UAB Molecular Genetic Testing

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Learning Objectives

- Describe the current situation around SARS CoV-2 mutation emergence and why it is important to monitor emerging variants
- Discuss how the University of Alabama implemented a next generation sequencing workflow to sequence samples to monitor the spread of variants across the state of Alabama
- Evaluate how NGS can be used to successfully perform variant analysis
- Discuss how the rapid turnaround time of NGS is making an impact compared to previous more lengthy turnaround times

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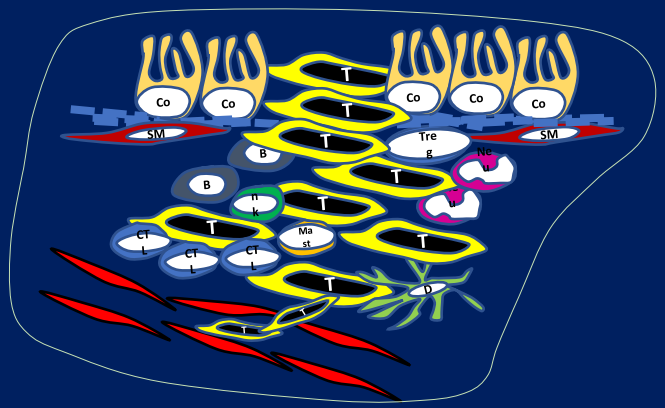
- Speaker was provided honorarium by Thermo Fisher Scientific for providing this presentation.

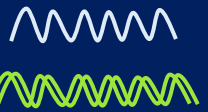
Outline

1. Overview of NGS Targeted Sequencing Workflow
2. Describe UAB evaluation of Genexus NGS platform for research
3. Review performance of Genexus

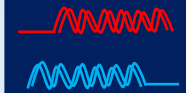


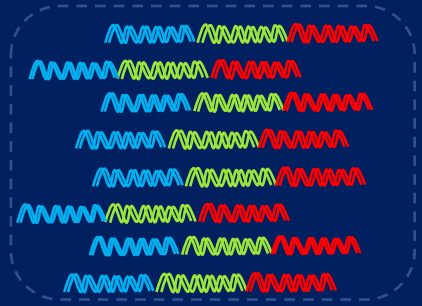
FFPE

RNA & DNA



NGS Library



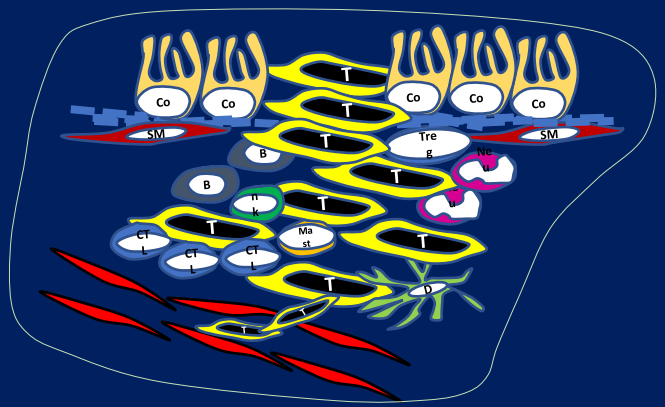


Sequence

Informatics

analysis

Identify & interpret variants



FFPE

RNA & DNA

NGS Library

1. pre-analytical

2. “wet lab”

3. “dry lab”

Informatics

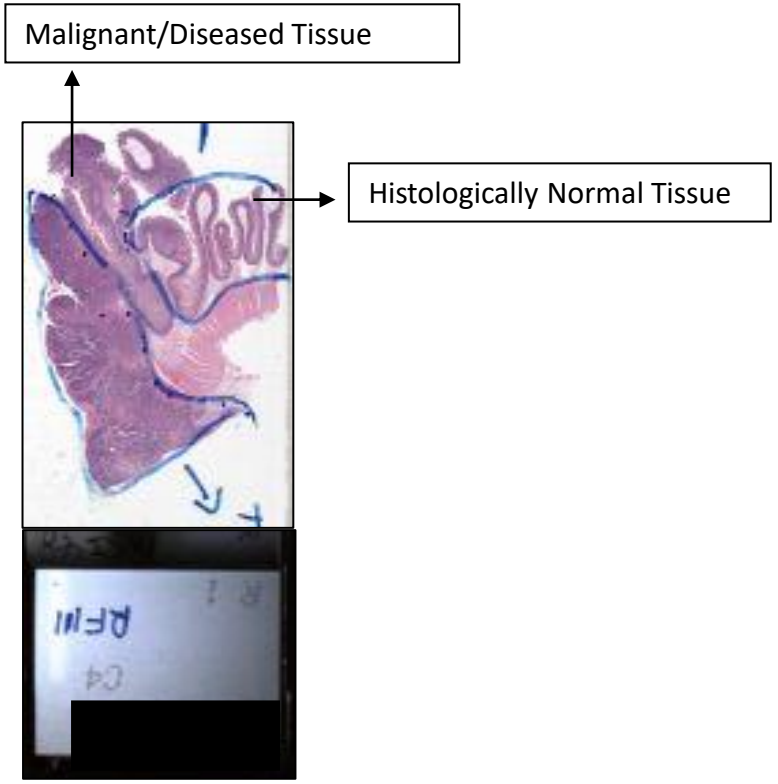
analysis

Sequence

Identify & interpret variants

Pre-Analytical Workflow

Considerations	
Source	***Fresh, FFPE***
Analyte	***DNA, RNA***
Target	***Malignant, Normal***
Yields	Quality, Quantity
Staffing	Pathologist, Technicians



Pre-Analytical Workflow

Considerations	
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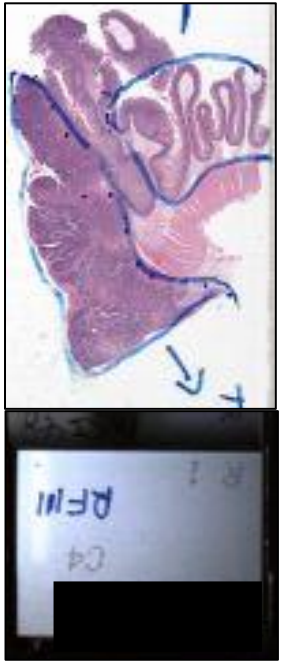
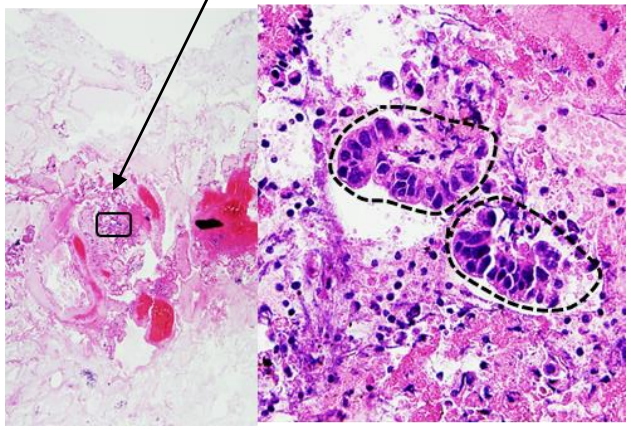


Miniscule Tissue Samples

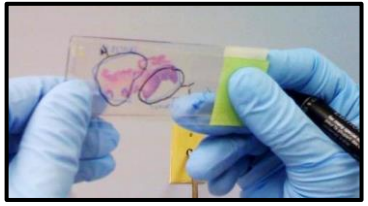
Low quality & quantity (1-10ng)



Wet Lab Analytical Phase

Several day process

DNA Prep | **Library Prep** | **Sequence**




Staffing ***Reagents*** ***Equipment*** ***Servers***

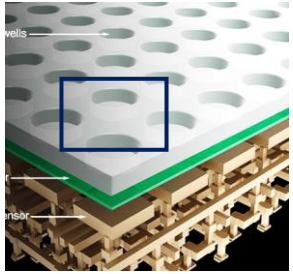
CONSIDERATIONS

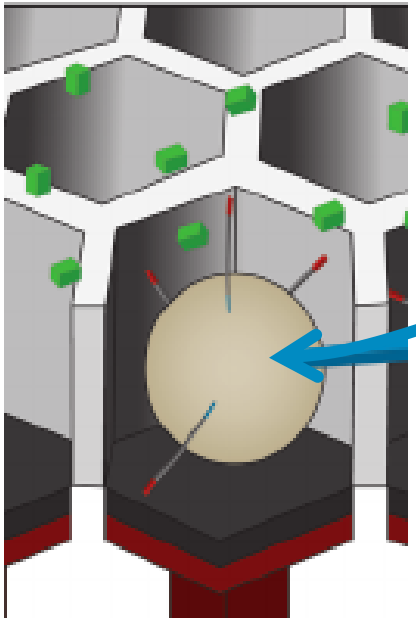
- Specialized Technologist
- DNA/RNA prep bottleneck
- Reagent Agreements
- Maintenance Contracts
- Specialized LIMS/Tracking
- IT and Data Storage

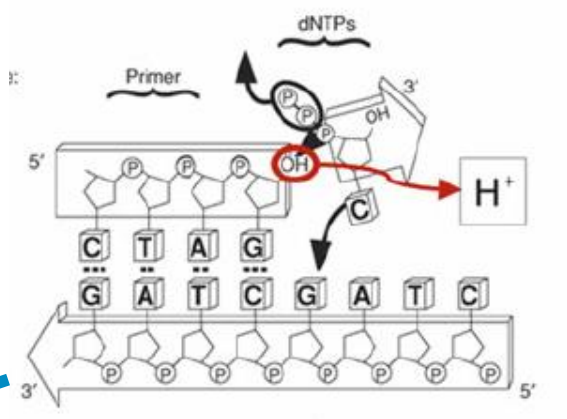
ION TORRENT SEQUENCING: *BASIC IDEA*

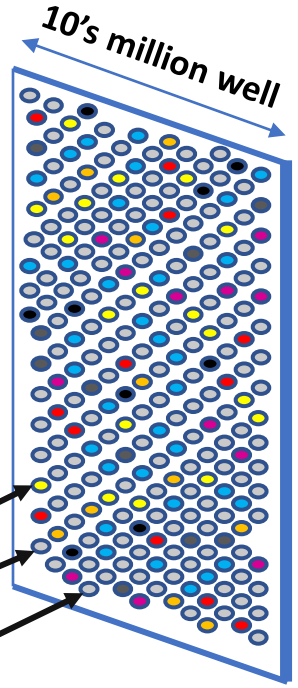
- Parallel Sequencing of Individual DNA Molecules in Isolated Reactions (Wells)
- Produce Massive Number of Unique Sequences (Reads)

Example of Ion Torrent NGS System





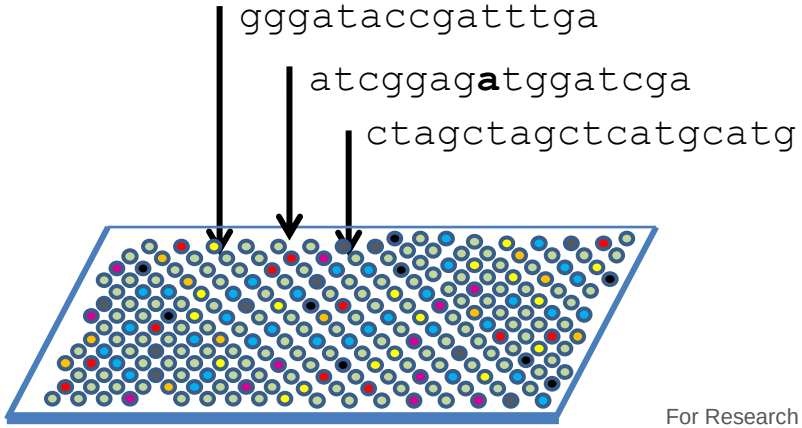




Individual Reads ~100 bp

```
gggataccgatttga
atcggagctggatcga
ctagctagctcatgcatg
```

DATA ANALYSIS: IDENTIFY VARIANTS IN THE SEQUENCES



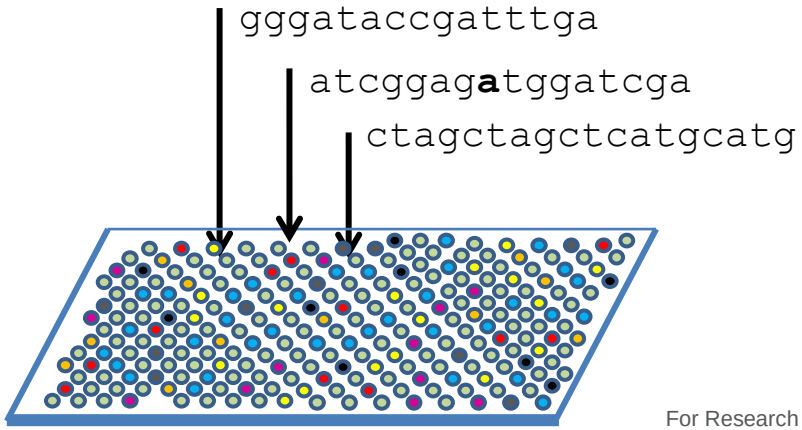
Reads generated by the sequencer

DATA ANALYSIS: IDENTIFY VARIANTS IN THE SEQUENCES

REFERENCE SEQUENCE ("GOLD STANDARD")

Alignment

gggataccgatttgacat tactaatcggagCtggatcgatac ctactagctagctcatgcatg



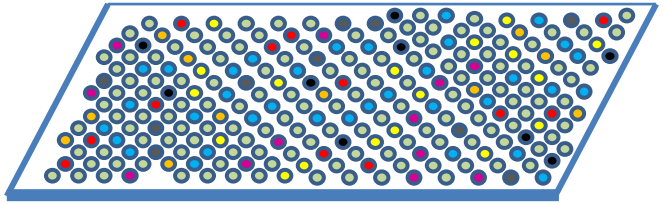
DATA ANALYSIS: IDENTIFY VARIANTS IN THE SEQUENCES

REFERENCE SEQUENCE ("GOLD STANDARD")

Alignment

gggataccgatttgacat **tactaatcggagCtggatcgatac** **ctactagctagctcatgcatg**

gggataccgatttga atcggag**a**tggatcga ctagctagctcatgcatg



DATA ANALYSIS: IDENTIFY VARIANTS IN THE SEQUENCES

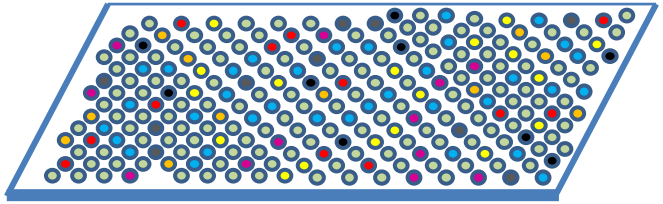
Alignment

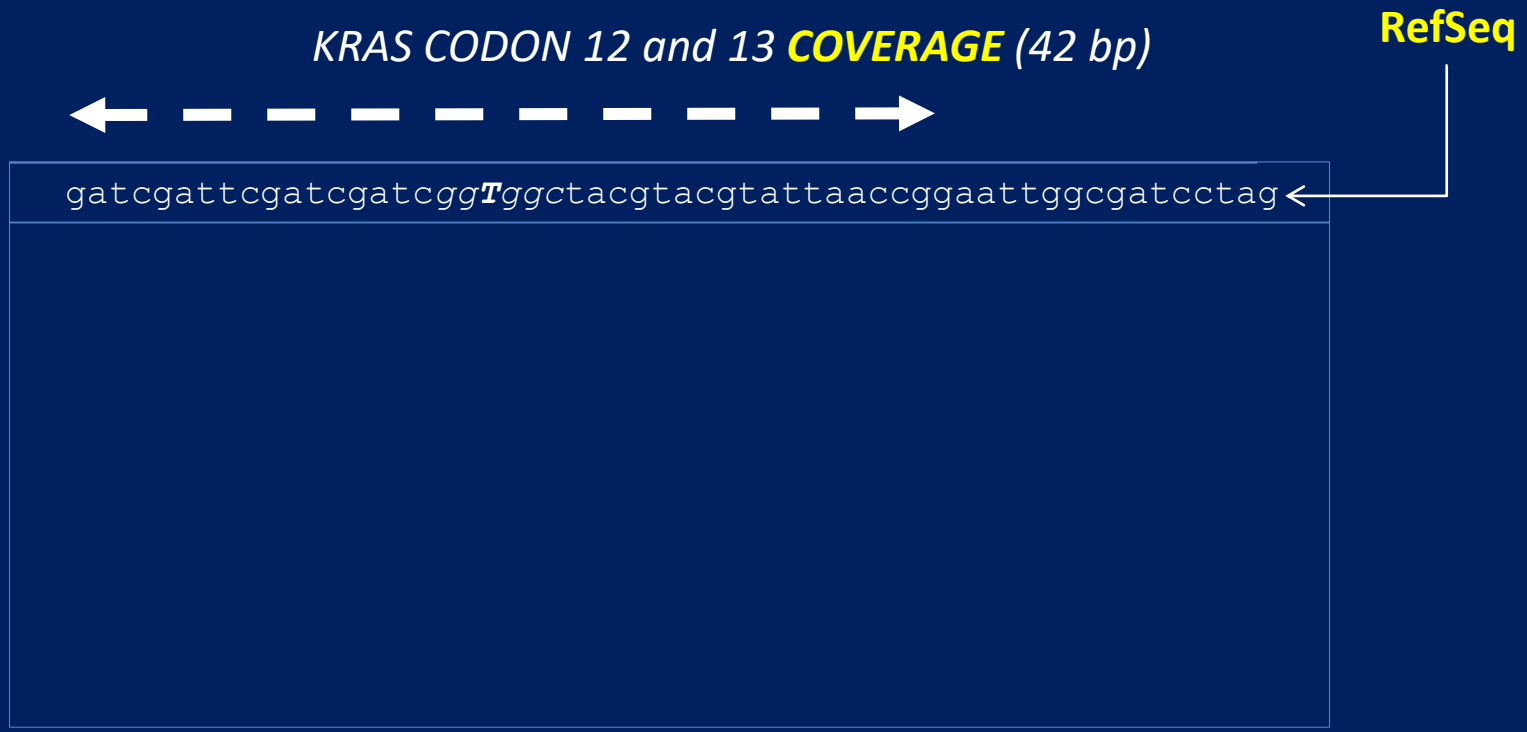
Variant calling

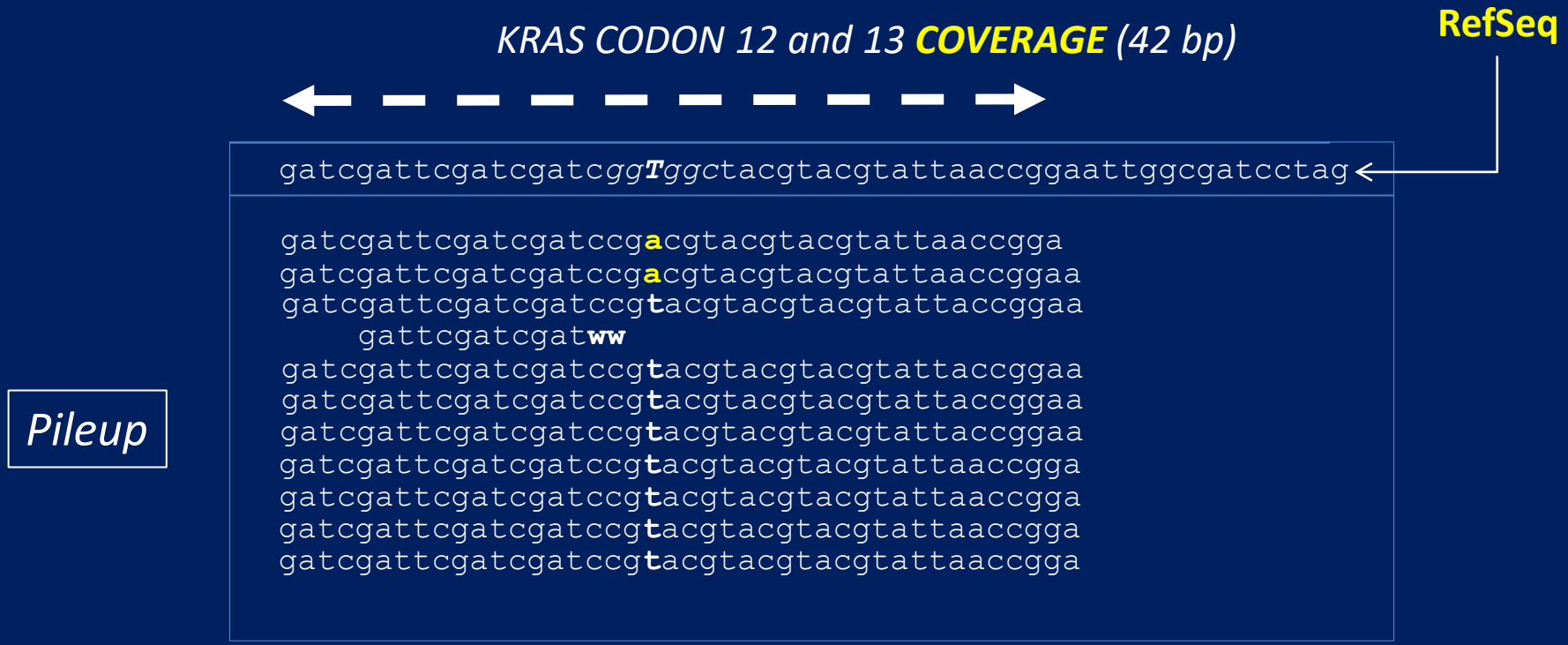
REFERENCE SEQUENCE ("GOLD STANDARD")

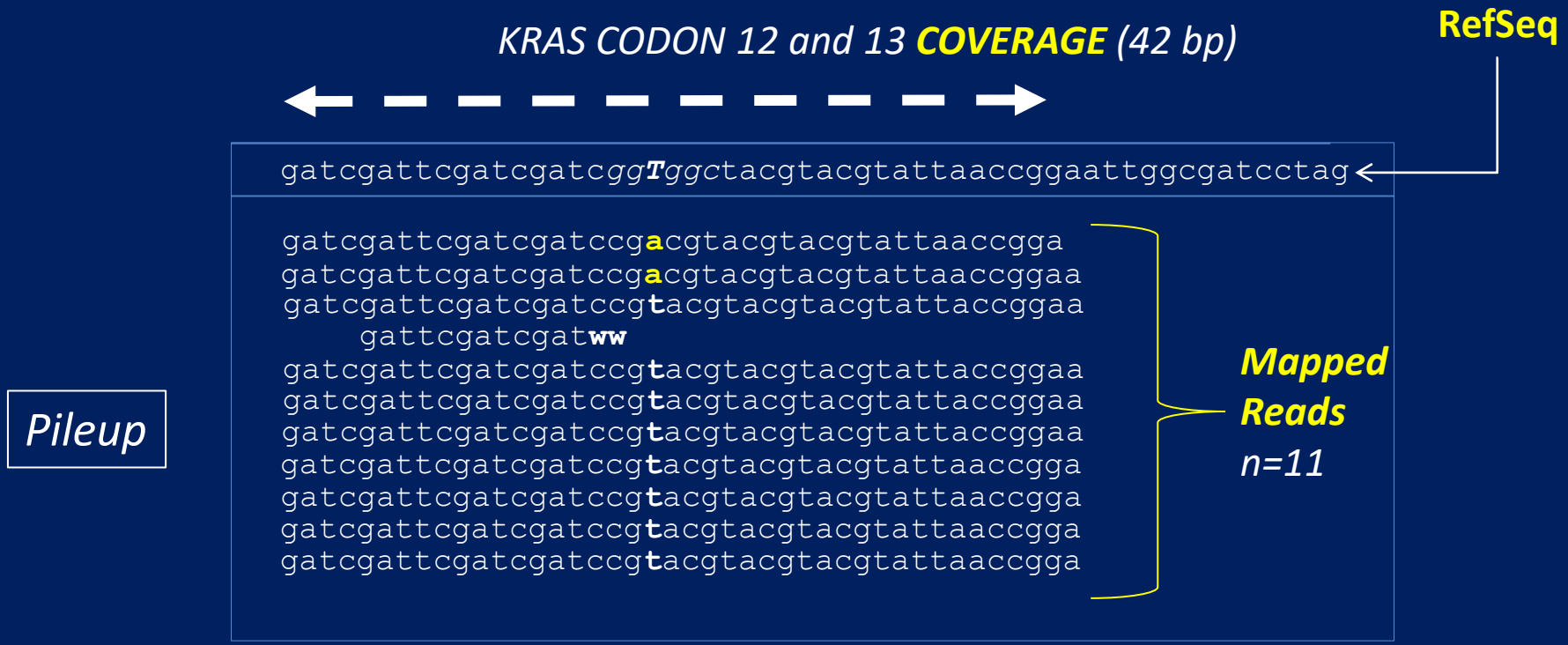
```
gggataccgatttgacat   tactaatcggagCtggatcgatac   ctactagctagctcatgcatg
gggataccgatttga        atcggagatggatcga          ctagctagctcatgcatg
```

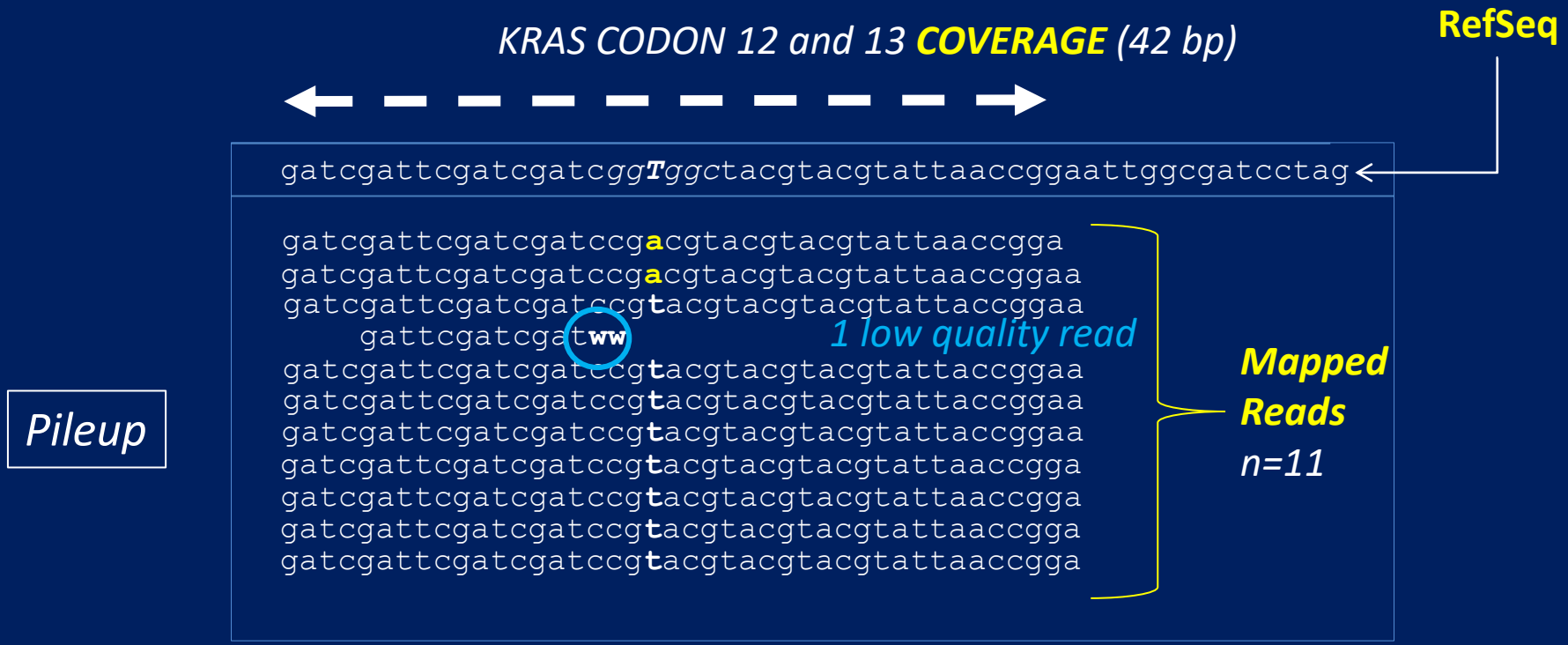
C>A (SNV) (***HOTSPOT VARIANT***)

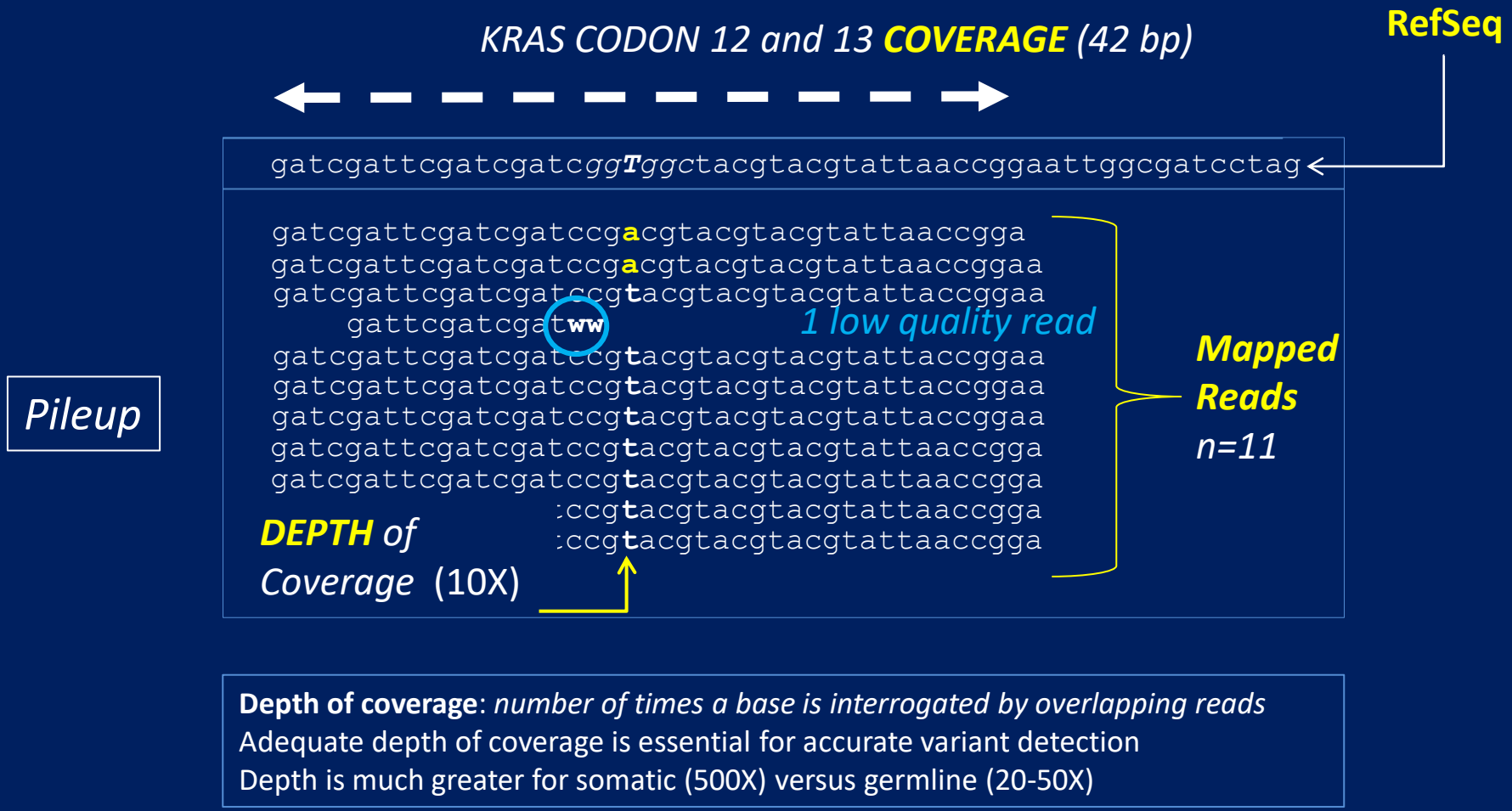




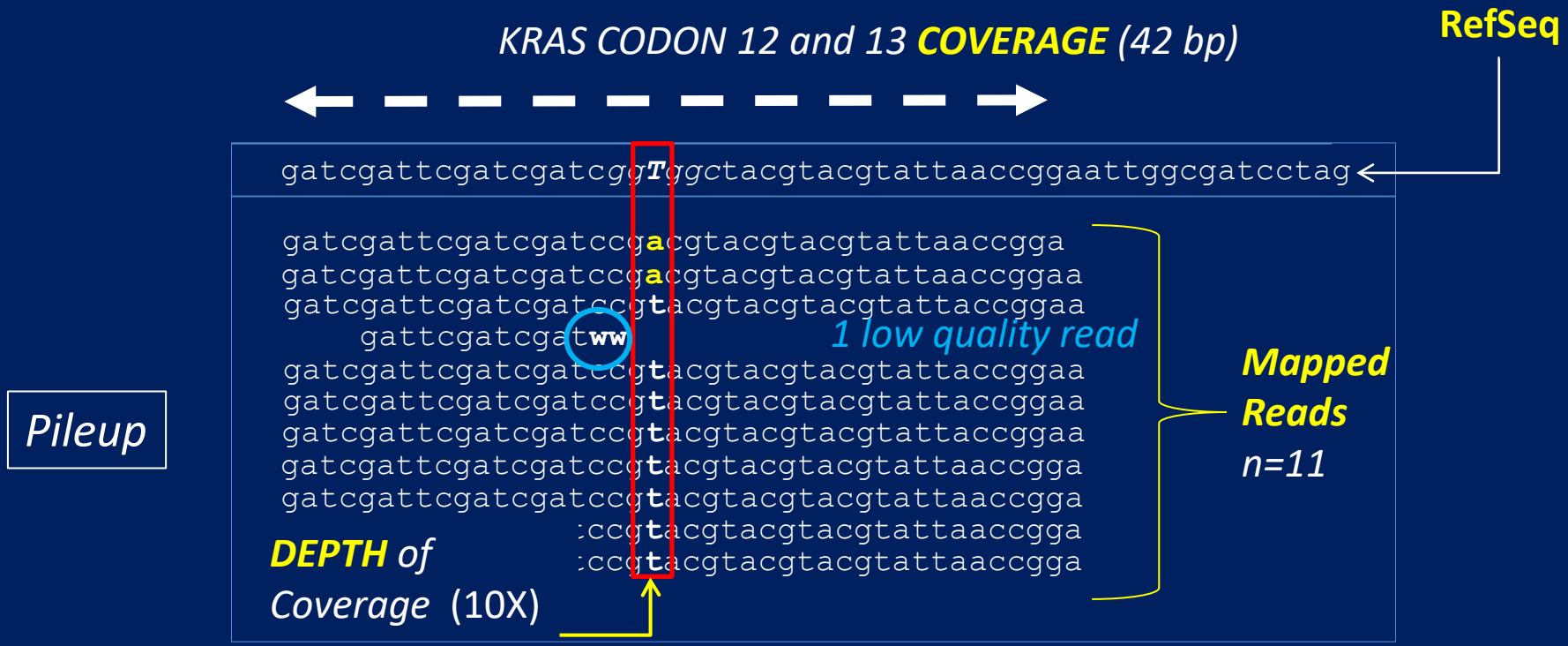


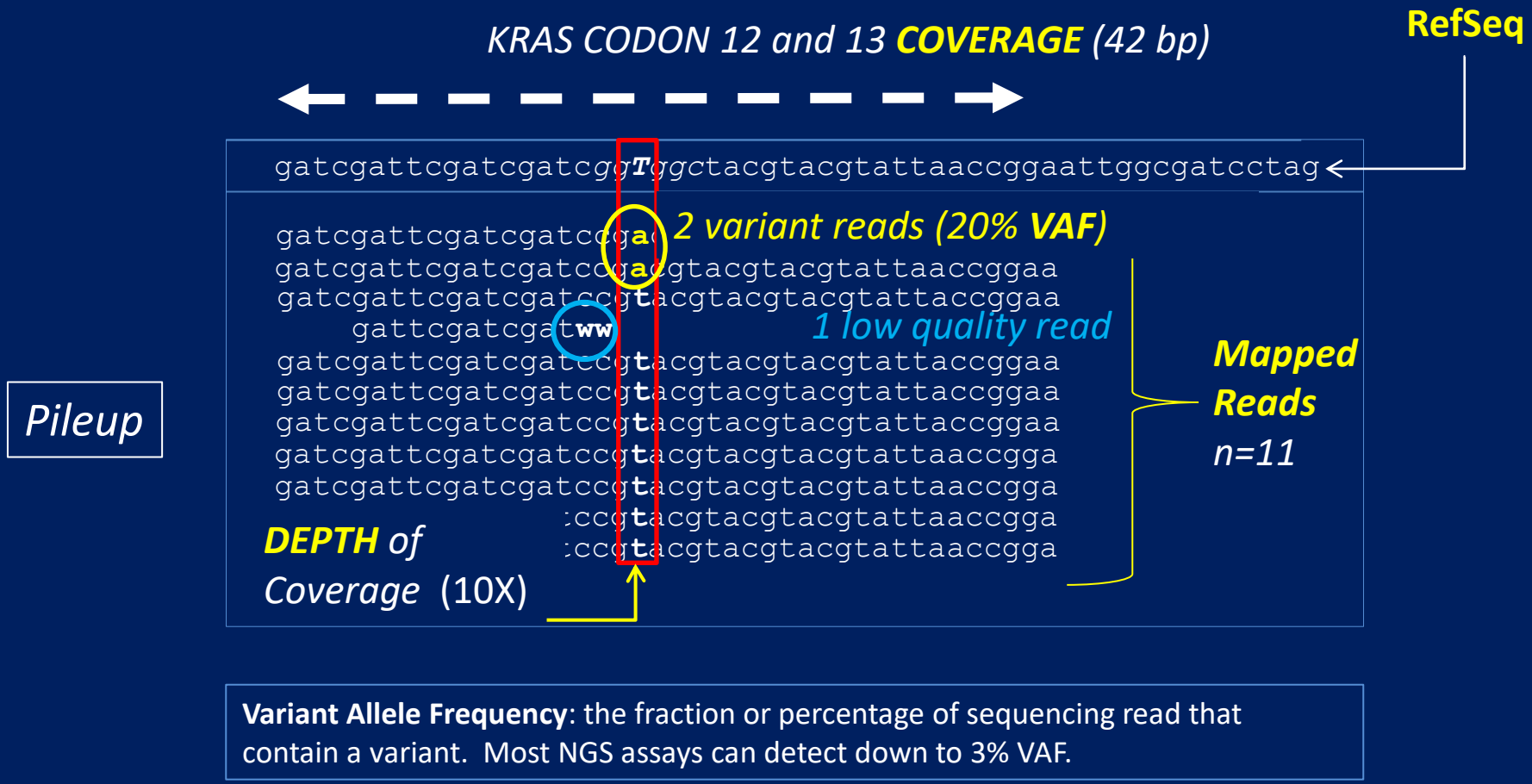






Depth of coverage: *number of times a base is interrogated by overlapping reads*
Adequate depth of coverage is essential for accurate variant detection
Depth is much greater for somatic (500X) versus germline (20-50X)





Variant Allele Frequency: the fraction or percentage of sequencing read that contain a variant. Most NGS assays can detect down to 3% VAF.

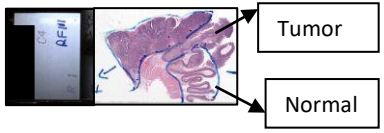
Molecular Diagnostic Workflow

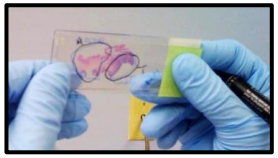
<u>Large investment</u> of time, personnel, and resources for NGS molecular analysis

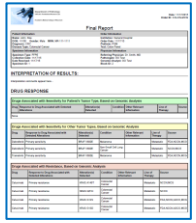
Several day process

Pre-analytical → Wet Lab Analytical Process → Post-analytical

DNA Prep | **Library Prep** | **Sequence** | **Pipeline** | **Variants** | **Review** | **Interpretation**







Surgical Pathologist
Molecular Pathologist
Molecular Technologists

Reagents

Equipment

Computers

Knowledge Databases

Expert Staffing

Reagent Agreements and Maintenance Contracts

IT and Informatics Support Data Storage and Backup

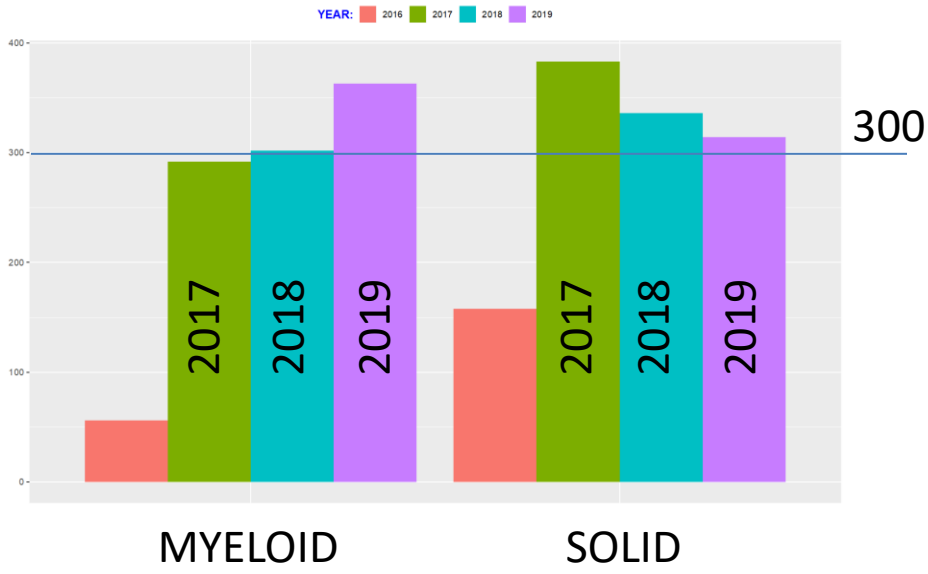
2. Describe UAB evaluation of Genexus NGS platform

Considerations for Changing Platforms

- Turnaround times
- Operating costs
- Content and coverage of panels
- Specimen requirements (quality and quantity)
- Performance

UAB HaloPlex HS NGS ASSAY for Research (2016-2021)

Genes (98)
Molecular Barcoding
myeloid (50) and solid tumors (48)
DNA: Bone marrow, blood and FFPE samples



- **50- 100ng DNA input**
- **Poor performance with small & low quality samples (QNS)**
- **Complex workflow**
- **Long turn around time (TAT)**
- **Expensive reagents ($)**

Not Optimized for Detection
- **Large indels (FLT3>100bp)**
- **CNV**
- **SV (Fusions, etc)**

Ion Torrent Genexus NGS System

- Cost savings (labor and reagent costs)
- 2 day turnaround
- FFPE tissue and liquid biopsy compatible
- SNV, Indel, CNV, Fusions detection
- Low input 10 ng of DNA/RNA required
- Little hands on time

Oncomine Precision Assay

- OPA covers 14,145 bp (9,828 exonic bp)
- Hotspot bed file covers 6,612 bp
- 46.74% of the whole panel, and 67.27% of the whole exonic panel)

IonTorrent Genexus: OPA DNA & RNA 664 samples/year (2016-2019)

Per case	Current	Genexus	Savings per year (664 annual samples)
Tech time (minutes)	149	10	**92,296 Minutes**
Reagents ($)	892.00	550.00	**$227,088.00**
Targets, DNA (n=)	92	50	
Targets, RNA (n=)	0	19	**New Test**
Targets, CNV (n=)	0	14	**New Test**
TAT (days)	7 – 21	2-3	**5-19 days faster**
QNS Samples	50ng-100ng	10ng	**Reduce waste**

Savings per year

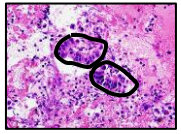
1,538 hours of hands on tech time

$227,088.00

Additional Gains

New assays (*replace FISH tests $$$)*

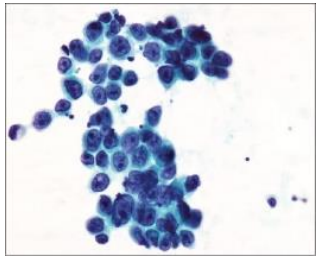
2-3 day TAT (attractive to users/clients)



Test more samples

Real Life Example

Testing archival research samples



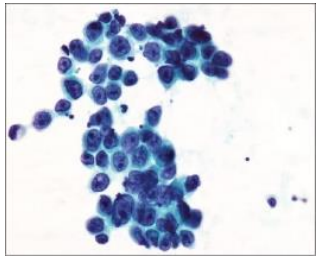
Pain points

- Long turnaround times
- Small samples fail testing
- Expensive reagents and labor intensive

	1	2	3	4	5	6
			QNS 10-15%			
HaloPlex NGS	DNA/RNA Prep	DNA/RNA Quant and QC	Library Prep	**Library Prep**	Sequencing	Informatics (VCF)

Real Life Example

Testing archival research samples



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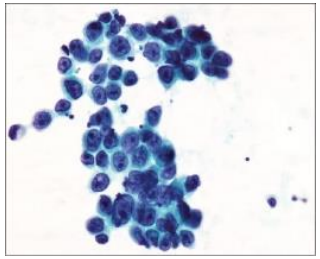
	1	2	3	4	5	6
				QNS 10-15%		
HaloPlex NGS	DNA/RNA Prep	DNA/RNA Quant and QC	Library Prep	**Library Prep**	Sequencing	Informatics (VCF)
Genexus	DNA Prep	RNA Prep	Library Prep & Sequencing	Informatics (VCF)		

Real Life Example

Testing archival research samples



Pain points

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	1	2	3	4	5	6
HaloPlex NGS	DNA/RNA Prep	DNA/RNA Quant and QC	Library Prep	**Library Prep**	Sequencing	Informatics (VCF)
Genexus	DNA Prep	RNA Prep	Library Prep & Sequencing	Informatics (VCF)		
Genexus	DNA & RNA Prep	Library Prep & Sequencing	Informatics (VCF)			

QNS 10-15%

Purification System & Genexus

Review performance of Genexus

Evaluation

OPA DNA/RNA: Detection of CNV, Indels, SNV, Fusion

Performance with Low Input Samples (“QNS”)

Pipeline

Comparing Genexus with Orthogonal Assay

Run (n=10)	DNA samples	RNA samples	Controls Samples
20210608	6		1
20210609	7		
20210621	13	13	2
20210704		4	2
20210707	5	5	2
20210711	5	5	2
20210723	6	6	1
20210725	12	12	3
20210820	15	0	0
20210920	17	0	6
SUM	**86**	**45**	**19**

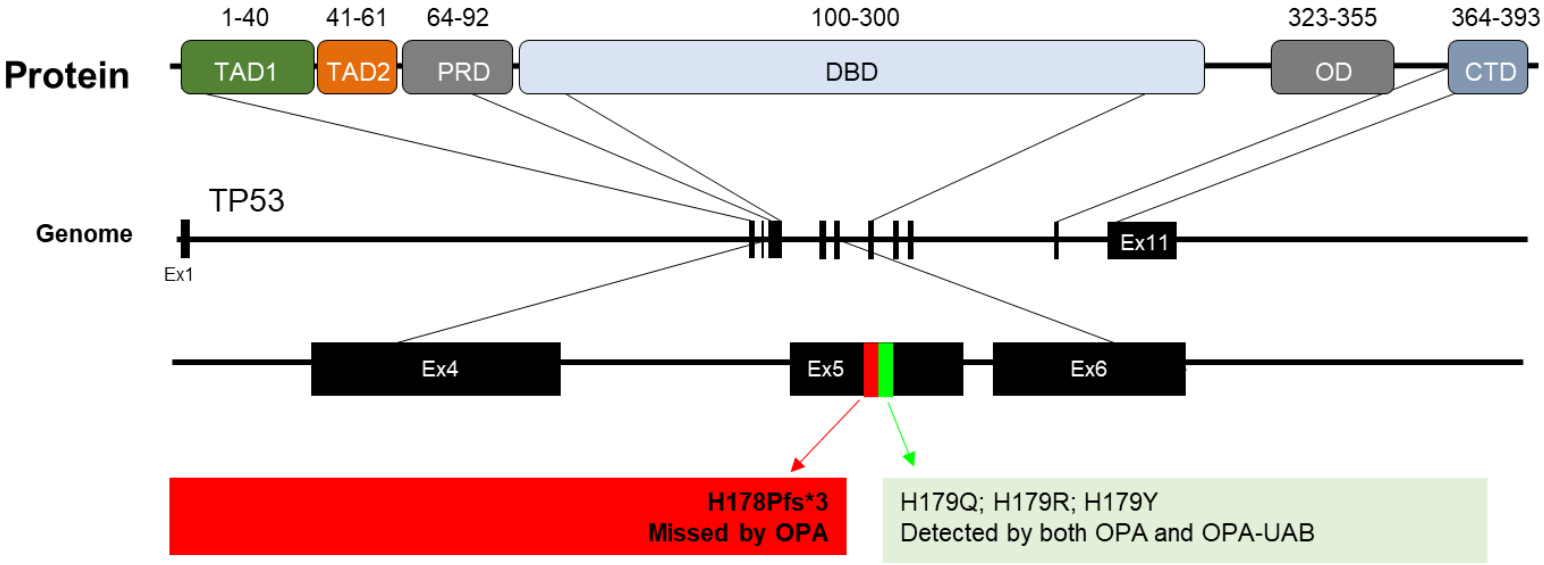
Total Samples	**150**		
Total Known Variants	**180**	**131 DNA/RNA Variants**	**49 CNV**
Concordance	**100%**		

UAB HaloPlex Low Input ("QNS") Samples

Sample	Orthogonal results	Genexus SNV/indels	CNV
1	TP53 R282G	*TP53 R282G (0.575)*	
2	TP53 R280T	*TP53 R280T (0.432)*	
3	KRAS G12V (cfDNA)	*KRAS G12V (0.139)*	
4	EGFR G719A, MDM2 Amp	*EGFR G719A (0.088)*, ROS1 L195M (0.052), CTNNB1 S37C (0.032), PTEN A126S (0.031), TP53 R273S (0.031) P151T (0.032)	
5	**EGFR E746_A750del**, TP53 G245S	*TP53 G245S (0.046)*	
6	MET Amp only	None	CD274 2.84 *MET 2.33*
7	BRAF V600E (Idylla)	BRAF V600E (0.603)	FGFR3 2.17
8	Inconclusive (sample depleted)	KRAS G12A (0.435)	KRAS 2.04
9	No mutation in EGFR KRAS (Sanger)	TP53 R248L (0.41)	MET 2.13
10	No mutation in EGFR KRAS (Sanger)	TP53 R248P (0.39)	
11	QNS	TP53 G154V (0.301)	
12	No mutation in EGFR KRAS (Sanger)	BRAF G469A (0.127), CTNNB1 D32N (0.056)	
13	No mutation in EGFR KRAS (Sanger)	None	
14	QNS	None	

Specimens with Missed Variants

- **Specimen #1 (SNV): TP53 H178Pfs*3** (VAF:0.42, DP:370)
  - TP53 H178Pfs*3 (**17:7578397..7578398**) is a cosmic hotspot **COSM8662124** [(Insertion - Frameshift) c.533dup (Insertion)]
  - OPA hotspot bed file covers adjacent TP53 mutations at positions:**p.H179Q/R/Y**



Specimens with Missed Variants

- **Specimen #2 (CNV)**
  - **BRAF Amplification** (2 additional copies)
  - BRAF is NOT in the list of list of 14 genes with reportable CNVs

Specimens with Missed Variants

- **Specimen #3 (Fusion)**
  - **NCOA4--RET** (Orthogonal test: 7 SS, 13 Reads)

- **Although, RET is part of the list of 18 genes with reportable fusions, the OPA RNA bed file covers only the following four NCOA4--RET fusions:**
  - *NCOA4--RET* chr10:51582218-51582272 -- chr10:43612031-43612058
  - *NCOA4--RET* chr10:51582873-51582939 -- chr10:43612031-43612058
  - *NCOA4--RET* chr10:51586363-51586411 -- chr10:43612031-43612058
  - *NCOA4--RET* chr10:51586363-51586411 -- chr10:43607546-43607607

Modification of Genexus Pipeline

- OPA covers **14,145 bp** (9,828 exonic bp)
- Hotspot bed file covers **6,612 bp**
  - 46.74% of the whole panel
  - 67.27% of the whole exonic panel

- OPA pipeline mainly focuses on current biologically significant targets
- Concerned that pipeline may miss variants of emerging significance
- Modify pipeline to analyze more of the covered regions

Modification to the Variant Calling Pipeline





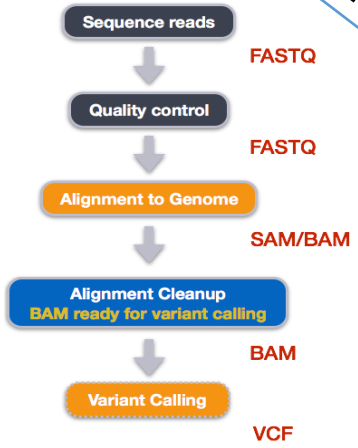
Specimen Accession → **Histo & tissue** → **DNA/RNA extraction** → **Targeted PCR, Library Prep and Genexus**

OPA Pipeline
Ion Reporter

OPA

2,769 variants ready for report without extensive interpretation

OPA-UAB Pipeline



OPA- UAB

All variants in the regions covered by Amplicon
Need variant classification

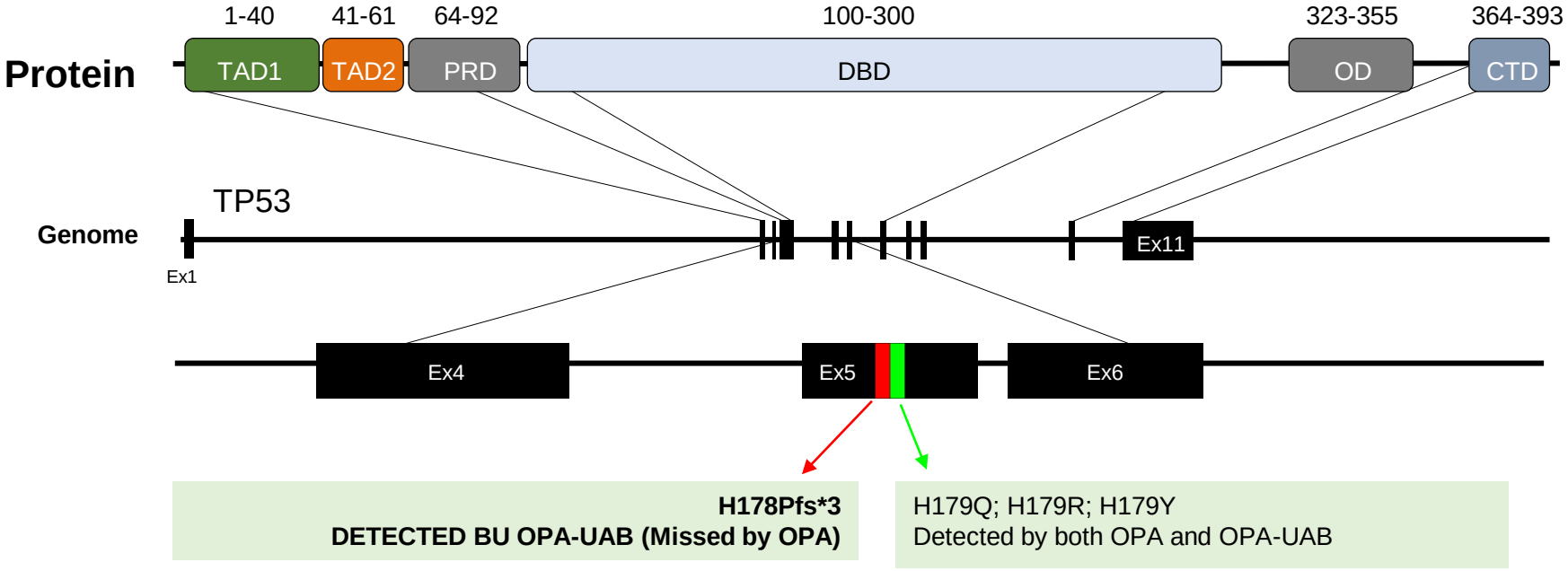
Pros:
- Lab developed, informatics staffing
- Expanded # of variants detected

Cons:
- Requires manual variants classification
- Generate variants of uncertain significance
- Lab developed, informatics staffing required

Yulong Fu, PhD

TP53

- OPA cover **293 mutations**
- Hundreds more in COSMIC and ClinVar;
- Mutations database is constantly expanding
- OPA-UAB can detect all variants, as long as covered by amplicons
- No extra material and wet lab are required compare to OPA



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Summary

- Genexus provides significant improvements in TAT, costs, labor, and sensitivity
- SNV, Indel, CNV, Fusion detection supported in a single, fast workflow with few hands on steps
- Very good performance compared with orthogonal testing
- “Out of the box” pipeline is convenient, fast and reliable for identifying variants in the hotspot bed file
- Option to use alternative pipelines for expanded variant detection