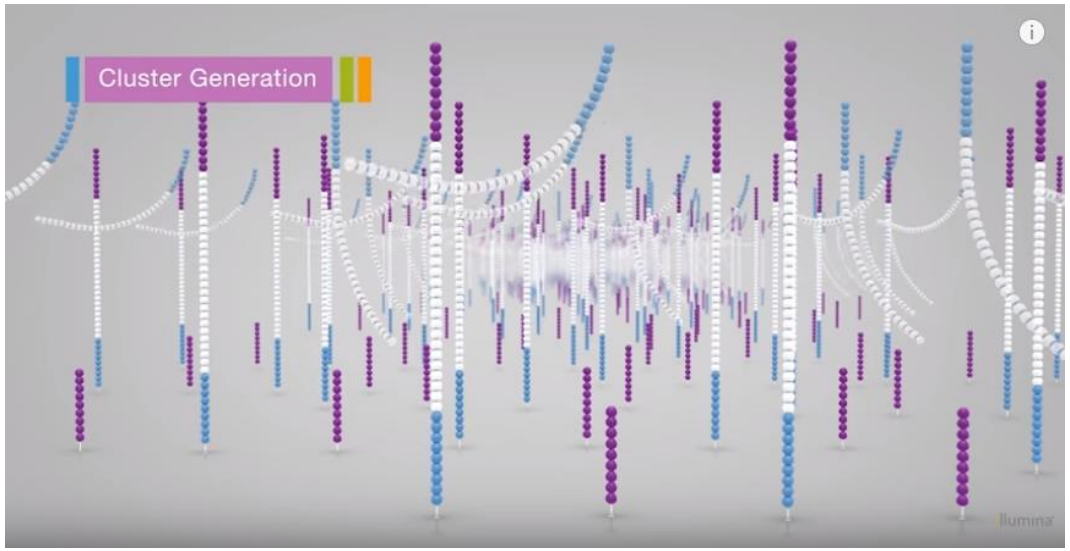




Clustering: final denaturing ready for sequencing

The reverse strands are cleaved and washed off, leaving only the forward strands.

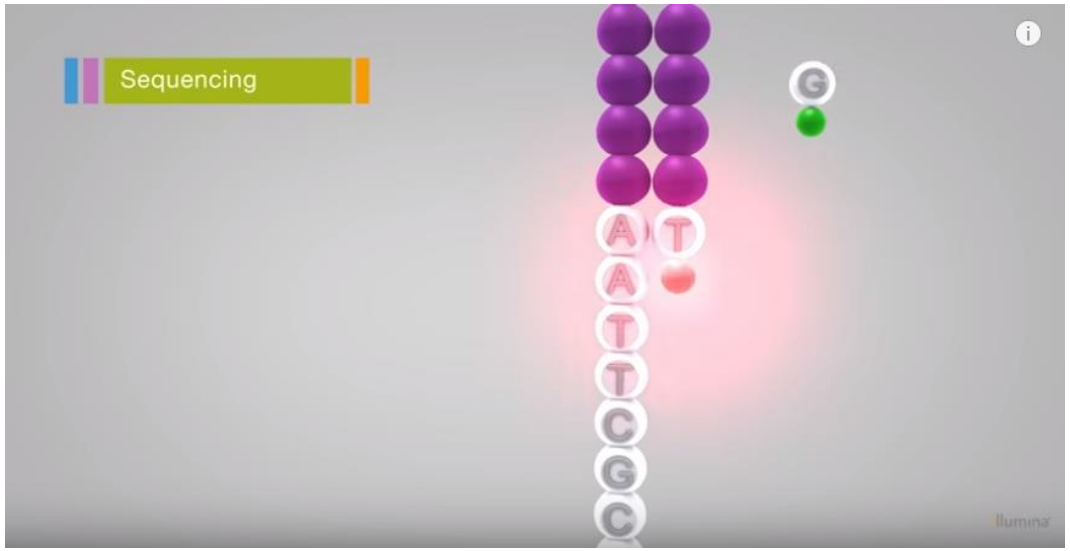
- The 3’ ends are blocked to prevent unwanted priming
- Cluster now ready for sequencing



Sequencing: first nucleotide extends from primer

Sequencing begins with the extension of the first sequencing primer to produce the first nucleotide.

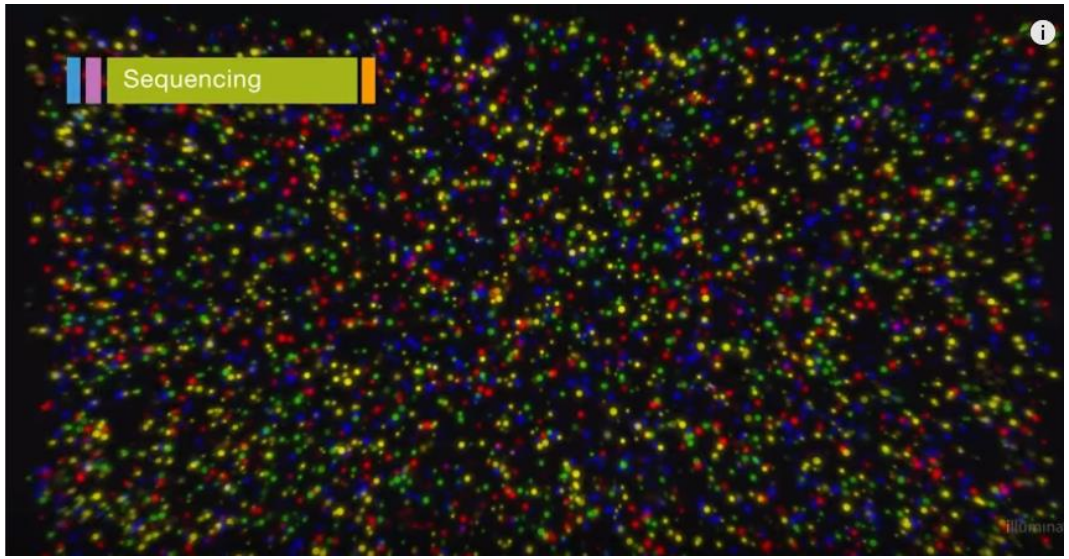
- With each cycle fluorescently labelled nucleotides compete for addition to the growing chain.
- Only one base is incorporated per cycle based on the template



Sequencing: generation of fluorescent signal

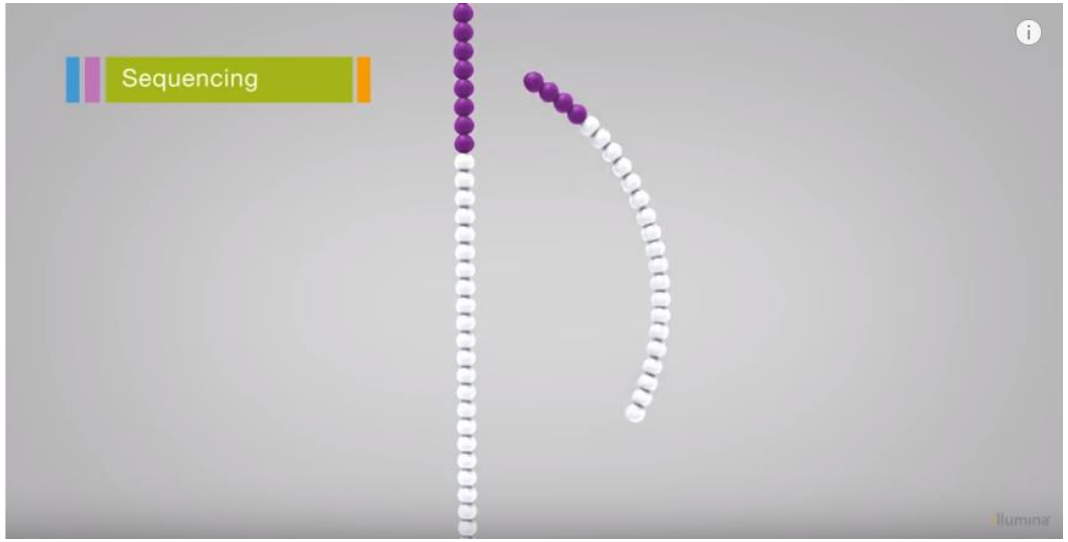
After the addition of each nucleotide, the clusters are excited by a light source and a characteristic fluorescent signal is emitted.

- The number of cycles determines the length of the read
- All clusters are sequenced in a parallel process



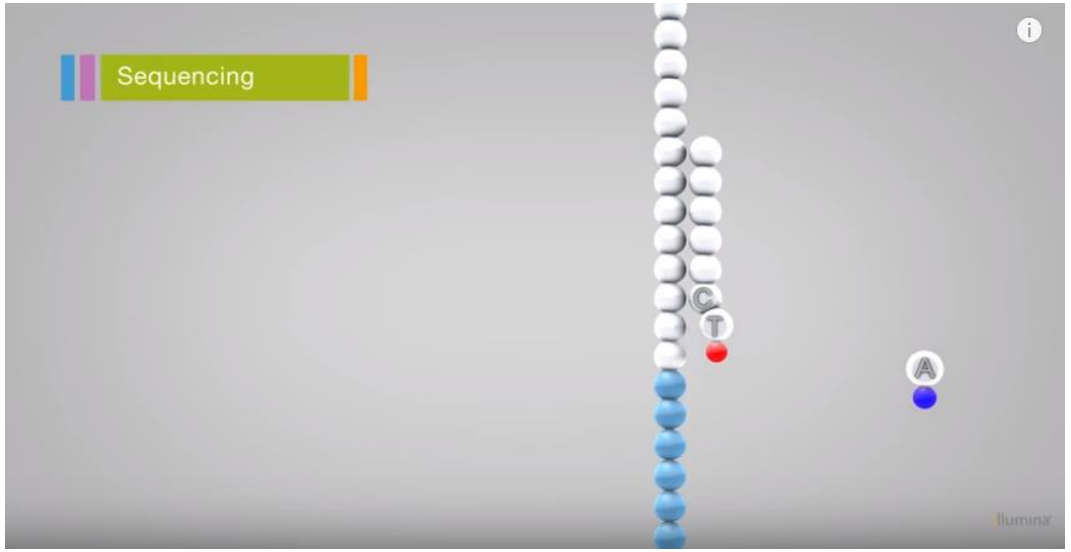
Sequencing: first read completion

After the completion of the first read, the read product is washed away

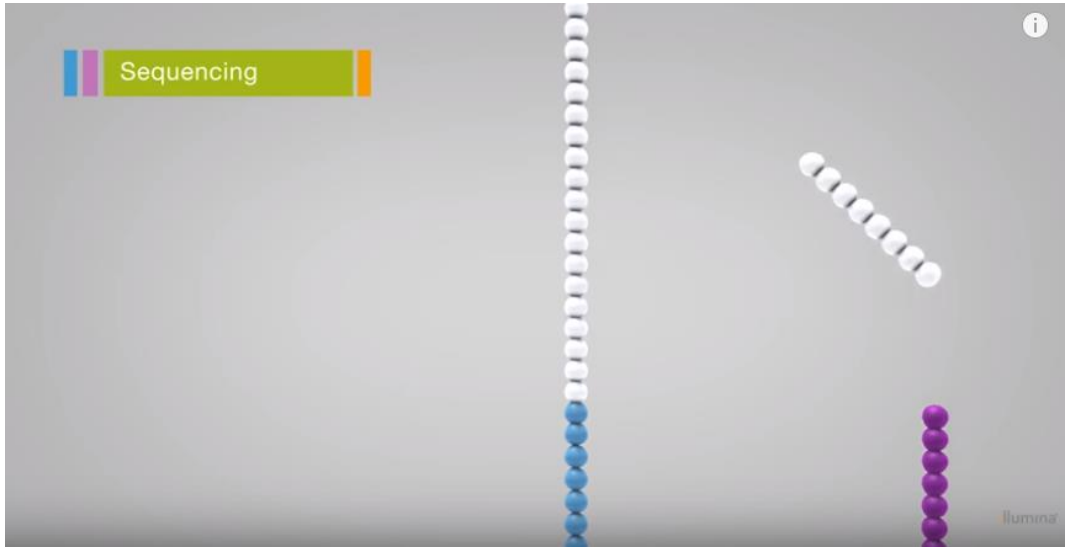


Sequencing: index read 1

- The index 1 read primer is introduced and hybridised to the template. The read is generated similar to the first read



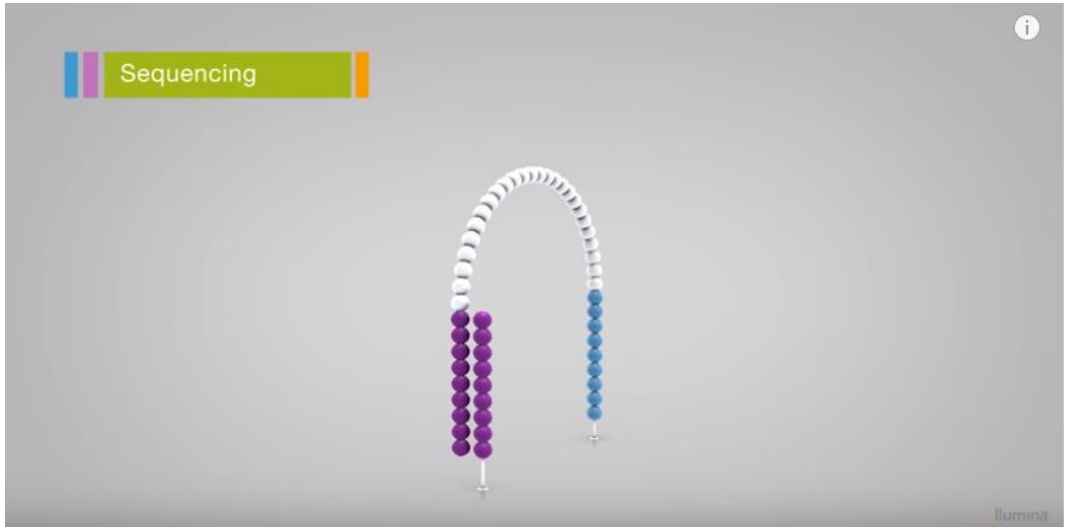
- After completion of the index read, the read product is washed off and the 3’ ends of the template are deprotected

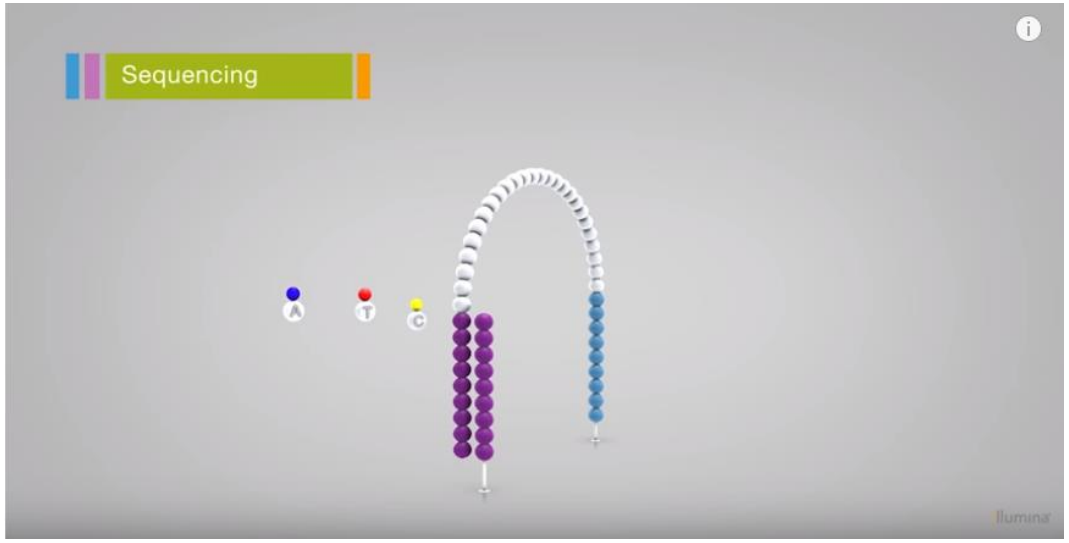


Sequencing: Index read 2

- The template now folds over and binds the second oligo on the flow cell

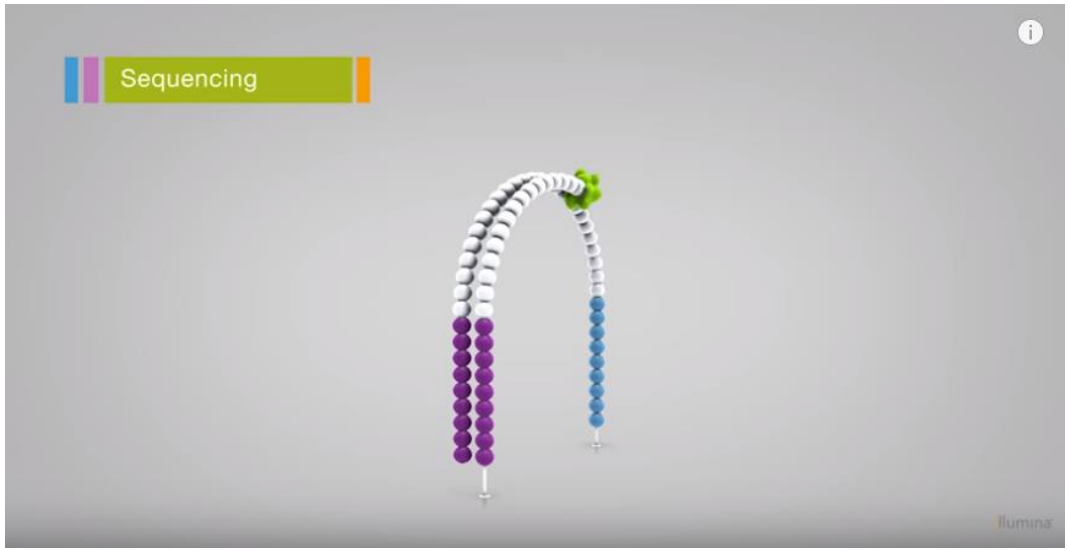
- Index 2 is read in the same manner as index one (for MiSeq)





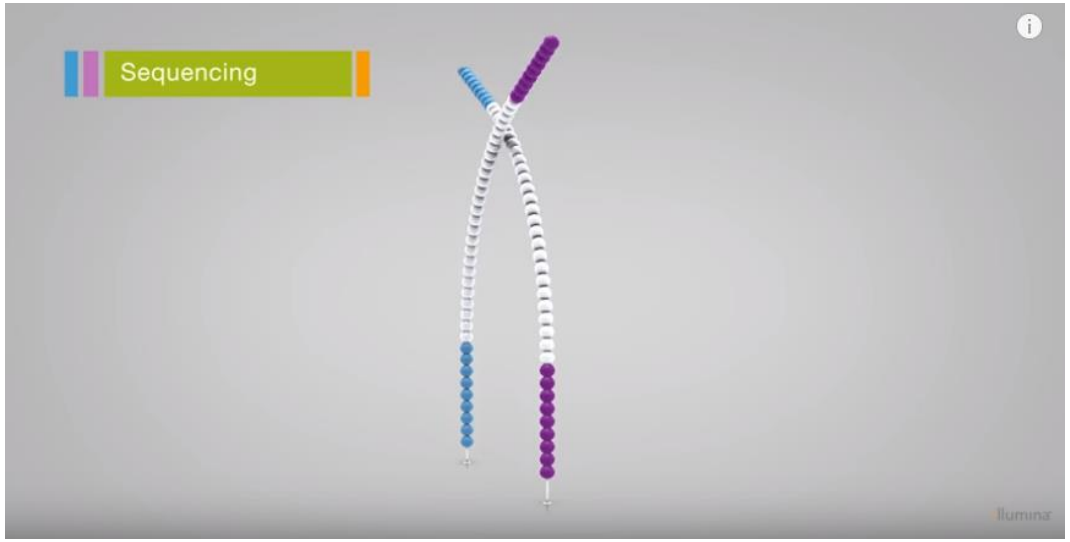
Sequencing: double stranded bridge formation

Polymerases extend the second flow cell oligo, forming a double stranded bridge

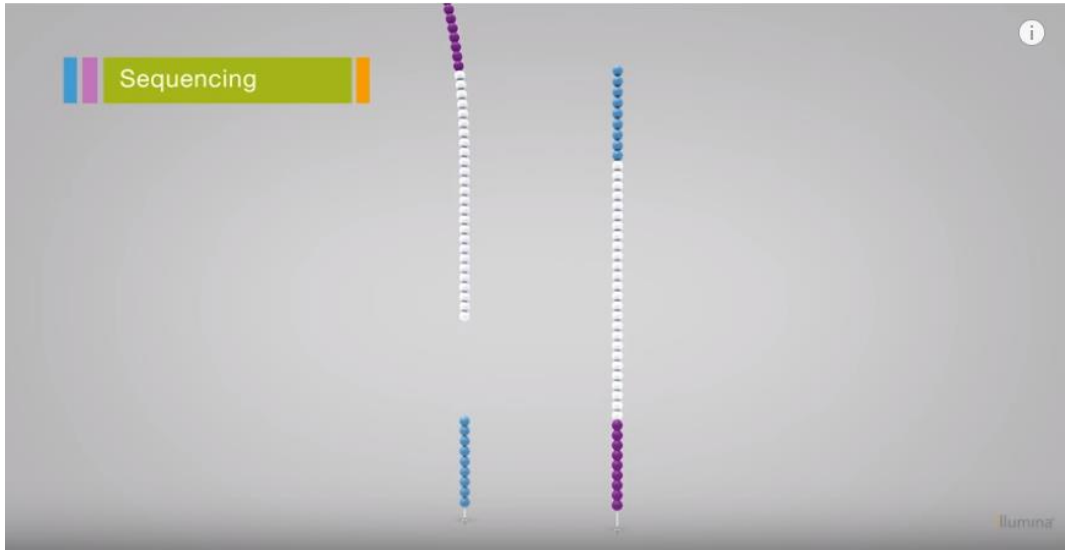


Sequencing: preparation for read 2

- This double stranded DNA is then linearised and the 3’ ends are blocked.



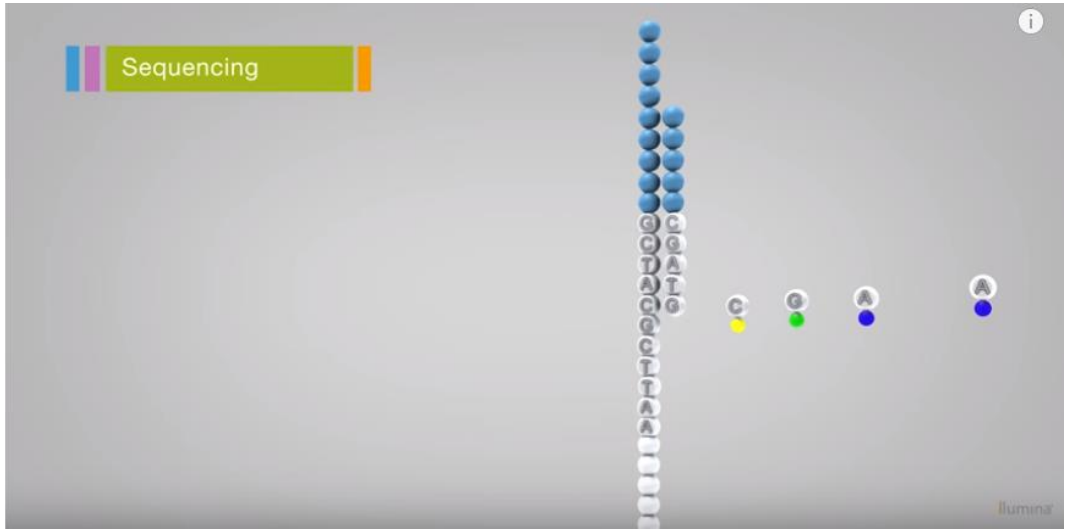
- The original forward strand is cleaved off and washed away, leaving only the reverse strand

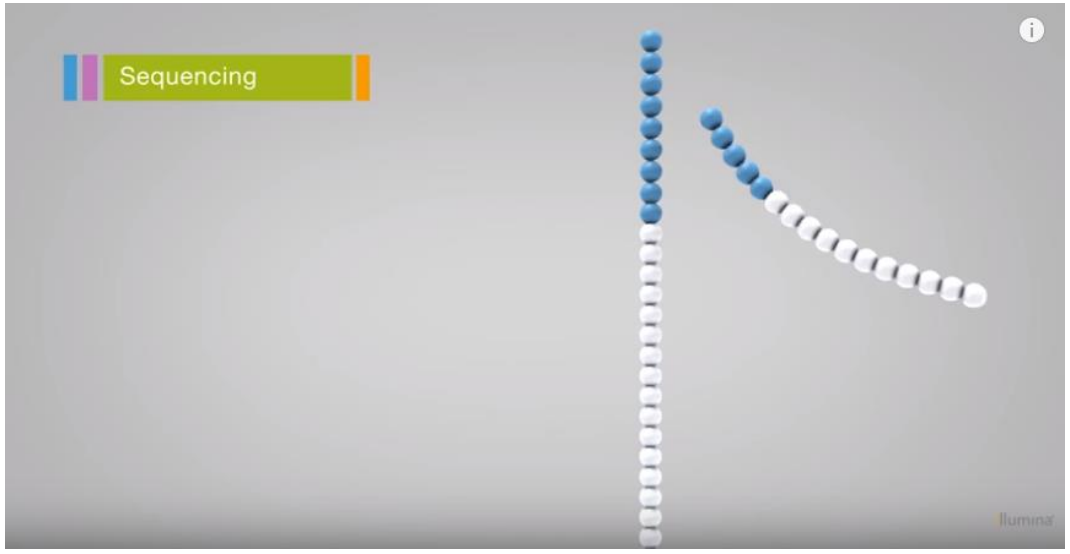


Sequencing: read 2

- Read 2 begins with the introduction of the read two sequencing primer. As with read one, the sequencing steps are repeated until the desired read length is achieved

- The read 2 product is then washed away

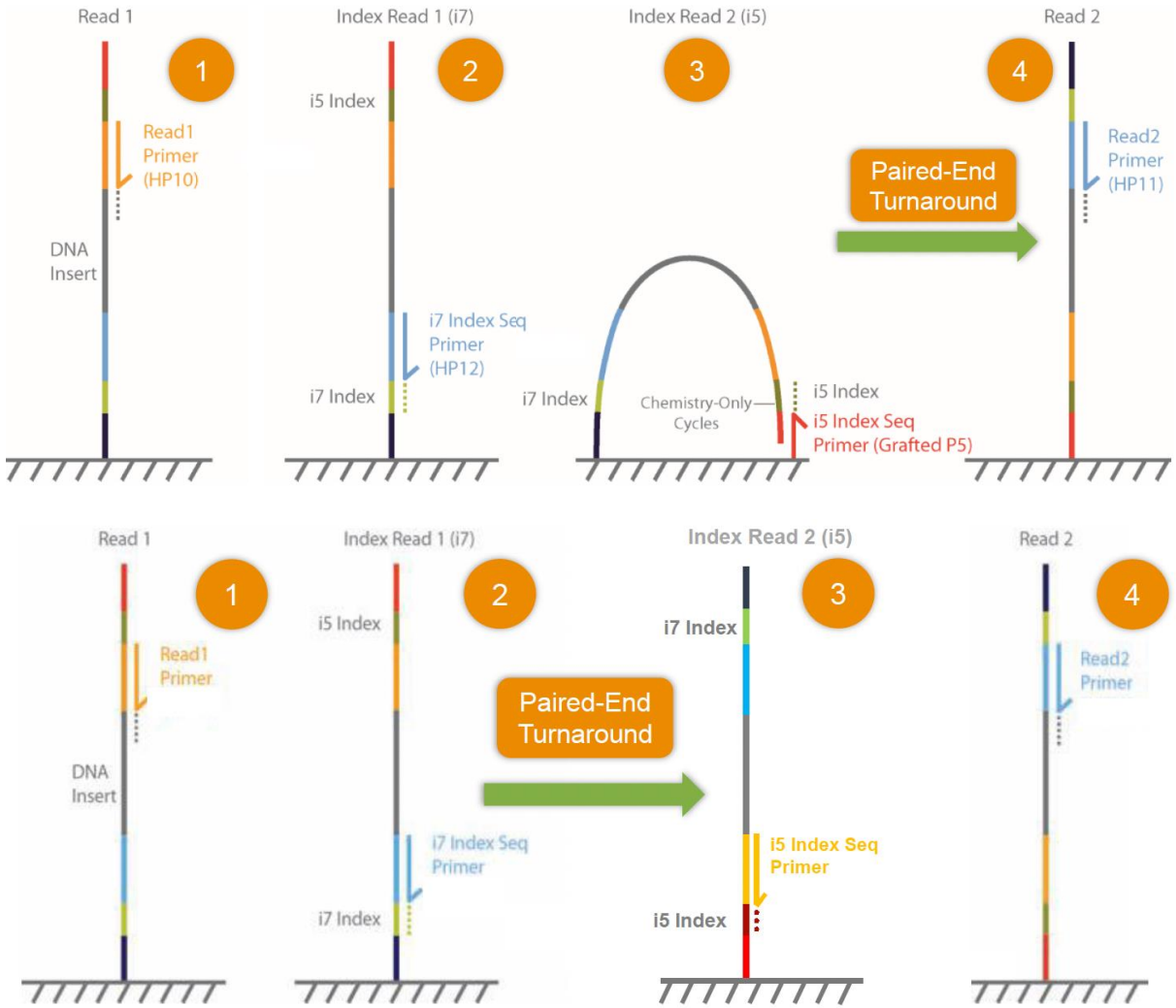




Dual-index sequencing on different Illumina instruments

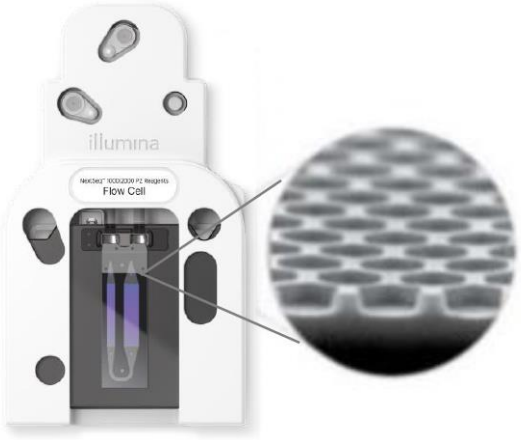
Dual indexing = 4 sequencing reads

1. Sequencing read 1 (i5 end)
2. Index 1
3. Index 2 - *read in different orientations on different sequencers*
   - Forward strand index 2 (MiSeq) – during paired-end turnaround
   - Reverse strand index 2 (iSeq-100, MiniSeq, NextSeq, NovaSeq) – after paired-end turnaround
   - Implications for demultiplexing
4. Sequencing read 2 (i7 end)



Random vs patterned flow cells

NextSeq 550 Flow Cell



NextSeq 1000/2000 Flow Cell

Random flow cell

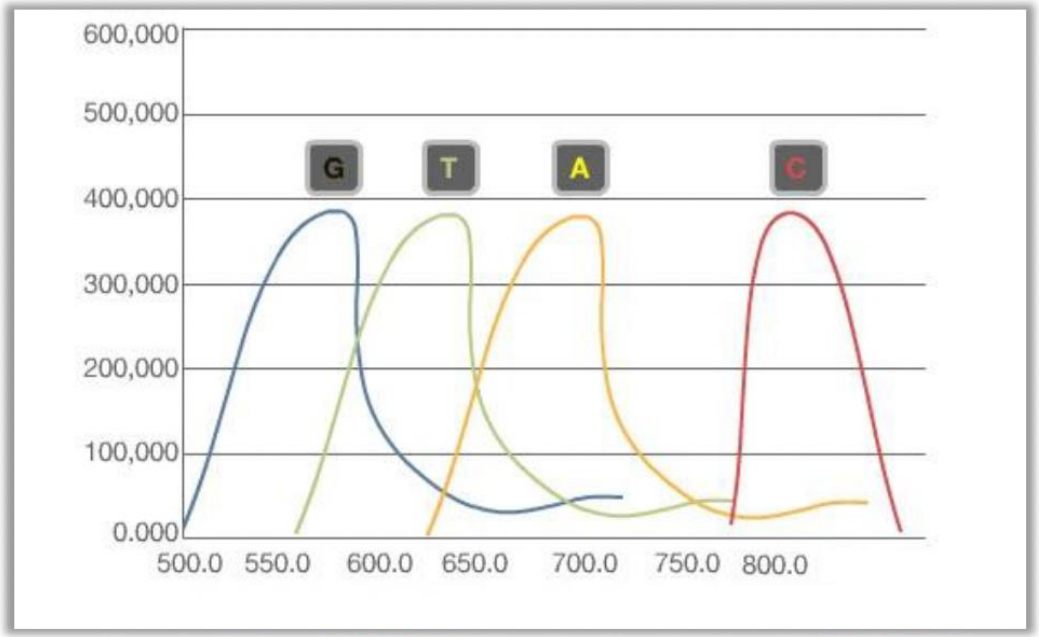
- MiSeq, NexSeq550, MiniSeq
- Fragments bind randomly spaced across the flow cell
- Map of clusters needs to be generated for identification
- More sensitive to overloading

Patterned flow cell

- NovaSeq, NextSeq1000/2000, iSeq 100
- Fragments bind in nanowells with defined cluster size and spacing
- Increase density
- Map already generated - quicker
- More sensitive to index-hopping

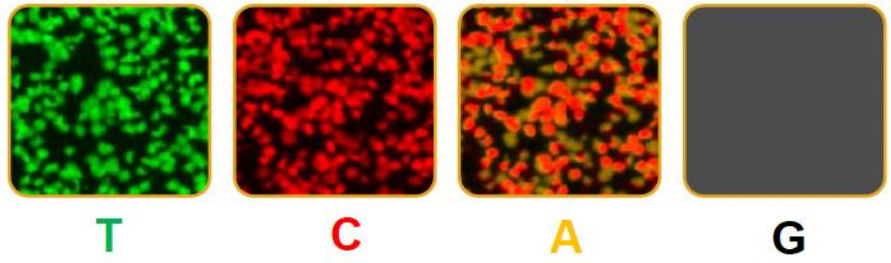
Four-colour chemistry

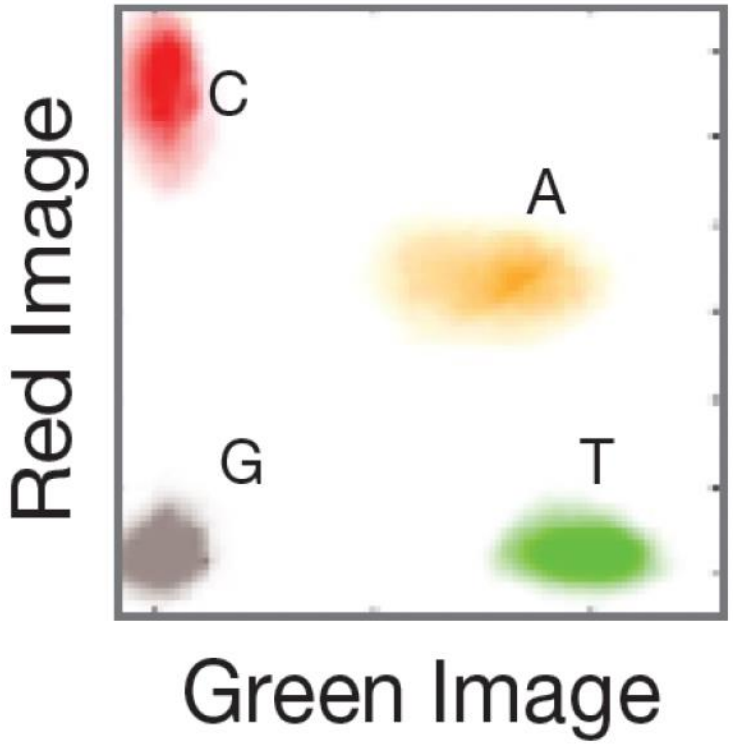
- Used by MiSeq
- 4 dyes – each base emits a unique wavelength
- 4 images collected on each sequencing cycle
- Each cluster appears in one image



Two-colour chemistry

- Used by NextSeq, MiniSeq, NovaSeq
- 2 dyes – red, green
- 2 images per cycle
- Clusters either appear in one, both or no images
- Runs of G's early in the read can cause problems!

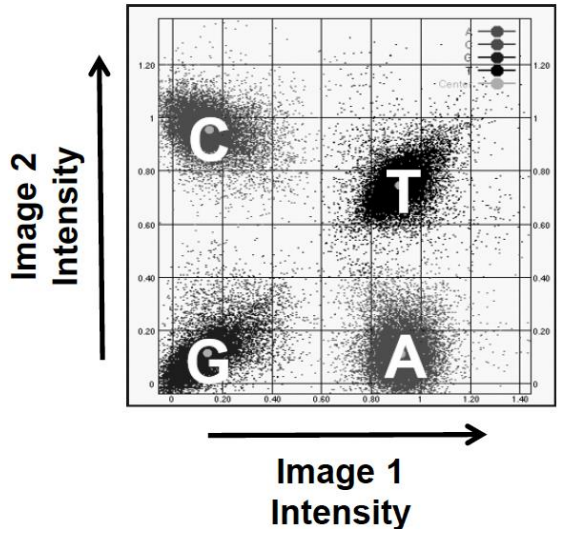


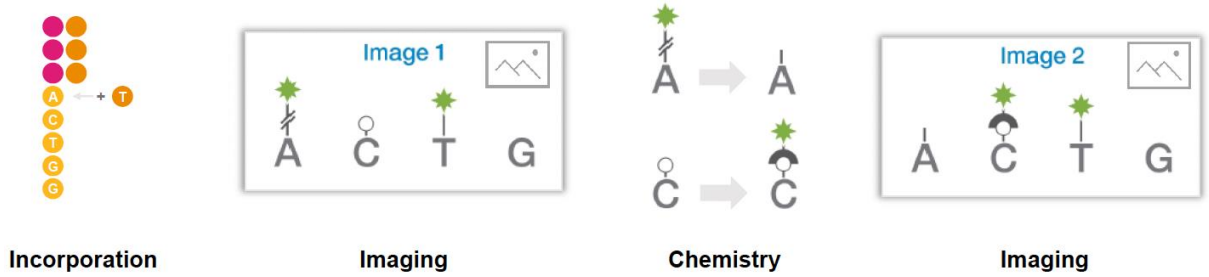


One-colour chemistry

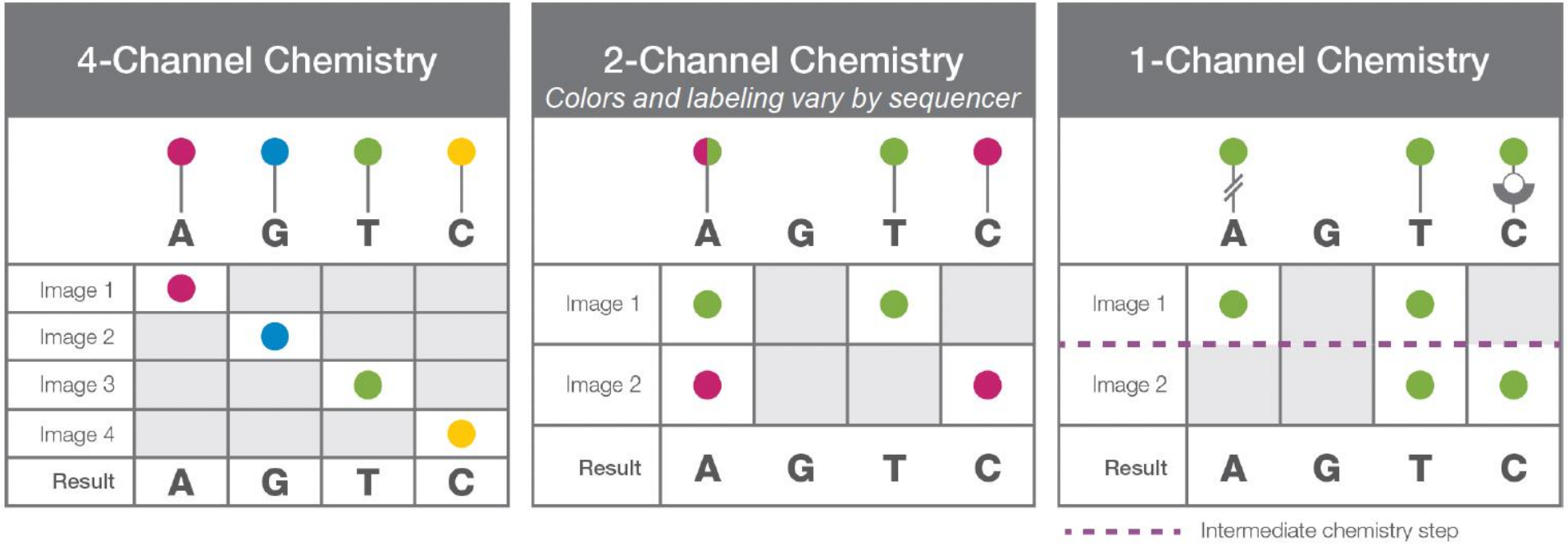
- iSeq-100
- Incorporate chemistry step
  - Metal-oxide-semiconductor (CMSO) technology
- single dyes
- 2 images per cycle – before and after chemistry
- Intensity in both images used to determine base

Base	Image 1	Image 2
T	ON	ON
A	ON	OFF
C	OFF	ON
G	OFF	OFF





Sequencing chemistry



Modifications in Illumina sequencing chemistry

- Decrease in number of dyes and images needed
- Faster sequencing and data processing
- Less tolerant of low-complexity regions

Sequencing metrics

Initial data analysis

How to tell if your run was good

Good Sequencing Run: means it meets the manufacturer's specifications.

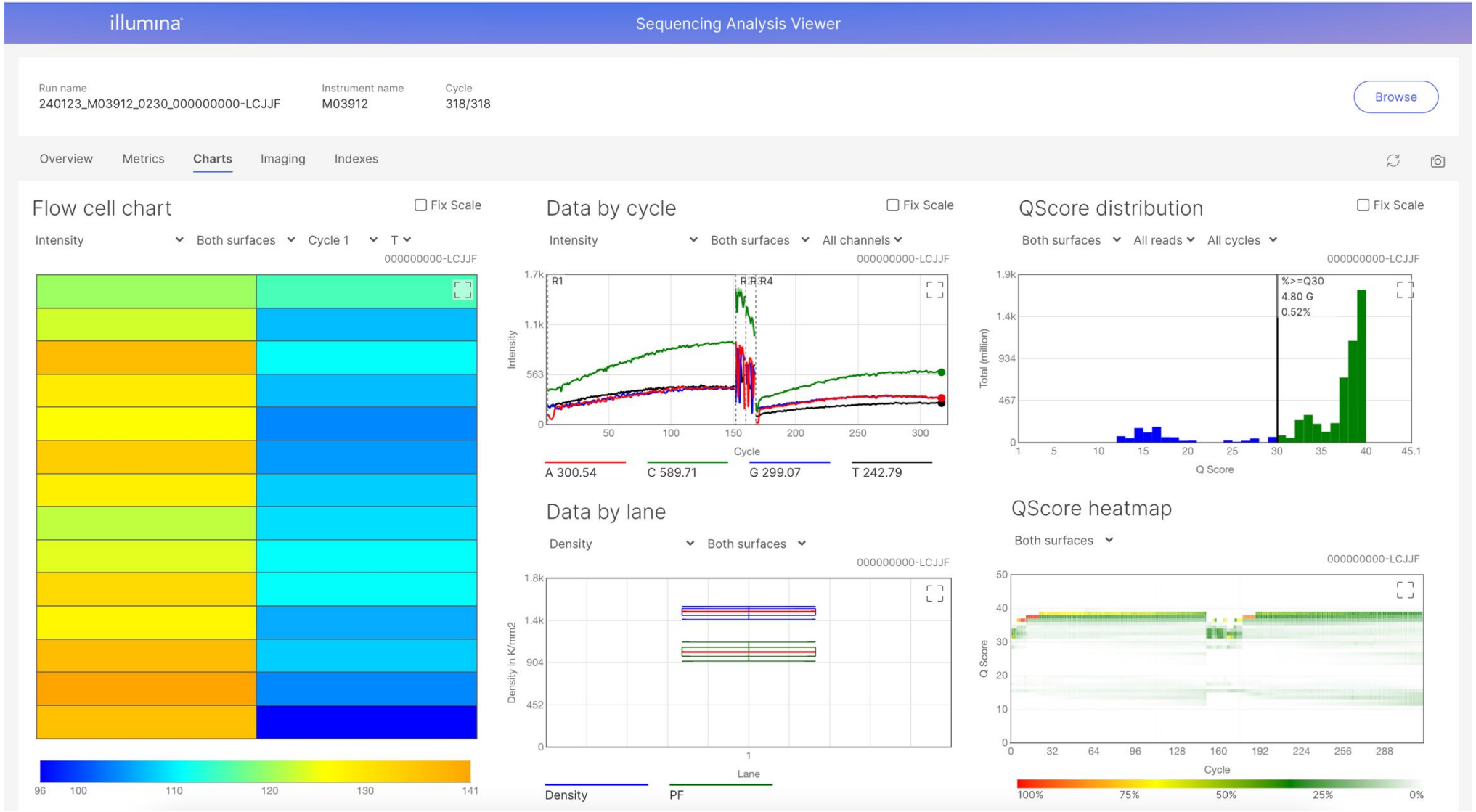
Specifications	MiSeq Reagent Kit V2				MiSeq Reagent Kit V3	
Read Length	1 x 36 bp	2 x 25 bp	2x 150 bp	2 x 250 bp	2 x 75 bp	2 x 300 bp
Output (bases)	540 - 610 Mb	750 - 850 Mb	4.5 - 5.1 Gb	7.5 -8.8 Gb	3.3 -3.8 Gb	13.2 -15 Gb
Q30 (%)	> 90%	> 90%	> 80%	> 75%	> 85 %	> 70%
Cluster Density (K/mm²)	600-1200				1000-1400	
Cluster Passing Filter (%)	>70%				>70%	

Tools: Illumina Sequencing Analysis Viewer (SAV)

Key metrics:

1. Yield (Gb)
2. % ≥ Q30
3. % Aligned and % error rate
4. Cluster Density (K/mm^2)
5. Clusters Passing Filter (%PF)

Sequence analysis viewer



Sequencing metrics

1. Yield (Gb)
2. % ≥ Q30
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Run summary

Level	Yield Total (GB)	Projected Yield (GB)	Aligned (%)	Error Rate (%)	Intensity Cycle 1	% Intensity Cycle 1	% >= Q30
Read 1	2.7	2.7	0.37	0.93	118.5	26.42	88.42
Read 2 (I)	0.13	0.13	0	NaN	940.32	209.67	86.85
Read 3 (I)	0.13	0.13	0	NaN	705.43	157.29	88.54
Read 4	2.7	2.7	0.34	1.31	29.68	6.62	79.59

Read 1

Lane	Tiles	Density (K/mm2)	% PF	Legacy Phasing / Prephasing Rate	Reads (M)	Reads PF (M)	% >= Q30	Yield (GB)	Cycle Error Rated	Aligned (%)	Error Rate
1	28	1,446.57 ± 40.41	70.18 ± 2.49	0.15 / 0.09	25.6	17.97	88.42	2.7	150	0.37 ± 0.01	0.93 ± 0.1

Read 2 (I)

Lane	Tiles	Density (K/mm2)	% PF	Legacy Phasing / Prephasing Rate	Reads (M)	Reads PF (M)	% >= Q30	Yield (GB)	Cycle Error Rated	Aligned (%)	Error Rate
1	28	1,446.57 ± 40.41	70.18 ± 2.49	0 / 0	25.6	17.97	86.85	0.13	0	NaN ± NaN	NaN ± NaN

1. Yield

The **total amount** of sequencing data generated in a run, measured in base pairs (Gigabases Gb)

Interpret:

- Should be within the expected range for the instrument and run type.
- Lower than expected yield may indicate issues with library prep, clustering, or flow cell loading.

Improving Yield:

- Optimize library loading concentration.
- Ensure proper denaturation and dilution steps

Sequencing Kit	Read Length	Total Time	Output
MiSeq Reagent Kit v2	2 x 25 bp	5.5 hours	750 – 850 Mb
	2 x 150 bp	24 hours	4.5 – 5.1 Gb
	2 x 250 bp	39 hours	7.5 – 8.5 Gb
MiSeq Reagent Kit v3	2 x 75 bp	21 hours	3.3 – 3.8 Gb
	2 x 300 bp	56 hours	13.2 – 15 Gb
MiSeq Reagent Kit v2 Micro	2 x 150 bp	19 hours	1.2 Gb
MiSeq Reagent Kit v2 Nano	2 x 150 bp	17 hours	300 Mb
	2 x 250 bp	28 hours	500 Mb

Output specifications for MiSeq cartridges

2. % ≥ Q30 (read accuracy)

Q score is logarithmically related to base calling **error possibility** P defined as:

$$Q = -10 \log P$$

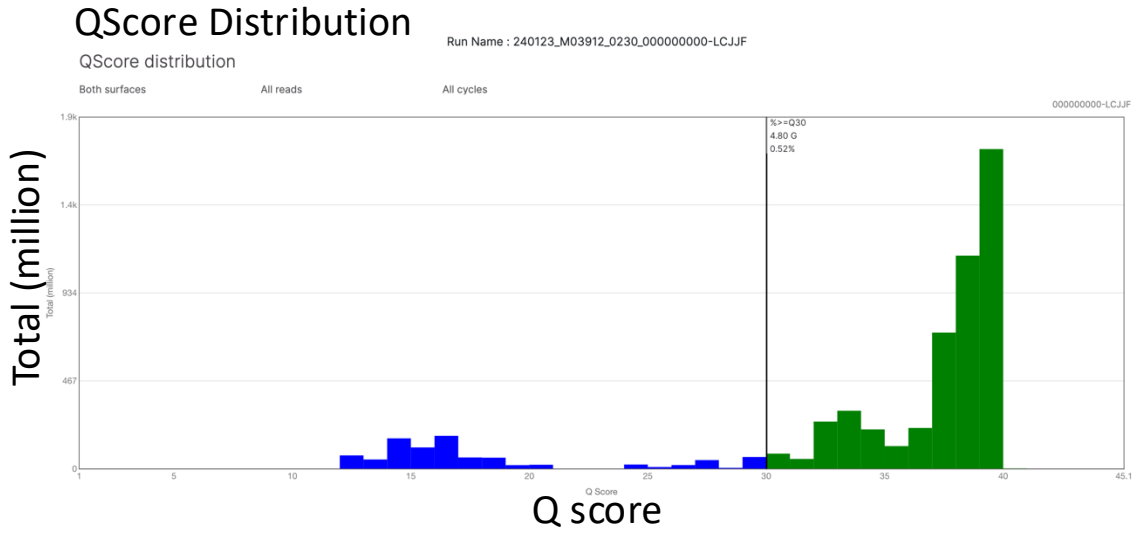
e.g. For every 1000 base calls, there is a likely one error - i.e., P = 0.001 then $\boldsymbol{Q=30}$

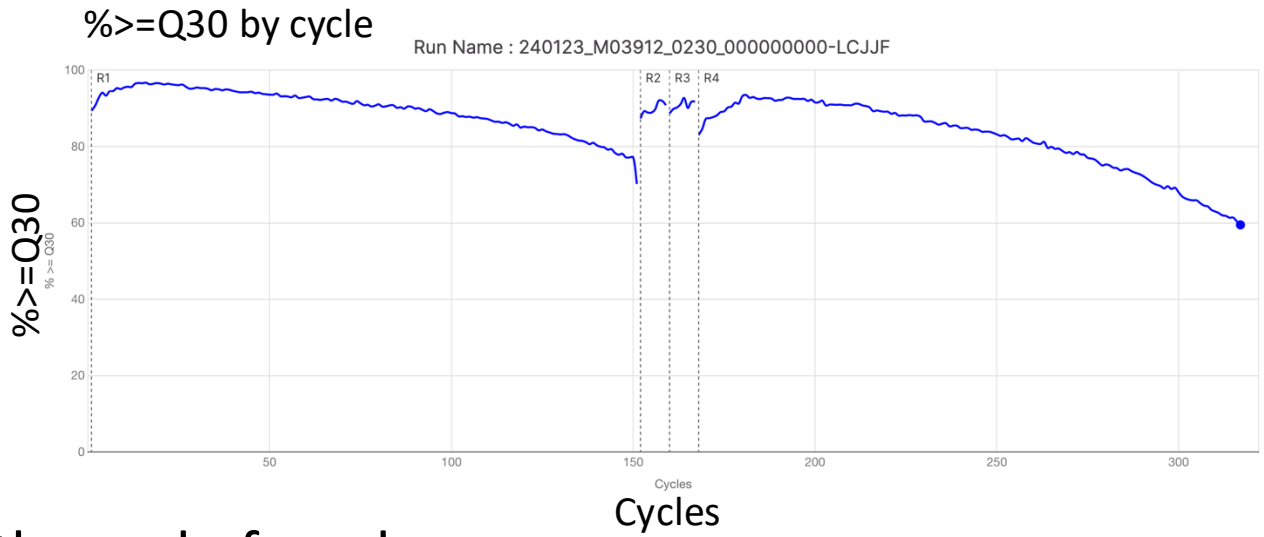
In other words, **Q30** describes the base calling accuracy = 99.9%

- Q30 is an average across the whole read length
- > = % Q30 refers to the percentage of bases in a sequencing run that have a $\boldsymbol{Q \geq 30}$

MiSeq Reagent Kit v2
> 90% bases higher than Q30 at 1 × 36 bp
> 90% bases higher than Q30 at 2 × 25 bp
> 80% bases higher than Q30 at 2 × 150 bp
> 75% bases higher than Q30 at 2 × 250 bp
MiSeq Reagent Kit v3
> 85% bases higher than Q30 at 2 × 75 bp
> 70% bases higher than Q30 at 2 × 300 bp
Optimal quality scores for MiSeq cartridges

2. % ≥ Q30 (read accuracy)





It is normal for accuracy to decreases towards the end of reads.

How to Improve:

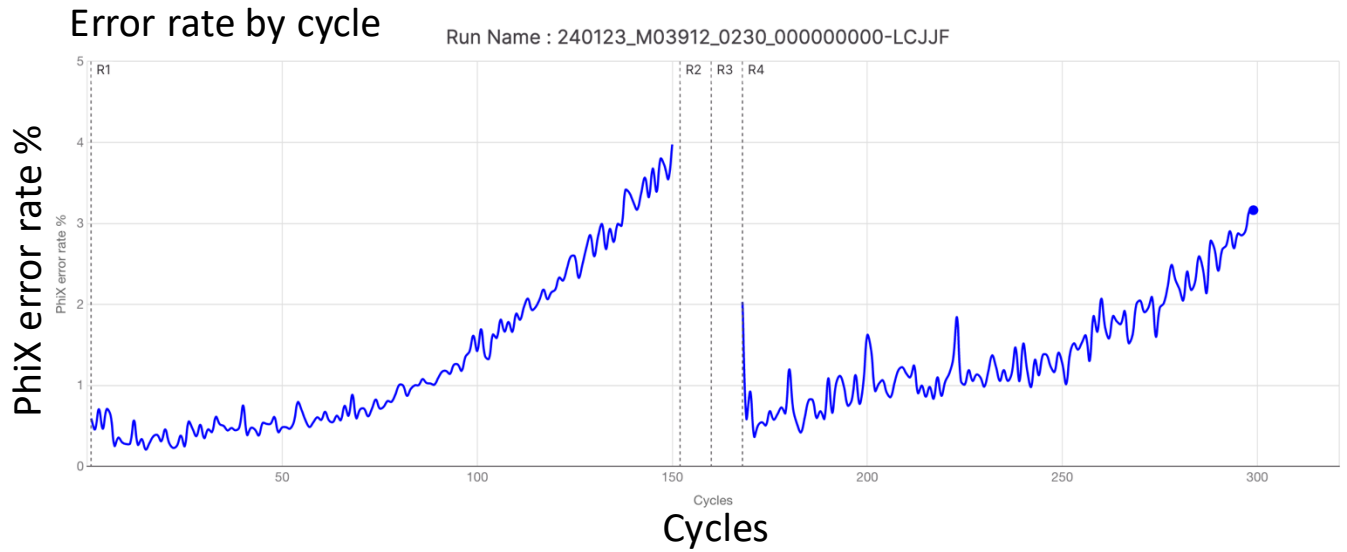
- Use high-quality reagents and clean lab practices
- Ensure proper cluster density
- Avoid overloading or underloading the flow cell

3. % Aligned and % error rate (PhiX)

Internal control: The PhiX Control v3 Library

- % Aligned: The percentage of reads that align to PhiX genome
- Error Rate: Is determined by the PhiX alignments, identifying any mismatches.

No PhiX No Aligned and no Error Rate will display in SAV



4. Cluster Density (K/mm²)

The number of clusters per mm² on the flow cell

Optimal Ranges : Varies with sequencer and cartridge version

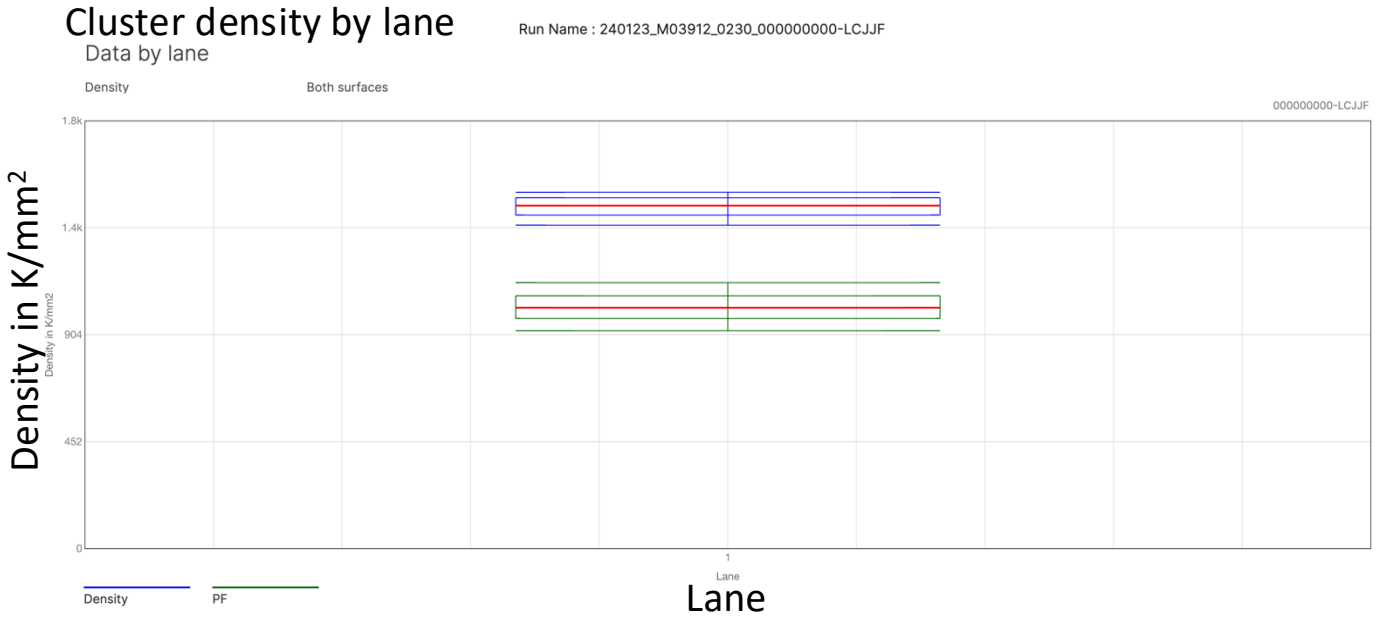
- Low cluster density = Under-utilization of sequencing capacity.
- High cluster density = Poor quality data due to overcrowding.

Optimizing Cluster Density:

- Titrate library concentration
- Use appropriate loading concentrations

Sequencer	Version	Raw Density (K/mm²)
MiniSeq	High and Mid	170-220
MiSeq	V2	1000-1200
	V3	1200-1400
NextSeq	V2 high and Mid	170-220
HiSeq 2500 Rapid	V1 and V2	850-1000
HiSeq 2500 High	V3	750-850
	V4	950-1050

Optimal cluster density for Illumina sequencers



5. Clusters Passing Filter (%PF)

%PF is the number of clusters that passed instrument's internal quality filter.

- Clusters that do not pass this filter removed from downstream analysis
- %PF range can vary depending on the type of flow cell used (patterned vs. non-patterned)

Interpret:

- Higher %PF: indicates better data quality (generally)
- Low % PF: suggests poor library prep (e.g. adapter dimers, short fragments); poor cluster formation

Cluster density and %PF

- Over-loading: High cluster density but low %PF
- Under-loading: Low cluster density but High %PF
- If not inversely related, consider an instrument or reagent issue.

Improvement:

- Perform thorough library QC, Optimize PCR amplification.

Day 2: Targeted enrichment

Workflow overview

Monday

Preparation of metagenomic libraries and sequencing on a MiSeq Illumina platform

Step 1: RNA library prep

(KAPA RNA HyperPrep kit)

Step 2: Library pooling

Step 3: MiSeq run

(Data to be analysed on Friday)

Tuesday

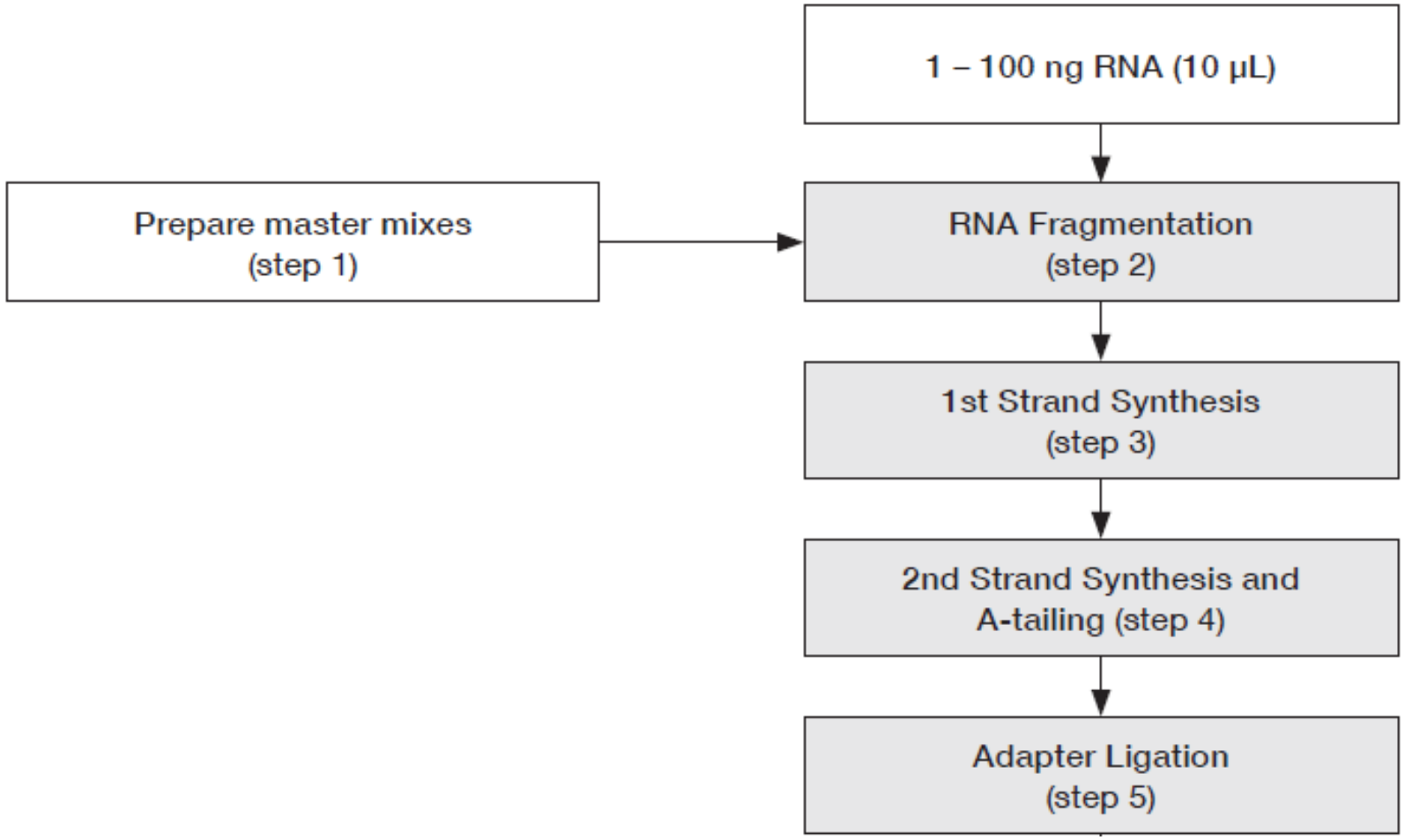
Target enrichment of HCV fragments and sequencing on a MiSeq Illumina platform

Step 4: Hybridisation of libraries with HCV-specific biotinylated probes

Step 5: Capture with streptavidin beads

Step 6: MiSeq run

(Data from a previous run of the same extracts will be provided)



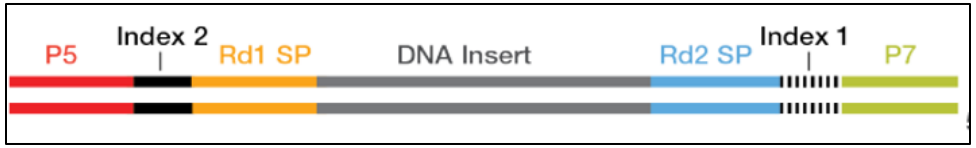
Total, poly(A)-enriched, or rRNA-depleted RNA

RNA is fragmented to the desired size using heat in the presence of Mg^{2+}

1st strand cDNA is synthesized with random primers

2nd strand cDNA synthesis converts cDNA:RNA hybrid to dscDNA, while marking the 2nd strand with dUTP. dAMP is then added to the 3'-end of dscDNA fragments

3'-dTMP adapters are ligated to 3'-dAMP library fragments



0.63X Bead-based Cleanup
(step 6)

Safe Stopping Point
Store at 2°C to 8°C for ≤24 hours

0.7X Bead-based Cleanup
(step 7)

Safe Stopping Point
Store at 2°C to 8°C for ≤1 week

Library Amplification
(step 8)

Adapter-ligated library DNA is amplified by PCR; dUTP-marked strand is not amplified

1X Bead-based Cleanup
(step 9)

Library QC

Library fragment size distribution is confirmed by electrophoresis. Library concentration is determined by qPCR (KAPA Library Quantification Kit).

Day 2: Targeted enrichment

Preparing probe-capture libraries

✓ Sample extraction

✓ Library preparation

❑ **Quality control and pooling of NGS libraries for capture-based target enrichment**

❑ Probe capture-based target enrichment

✓ Quantification and dilution of pooled libraries (Qubit and TapeStation)

✓ Miseq run setup

✓ MiSeq sequencing metrics

Pooling strategies for capture-based enrichment

- Multiplexing prior to probe capture decreases the cost per sample - **samples need to be barcoded prior to capture!**

- Suppliers typically recommend 8-12 libraries per hyb reaction- 6-60 samples can be combined depending on VL and sample type, **but you need to test.**

- Follow recommendations for total DNA input- you can adjust for lower inputs but keep in mind it can result in poorer results… more tests!

Pooling strategies for capture-based enrichment

- Pool an equal ratio of each sample, using:
  - Mass - only recommended if the library size distribution the same amongst different libraries
  - Molarity - will avoid under-representation of libraries with larger fragment sizes
  - Viral load - if available this is the best way to pool as samples with high viral low can take over the pool!

- Avoiding within-capture cross-contaminations:
  - Multiplex samples with similar viral load
  - Include negative controls
  - Use UDIs

Pooling strategies for capture-based enrichment

- Worked example – final pool with 700 ng of DNA

Kapa RNA HyperPrep libraries	Concentration (ng/μl)	Amount required per library (ng)	Volume for 70 ng (μl)
Library 1	8	70	8.8
Library 2	9.2	70	7.6
Library 3	20	70	3.5
Library 4	35.1	70	2.0
Library 5	31.4	70	2.2
Library 6	16.3	70	4.3
Library 7	17	70	4.1
Library 8	11.2	70	6.3
Library 9	4.9	70	14.3
Library 10	10.1	70	6.9
Total	11.7	700	60.0

Pooling strategies for capture-based enrichment

Calculate the volume required for your pool:

- Best practice to re-check the concentration of the final pool prepared: Pool = 12.5 ng/ml
- Require 500 ng of pool for probe capture (500-1500ng)
- Vol= ?

$$v = \frac{\text{total mass of DNA required (ng)}}{\text{concentration of DNA (ng/µl)}}$$

$$v = \frac{500\text{ng}}{12.5\ (\text{ng/µl})}$$

$$v = 40\ \text{µl}$$

Day 2: Targeted enrichment

Preparing probe-capture libraries

✓ Sample extraction

✓ Library preparation

✓ Quality control and pooling of NGS libraries for capture-based target enrichment

❑ **Probe capture-based target enrichment**

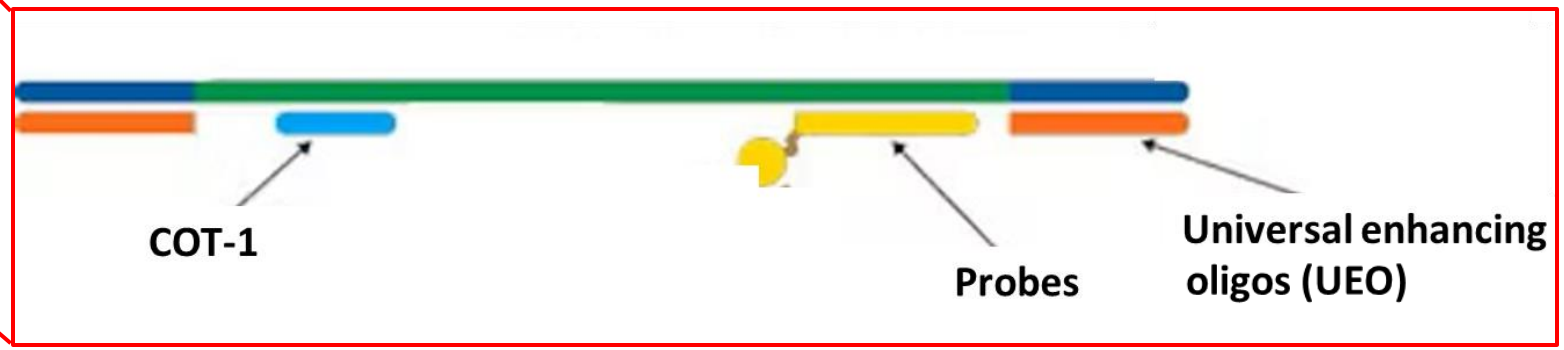
✓ Quantification and dilution of pooled libraries (Qubit and TapeStation)

✓ Miseq run setup

✓ MiSeq sequencing metrics

Day 2: Targeted-enrichment

- Hybridization rxn setup
- Hybridization
- Capture bead binding
- Capture bead washes
- Post-capture amplification
- Bead cleanup



What happens during the hyb step?

Multiplexed DNA libraries are combined with COT DNA - this binds to human repetitive DNA sequences preventing non-specific probe binding.

What happens during the hyb step?

Concentrate sample by bead purification (high ratio) and elute in Universal Enhancing Oligo/ Blocking oligo - they hybridize with Illumina's indexed adaptors preventing unwanted interactions with adaptors from other DNA molecules and consequent off-target effects

Add hybridization mix (hybridization buffer + hybridization component H)

What happens during the hyb step?

Combine with capture probe

- Denature at 95°C- to separate the DNA strands
- Hybridize at 55°C for 15min-72hr depending on protocol

Hybridisation

- Thaw the following reagents and keep on ice until required.
  - Universal Enhancing Oligos (Labelled UEO, Green lid)
  - COT DNA (1mg/mL) (Labelled COT, Blue lid)
  - 2X Hybridization Buffer (Labelled Hyb B, Pink lid)
  - Hybridization Component H (Labelled CH, Red lid)
  - HCV Target Enrichment Probe (Labelled HCV, Yellow lid)
- Preparation of other reagents and equipment:
  - Equilibrate Ampure XP beads at room temperature.
  - Fresh 80% ethanol solution.
  - DNA library pool prepared as described in the section “Quality control and pooling of NGS libraries for capture-based target enrichment”

Note: *this will be provided for you*

Hybridisation

Step 1. Prepare the Hybridisation Sample

- Add 20 µl of COT Human DNA (1 mg/ml) to a new 1.5 ml tube (low DNA binding tube).
- Add 40 µl of the DNA library pool to the 1.5 ml tube containing 20 µl of COT Human DNA to give a total volume of 60 µl
- Add 2X sample volume of Ampure XP beads (or KAPA HyperPure Beads) to each tube = 120 µl Ampure
- Mix thoroughly by vortexing for 10 seconds and perform a quick spin (**do not pellet beads**, ensure solution remains homogenous).

Concentrate using bead clean-up- **Alerts**:

- 1 EtOH wash
- elute in 13.4 µl of Universal Enhancing Oligos

Hybridisation

Step 2. Prepare the Hybridization Master Mix

- Add 43 µl of the Hybridization Master Mix to the bead-bound DNA mixture resuspended in Universal Enhancing Oligos from step 1.
- Mix thoroughly and perform a quick spin (do not pellet beads, ensure solution remains homogenous).
- Incubate at room temperature for 2 minutes.
- Place the sample on the magnet to collect the beads. Incubate until the liquid is clear.
- Transfer 56.4 µl of the eluate (entire volume) into a new tube containing 4 µl of the HCV Target Enrichment Probe

Lid Colour	Aliquot label	Component	Master mix volume (inc. 10% excess)
Pink	Hyb B	Hybridisation buffer (2X)	30.8 µl
Red	CH	Hybridisation Component H	13.2 µl
		PCR Grade water	3.3 µl
		TOTAL:	47.3 µl

Step 3. Perform the hybridization incubation

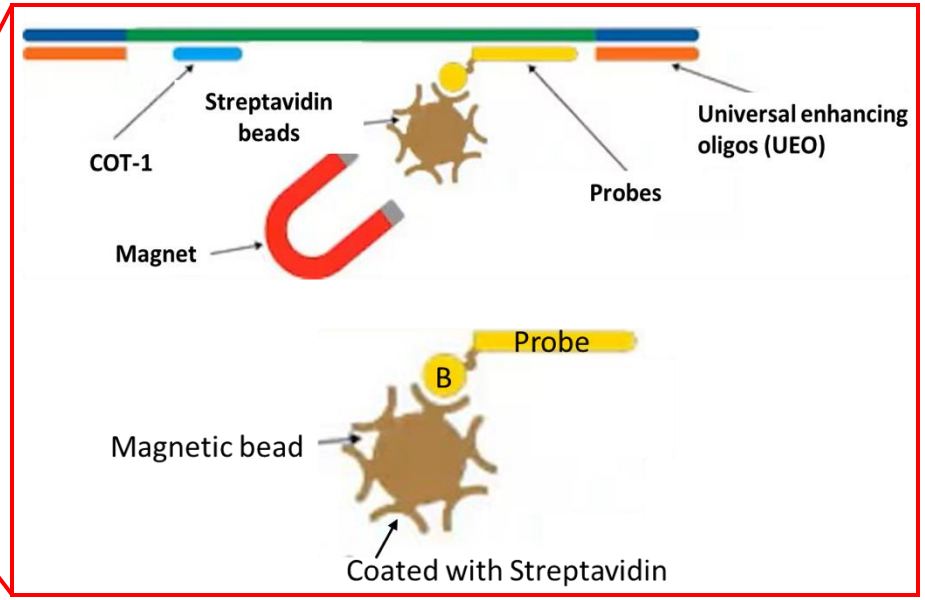
Step	Temp.	Duration
Denaturation	95°C	5 min
Hybridisation	55°C	Hold*

Time constrain alert! We are only leaving them for the break and the time it takes to prepare the capture beads. Normally 24 hours for this protocol.

Coffee break

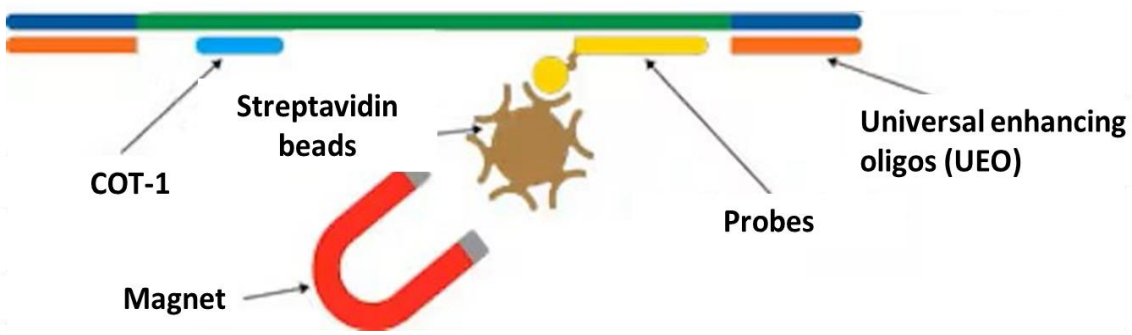
Day 2: Targeted-enrichment

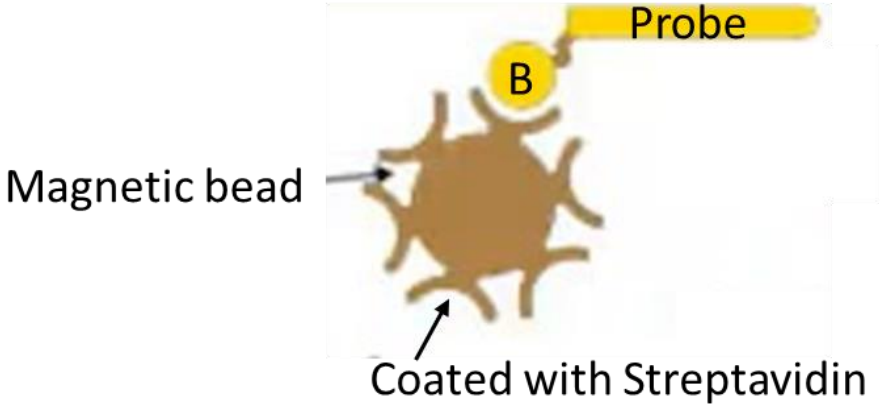
- Hybridization rxn setup
- Hybridization
- Capture bead binding
- Capture bead washes
- Post-capture amplification
- Bead cleanup



KAPA HyperCap Workflow v3.0 Roche Briefing

What happens during the capture step





1. Capture beads (streptavidin magnetic beads)- wash & replace their buffer (3X bead wash buffer)

2. Incubate hyb with capture beads to capture biotinylated probes- **HCV libraries bound to beads & supernatant with non-specific DNA**

3. Requires careful and stringent washing to remove non-specific interactions; temperature when adding beads/initial washing is also important

4. Elute enriched library from the capture beads with water and heat

HCV enriched libraries= supernatant after PCR!

Bead capture and washes

Washing and Recovering Captured DNA Library Pool

Step 1. Prepare Hybridization Wash Buffer and Bead Wash Buffers.

Note: *These have been pre-prepared for you by diluting the stock buffers with PCR grade water.*

- To pre-warm 1X Stringent Wash Buffer put two aliquots of 200 µl in 0.2 ml tubes and place the tubes into a thermocycler set to +55°C.
  - ***Note:*** *for the practical demonstration we will use 1.5 mL tubes and heat blocks, but in real situations 0.2 mL tubes and thermocyclers give more efficient heat transfer.*
- To pre-warm the 1X Wash Buffer I, make one aliquot of 100 µl into a 0.2 ml tube and place the tube into a thermocycler set to +55°C.
- Pre-warm the buffers for a minimum of 15 minutes. It is extremely important that the temperature remains at +55°C.

Bead capture and washes

Step 2. Prepare the Capture Beads

- Allow the Streptavidin Capture Beads (CB – White label) to equilibrate to room temperature prior to use, vortex the Capture Beads for 15 seconds before immediate use to ensure a homogeneous mixture.
- Aliquot 50 µl of beads for each capture into a 0.2 ml or single 1.5 ml tube (*i.e*. for one capture use 50 µl beads and for four captures use 200 µl beads *etc*.). ***Note:*** *Beads for four captures can be prepared in a single 0.2 ml tube or up to twelve captures can be prepared in a single 1.5 ml tube.*
- Place the sample on a magnet to collect the supernatant. Incubate until the liquid is clear. Remove and discard the supernatant being careful not to disturb the beads.
- Add 2X the initial volume of beads of 1X BWB (e.g. for one capture use 100 µl), mix thoroughly by vortexing, quick spin, incubate on magnet until the liquid is clear, remove and discard the supernatant being careful not to disturb the beads.
- Repeat this wash step
- Add 1X the initial volume of beads of 1X Bead Wash Buffer (*i.e*. 50 µl), mix thoroughly by vortexing, quick spin
- Aliquot 50 µl of resuspended beads into a new tube/well for each capture. Incubate on magnet to until the liquid is clear.
- Carefully remove and discard the supernatant. The Capture Beads are now ready to bind the hybridized DNA.

Time Alert! Proceed immediately to the next step.

Bead capture and washes

Step 3. Bind Hybridized DNA to the Capture Beads

- Transfer the hybridization samples to the Capture Beads.
- Mix thoroughly by pipetting up and down ten times and perform a quick spin. **Take Care!**
- Incubate the hybridization reaction by placing the sample in a thermocycler set to +55°C for 15 minutes, with the thermocycler lid temperature set to +105°C.

Note: normally we would use a thermocycler but for demonstration purposes leave it in your thermomixer

- **Proceed immediately to the next step.**

Temperature alert! *the following steps which should be performed quickly and carefully to prevent the beads from drying or the temperature from varying which can have effect the yield.*

Bead capture and washes

Step 4. Wash the Capture Beads Plus Bound DNA

- Add **100 µl of pre-warmed WBI** to the Hyb reaction, mix thoroughly by vortexing (10 s), perform a very quick spin.
- Place on a magnet, remove supernatant, immediately add **200 µl of pre-warmed SWB**
- Mix well (vortex 10s), perform a quick spin incubate at +55°C for 5 minutes
- Place on a magnet, remove supernatant, immediately add **200 µl of pre-warmed SWB**
- Mix well (vortex 10s), perform a quick spin incubate at +55°C for 5 minutes
- Place on magnet, remove supernatant, **add 200 µl of room-temp WBI,** mix well, quick spin, room-temp for 1 min
- Place on magnet, remove supernatant, **add 200 µl of room-temp WBII,** mix well, quick spin, room-temp for 1 min
- Place on magnet, remove supernatant, **add 200 µl of room-temp WBIII,** mix well, quick spin, room-temp for 1 min
- Place on a magnet, remove and discard the supernatant, add 20 µl “water”

Alerts!

- *Today the water is replaced with –pre-enriched libraries as your hyb reaction was too short*
- *Make sure you don’t discard the beads – we will perform on-bead PCR*

Day 2: Targeted-enrichment

Hybridization rxn setup

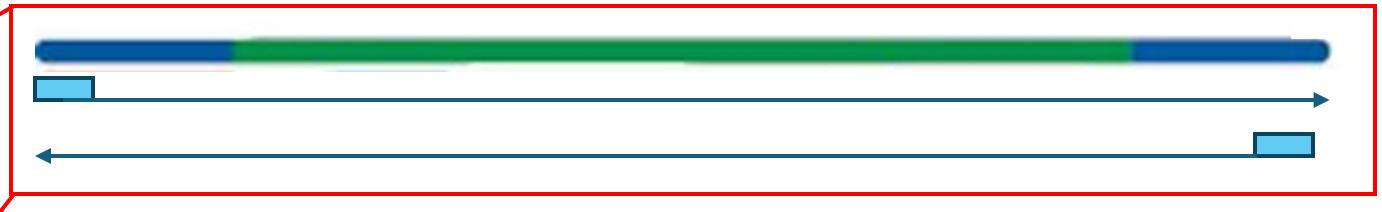
Hybridization

Capture bead binding

Capture bead washes

Post-capture amplification

Bead cleanup



KAPA HyperCap Workflow v3.0 Roche Briefing

What happens during on-bead PCR

The interaction between biotin and streptavidin is very strong

➢needs high temperatures for efficient elution

Your library is now very pure (HCV fragments) but corresponds to very low amounts

➢needs amplification to have enough material to load in the sequencer

Solution: on-bead PCR with universal primers against the universal ends of the library

- **p5** and **p7**

On-bead PCR

- **Amplifying Captured DNA Library Pool using LM-PCR**
- **Step 1. Set post-capture PCR**
- Prepare the LM-PCR Master Mix
- Add 30 µl of Post-Capture PCR Master Mix to a 0.2 mL tube or well of a PCR plate.
- Transfer 20 µl of the bead-bound DNA as template into the tube with the 30 µl Post-Capture PCR Master Mix
- Mix thoroughly by pipetting up and down several times ensure a homogenous mixture of beads.
- Perform the Post-Capture PCR Amplification

*cycle number can be adjusted according to the capture target size

Lid colour	Aliquot Label	Component	Volume per reaction
Green	HiFi	KAPA HiFi HotStart Ready-mix (2X)	25 µl
Blue	LM-PM	Post-LM PCR Oligos (5 µM)*	5 µl
		Total	30 µl

Step	Temp.	Duration	Cycles
Initial denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	11*
Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Final extension	72°C	1 min	1
HOLD	4°C	∞	1

Lunch Break!

PCR clean up and QC

Step 2. Library amplification clean-up

- Finally, clean up the library with the HyperPure beads:
  - **Alert 1-** Don't forget you still have the capture beads there! So, first of all, you need to remove them: place the PCR rx in a magnet, and once the supernatant is clear you can transfer it to a new tube
  - **Alert 2-** this supernatant is the product of 2 days of hard work, don't discard it by mistake ☺
  - Add 50ul of HyperPure beads to the 50ul of PCR reaction (top up with water if necessary)

- The rest you know, QC and load the sequencer as yesterday ☺

Day 2: Targeted enrichment

Preparing probe-capture libraries

- ✓ Sample extraction
- ✓ Library preparation
- ✓ Quality control and pooling of NGS libraries for capture-based target enrichment
- ✓ Probe capture-based target enrichment
- ✓ Quantification and dilution of pooled libraries (Qubit and TapeStation)
- ✓ Miseq run setup
- ✓ MiSeq sequencing metrics

Next steps

Friday you will analyze the results as part of your group projects:

- ❑ Metagenomics you will be analysing your own libraries that you prepared yourselves!
- ❑ Target enrichment you will be using data from a previous run of the same extracts for analysis

Goodbye from the Illumina wet-lab team!