Next Generation Sequencing of Hematologic Neoplasms

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Faculty Disclosures:

Consulting: Bio-Rad Laboratories

Learning Objectives:

1. List the components of a NGS pipeline for testing of hematologic neoplasms

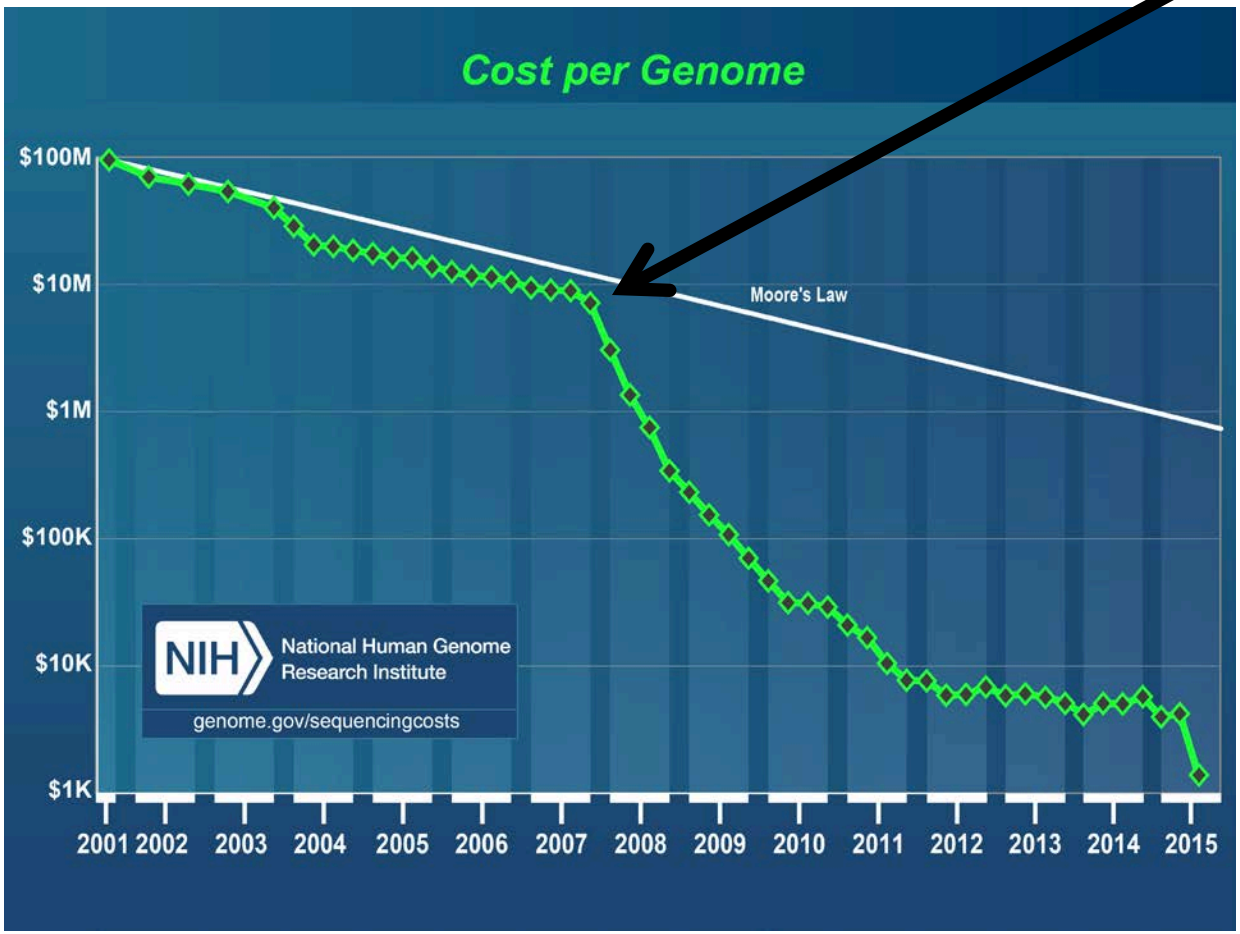
2. Describe the clinical utility of NGS technology in the context of testing of hematologic neoplasms

Outline

- NGS background
- Overview of types of clinical NGS tests
- NGS panels
- Single gene tests
  - Lymphoid clonality testing by NGS
  - *BCR-ABL1* kinase domain sequencing
- Copy number variant (CNV) detection by NGS
- Detection of translocations by NGS

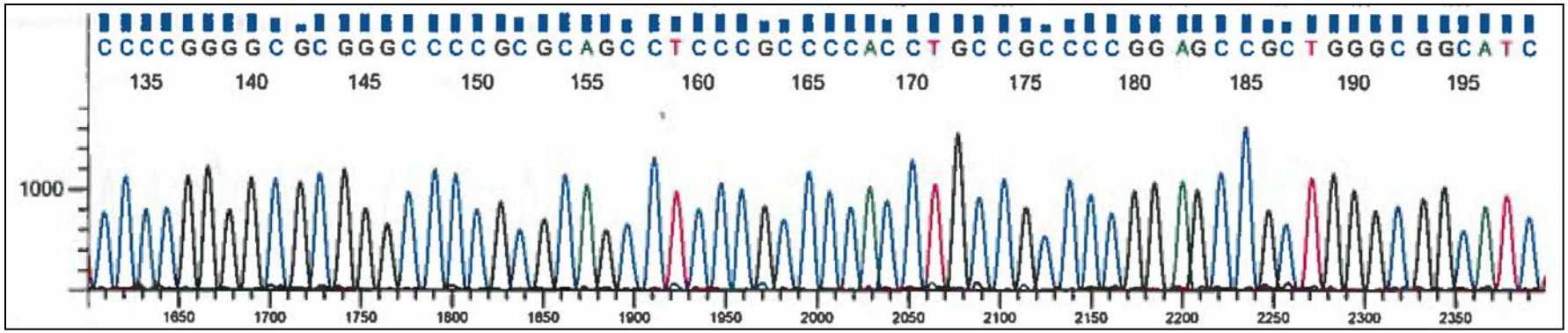
Next Generation Sequencing (NGS)

Impact of NGS



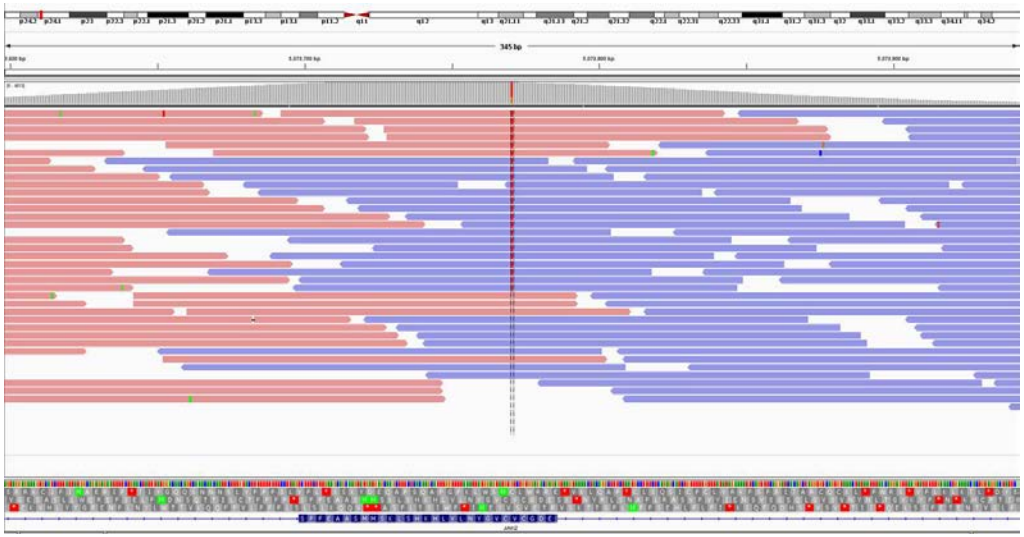
1st generation sequencing - Sanger sequencing

- utilizes chain terminating dideoxynucleotides
- slow and laborious, method has been relatively unchanged for ~30 years
- data = mixture of sequences
- sequence data can be reviewed manually
- poor sensitivity for detection of variants (~15-20%)
- relatively long contiguous sequence can be generated (>600bp)



NGS - also known as massively parallel sequencing

- parallel single molecule sequencing
- millions of small fragments of DNA are immobilized on a solid surface, amplified (copied), and sequenced simultaneously
- during sequencing a signal (light, pH change) is detected when a base is incorporated
- short contiguous sequences (reads) are generated
- reads are aligned to a reference sequence and analyzed
- analysis is computationally intense

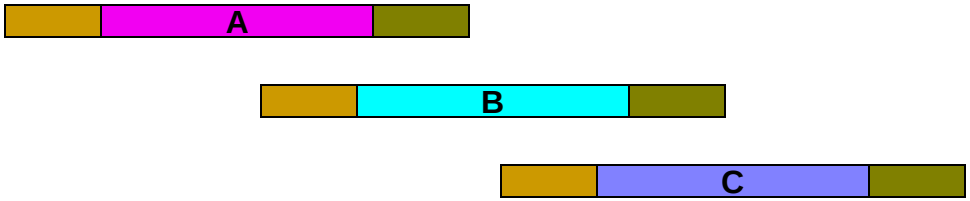


NGS - workflow

1) DNA extraction
2) sequencing library preparation
3) target enrichment by PCR or hybrid capture
4) hybridization of library fragments to a solid surface (i.e. flow cell)
5) clonal amplification of library fragments
6) massively parallel sequencing
7) Generation of fastq files (raw data)

Overview: Library to sequencing….

Library: Multiple DNA fragments + Adaptors



One Fragment

↓

One Cluster

↓

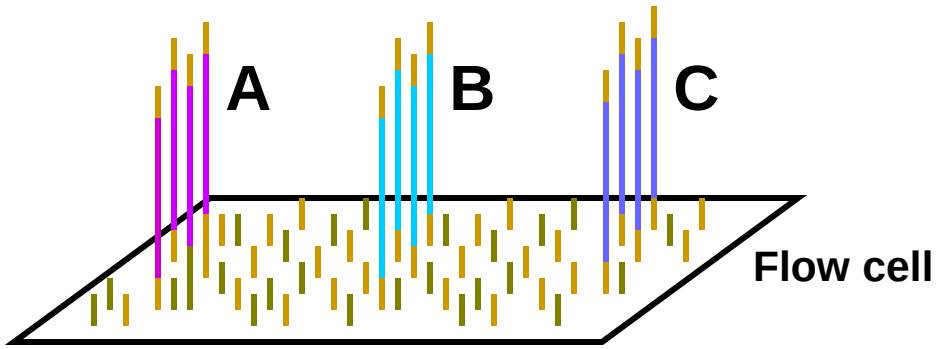
One Read

or

2 Paired End Reads

Hybridization of Library Fragments

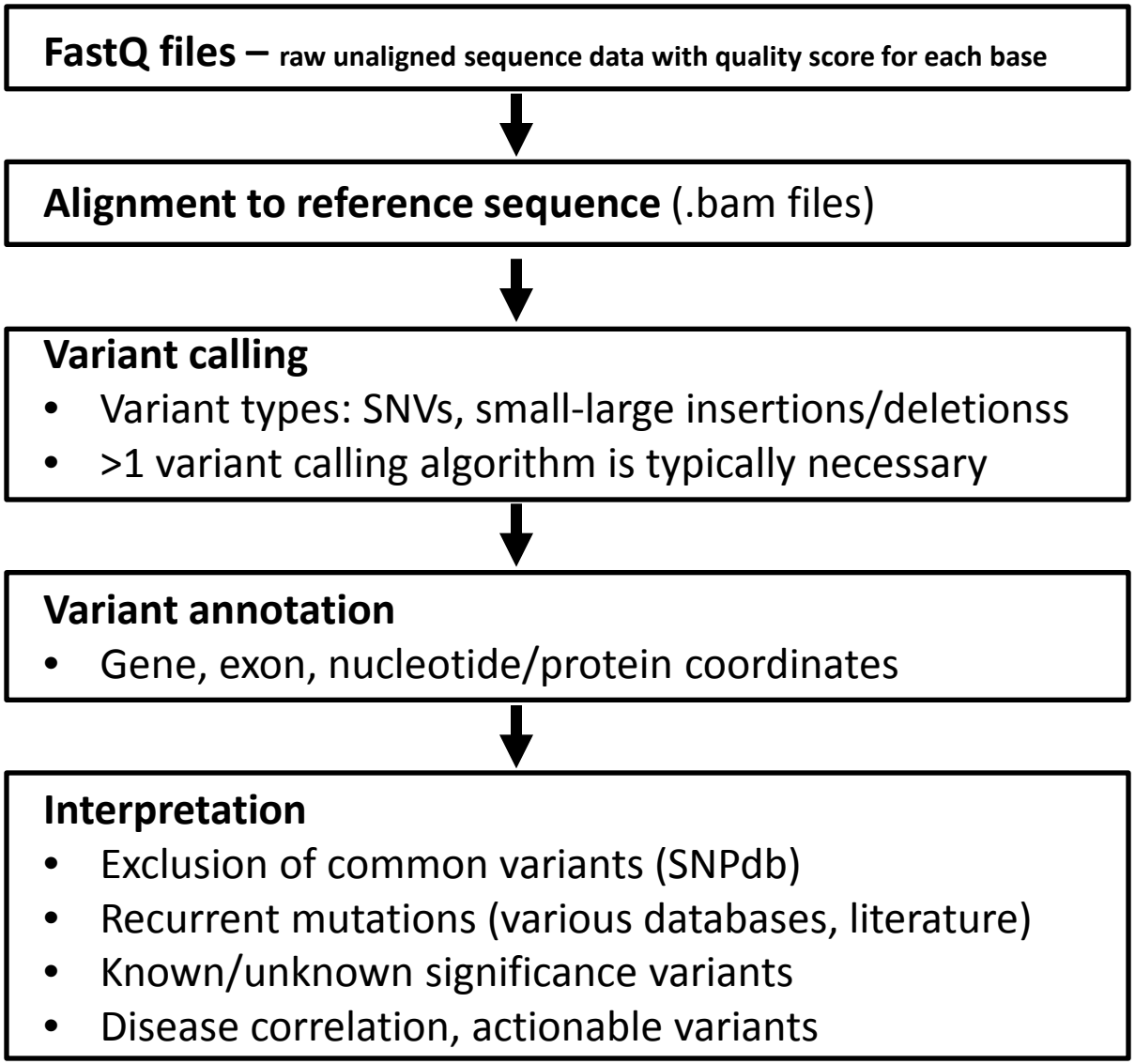
Clonal Amplification of Each Fragment



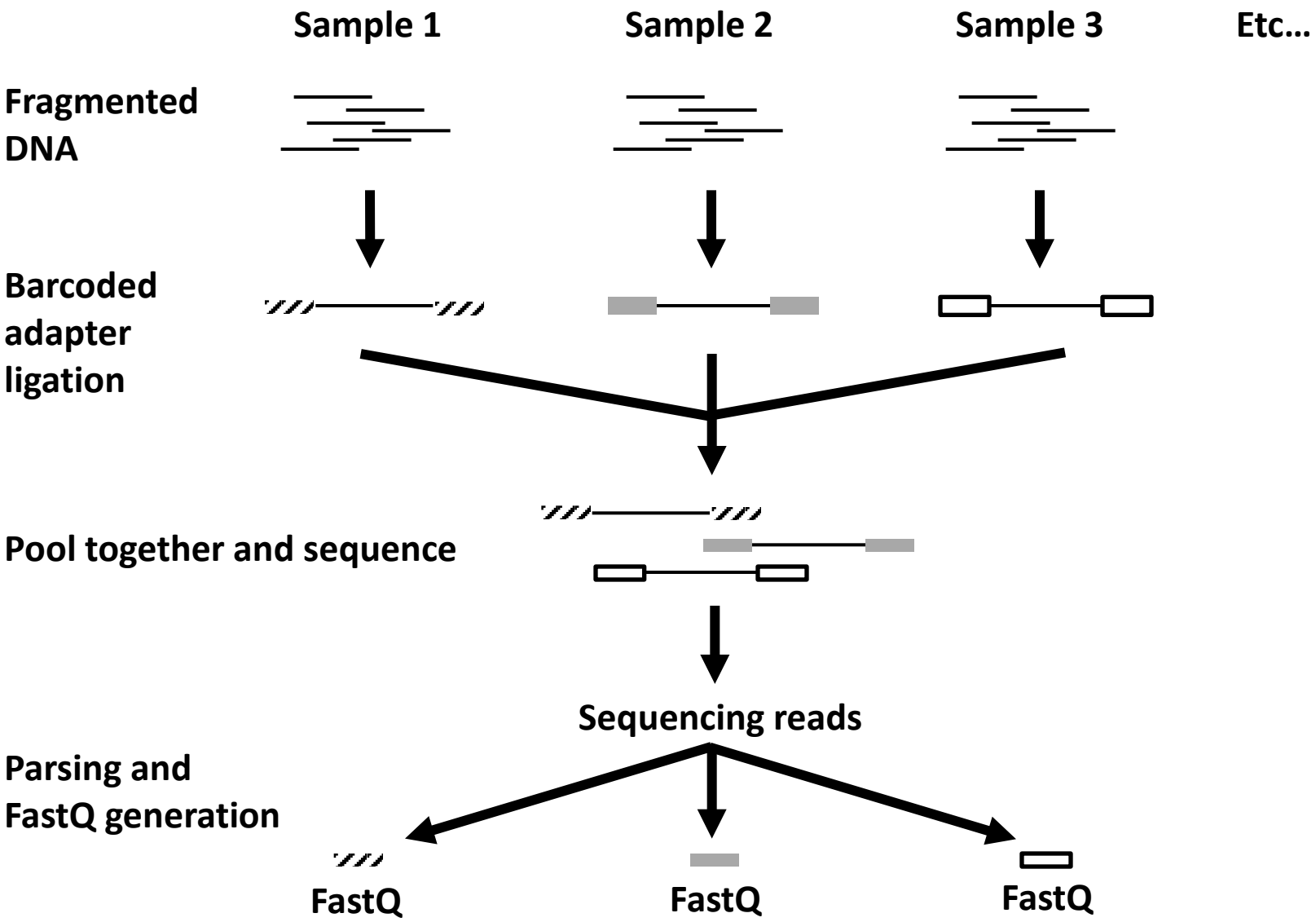
Sequencing of the Clonal Amplicons (paired-ends):

Read A, Read B, Read C

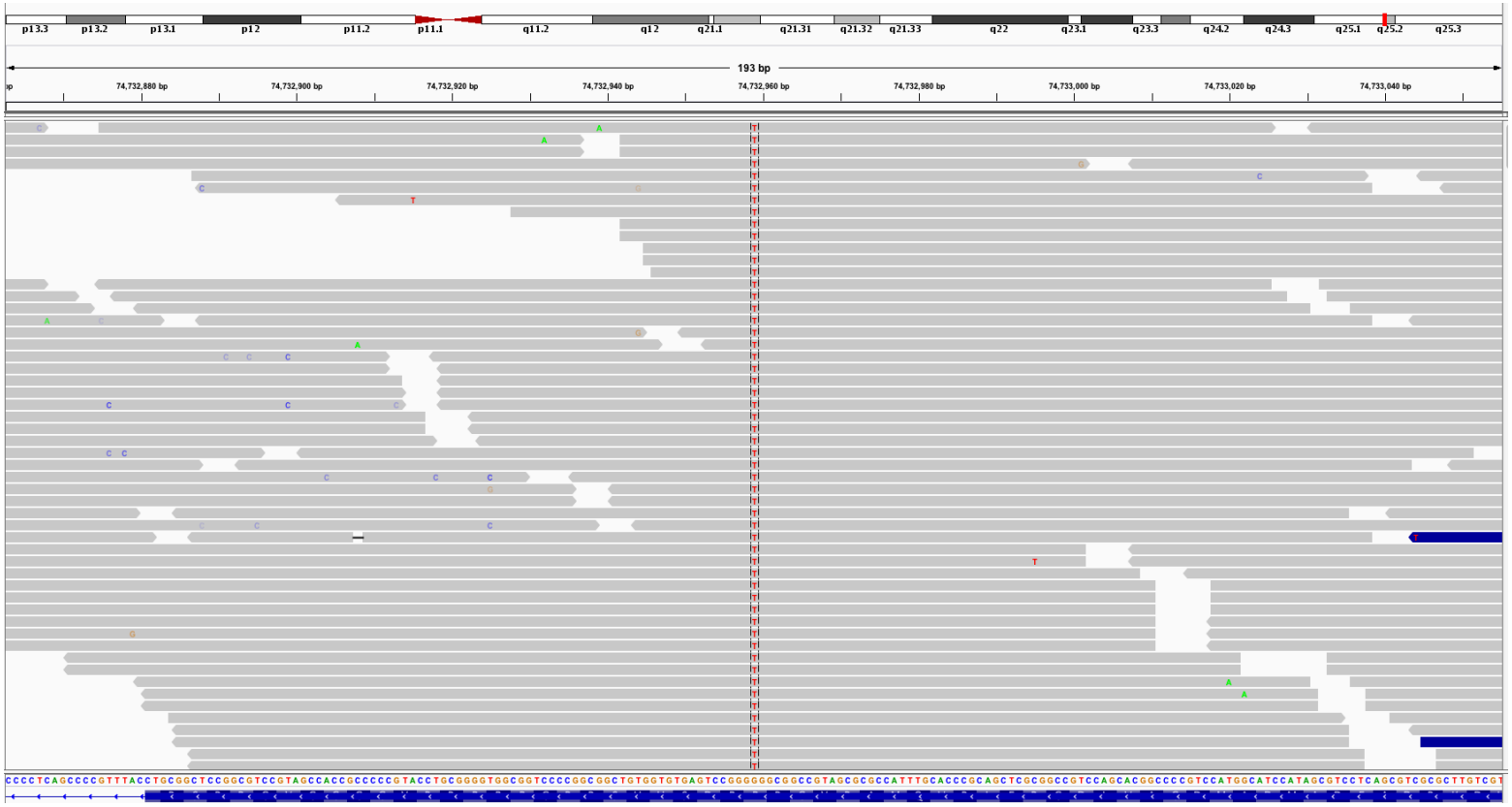
Bioinformatics Workflow



Sample multiplexing for NGS



IGV: Integrative Genomics Viewer

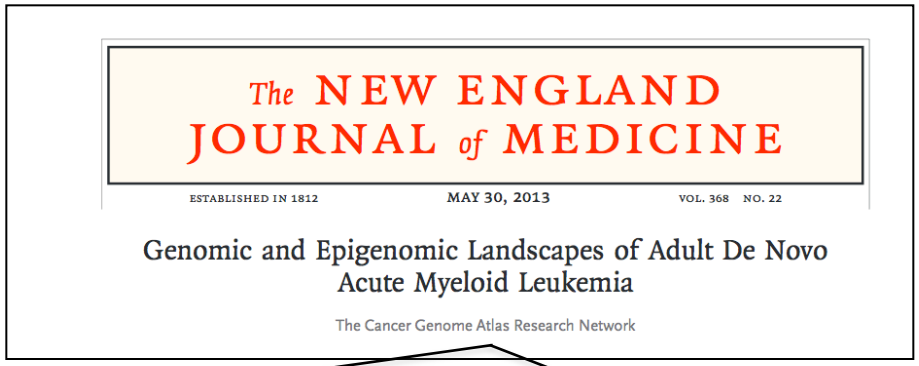


Sequence data is aligned to a reference genome

Comparison of NGS applications

NGS Application	Cost/Time	Sensitivity (depth of coverage)	Portion of genome sequenced (breadth of coverage)	Suitable for MRD detection?
Whole genome sequencing	++++	+	++++	No
Whole exome sequencing	+++	++	+++	No
Mutation panels	++	+++	++	No
Single gene tests	+	++++	+	Yes

The power of NGS



The NEW ENGLAND JOURNAL of MEDICINE

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Genomic and Epigenomic Landscapes of Adult De Novo Acute Myeloid Leukemia

The Cancer Genome Atlas Research Network

-Study performed by the Cancer Genome Atlas Research Network

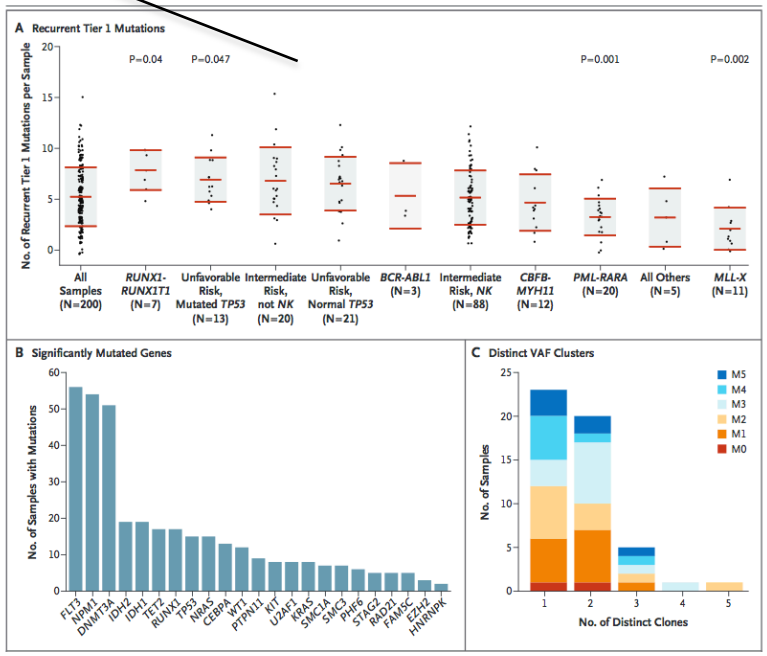
-200 cases of *de novo* adult AML subjected to whole genome (50) or whole exome (15) sequencing

-Tier 1 – coding changes or splice sites

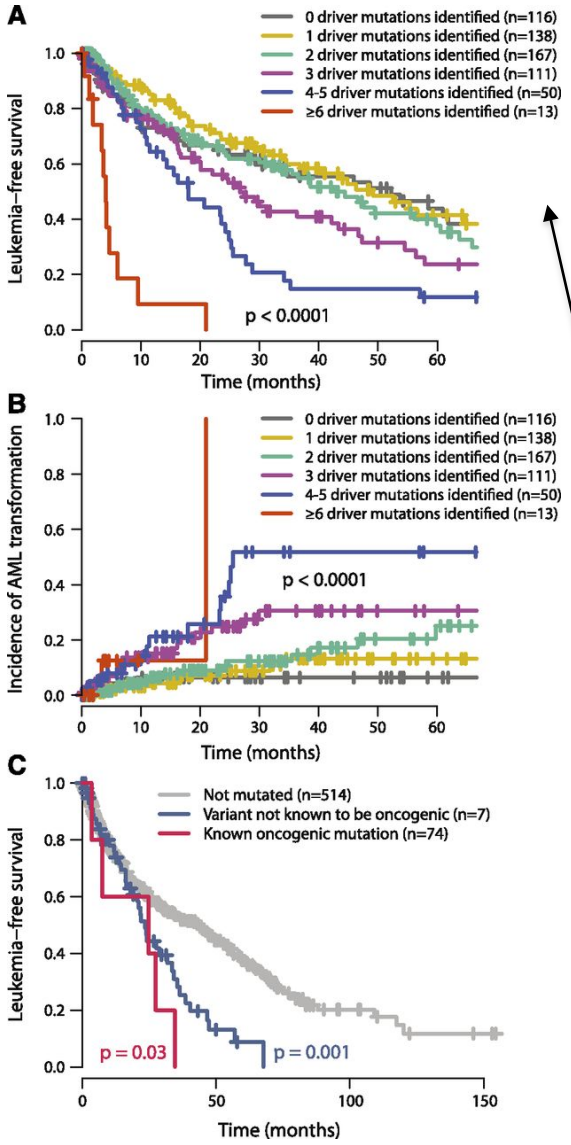
-average of 13 overall (all tiers) mutations per case

-23 genes significantly mutated (>5% of cases)

-majority of cases demonstrated more than 1 clone based on distinct clusters of variant allele frequencies (VAFs)



Clinical impact of somatic mutations



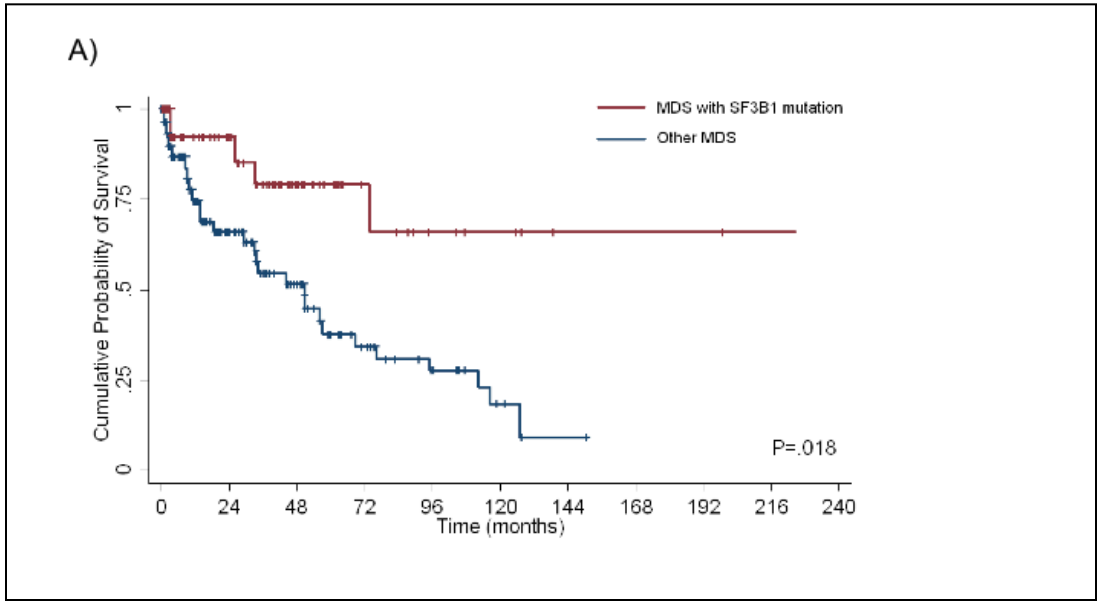
-738 patients with MDS, MDS-MPN
-111 cancer associated genes were sequenced by NGS (gene panel)
-78% of patients had 1 or more oncogenic mutations
-No systematic differences between DNA derived from bone marrow or peripheral blood

Higher overall number of oncogenic mutations correlated with worse outcome

Papaemmanuil E et al. Blood 2013;122:3616-3627

Clinically important information is derived from large scale genetic analysis by NGS: The example of MDS

- *SF3B1* mutations are associated with favorable outcome



Malcovati L et al. Blood 2014;124:1513-1521

308 pts w/ myeloid neoplasms
- MDS: 245
- MDS/MPN: 34
- AML-MDS: 29

111 gene mutation panel

*Almost all patients with RARS (refractory anemia with ring sideroblasts) had an *SF3B1* mutation

Clinical applications of NGS in hematology

- Clinical applications:
  - Whole genome sequencing (entire genome - ~3B base pairs)
  - Whole exome sequencing (~30M base pairs)
    - Sequencing limited to protein coding regions representing ~1% of genome
  - Mutation panels
    - Myeloid
      - AML prognostic markers – *FLT3, NPM1, CEBPA, ASXL1, IDH1/2*
      - Myelodysplastic syndromes (MDS) – cohesin and spliceosome genes frequently mutated
      - Myeloproliferative neoplasms (MPNs) – *JAK2, CALR, MPL, ASXL1*
      - Pan myeloid panels
    - Lymphoid
      - Diffuse large B cell lymphoma (BCR pathway mutations)
      - Mutations associated with T cell lymphoproliferative disorders (JAK-STAT pathway mutations)
      - Pan lymphoid panels
    - Congenital disorders – bone marrow failure syndromes, congenital hemolytic anemias
  - Detection of complex genomic abnormalities - copy number variants (CNVs) and translocations
  - Analysis of single genes with high complexity
    - Ex. lymphoid clonality and *IGH* or *TRG*/*TRB* genes

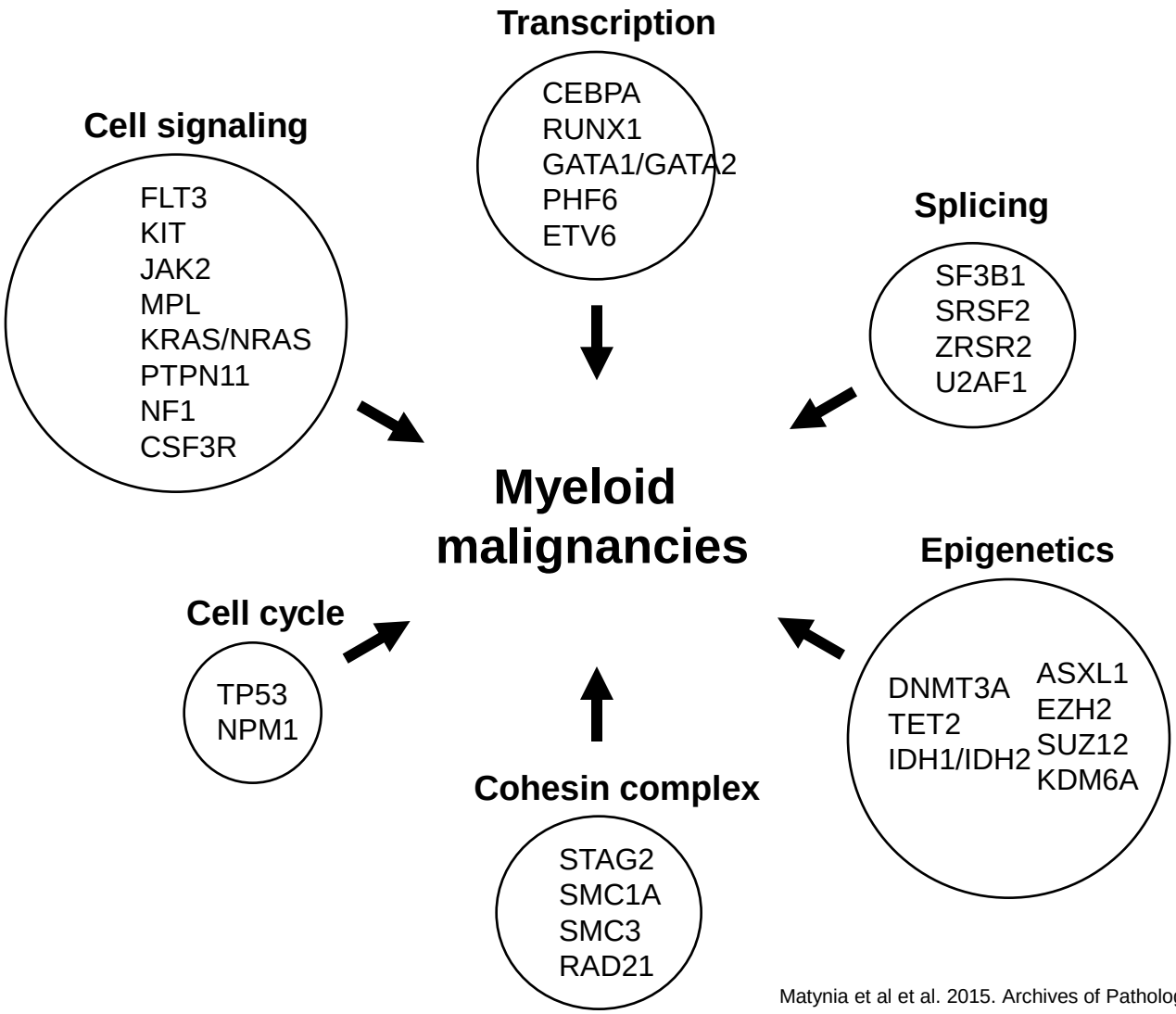
Whole genome sequencing

- Many of the biomarkers we now know to be important were discovered in whole genome sequencing studies (ie. *DNMT3A*, *IDH1/2*, etc)
- Not routinely performed in the clinical lab
  - Would need paired normal tissue for tumors
  - Time consuming
  - Expensive
  - Yields relatively low coverage (~30X) so results may be difficult to interpret, especially with low tumor burden

- Benefit: Not limited to selected targets

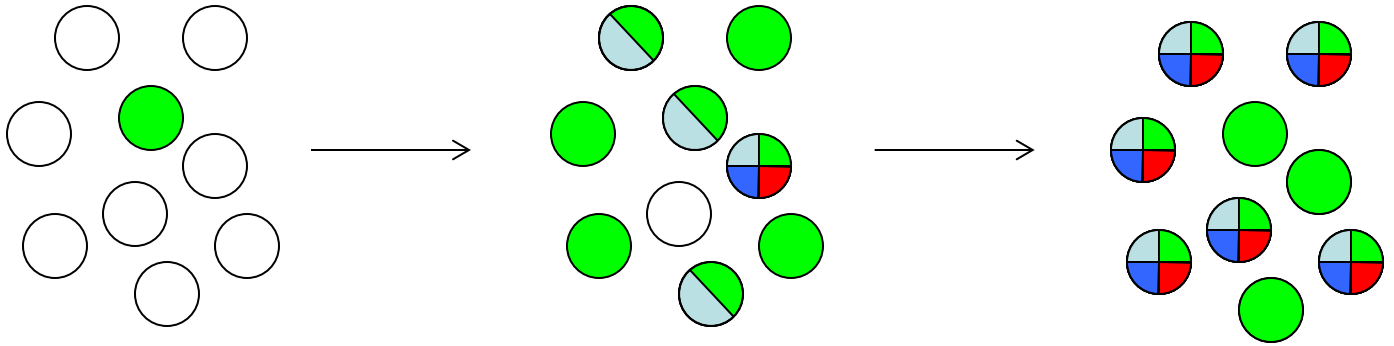
Spectrum of mutations in myeloid malignancies

AML, MDS, MPN and MDS/MPN overlap disorders



Matynia et al et al. 2015. Archives of Pathology and Laboratory Medicine.

There is often a complex subclonal architecture in myeloid malignancies



Pre diagnosis

Ex. clonal hematopoiesis of uncertain significance (CHIP)

Diagnosis

Relapse

Matynia et al et al. 2015. Archives of Pathology and Laboratory Medicine.

Variant Associations

Gene	MPN	MDS	MDS/MPN	De novo AML	Secondary AML	Effect *
JAK2	++	-	+	-	-	Gain
MPL	+	-	-	-	-	Gain
CALR	++	-	+	-	-	Gain
FLT3	-	-	-	++	-	Gain
NPM1	-	-	+	++	-	Gain
CEBPA	-	-	-	+	-	Loss
RUNX1	-	+	++	+	-	Loss
KIT	+	-	-	+	-	Gain
CSF3R	+	-	+	-	-	Gain
DNMT3A	+	+	+	++	-	Loss
TET2	+	++	++	++	+	Loss
IDH1/2	+	+	+	++	+	Gain
SF3B1	-	+	+	-	+	Unknown
SRSF2	-	+	++	+	++	Unknown
STAG2	-	+	-	-	++	Loss
ASXL1	++	++	++	+	++	Unknown
EZH2	+	+	+	-	++	Loss
TP53	+	+	+	+	+	Loss

From: Tietz textbook of Clinical Chemistry and Molecular Diagnostics, 6th Edition

Mutation panels: Variant reporting

- ***Tiered strategy***
  - ***A variety of systems are in use and this area currently lacks a uniform standard***

NRAS c.37G>C, p.Gly13Arg

Higher tiers – more likely to be pathogenic or actionable

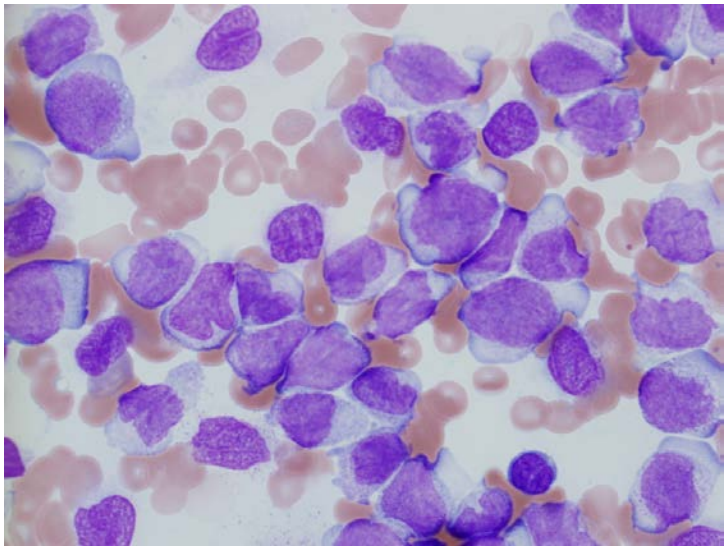
Variants of unknown significance (VUSs)

TET2 c.5284A>G, p.Ile1762Val

Lower tiers – less likely to be pathogenic or likely or known germline polymorphism

Clinical Scenario #1

- 52 year-old female presented with easy bruising and fatigue
  - CBC: WBC – 33 K/uL, Hgb – 9.6 g/dL, Platelets – 12,000 K/uL
  - Flow cytometry on BM aspirate: large CD34 negative atypical myeloid blast population (48% of leukocytes)
  - BM morphology – Acute myeloid leukemia
  - Cytogenetics/FISH – normal karyotype



Clinical scenario #1 -mutations

Mutation panel testing by NGS:

Tier 1 variants:

1. NPM1 c.860_863dup, p.Trp288fs

-Variant frequency 35.5%

-Associated with good prognosis except when a FLT3-internal tandem duplication mutation is present.

2. FLT3 c.1802_1803ins45, p.Leu601_Lys602ins15

-Variant frequency 30.0%

-Associated with early relapse and poor overall survival.

3. DNMT3A c. 2645G>A, p.Arg882His

-Variant frequency 41.2%

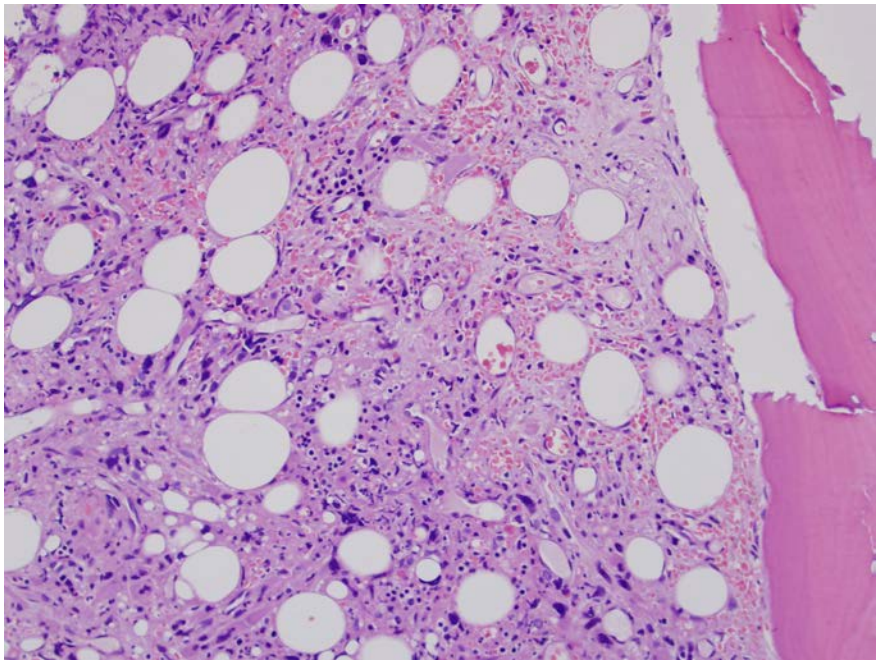
-Commonly seen with NPM1 mutations in patients with CN-AML

-DNMT3A R882 mutations are associated with poor outcome when compared to NPM1 mutated AML patients without DNMT3A mutations

Conclusion – Poor prognosis; patient should proceed to BM transplant

Clinical scenario #2

- 75 y/o male with complaint of fatigue and history of primary myelofibrosis
- CBC:
  - WBC: 40.05 k/uL
  - Hgb: 14.9 g/dL
  - MCV: 76.5 fL
  - Plts: 205 k/uL
- Cytogenetics: 46, XY, inv(12)



Clinical scenario #2 - Mutations

1. *JAK2* c.1849G>T, p.Val617Phe
   - Variant frequency: 92.4% ← Dominant clone, VAF implies LOH @ 9p
2. *NRAS* c.37G>C, p.Gly13Arg
   - Variant frequency: 16.5% ←
3. *NRAS* c.183A>C, p.Gln61His
   - Variant frequency: 8.6% ← Subclone(s) implied by VAFs
4. *ASXL1* c.2275_2284del, p.Gln760fs
   - Variant frequency: 8.3% ←

Variant frequencies illustrate complex underlying clonal architecture

Panel-based NGS testing

Mutation panel testing by NGS

Pros

1. Variants are reported together, at the same time, on a single report
2. Interpretation takes into account all variants identified
3. Cost is less compared to multiple single gene tests
4. Variant frequencies provide information on subclonal structure
5. Pattern and identity of mutations facilitates accurate subclassification and prognostication
6. Detection of certain variants allows for the use of targeted therapies

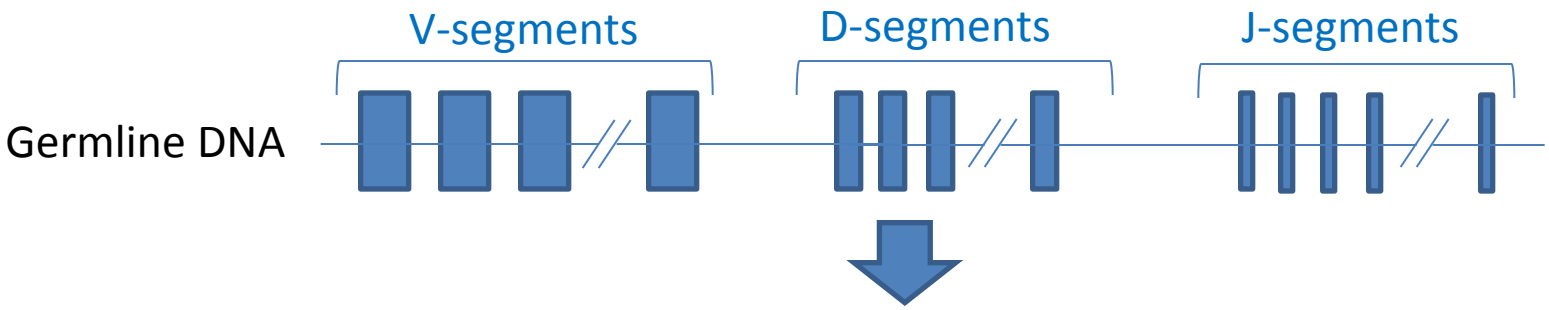
Cons

1. May not be reimbursed by payers
2. Variants of unknown significance – what to do?
3. Subclone information not currently actionable

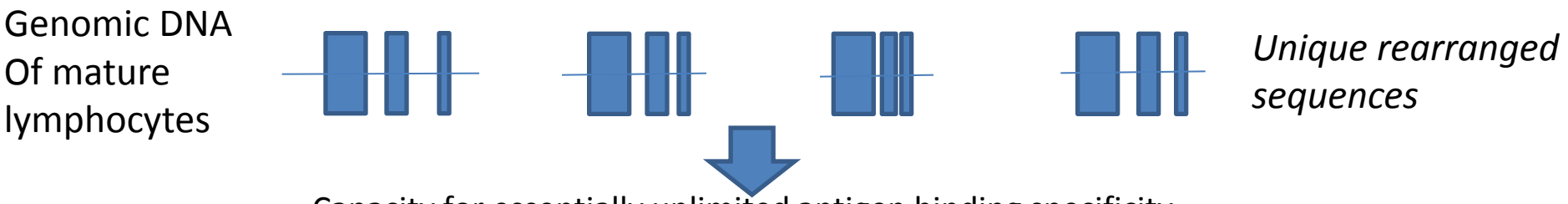
Lymphoid clonality testing by NGS

T/B cell antigen receptors – target for clonality testing

- B cells
  - B-cell receptor - surface expressed immunoglobulin
- T cells
  - T cell receptor

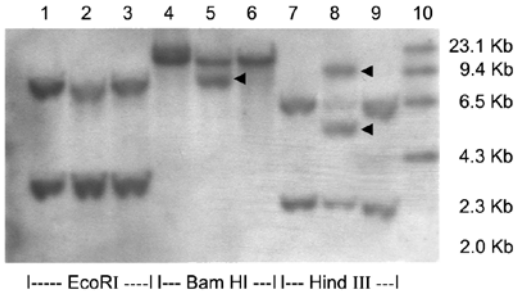


Somatic recombination (V-D-J joining) and addition of N and P nucleotides – junctional diversity



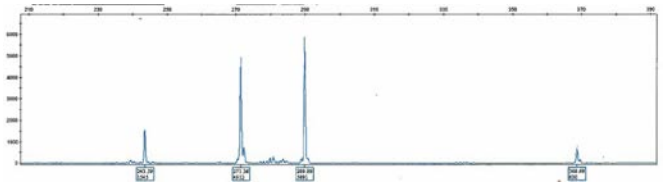
Capacity for essentially unlimited antigen binding specificity

Evolution of Clonality Testing



Southern blotting

-slow and laborious
-large amounts of input DNA required
-relatively insensitive



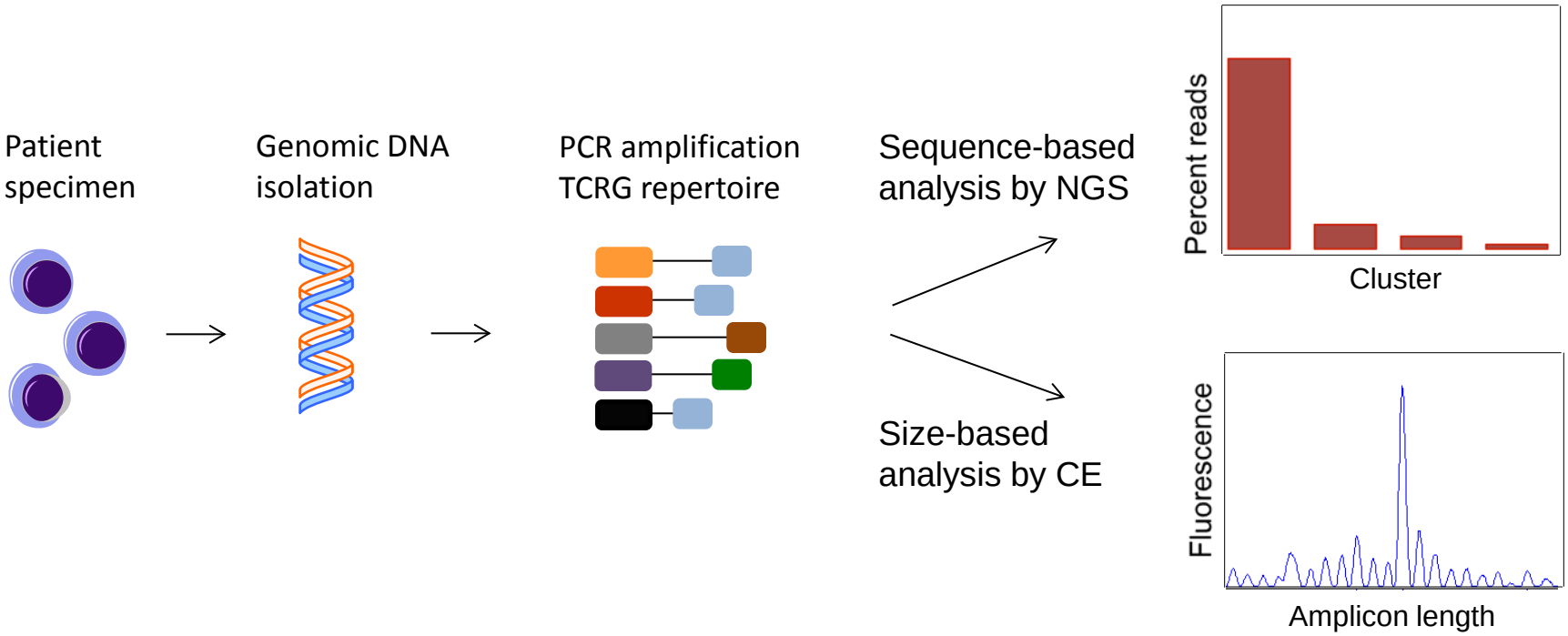
PCR/capillary electrophoresis

-most commonly used method
-fast and inexpensive
-much less DNA required
-better sensitivity (~10%)
-sensitivity too low for MRD detection
-subjective interpretation

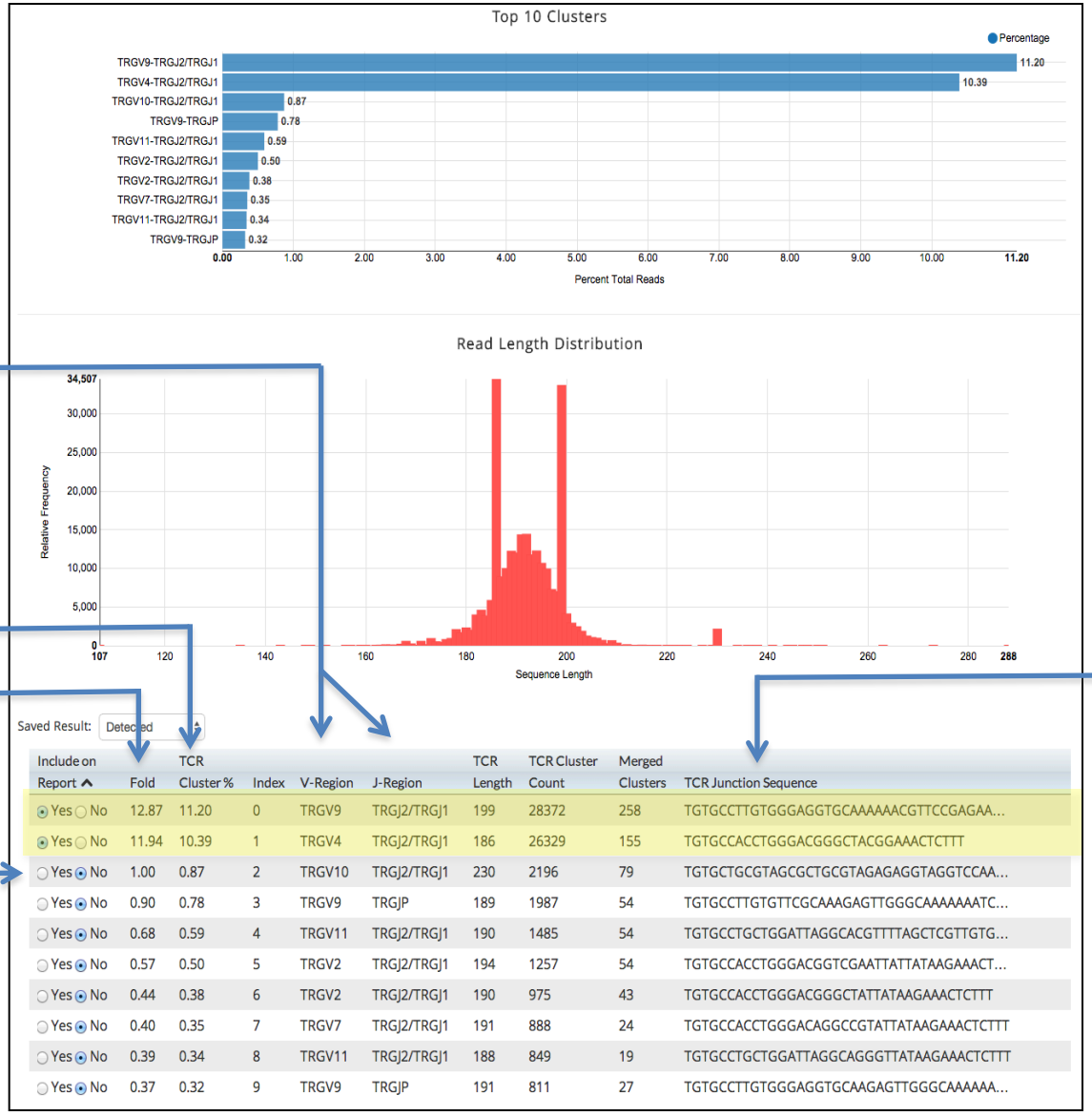
Next generation sequencing

-more expensive
-little DNA required
-similar sensitivity for initial clone identification
-very sensitive method for detection of a known clone - MRD
-less subjective

T cell clonality testing: NGS workflow



T cell clonality testing by NGS: Data format



Include on Report	Fold	TCR Cluster %	Index	V-Region	J-Region	TCR Length	TCR Cluster Count	Merged Clusters	TCR Junction Sequence
Yes No	12.87	11.20	0	TRGV9	TRGJ2/TRGJ1	199	28372	258	TGTGCCTTGTGGGAGGTGCAAAAAACGTTCCGAGAA...
Yes No	11.94	10.39	1	TRGV4	TRGJ2/TRGJ1	186	26329	155	TGTGCCACCTGGGACGGGCTACGGAAACTCTTT
Yes No	1.00	0.87	2	TRGV10	TRGJ2/TRGJ1	230	2196	79	TGTGCTGCGTAGCGCTGCGTAGAGAGGTAGGTCCAA...
Yes No	0.90	0.78	3	TRGV9	TRGJP	189	1987	54	TGTGCCTTGTGTTCGCAAAGAGTTGGGCAAAAAAATC...
Yes No	0.68	0.59	4	TRGV11	TRGJ2/TRGJ1	190	1485	54	TGTGCCTGCTGGATTAGGCACGTTTTAGCTCGTTGTG...
Yes No	0.57	0.50	5	TRGV2	TRGJ2/TRGJ1	194	1257	54	TGTGCCACCTGGGACGGTCGAATTATTATAAGAAACT...
Yes No	0.44	0.38	6	TRGV2	TRGJ2/TRGJ1	190	975	43	TGTGCCACCTGGGACGGGCTATTATAAGAAACTCTTT
Yes No	0.40	0.35	7	TRGV7	TRGJ2/TRGJ1	191	888	24	TGTGCCACCTGGGACAGGCCGTATTATAAGAAACTCTTT
Yes No	0.39	0.34	8	TRGV11	TRGJ2/TRGJ1	188	849	19	TGTGCCTGCTGGATTAGGCAGGGTTATAAGAAACTCTTT
Yes No	0.37	0.32	9	TRGV9	TRGJP	191	811	27	TGTGCCTTGTGGGAGGTGCAAGAGTTGGGCAAAAAA...

V- and J-segments used in the rearrangement

% of total reads represented by cluster

Fold difference in frequency compared to background

Background defined as third rearrangement

Unique sequence between V- and J-segments

Positive case

T cell clonality testing by NGS: Advantages

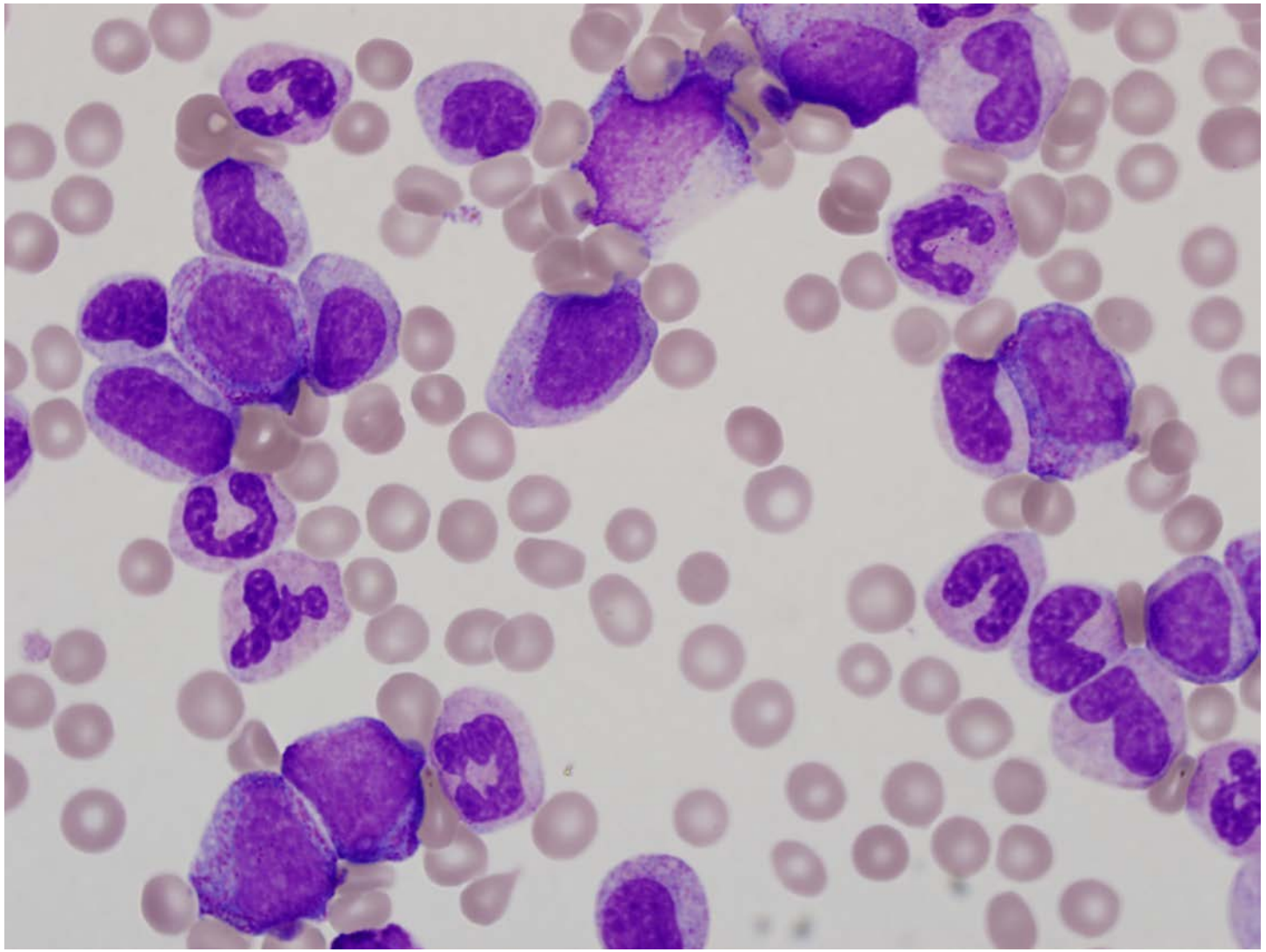
- Removes subjectivity from interpretation
- Overcomes some of the common pitfalls of electrophoresis based analysis
  - Additive effects of rearrangements which yield same sized amplicons
- Allows for detection of minimal residual disease based on unique TCR sequence(s)
  - MRD detection to as few as 0.004% tumor cells

Antigen receptor sequencing – general uses

- Many publications have shown that IGH sequencing can be used as a very sensitive and specific marker for MRD in B-lymphoblastic leukemia and myeloma

Use of NGS to assess for *BCR-ABL1* kinase domain mutations in chronic myelogenous leukemia (CML)

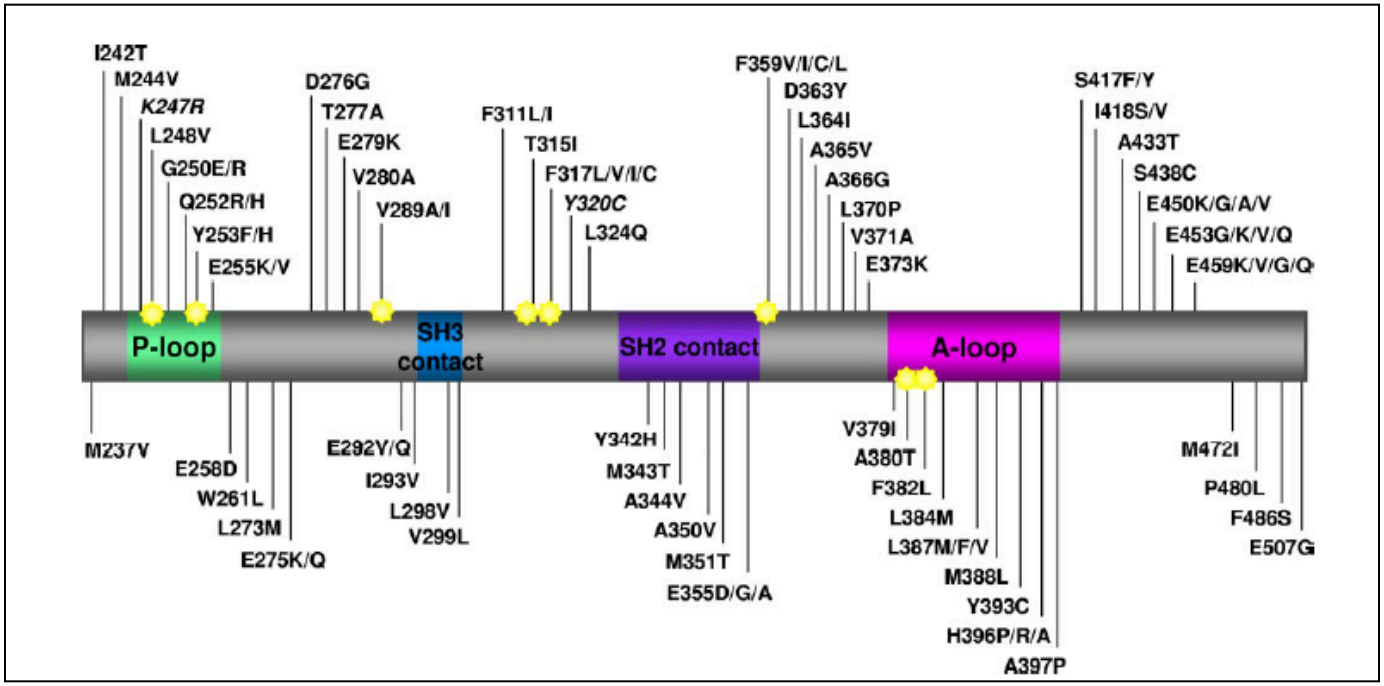
Peripheral blood smear from a patient with chronic myelogenous leukemia, *BCR-ABL1* positive



BCR-ABL1 sequencing by NGS

- t(9;22)(q34;q11.2);*BCR-ABL1*
  - Defining genetic abnormality in chronic myelogenous leukemia (CML)
  - Present in a subset of lymphoblastic leukemia patients (Ph+ ALL)
- Tyrosine kinase inhibitors (TKIs)
  - Multiple TKIs now available for treatment (ie. imatinib)
  - A subset of patients develop acquired resistance mutations in drug binding sites in the kinase domain
  - Different TKIs have varying levels of effectiveness in the context of these mutations
  - T315I mutation confers resistance to most currently available TKIs (1 available in US to treat T315I+ patients)

BCR-ABL1 kinase domain mutations



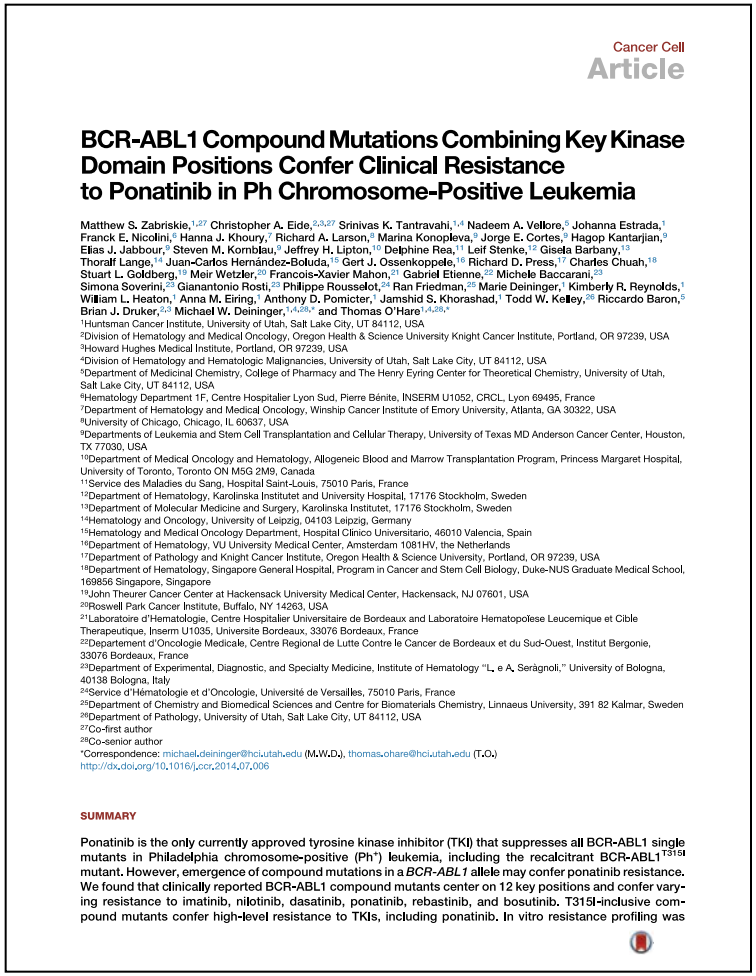
From: Soverini S et al. 2011. Blood, 118: 1208-1215

- Why use NGS for *BCR-ABL1* kinase domain mutation testing?
  - Better sensitivity vs Sanger sequencing
  - More comprehensive coverage (SH2/SH3 and kinase domain)
  - Ability to detect compound mutations which have uncertain resistance profiles

BCR-ABL1 compound mutations

- Compound mutation = more than 1 mutation in the same *BCR-ABL1* sequence
- Polyclonal mutations = more than 1 mutation in different *BCR-ABL1* sequences
  - This distinction may have therapeutic consequences

BCR-ABL1 compound mutations



Cancer Cell
Article

BCR-ABL1 Compound Mutations Combining Key Kinase Domain Positions Confer Clinical Resistance to Ponatinib in Ph Chromosome-Positive Leukemia

Matthew S. Zabriskie,[1,27] Christopher A. Eide,[2,3,27] Srinivas K. Tantravahi,[1,4] Nadeem A. Vellore,[5] Johanna Estrada,[1] Franck E. Nicolini,[6] Hanna J. Khoury,[7] Richard A. Larson,[8] Marina Konopleva,[9] Jorge E. Cortes,[9] Hagop Kantarjian,[9] Elias J. Jabbour,[9] Steven M. Kornblau,[9] Jeffrey H. Lipton,[10] Delphine Rea,[11] Leif Stenke,[12] Gisela Barbany,[13] Thoralf Lange,[14] Juan-Carlos Hernández-Boluda,[15] Gert J. Ossenkoppele,[16] Richard D. Press,[17] Charles Chuah,[18] Stuart L. Goldberg,[19] Meir Wetzler,[20] Francois-Xavier Mahon,[21] Gabriel Etienne,[22] Michele Baccarani,[23] Simona Soverini,[23] Gianantonio Rosti,[23] Philippe Rousselot,[24] Ran Friedman,[25] Marie Deininger,[1] Kimberly R. Reynolds,[1] William L. Heaton,[1] Anna M. Eiring,[1] Anthony D. Pomicter,[1] Jamshid S. Khorashad,[1] Todd W. Kelley,[26] Riccardo Baron,[5] Brian J. Druker,[2,3] Michael W. Deininger,[1,4,28,*] and Thomas O'Hare[1,4,28,*]
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SUMMARY

Ponatinib is the only currently approved tyrosine kinase inhibitor (TKI) that suppresses all BCR-ABL1 single mutants in Philadelphia chromosome-positive (Ph+) leukemia, including the recalcitrant BCR-ABL1^{T315I} mutant. However, emergence of compound mutations in a *BCR-ABL1* allele may confer ponatinib resistance. We found that clinically reported BCR-ABL1 compound mutants center on 12 key positions and confer varying resistance to imatinib, nilotinib, dasatinib, ponatinib, rebastinib, and bosutinib. T315I-inclusive compound mutants confer high-level resistance to TKIs, including ponatinib. In vitro resistance profiling was

-100 specimens from 64 patients on clinical trial or in expanded access program for new TKI (ponatinib)

-Compound mutations were centered on 12 key residues

-Clinical ponatinib failure attributed to T315I inclusive compound mutants:

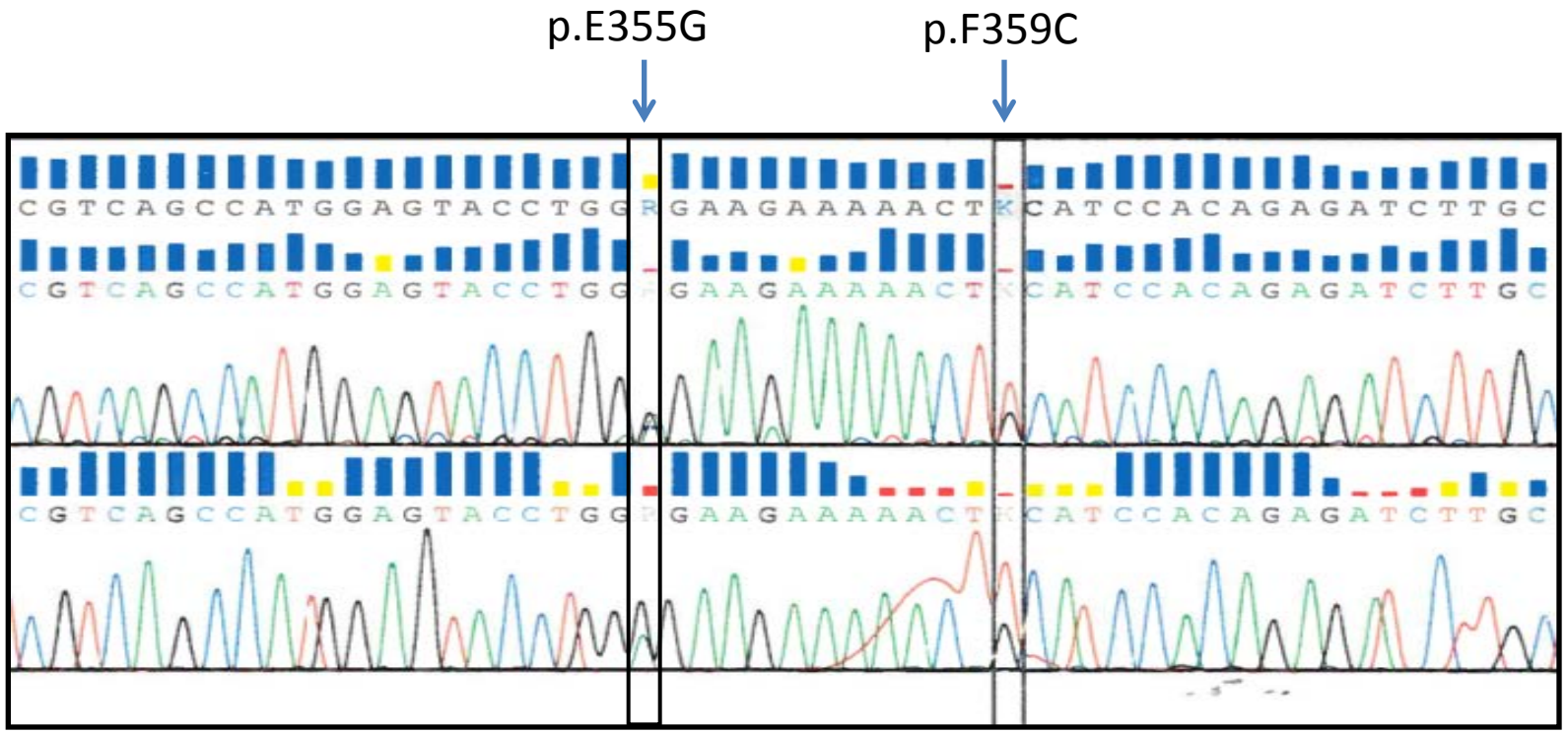
- E255V/T315I
- T315I/F359C
- Y253H/T315I
- T315I/H396R
- T315I/E453K

-Highest IC50 for ponatinib seen with E255V/T315I

-Some compound mutants displayed differential sensitivity to other TKIs ex. Y253H/E255V and dasatinib

-There is clinical utility to knowing the configuration when multiple mutations are detected

Sanger sequencing of the kinase domain



E355G----------

----------F359C-

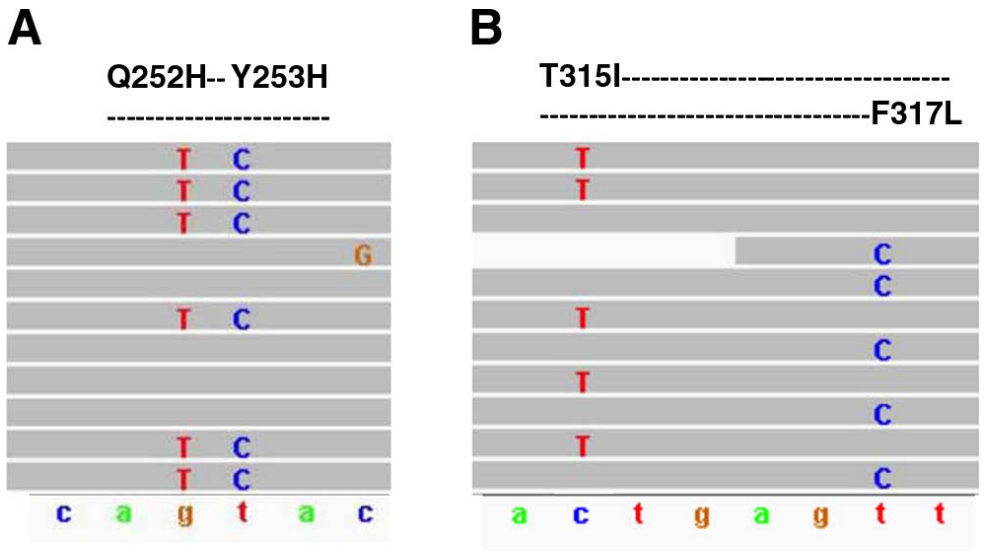
OR

E355G---F359C-

?

NGS of the *BCR-ABL1* kinase domain

Integrated genomics viewer



Configuration = compound

Configuration = polyclonal

From: Szankasi et al (2015), Annals of Hematology, in press

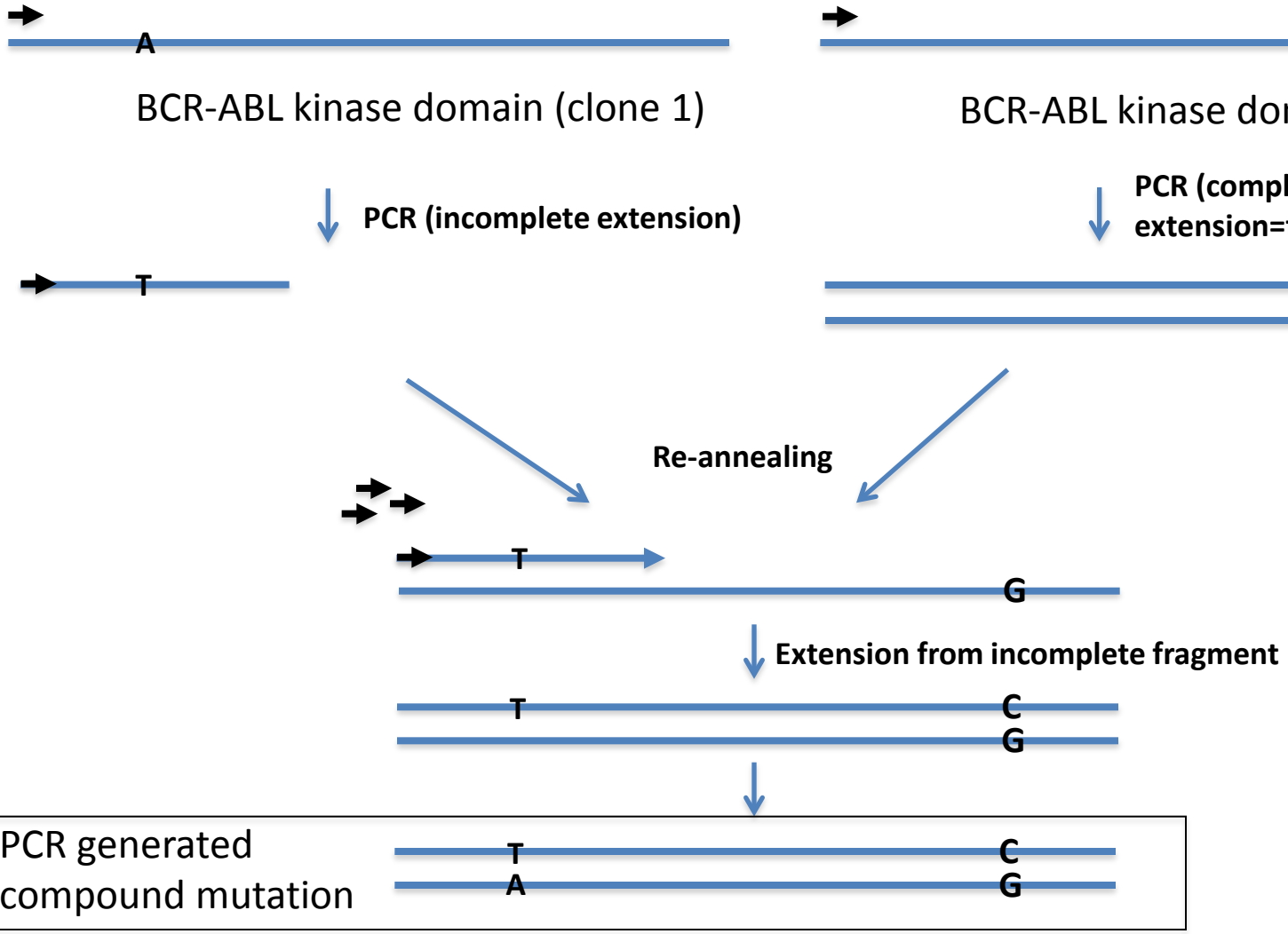
Detecting *BCR-ABL1* compound mutations: Limitations

- Informative reads are necessary – span both mutated positions
- Short read lengths impede detection of compound mutations due to lack of informative reads
  - Illumina: 300bp (600bp with paired end sequencing)
  - Ion Torrent PGM: ~120-130bp mean read length
    - Limited to analysis of mutations 30-50 codons apart
  - Roche GS FLX+ (454): long read lengths (up to 1kb)
    - Kastner *et al* (2014) *European Journal of Cancer*, 50:793-800
    - Able to sequence entire *BCR-ABL1* kinase domain in a single read (long range NGS)

Detecting BCR-ABL1 compound mutations: Limitations

- PCR recombination yields artificial compound mutations
  - PCR recombination: results from recombination and extension of a PCR product incompletely extended in a prior cycle
  - More PCR recombination with increasing distance between mutated positions
  - We have observed up to 10% artificial compound mutations at the limit of Ion Torrent read length

PCR recombination



PCR recombination

BCR-ABL kinase domain (compound)

BCR-ABL kinase domain (wild type)

PCR generated
polyclonal mutations

NGS for *BCR-ABL1* kinase domain mutation testing – take home points

- More sensitive than Sanger sequencing (<5% vs 15-20%)
- Mutant frequencies can be reported
- Possible to determine the configuration of multiple mutations (compound vs polyclonal) in certain circumstances
- Beware of artificial compound mutations from PCR recombination!

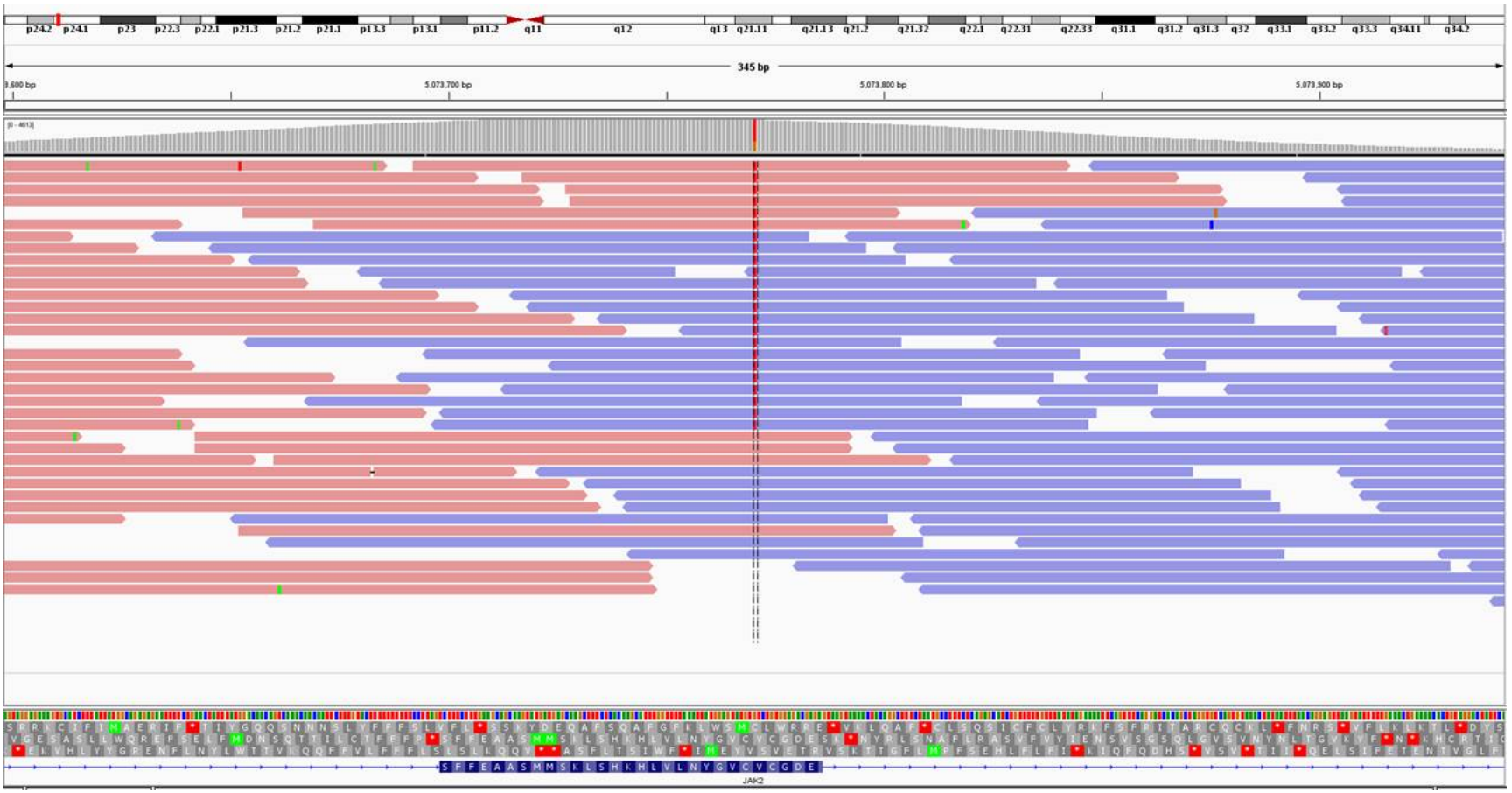
Detecting structural variants by NGS

Copy Number Variants

- Variation in the number of copies of one or more segments of DNA
- Common in myeloid malignancies
- Incidence
  - AML 50%
  - MDS 70%
- Clinical significance
  - 5q, 7q, 12p, 17p deletions and trisomy 8
  - the number of CNVs is an independent predictor of poor overall survival in MDS
- **Current methods for CNV detection:**
  - **Karyotype**
  - **FISH**
  - **Microarray**

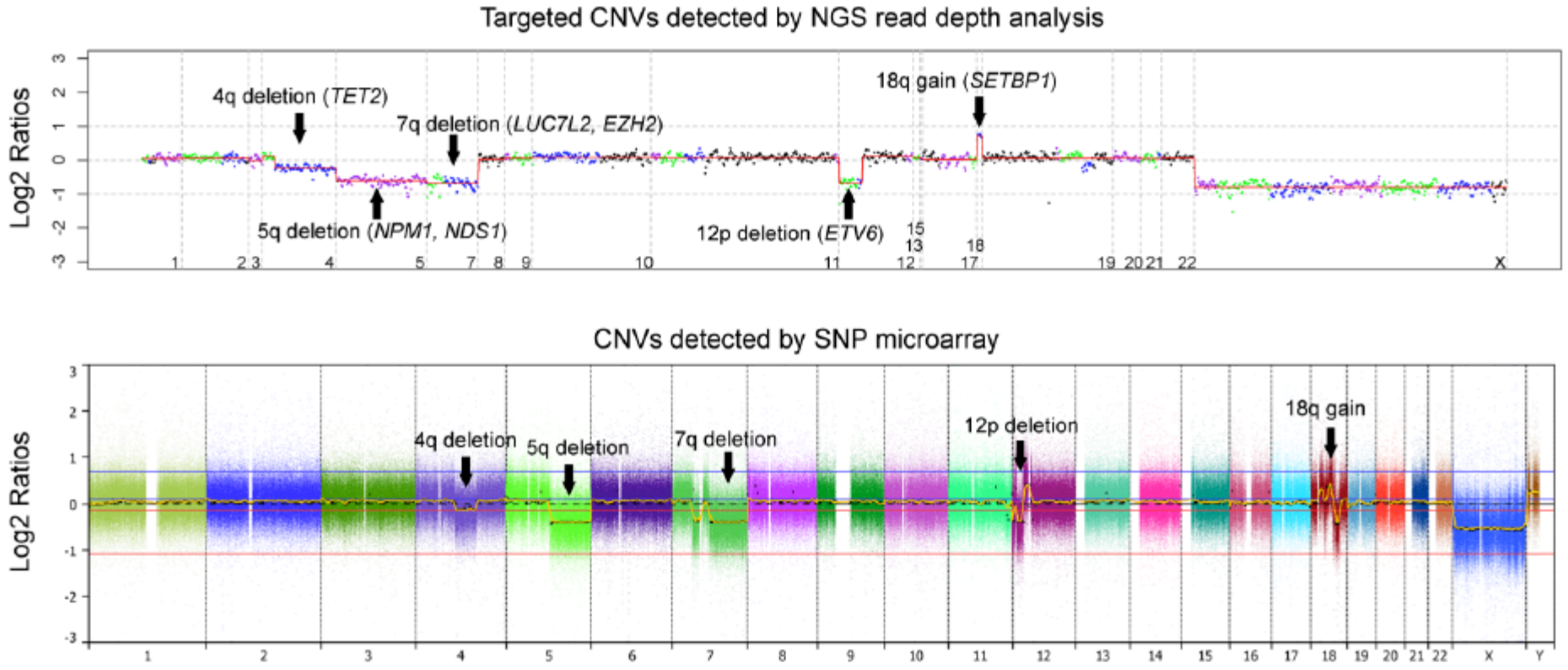
Copy Number Variants

Read depth
- the total number of bases sequenced and aligned at a given reference base position



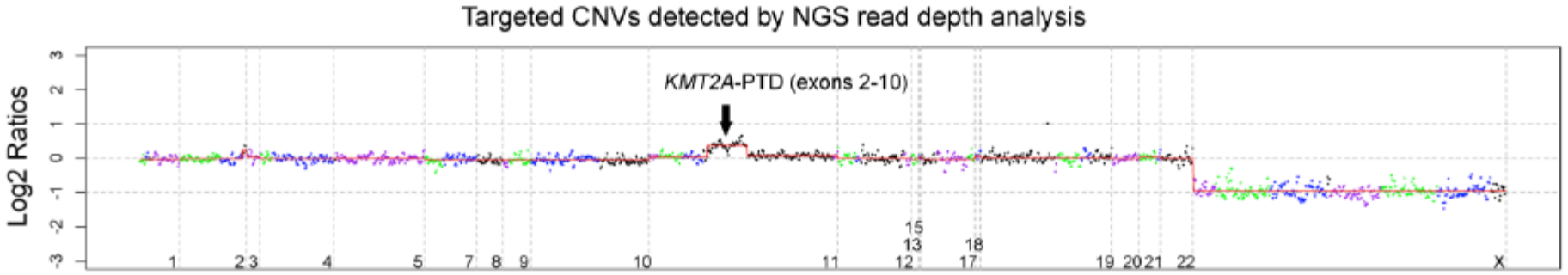
Read depth -> Copy number?

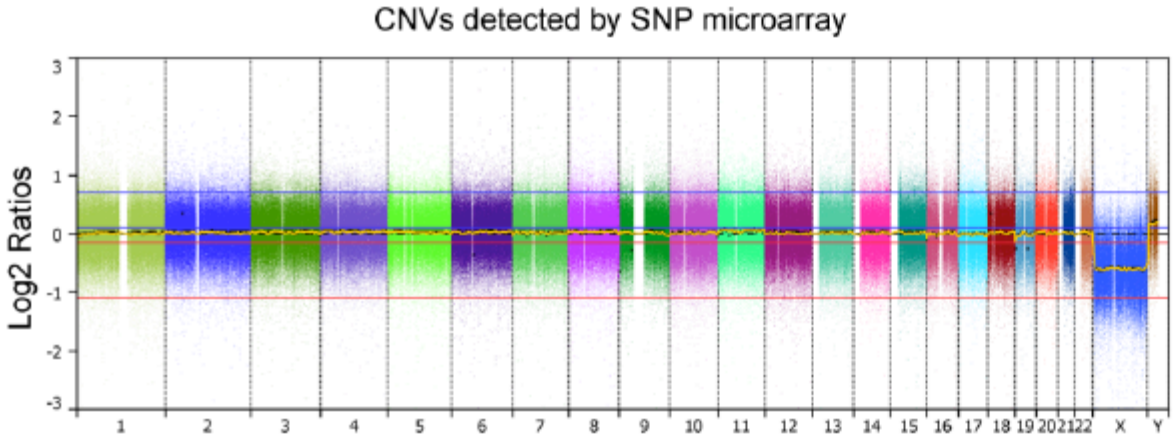
Comparison of targeted CNVs detected by NGS to SNP microarray analysis

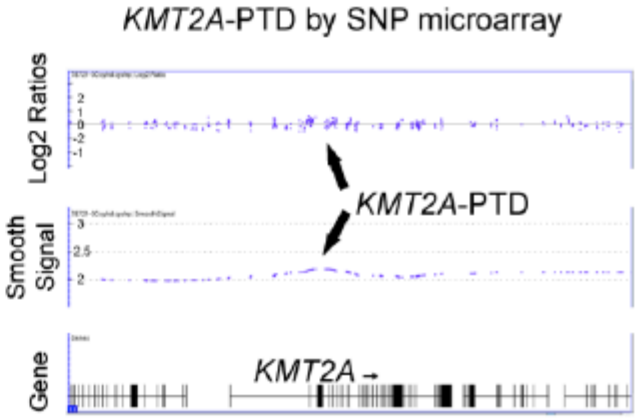


Shen and Szankasi, et al, *British Journal of Haematology*, in press

Exon level CNVs detected by NGS

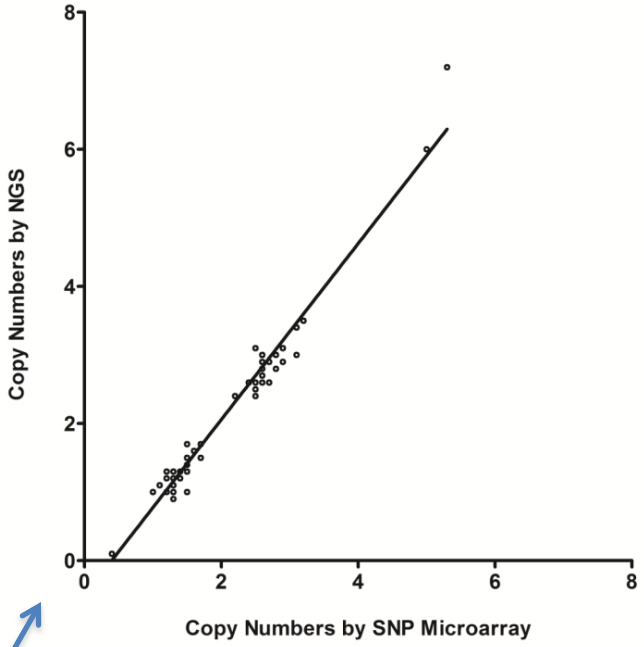




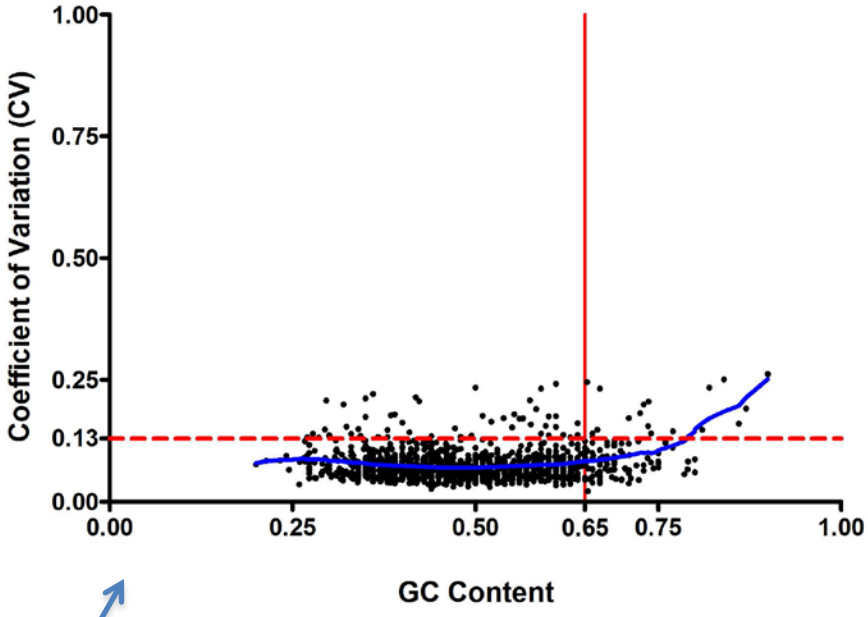


Shen and Szankasi, et al, *British Journal of Haematology*, in press

Copy Number Variants by NGS



Copy numbers by NGS using read depth data compare very favorably to copy numbers by SNP microarray



…but beware variation in read depths in high GC regions

Shen and Szankasi, et al, *British Journal of Haematology*, in press

CNVs by NGS

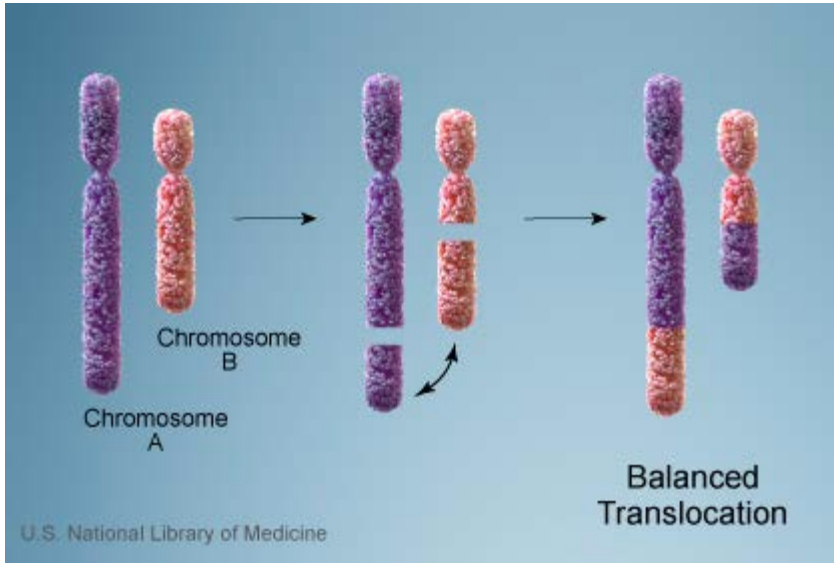
- NGS method demonstrates excellent concordance with gold standard (SNP microarray)

- Provides the opportunity for detection of both mutations and CNVs using a single assay (lower cost!)

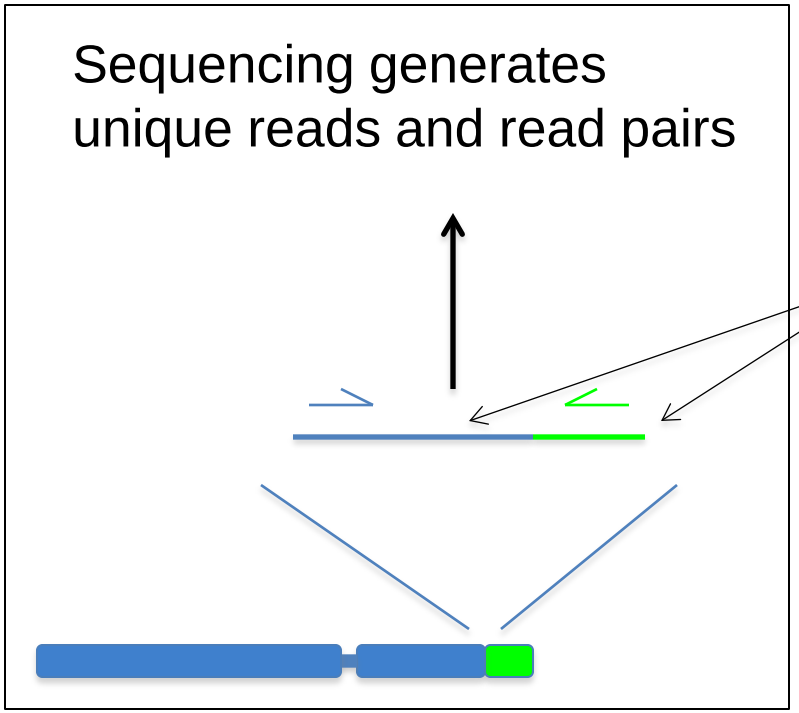
Translocations

Detection translocations by NGS in B cell lymphoproliferative disorders

- Balanced translocations – chromosomes are rearranged but no genetic information is gained or lost; not detectable by SNP arrays or CNV testing
- Balanced translocations in B cell lymphoma often involve the *IGH* locus
  - *IGH-MYC*
  - *IGH-BCL2*
  - *IGH-CCND1*
  - Myeloma – *IGH-MAF, IGH-CCND3*, *IGH-FGFR3*
- Advantage of NGS?
  - Assess for both translocations and mutations in a single assay
  - More comprehensive coverage of translocations than FISH
  - Less costly than multiple FISH assays for different translocations



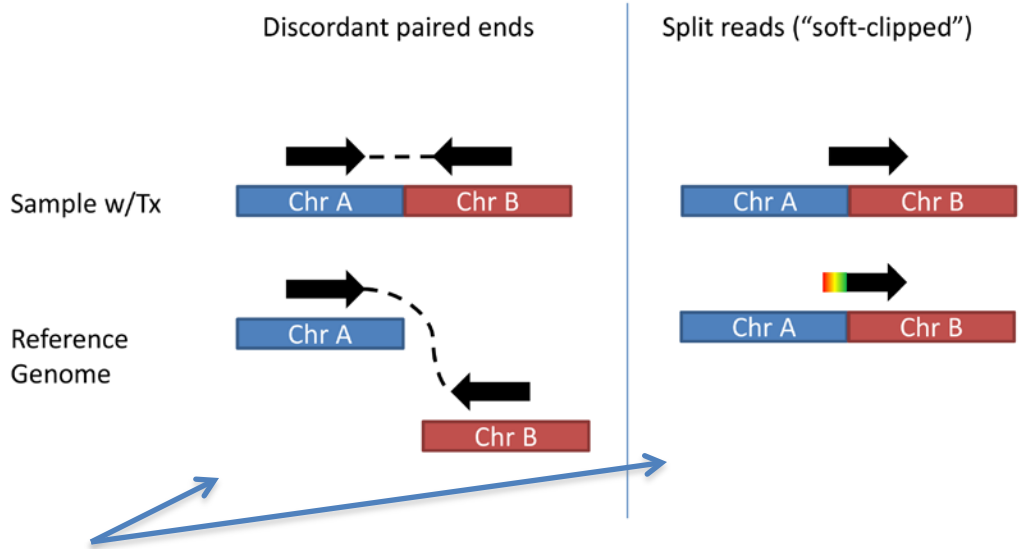
Detecting Translocations by NGS



generated reads would align to different chromosomes

flagged if they are derived from paired-end sequencing

Detection translocations by NGS

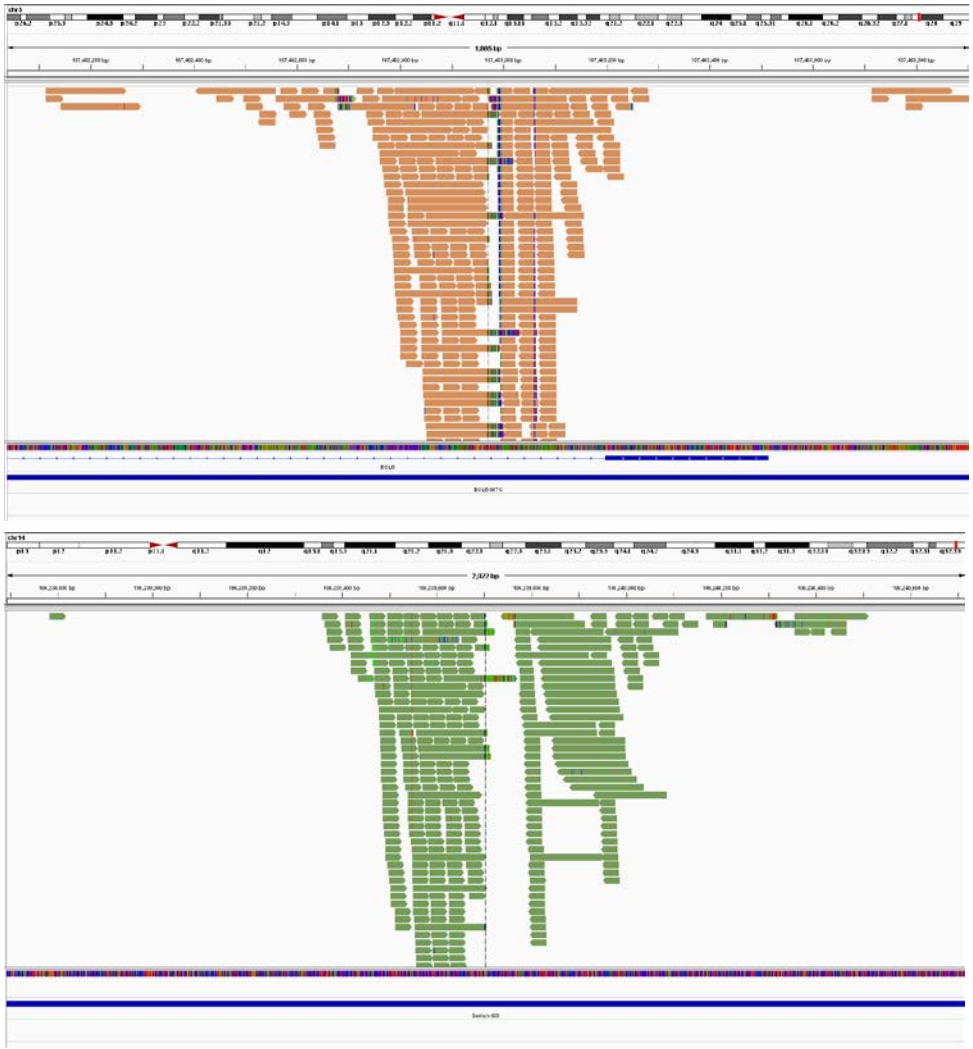


Paired end sequencing – a fragment is sequenced from both ends; alignment to reference genome will not be contiguous if a translocation is present; split reads will also be identified

Need informatics algorithm that can handle this kind of data!

Detecting translocations by NGS

BCL6 breakpoint



IGH breakpoint

Discordant paired ends - flagged because they align to different genes

t(3;14) BCL6.MTC – IGH.S-gamma 3

Conclusions

- NGS is revolutionizing pathology and laboratory medicine
- Allows for true personalized medicine
- Facilitates use of targeted therapeutic strategies
- Costs are rapidly decreasing while the technology continues to improve
- Challenges remain
  - Cost and reimbursement
  - Data analysis
  - Variant interpretation
  - Other aspects of testing (ie. PCR) can affect the results!
- Today – panels and genetically complex single gene analysis; detection of targeted structural variants
- Future – routine comprehensive whole genome analysis of tumors