Practical Considerations for Implementation of Clinical Sequencing

Emily Winn-Deen, Ph.D.

April 2017

RXDXAdvisors

1. DEFINE THE CLINICAL PROBLEM TO BE ADDRESSED

RXDXAdvisors

Targeted Gene Panels

- Focused panels
  - Gene or disease-based
  - Can provide a definitive genetic diagnosis
  - Lowest cost with good reimbursement
- Broad panels
  - Combines genes of known significance with genes of research interest
  - Medium cost
  - Reimbursement for research content can be an issue

RxDx Advisors

Clinical Whole Genome/Exome Sequencing

- Indications for Use:
  - Diagnosis refractory cases
    - Particularly helpful in pediatrics
  - Cancer/tumor characterization
    - Particularly when tumor does not respond to standard of care
- Issues:
  - Counseling is critical
    - Takes 6-10 hours per person
  - Interpretation can be time consuming
    - Still debating how much information should be reported
      - Yes: medically actionable results
      - No: variants of unknown significance (VUS)
      - It depends: Late onset/untreatable disorders

RxDxAdvisors

Clinical Whole Genome/Exome Sequencing

- Advantages of WES
  - Lower cost
  - Can afford to use deeper coverage
  - Easier to interpret
- Advantages of WGS
  - Hypothesis-free approach
  - No bias from selection method
  - Intragenic regions can have significance

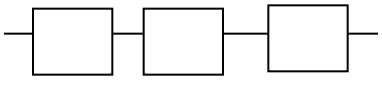
RxDxAdvisors

2. CHOOSE A SEQUENCING TECHNOLOGY

RXDXAdvisors

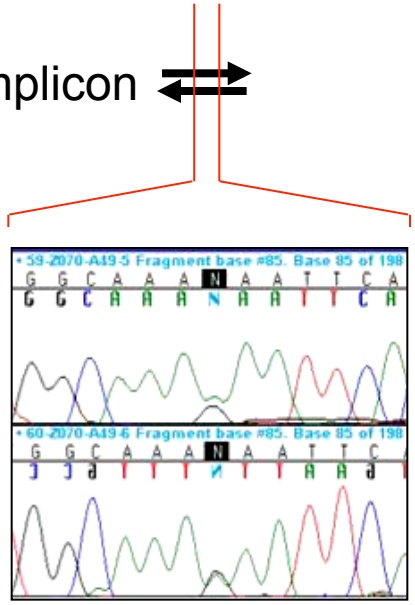
Sequencing Method Comparison

A. Bi-directional Sanger Sequencing



gene

PCR amplicon

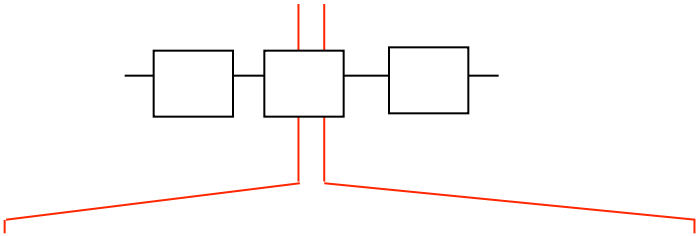


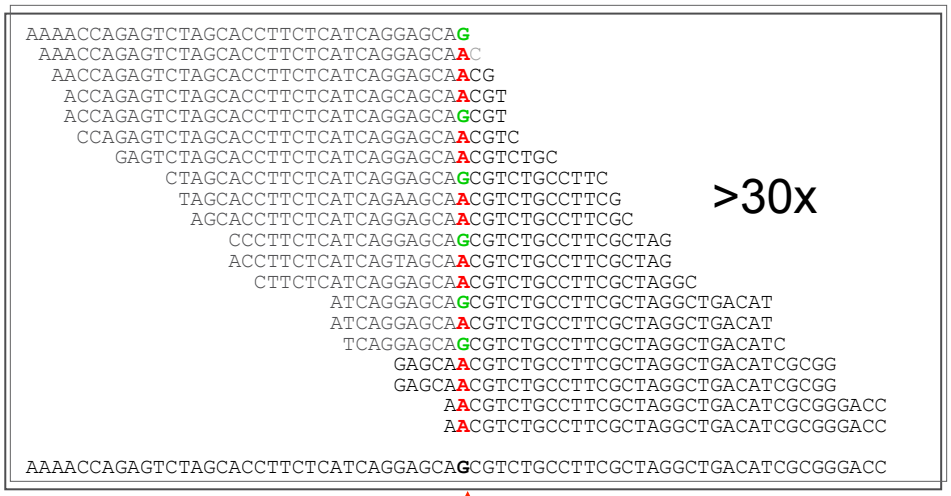
sequence

A
G

variant

B. Sequencing by Synthesis (SBS)





>30x

A
G

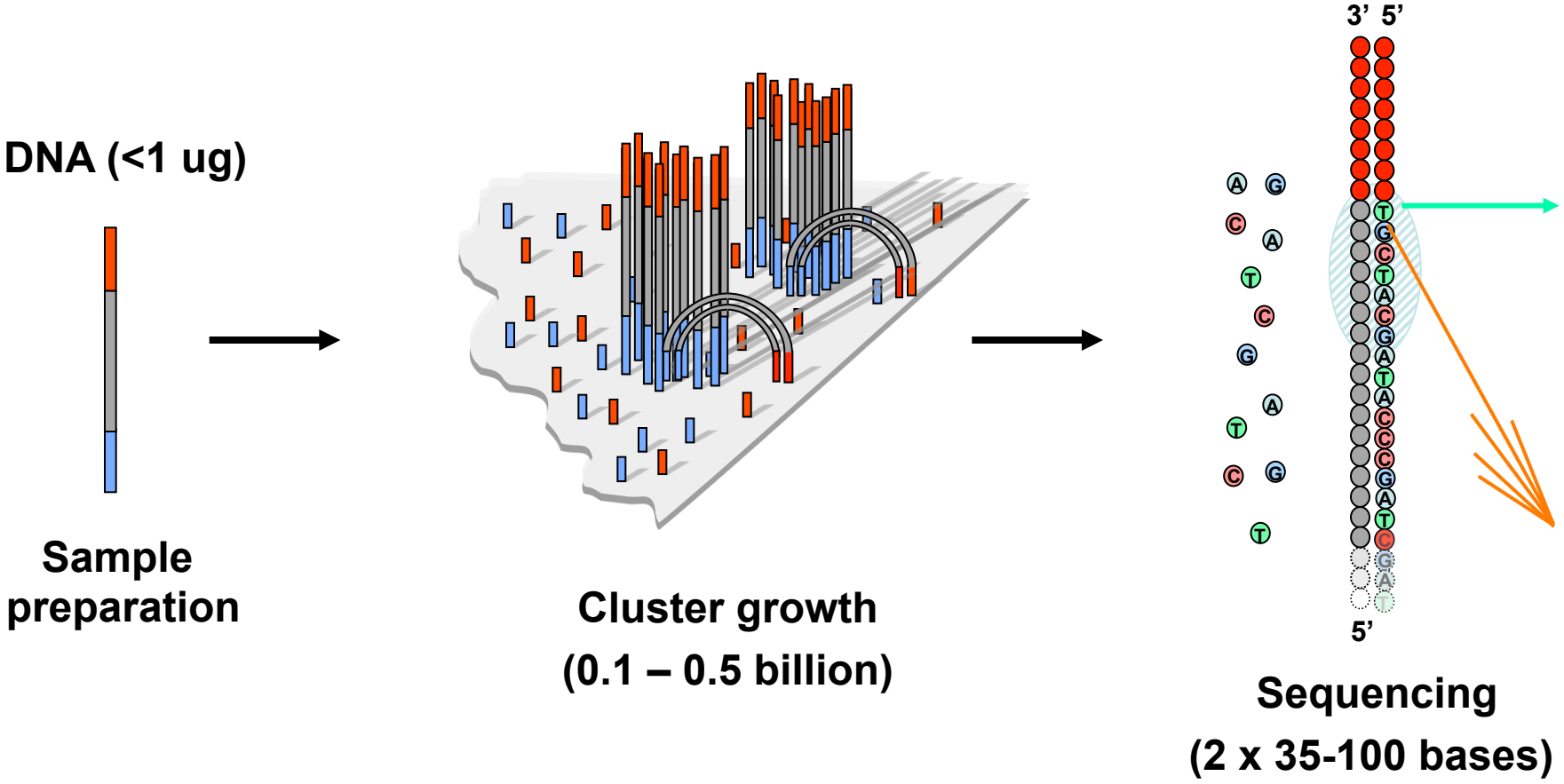
variant

RxDxAdvisors

Sanger vs. SBS Sequencing

Characteristic	Sanger	SBS
Through-put	One gene segment is sequenced on one direction in each capillary	Many gene segments are simultaneously sequenced in both directions using paired end reads
Signal Purity	Signal is averaged across a population of molecules	Signal is generated for each molecule separately
Heterozygote Detection	Decrease in base signal intensity and appearance of a second base signal.	Accumulation of sequence reads from each allele
Limit of Minor Allele Detection	15-20%	1-5%, minor allele detection limit is dependent on the number of clusters sequenced (depth of coverage)
Quality Scoring	Qualitative measure of peak height relative to surrounding sequence	Quantitative measure per base relative to signal/noise, sequence alignment and number of sequences reads

Illumina Sequencing by Synthesis



RXDXAdvisors

Illumina Family of Sequencers

NovaSeq **MiniSeq** **MiSeq**

Illumina sequencers all use the same basic sequencing chemistry and provide options for every budget and test volume.

RxDxAdvisors

Illumina Family of Sequencers

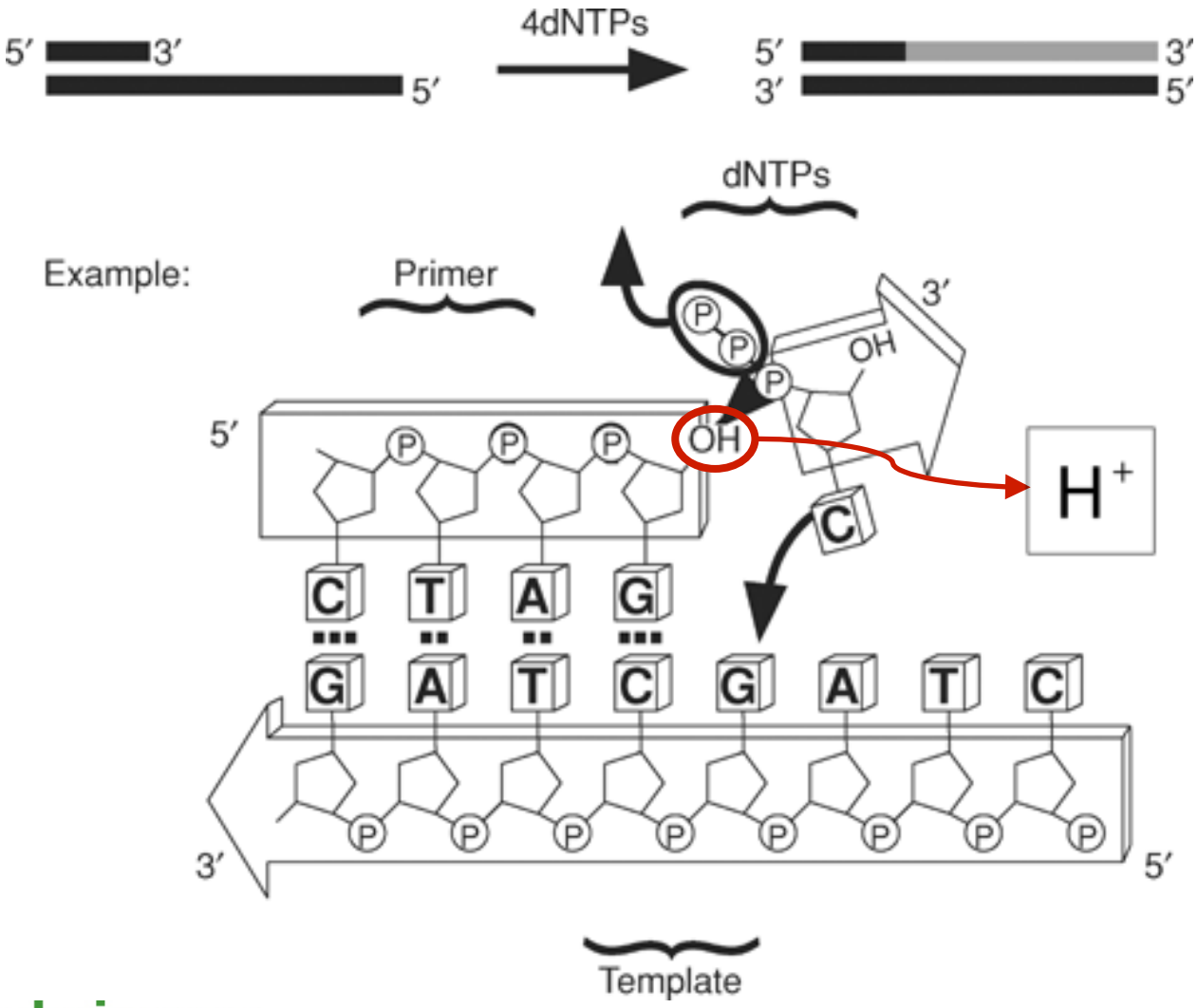
NextSeq

HiSeq 2500

HiSeq X-Ten

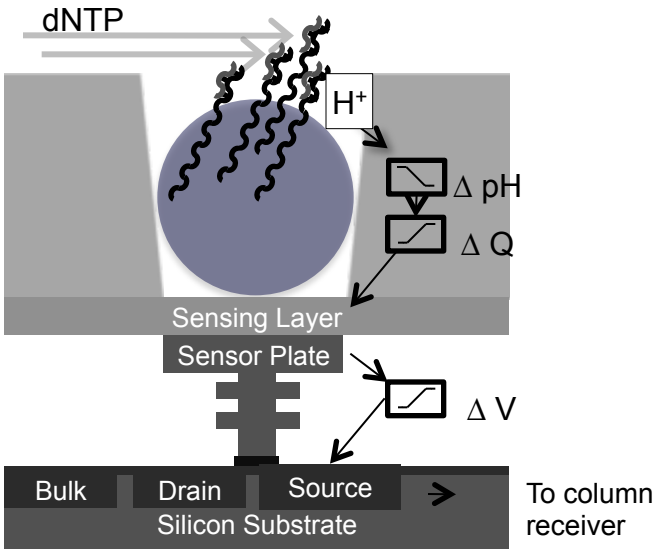
RXDXAdvisors

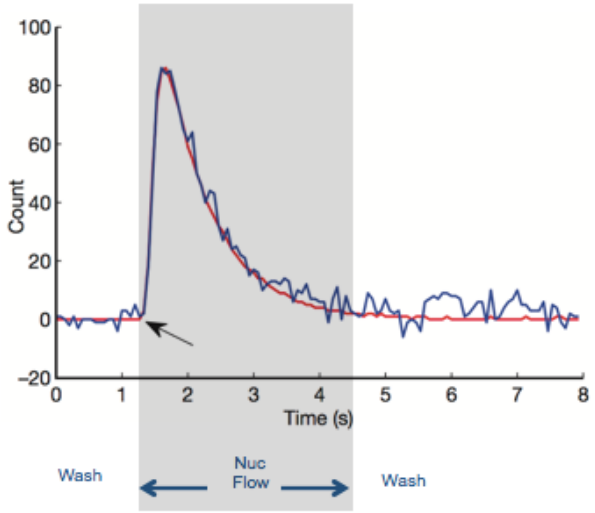
Ion Torrent Natural Nucleotide Chemistry



RXDXAdvisors

ION Torrent - Fast Direct Detection





Rothberg J.M. *et al Nature doi:10.1038/nature10242*

- Natural nucleotides flow sequentially over the semiconductor chip
- Direct detection of H^+ ions released during natural DNA extension
- Only a few seconds per incorporation

RXDXAdvisors

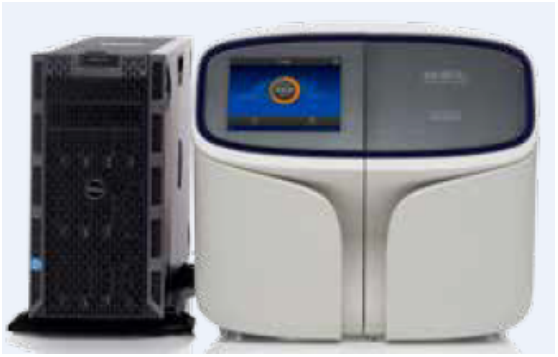
Ion Torrent Sequencer Family

Ion PGM

Off-board data analysis on Ion Proton server

Ion S5

On board data analysis



Ion S5 XL

Off-board data analysis

Ion Proton

Data analysis on Ion Proton server

RXDXAdvisors

3. CHOOSE A SEQUENCING WORKFLOW

RxDx Advisors

Patient Sample for Germline Genetics and Hematologic Cancers

- White blood cells isolated from K_2EDTA anticoagulated whole blood
- Incoming inspection
  - Make sure blood was drawn in the correct anticoagulant tube. Heparin is a powerful inhibitor of polymerase and heparinized blood should not be used for any molecular test.
  - Make sure blood is not hemolyzed. Hemoglobin is a powerful inhibitor of polymerase and grossly hemolyzed blood should be rejected.

RxDx Advisors

STOP if sample fails incoming QC

Patient Sample for Solid Tumors

- Types of samples for solid tumor analysis
  - Biopsy or fine needle aspirate
  - Tumor tissue (fresh, frozen, or FFPE)
  - Circulating tumor cells (CTCs)
  - Circulating tumor DNA (ctDNA)
- Incoming inspection
  - Tissue
    - Make sure the tumor area is at least 10% of the total sample. If the pathologist has indicated the area of cancer cells, consider macro-dissection to enrich for tumor cells prior to DNA or RNA isolation
  - CTCs
    - Make sure sufficient CTCs have been separated from other blood cells and collected as required for the DNA or RNA isolation
  - ctDNA
    - Make sure ctDNA was collected in a tube which stabilizes the ctDNA prior to isolation

RxDxAdvisors

STOP if sample fails incoming QC

Nucleic Acid Isolation and QC

- DNA
  - Isolation and purification
    - Select the appropriate DNA isolation procedure for the type and quantity of sample
  - Quality Control
    - Check DNA purity using the A260/A280 ratio
    - Check fragment size to assure damage due to the isolation process is minimized
- RNA
  - Isolation and purification
    - Select the appropriate RNA isolation procedure for the type and quantity of sample
  - Quality Control
    - Check RNA purity using the A260/A280 ratio
    - Check fragment size to assure damage due to the isolation process is minimized

RXDXAdvisors

STOP if isolated nucleic acid fails QC

Library Preparation and QC

- DNA fragmentation and adaptor ligation
  - Fragmentation by shearing (Covaris) or enzymatic digestion
    - Select the appropriate DNA shearing procedure for the downstream sequencing chemistry
  - Quality Control
    - Check fragment size to assure size distribution is appropriate for the downstream sequencing chemistry prior to adaptor ligation
- DNA pull-out by hybridization and adaptor ligation
  - Fragmentation and hybridization
    - Fragment sample as needed to assure efficient hybridization
    - Optimize hybridization temperature/time for selective and even pull-out of desired genomic regions
  - Quality Control
    - Check fragment size to assure size distribution is appropriate prior to initiating hybridization
    - Check for efficiency of hybridization procedure prior to adaptor ligation
- PCR amplification of selected regions and adaptor ligation
  - Amplification of selected regions
    - Amplify DNA under appropriate cycling conditions for selective amplification of desired genomic regions
  - Quality Control
    - Check for efficiency of amplification across all regions prior to adaptor ligation

RxDxAdvisors

STOP if isolated library fails QC

MiSeq Workflow

DNA Isolation
(From Blood or FFPE)
1.5 hours
(15-30 min hands on)

PCR or Selective Pull-out
1.5 hours
(15-30 min hands on)

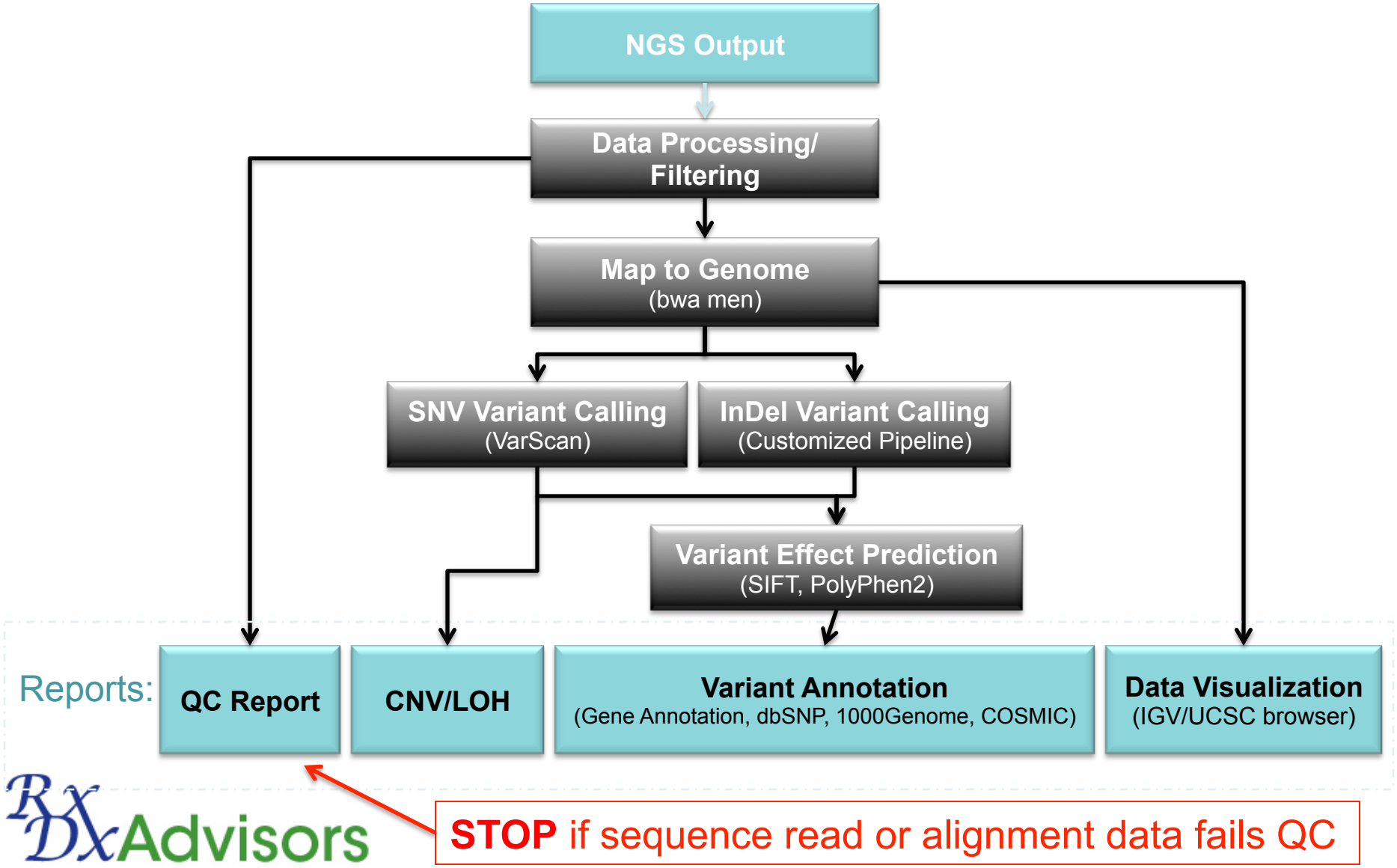
Cluster Generation and Paired-End Sequencing
22.6 hours*
(20 minutes hands on)

Alignment and Variant Calling
2 hours
(fully automated)

RxDxAdvisors

*2 x 100 bp run

Illumina NGS Bioinformatic Analysis



RxDxAdvisors

Ion AmpliSeq™ Cancer Workflow

01 Construct Library

Single tube amplification for hundreds of amplicons

3.5 hour library construction

Starting with 10ng of FFPE DNA

Comprehensive coverage of 739 known mutations

+ ION KITS

02 Prepare Template

Compatible with Ion OneTouch or Ion Xpress Template Kits

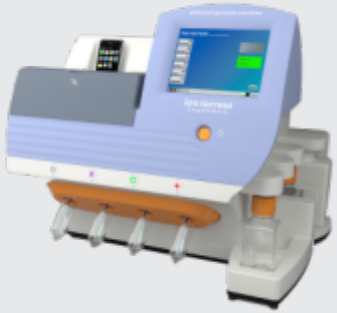
4 hour amplification

+ ION ONE-TOUCH™ SYSTEM

03 Run Sequence

1.5 hour sequencing run

Compatible with all Ion Chips



+ ION PGM™ SEQUENCER

04 Analyze Data

Single day workflow from DNA to Variants- 1 hour analysis

Ion AmpliSeq Variant Caller Software

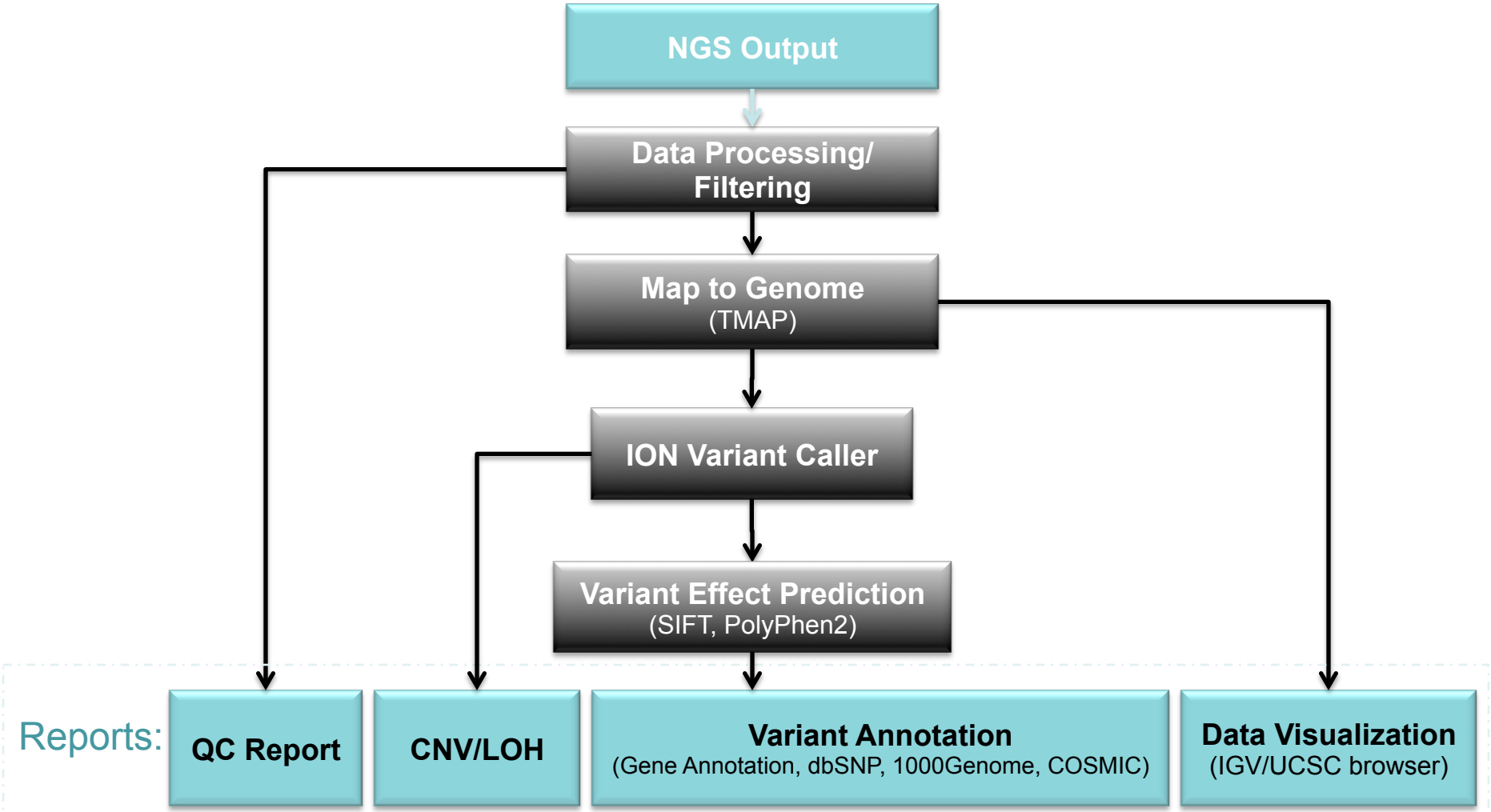
Detection of allele frequencies at 5% with 99% confidence

+ TORRENT SERVER

For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.

RXDXAdvisors

Ion Torrent NGS Bioinformatic Analysis



RxDxAdvisors

Clinical Whole Genome/Exome Sequencing

- Interpretation Challenges
  - There is no single “reference” genome to map individuals against
    - All healthy people have both benign single nucleotide and copy number variants
  - Curated clinical variant databases are continuously under development and bioinformatic analysis pipelines need to be updated regularly
  - Even with automated annotation tools, previously unknown variants continue to be discovered and require human intervention for interpretation

RxDxAdvisors

Interpretation of Sequencing Data

- Known Pathogenic
  - Sequence variation is previously reported and is a recognized cause of the disorder.
  - Reported when there exists credible documentation of a sequence variation and a clinical outcome.
  - At a minimum, this includes all of the known pathogenic variants in the 59 genes on the ACMG recommendation list.
- Likely Pathogenic
  - Sequence variation is previously unreported and is of the type which is expected to cause the disorder.
  - Insertions, deletions, frame shifts, and changes at the invariant splice site AG/GT nucleotides can disrupt normal protein synthesis or regulation of cellular processes such as transcription and translation.

American College of Medical Genetics Guidelines, 2008 and 2015

Interpretation of Sequencing Data

- Likely Benign
  - Sequence variation is previously unreported and is probably not causative of disease.
  - These variations represent base changes that do not change the coding sequence or effect known processing or regulatory pathways, or are found in addition to a known pathologic change (in autosomal dominant disorders).
- Benign
  - Sequence variation is previously reported and is a recognized neutral variant.
  - Reported when evidence is available that the sequence variation has been consistently observed in a normal population without association to disease or predisposition.

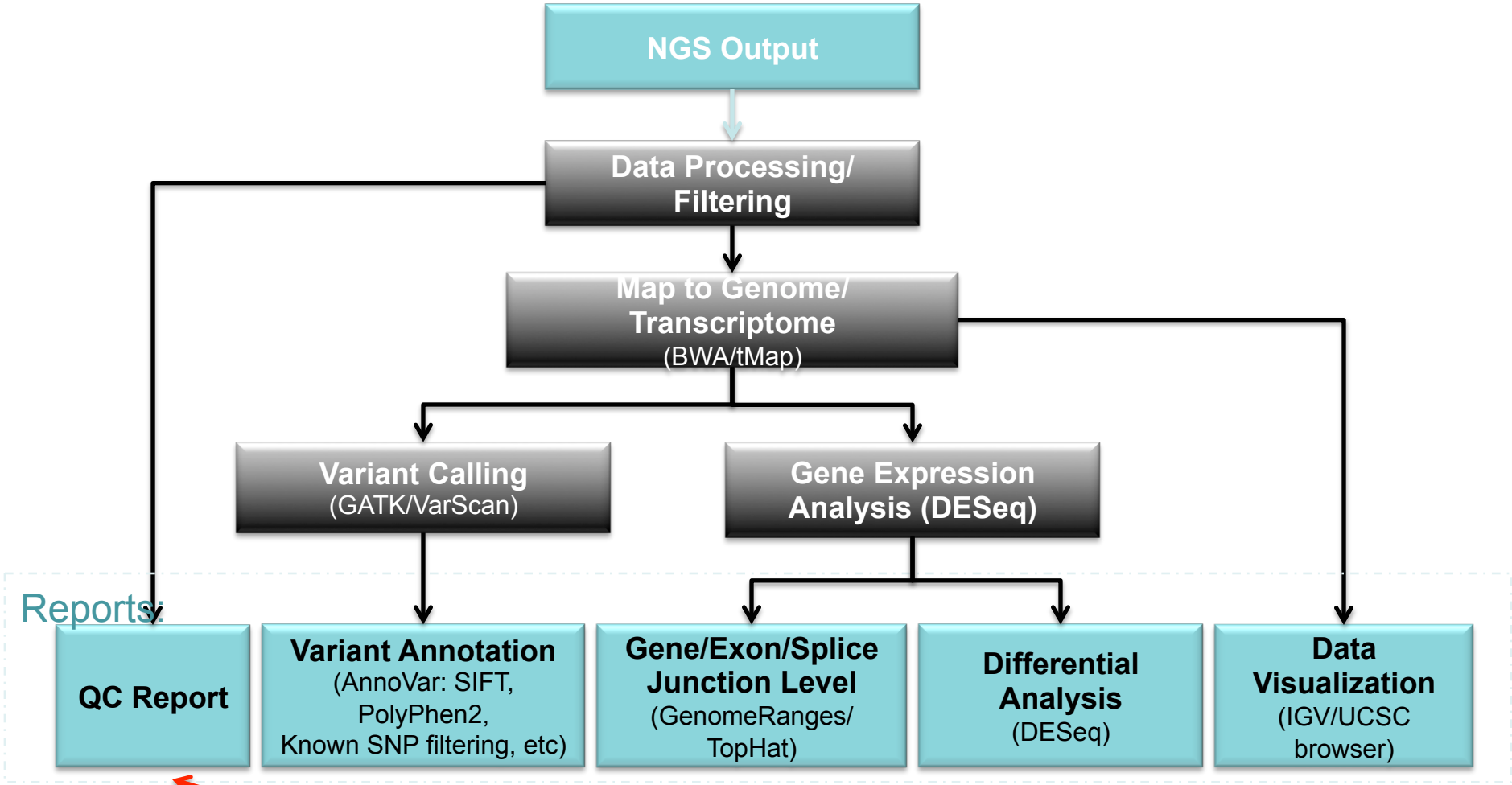
American College of Medical Genetics Guidelines, 2008 and 2015

Interpretation of Sequencing Data

- Uncertain Significance
  - Sequence variation is previously unreported and is of the type which may or may not be causative of the disorder.
  - Sequence variation is not known or expected to be causative of disease, but is found to be associated with a clinical presentation.
  - Missense changes, in-frame deletions or insertions, or variants in a splice consensus sequence or that may produce cryptic splice sites, affect regulatory processes, interrupt exonic splicing enhancers and suppressor sites, or participate in other mechanisms that may be associated with disease. Further analysis may be warranted to clarify the clinical significance of such changes.
  - Variants in this category have been suggested to contribute to disease as low-penetrance mutations, which alone or in combination may or may not predispose an individual to disease or modify the severity of a clinical presentation in complex disorders. Variants in this category should be reported with appropriate caveats that the variants are not definitive mutations and medical management decisions should not be made on the presence of the variants alone.

American College of Medical Genetics Guidelines, 2008 and 2015

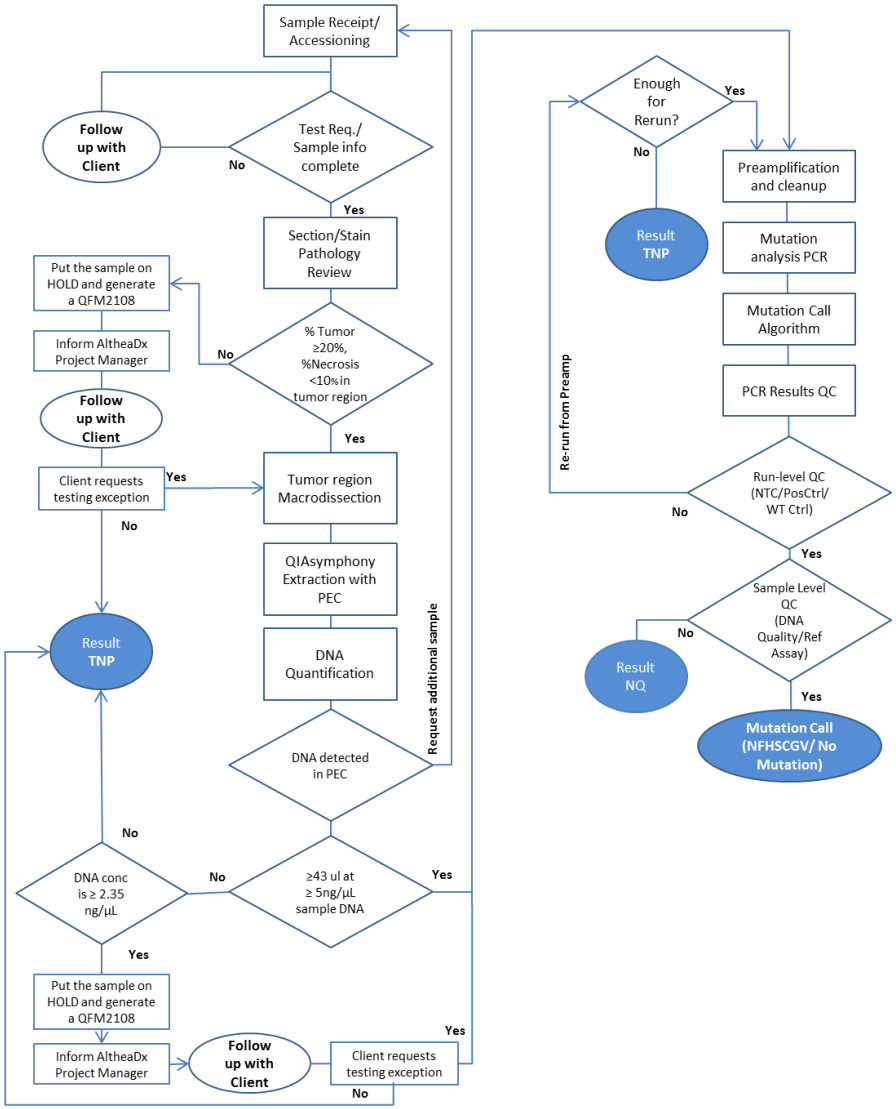
Gene Fusion Analysis Pipeline



STOP if sequence read or alignment data fails QC

RxDxAdvisors

CLIA Oncology NGS Workflow Example



RxDxAdvisors

2016 European Cost for Clinical Sequencing

Calculation of the NGS costs per sample (in Euros) for clinical grade sequencing

	Targeted Gene Panel	Whole Exome	Whole Genome
Sequencing platform	NextSeq500	HiSeq4000	HiSeqXS
Capital costs per year	47,073.80	205,847.04	1,288,702.85
Capital costs per sample	1.89	35.19	175.33
Maintenance costs per year	22,640.00	71,909.00	529,509.00
Maintenance costs per sample	0.91	12.29	72.04
Reagent costs per sample (including blood draw & DNA isolation)	289.35	601.09	1127.59
Lab personnel costs	8.97	70.08	70.08
Data processing, storage, interpretation & report costs	31.78	73.40	223.97
Total costs per sample	**€332.90**	**€791.75**	**€1669.02**

RxDxAdvisors

Van Nimwegen *et al.*, Clin. Chem. 62:1458-64 (2016)

Clinical Sequencing Lab Pricing - 2017

- HIV drug resistance mutations
  - CPT code 87901 - $359.69 (2016 fee schedule)
- Whole Exome (including interpretation)
  - Ambry Genetics - $5800
  - Personalis- $2000-3500
  - Invitae - $2500 (proband only), $4500 (duo or trio)
- Whole Genome (including interpretation)
  - Veritas – myGenome (personal genome, not clinical), $999
  - Illumina - TruGenome clinical genome, $9500
- Cancer Panels (including interpretation)
  - Myriad Genetics – BRCA1and BRCA2, Medicare CPT 81211, $2795
  - Invitae - Hereditary Breast Cancer Panel (14 genes) – Medicare CPT 81432, $925 (Palmetto GBA)
  - Foundation Medicine – FoundationOne Panel (343 genes) - $2800 average reimbursement

RxDxAdvisors