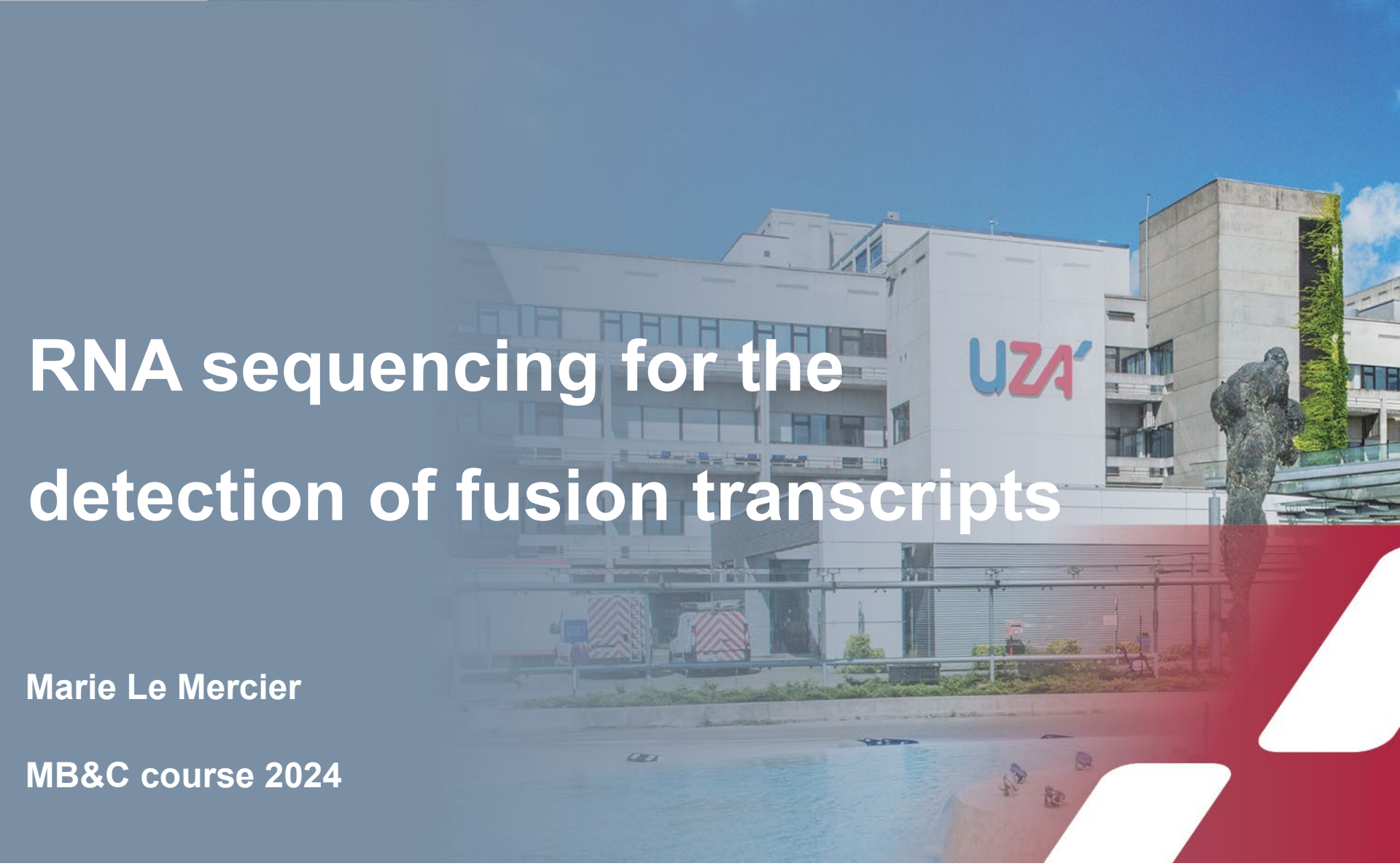
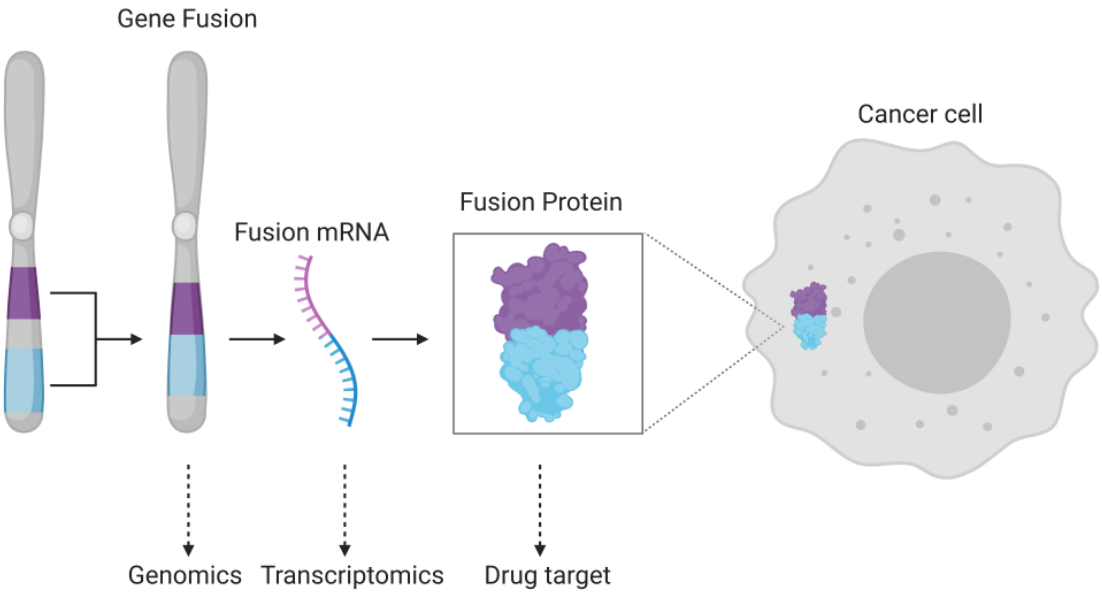


RNA sequencing for the detection of fusion transcripts

Marie Le Mercier

MB&C course 2024

Gene fusion in cancer



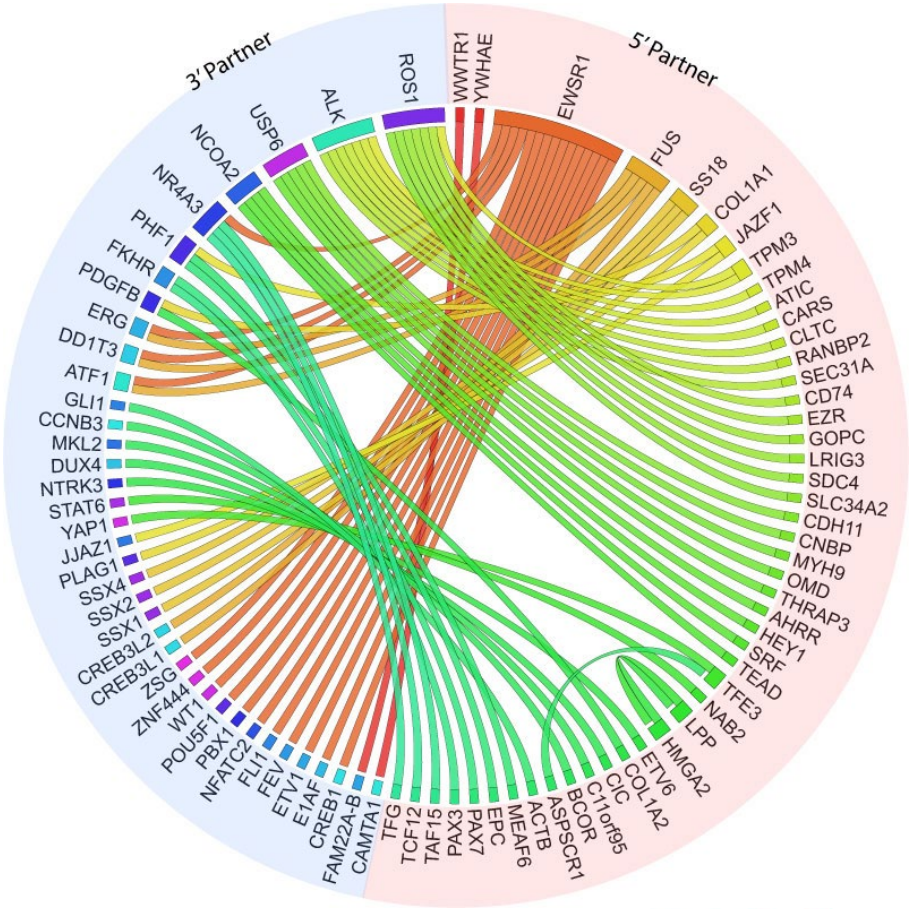
- Novel gene formed by fusion of two distinct wild type genes
- Produced by somatic genome rearrangements
- >10.000 gene fusions identified in human cancers
- Strong driver alterations

Gene fusions: Diagnostic markers

- Soft-Tissue Sarcomas
  - Many different translocations associated with different histological subtypes

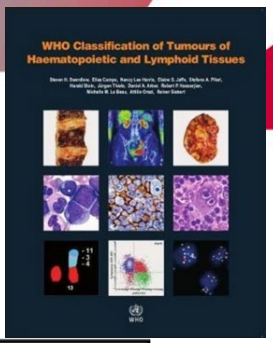
Table 2. Chromosomal Translocations in Soft-Tissue Sarcomas.*

Type of Tumor	Translocation	Genes Involved
Synovial sarcoma	t(X;18)(p11.2;q11.2)	*SSX1* or *SSX2*, *SYT*
Myxoid or round-cell liposarcoma	t(12;16)(q13;p11)	*CHOP*, *TLS*
	t(12;22)(q13;q11-q12)	*CHOP*, *EWS*
Ewing's sarcoma or peripheral primitive neuroectodermal tumor	t(11:22)(q24;q12)	*FLI1*, *EWS*
	t(21:22)(q22;q12)	*ERG*, *EWS*
	t(7;22)(p22;q12)	*ETV1*, *EWS*
	t(2;22)(q33;q12)	*FEV*, *EWS*
	t(17;22)(q12;q12)	*E1AF*, *EWS*
Desmoplastic small round-cell tumor	t(11;22)(p13;q12)	*WT1*, *EWS*
Alveolar rhabdomyosarcoma	t(2:13)(q35;q14)	*PAX3*, *FKHR*
	t(1;13)(p36;q14)	*PAX7*, *FKHR*
Extraskeletal myxoid chondrosarcoma	t(9;22)(q21-31;q12.2)	*CHN*, *EWS*
	t(9;17)(q22:q11)	*CHN*, *RBP56*
Clear-cell sarcoma	t(12;22)(q13;q12)	*ATF1*, *EWS*
Alveolar soft-part sarcoma	t(X;17)(p11;q25)	*TFE3*, *ASPL*
Dermatofibrosarcoma or giant-cell fibroblastoma	t(17;22)(q22;q13)	*COL1A1*, *PDGFB1*
Infantile fibrosarcoma	t(12;15)(p13;q25)	*ETV6*, *NTRK3*
Low-grade fibromyxoid sarcoma	t(7;16)(q34;p11)	*FUS*, *BBF2H7*



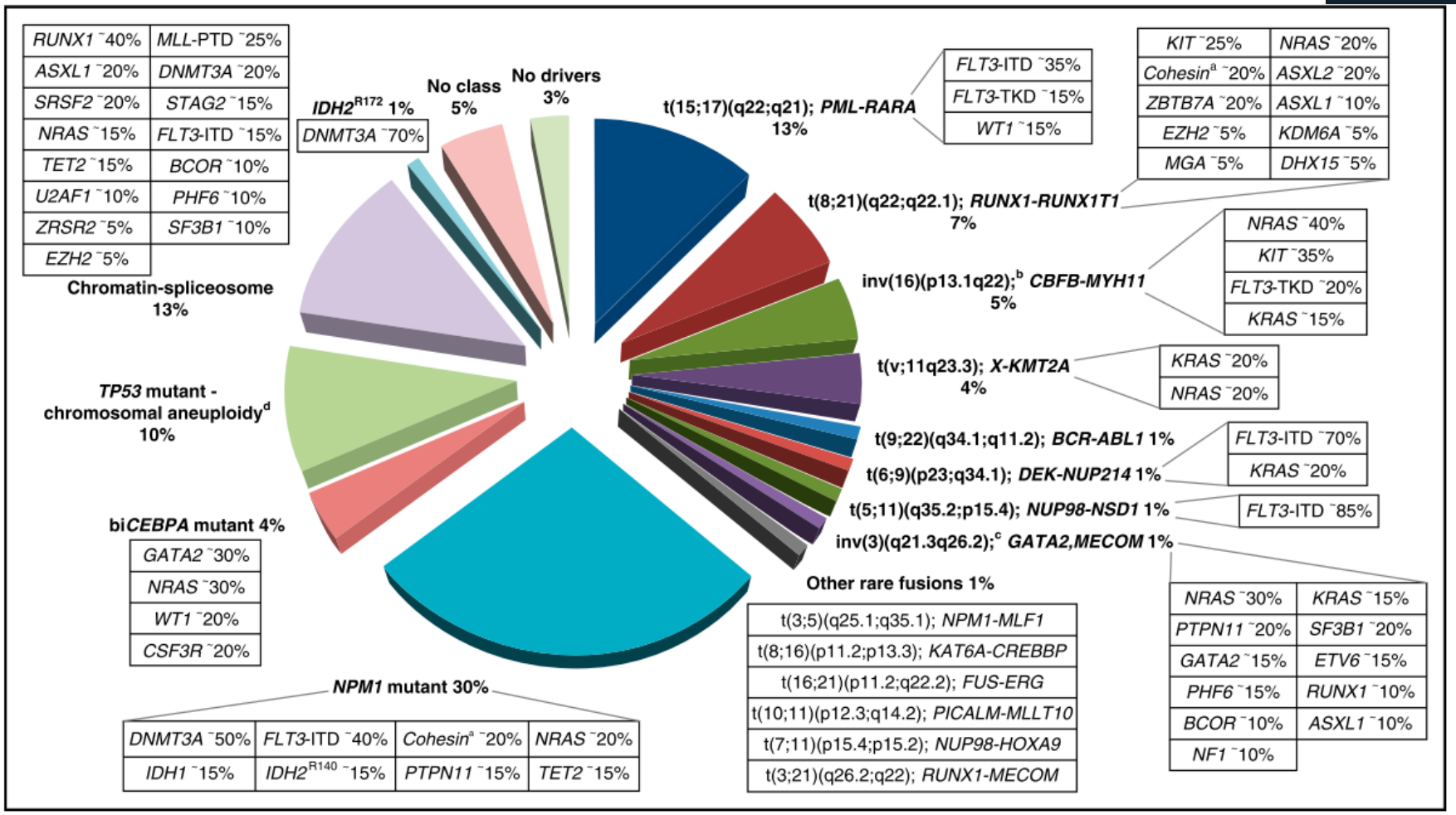
Clark et al., NEJM 2005

Gene fusions: Diagnostic/prognostic markers



- Genomic Classification in AML

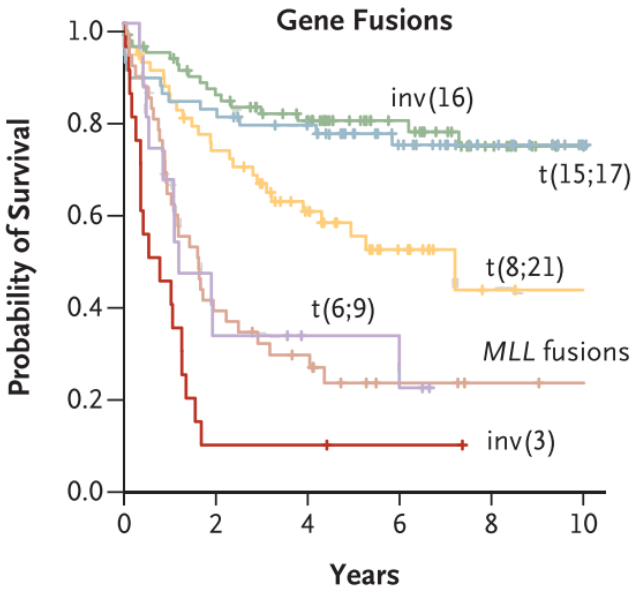
WHO 2017



Döhner et al., Blood 2017

Gene fusions: Diagnostic/prognostic markers

- Prognosis & Risk Category



Papaemmanuil et al., NEJM 2016

Risk Category[b]	Genetic Abnormality
Favorable	• t(8;21)(q22;q22.1)/*RUNX1*::*RUNX1T1*[b] • inv(16)(p13.1q22) or t(16;16)(p13.1;q22 • Mutated *NPM1*[b,d] without *FLT3*-ITD • bZIP in-frame mutated *CEBPA*[e]
Intermediate	• Mutated *NPM1*[b,d] with *FLT3*-ITD • Wild-type *NPM1* with *FLT3*-ITD • t(9;11)(p21.3;q23.3)/*MLLT3*::*KMT2A*[b,f] • Cytogenetic and/or molecular abnormalit
Adverse	• t(6;9)(p23;q34.1)/*DEK*::*NUP214* • t(v;11q23.3)/*KMT2A*-rearranged[g] • t(9;22)(q34.1;q11.2)/*BCR*::*ABL1* • t(8;16)(p11;p13)/*KAT6A*::*CREBBP* • inv(3)(q21.3q26.2) or t(3;3)(q21.3;q • t(3q26.2;v)/*MECOM*(*EVI1*)-rearrang • -5 or del(5q); -7; -17/abn(17p) • Complex karyotype,[h] monosomal karyotype[i] • Mutated *ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1, or ZRSR2*[j] • Mutated *TP53*[k]

Allogenic HCT not recommended

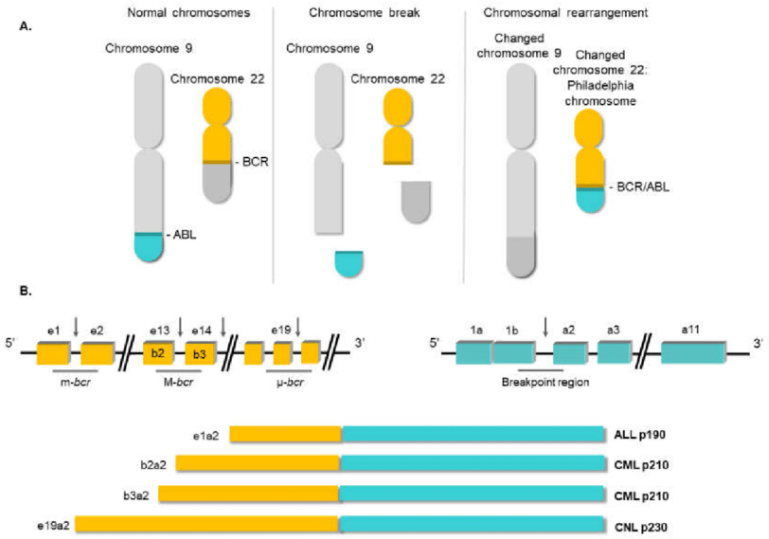
Allogenic HCT recommended for most cases

Allogenic HCT recommended

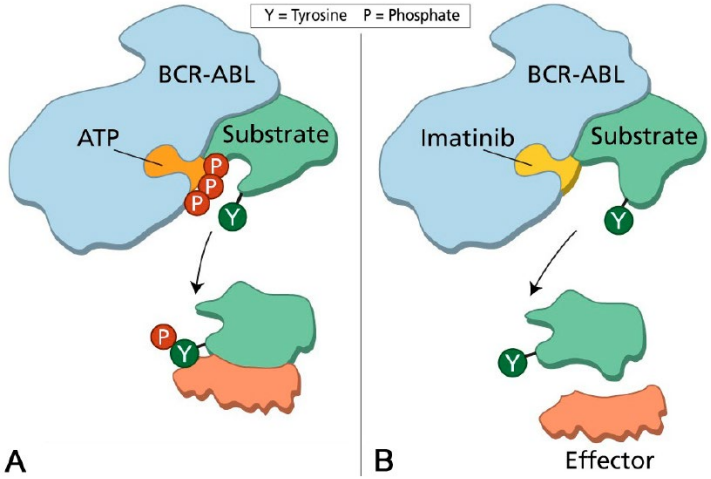
Döhner et al., Blood 2022

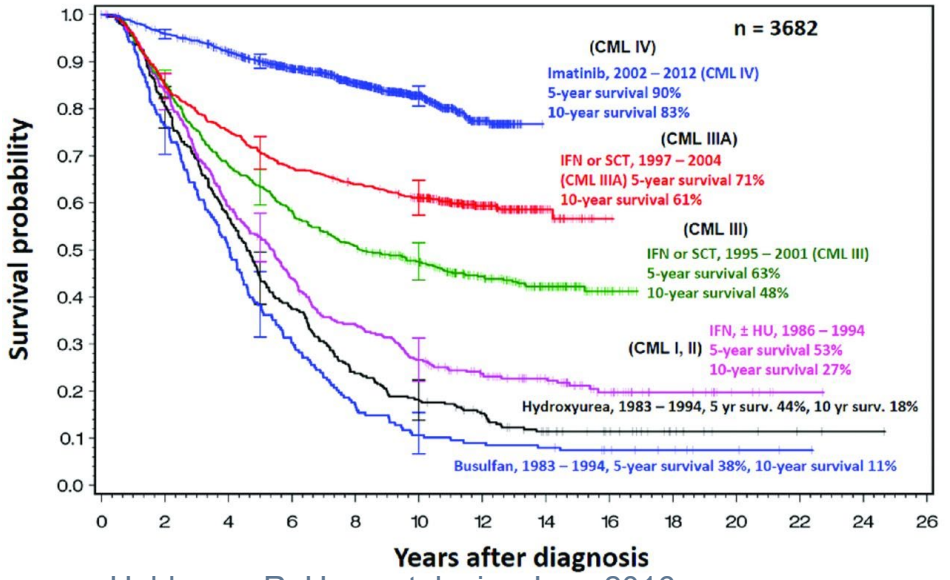
Gene fusions: Targetable alterations

- Imatinib for treatment of Phi+ CML/ALL



T(9;22) BCR-ABL1 - Philadelphia chromosom (Ph)

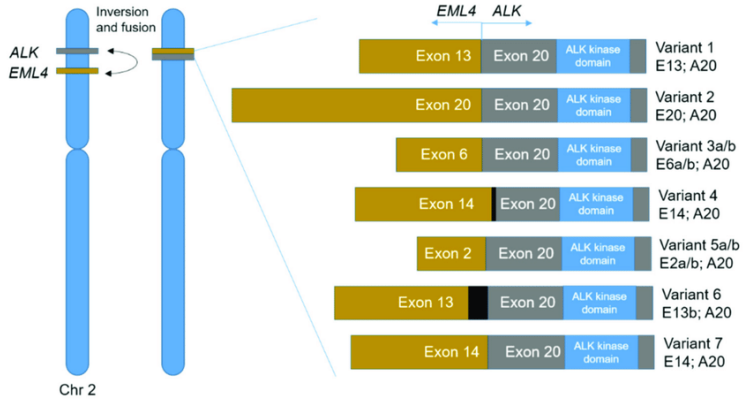


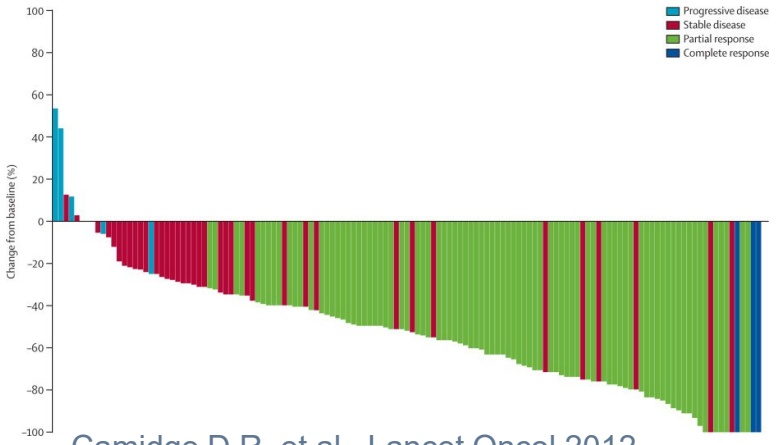


Hehlmann R. Haematologica June 2016

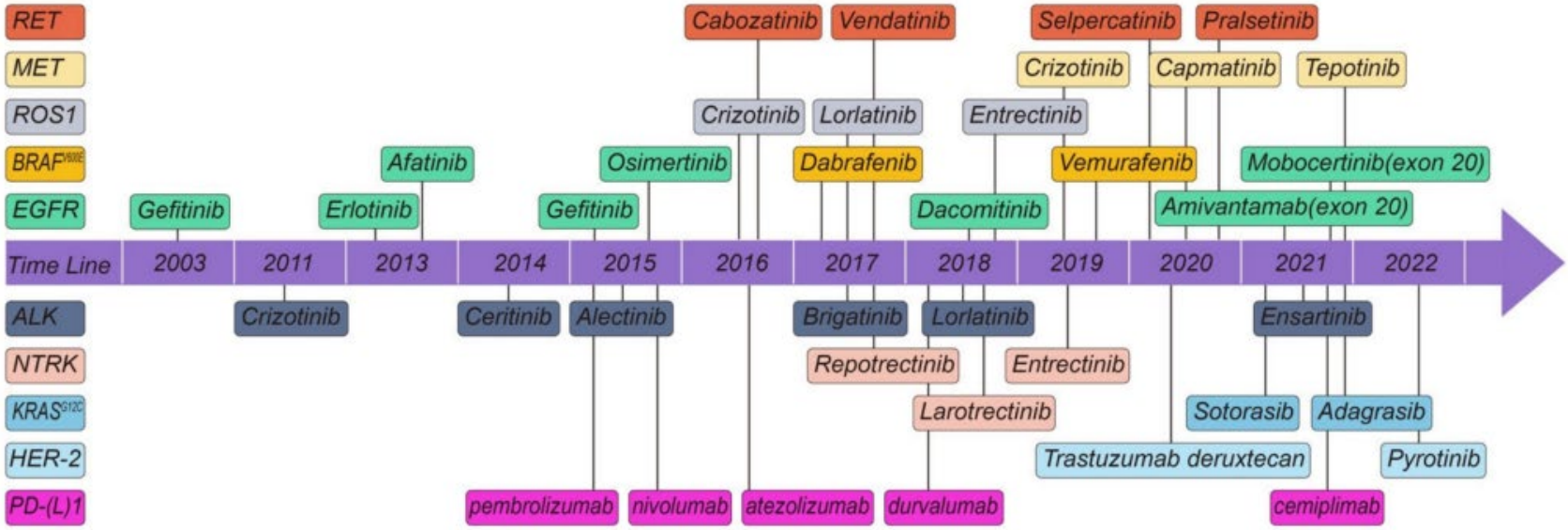
Gene fusions: Targetable alterations

- Non small cell lung cancer





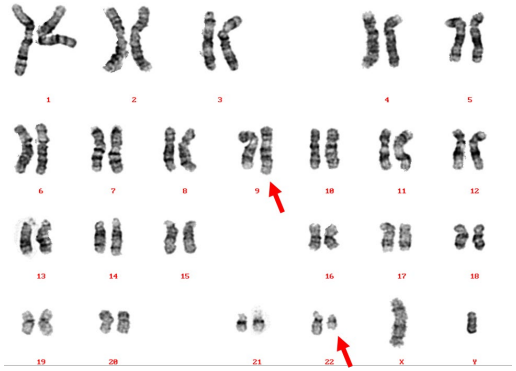
Camidge D.R. et al., Lancet Oncol 2012



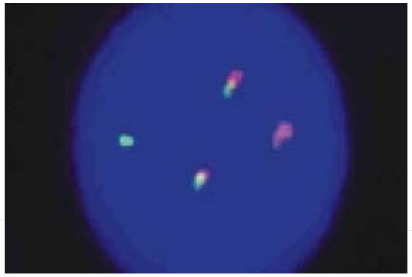
Guo H et al., Cells 2022

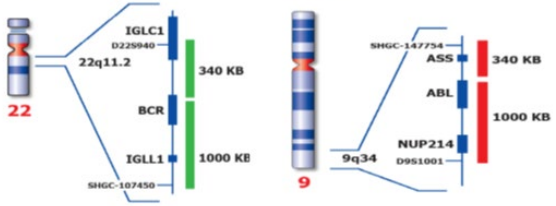
Classical detection methods

Karyotype



FISH





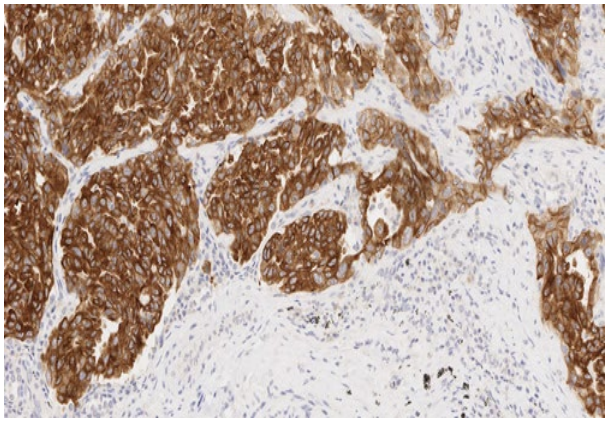
Karyotype & in situ-hybridization (F)ISH:

- Still Gold standard in certain discipline (BCR-ABL1, PML-RARA, ALK-EML4….)

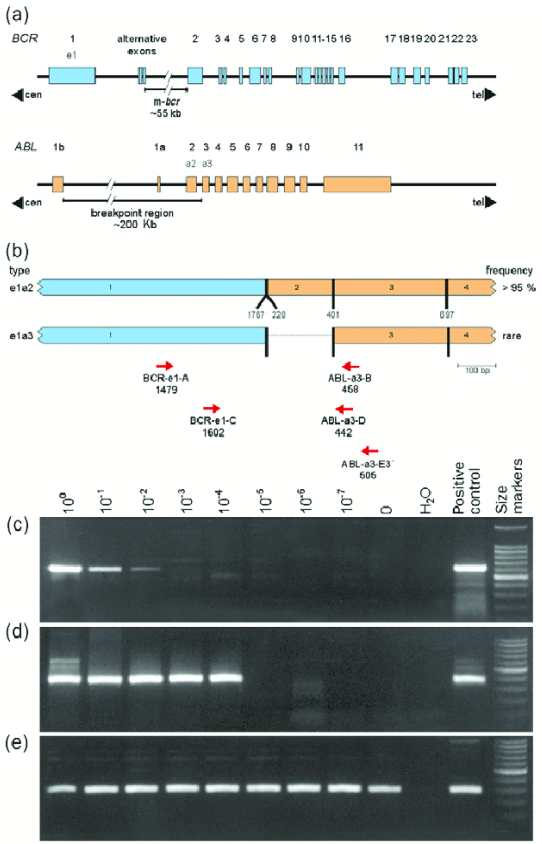
- Necessity of cell culture (Karyotype)
- One FISH per gene, Only for known targets
- Time consuming
- Cost

Classical detection methods

IHC



RT-PCR



- Fast, sensitive and affordable
- One target and only for Known targets

Translocation Screening

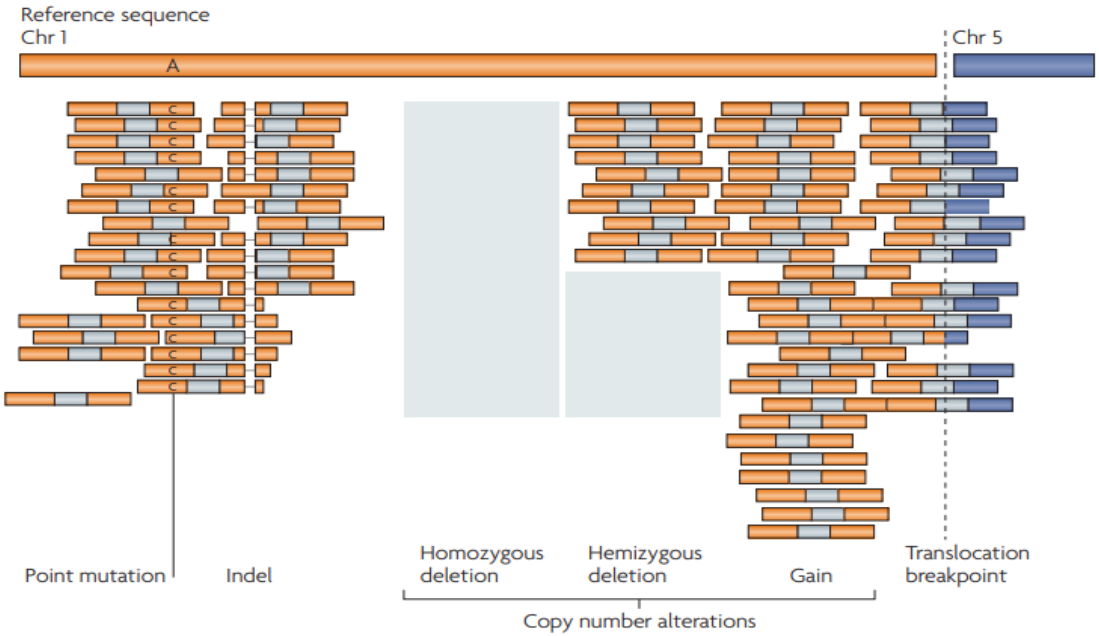
- RT-qPCR HemaVision®
  - CE/IVD Kit
  - Screening for 28 different fusion transcripts
  - Acute Leukemia (AML, ALL)

  - Still a limited in number of targets
  - Only Known fusion transcript

=> High Throughput = NGS

Tube	Translocation	Fusion Gene	Fw primer - Rev primer	Fluorochrome	
1	t(15;17)(q24;q21)	PML-RARA (bcr2, V)	PML ex5-RARA ex3	FAM	CY5
	inv(16)(p13;q22)	CBFB-MYH11	CBFB ex3-MYH11 ex30	ROX	CY5
2	inv(16)(p13;q22)	CBFB-MYH11	CBFB ex4-MYH11 ex34	FAM	CY5
	t(8;21)(q22;q22)	RUNX1-RUNX1T1	RUNX1 ex6-RUNX1T1 ex3	ROX	CY5
3	t(15;17)(q24;q21)	PML-RARA (bcr1, L)	PML ex6a-RARA ex3	FAM	CY5
	t(9;11)(p21.3;q23.3)	KMT2A-MLLT3	KMT2A ex7-MLLT3 ex7	ROX	CY5
4	t(15;17)(q24;q21)	PML-RARA (bcr3, S)	PML ex3-RARA ex3	FAM	CY5
	t(9;11)(p21.3;q23.3)	KMT2A-MLLT3	KMT2A ex8-MLLT3 ex11	ROX	CY5
5	t(11;19)(q23.3;p13.1)	KMT2A-ELL	KMT2A ex7-ELL ex3	FAM	CY5
	t(16;21)(p11;q22)	FUS-ERG	FUS ex6-ERG ex12	ROX	CY5
6	t(12;22)(p13;q11-12)	ETV6-MN1	ETV6 ex2-MN1 ex2	FAM	CY5
	t(6;9)(p23;q34)	DEK-NUP214	DEK ex9-NUP214 ex19	ROX	CY5
7	Reference gene	GUS	GUS ex11-GUS ex12	FAM	CY5
8	Reference gene	B2M	B2M ex2-B2M ex4	FAM	CY5
9	t(1;11)(p32;q23.3)	KMT2A-EPS15	KMT2A ex8+9-EPS15 ex3	FAM	CY5
	t(6;11)(q27;q23.3)	KMT2A-AFDN	KMT2A ex8+9-AFDN ex2	ROX	CY5
10	t(1;19)(q23;p13)	TCF3-PBX1	TCF3 ex16-PBX1 ex3	FAM	CY5
	t(12;21)(p13;q22)	ETV6-RUNX1	ETV6 ex5-RUNX1 ex4b	ROX	CY5
11	t(11;19)(q23.3;p13.3)	KMT2A-MLLT1	KMT2A ex8+9-MLLT1 ex2	FAM	CY5
	t(4;11)(q21;q23.3)	KMT2A-AFF1	KMT2A ex8+9-AFF1 ex9	ROX	CY5
12	t(17;19)(q22;p13)	TCF3-HLF	TCF3 ex14-HLF ex4	FAM	CY5
	del(1)(p32)	STIL-TAL1	STIL ex1-TAL1 ex2	ROX	CY5
13	t(9;22)(q34;q11)	BCR-ABL1 (m-bcr, P190)	BCR ex1-ABL1 ex3	FAM	CY5
	t(9;9)(q34;q34)	SET-NUP214	SET ex9-NUP214 ex19	ROX	CY5
14	t(11;19)(q23.3;p13.3)	KMT2A-MLLT1	KMT2A ex7-MLLT1 ex9	FAM	CY5
	t(9;22)(q34;q11)	BCR-ABL1 (M-bcr, P210)	BCR ex12-ABL1 ex3	ROX	CY5
15	t(9;22)(q34;q11)	BCR-ABL1 (µ-bcr, P230)	BCR ex19-ABL1 ex3	FAM	CY5
	t(11;17)(q23;q21)	ZBTB16-RARA	ZBTB16 ex3-RARA ex3	ROX	CY5
16	Reference gene	ABL1	ABL1 ex2-ABL1 ex3	FAM	CY5
17	t(9;12)(q34;p13)	ETV6-ABL1	ETV6 ex2+5-ABL1 ex3	FAM	CY5
	t(5;12)(q33;p13)	ETV6-PDGFRB	ETV6 ex2+5-PDGFRB ex12	ROX	CY5
18	t(10;11)(p12;q23.3)	KMT2A-MLLT10	KMT2A ex8+9-MLLT10 ex18	FAM	CY5
	t(1;11)(q21;q23.3)	KMT2A-MLLT11	KMT2A ex8+9-MLLT11 ex2	ROX	CY5
19	t(X;11)(q13;q23.3)	KMT2A-FOXO4	KMT2A ex7-FOXO4 ex2	FAM	CY5
	t(11;17)(q23.3;q21)	KMT2A-MLLT6	KMT2A ex7-MLLT6 ex12	ROX	CY5
20	t(3;21)(q26;q22)	RUNX1-MECOM	RUNX1 ex6-MECOM ex2	FAM	CY5
	t(10;11)(p12;q23.3)	KMT2A-MLLT10	KMT2A ex7-MLLT10 ex7	ROX	CY5
21	t(5;17)(q35;q21)	NPM1-RARA	NPM1 ex4-RARA ex3	FAM	CY5
	t(3;5)(q25.1;q35)	NPM1-MLF1	NPM1 ex4-MLF1 ex3	ROX	CY5
22	t(10;11)(p12;q23.3)	KMT2A-MLLT10	KMT2A ex7-MLLT10 ex11	FAM	CY5
	t(3;21)(q26;q22)	RUNX1-MECOM	RUNX1 ex6-MECOM ex6	ROX	CY5
23	t(10;11)(p12;q23.3)	KMT2A-MLLT10	KMT2A ex8-MLLT10 ex10	ROX	CY5
24	-	-	-	-	-

NGS: DNA Sequencing



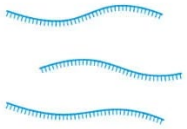
Meyerson et al., Nat Rev Genet 2010

- Large introns
- Repetitive sequences
- No discrimination between expressed and unexpressed gene fusions

NGS: RNA Sequencing -> cDNA sequencing

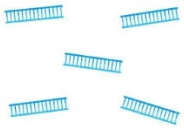
RNA Sequencing

1. Isolate RNA from samples

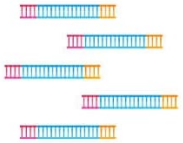


2. Fragment RNA into short segments

3. Convert RNA fragments into cDNA

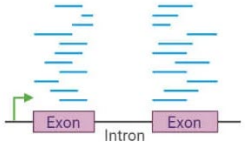


4. Ligate sequencing adapters and amplify

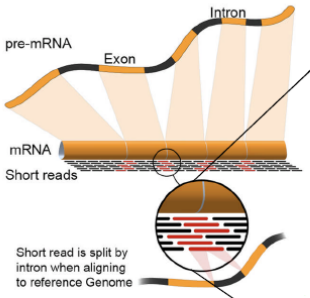


5. Perform NGS sequencing

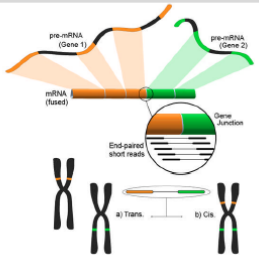
6. Map sequencing reads to the transcriptome/genome



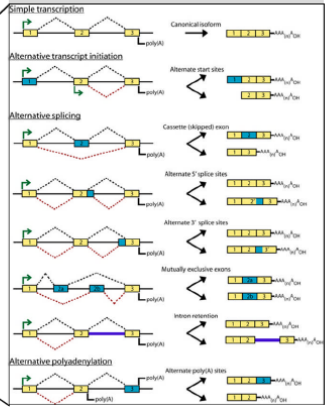
Gene Expression



Gene Fusions

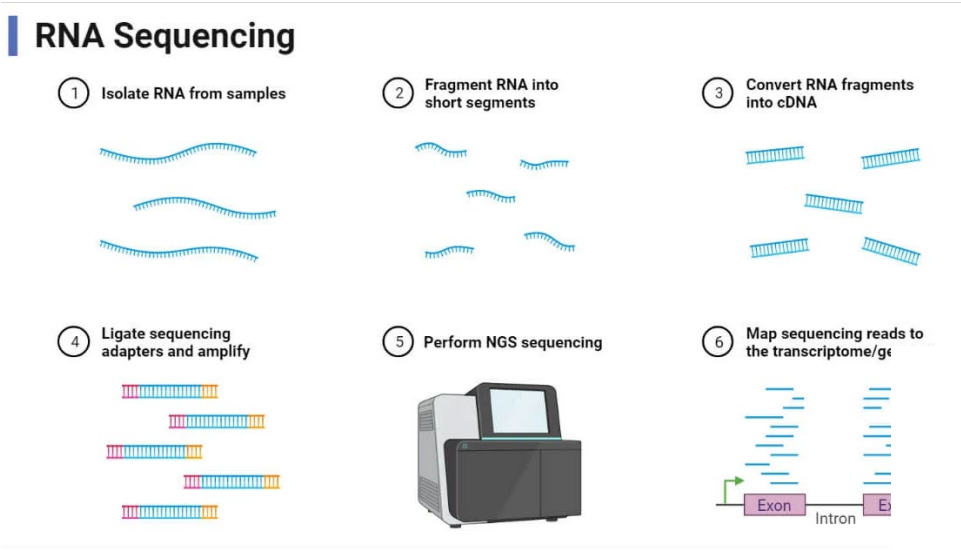


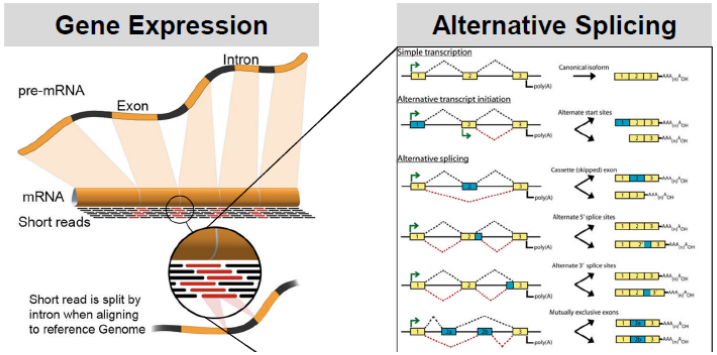
Alternative Splicing

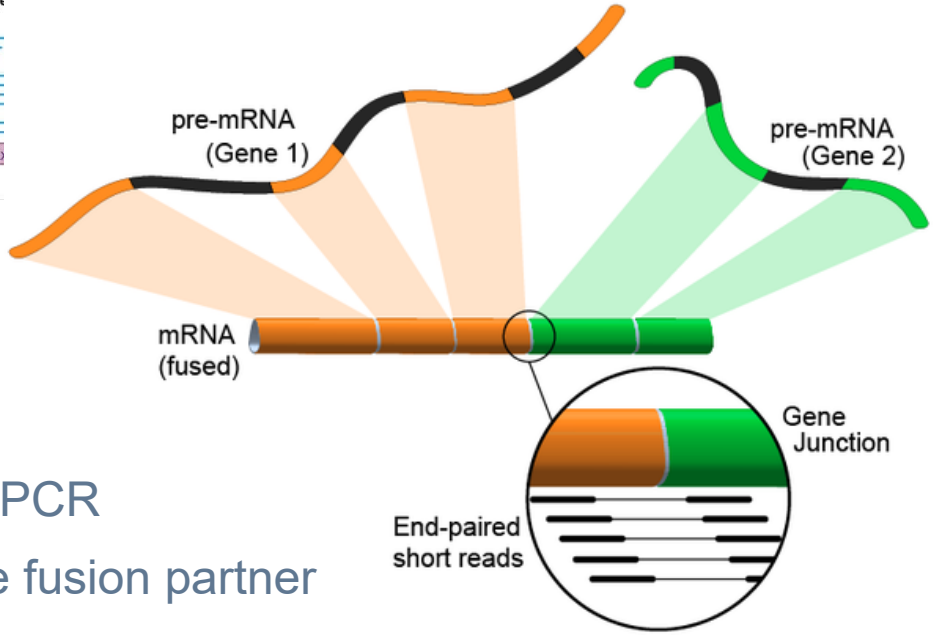


And RNA-editing, Allele specific expression, Transcript Discovery…

NGS: RNA Sequencing -> cDNA sequencing

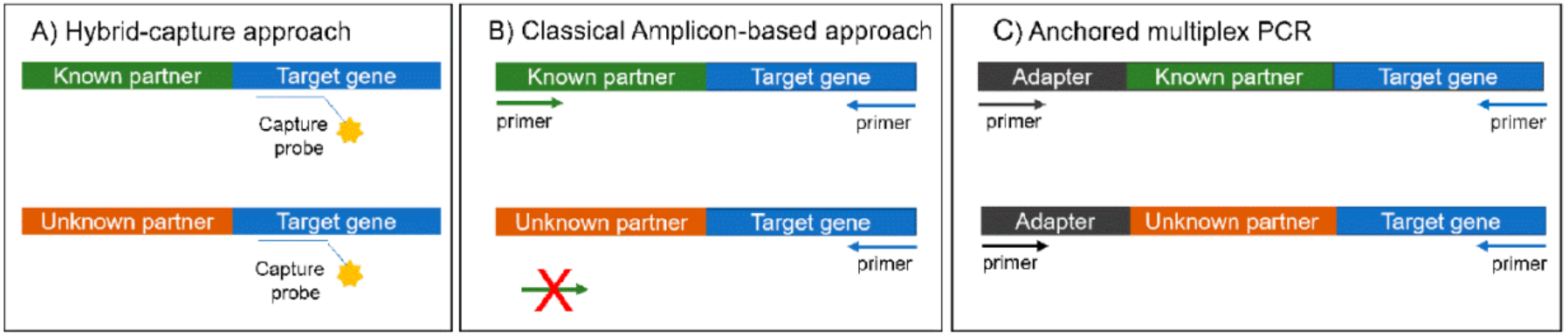






- RNAseq (Targeted)
  - Much bigger panels than with RT-PCR
  - Not always necessary to know the fusion partner

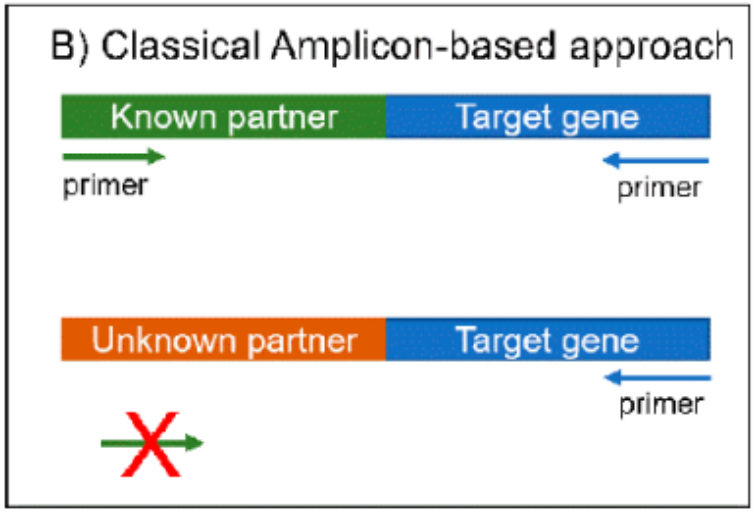
RNAseq Targeted panels



Bruno R et al., Diagnostics 2020

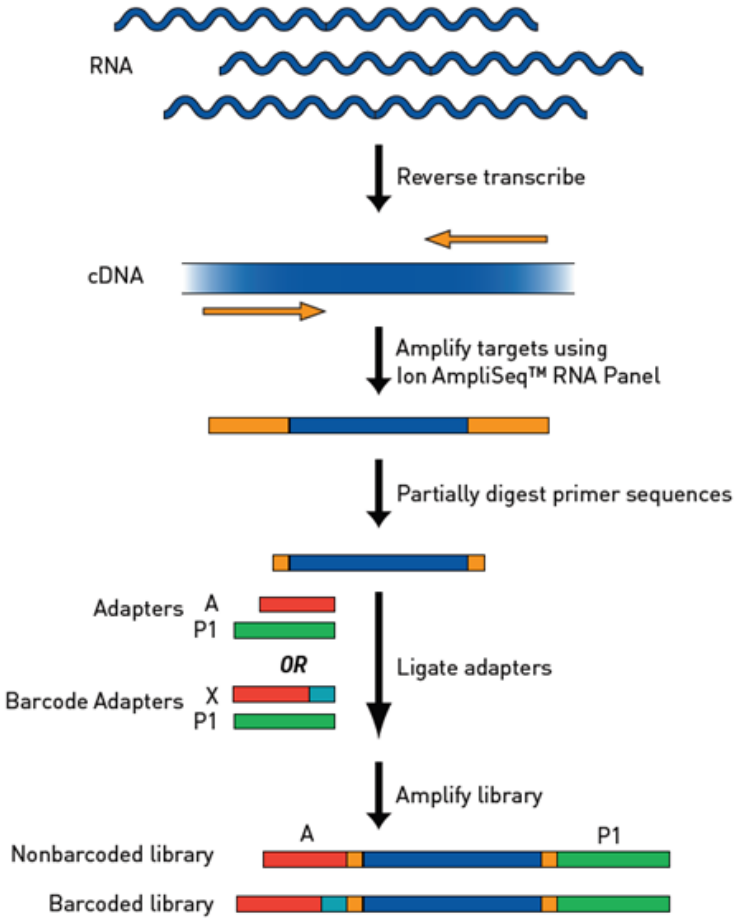
- Large choice of commercially available panels.
  - Amplicon-based approach = AmpliSeq (Thermofischer)…
  - Anchored multiplex PCR = Archer (IDT Technologies)…
  - Hybrid-Capture = Trusight (Illumina), SureSelect (Agilent)….

Amplicon-based approach



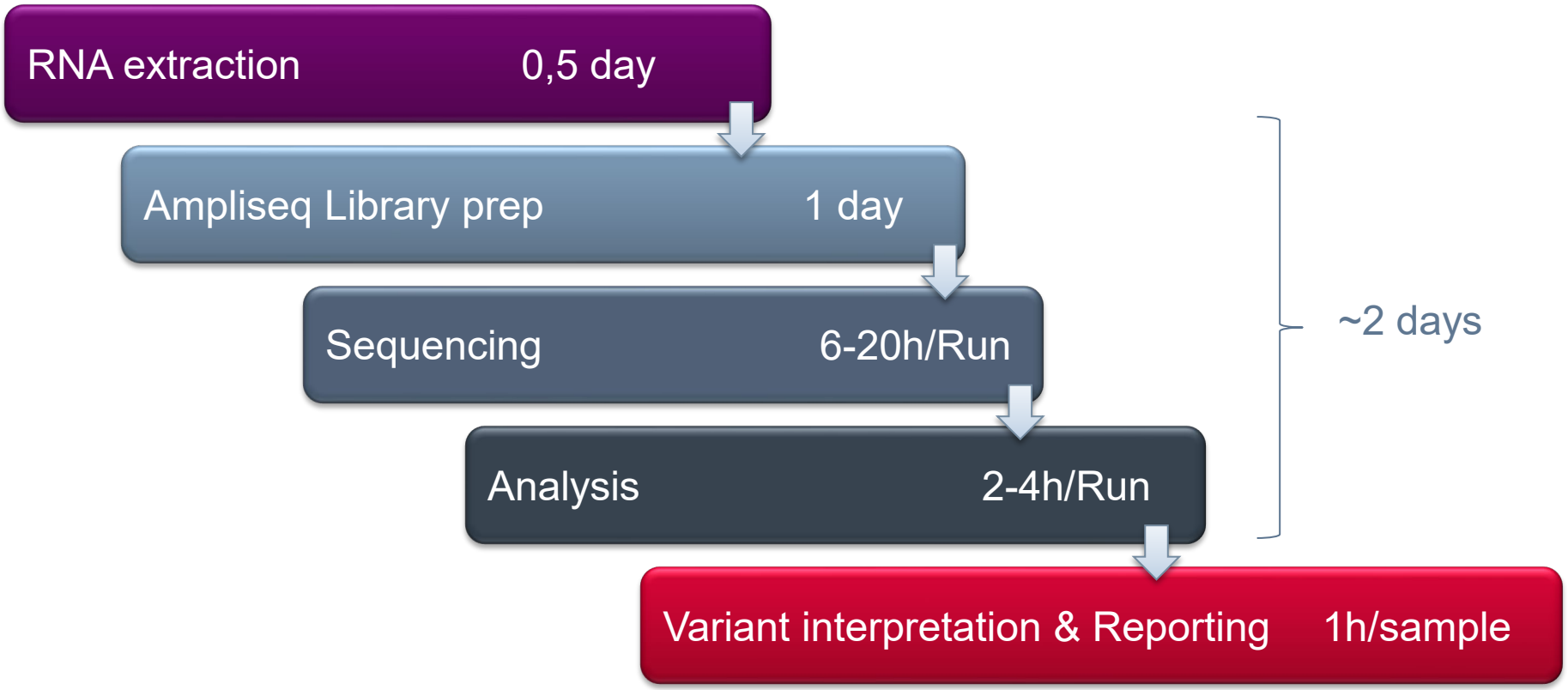
- Based on multiplex RT-PCR
- Primers flanking exons fusion combinations
- Detections of known fusion transcripts (both partners)
- Commercial & Custom panels

Amplicon-based approach: AmpliSeq™



- Sequencing on ThermoFischer or on Illumina platforms (AmpliSeq for Illumina)
- Several commercial (CE/IVD) panels
- Possible custom panels
- Include 5 positives expression controls
  - control of RNA quality and assay performance
- Expression imbalances between 3' and 5' regions
  - Detection of new fusion transcripts
- **Low input (from 10 ng RNA)**

Amplicon-based approach: AmpliSeq™



Analysis

Analysis Results

Download | Visualize | Select

Analysis Name: Ct_LFP_Hor_RNA **Fusion Sample QC**: PASS,[TotalMappedFusionPanelReads>20000... **Fusion Overall Call**: POSITIVE ,[3pGene=RET,IsoformsDetected=C... **Total Mapped Fusion Panel Reads**: 139059 **Total Unmapped Reads**: 2778

Fusions

Search | Go | Preferences

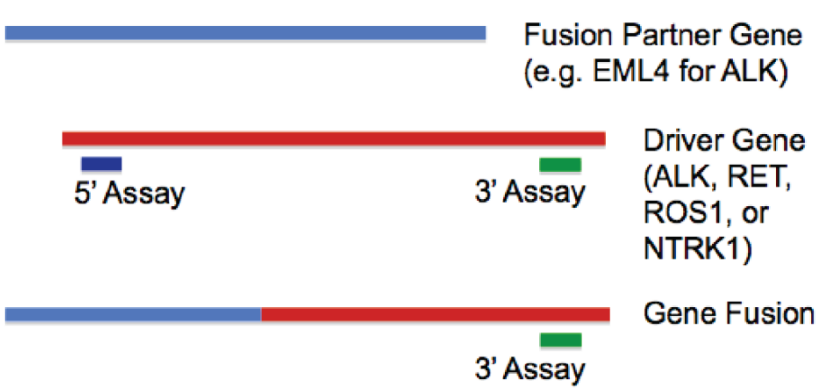
Classification	Locus	Type	Genes (Exons)	Read Counts	Detection	3'/5' Imbalance	COSMIC/NCBI	Variant ID	Read Counts Per Million
Unclassified	chr4:25665952 - chr6:117650609	FUSION	SLC34A2(4) - ROS1(32)	18944	Present		COSF1197	SLC34A2-ROS1.S4R32.COSF1197	136229.945563
Unclassified	chr10:61665879 - chr10:43612031	FUSION	CCDC6(1) - RET(12)	5165	Present		COSF1271	CCDC6-RET.C1R12.COSF1271	37142.507856
Unclassified	chr2:42522656 - chr2:29446394	FUSION	EML4(13) - ALK(20)	2482	Present		COSF408.1	EML4-ALK.E13A20.COSF408.1	17848.539109
Unclassified	chr4:25665952 - chr6:117645578	FUSION	SLC34A2(4) - ROS1(34)	1149	Present		COSF1198	SLC34A2-ROS1.S4R34.COSF1198	8262.67987
Unclassified	chr6:170871321	EXPR_CONTI	TBP	23122	Present			TBP.ENCTRL.E3E4	166274.74669
Unclassified	chr12:53585786	EXPR_CONTI	ITGB7	323	Present			ITGB7.ENCTRL.E14E15	2322.75509
Unclassified	chr8:128751265	EXPR_CONTI	MYC	22329	Present			MYC.ENCTRL.E2E3	160572.131254
Unclassified	chr11:118960975	EXPR_CONTI	HMBS	1919	Present			HMBS.ENCTRL.E8E9	13799.897885
Unclassified	chr1:156104319	EXPR_CONTI	LMNA	33742	Present			LMNA.ENCTRL.E3E4	242645.208149
Unclassified	chr10:43606730, chr10:43622086	ASSAYS_5F	RET	906,7168	NoCall	0.076896		RET.5p_NM_020975.4.e6e7,RET.3p_NM_020975.4.	6515.220158,51546.465889
Unclassified	chr1:156834532, chr1:156851323	ASSAYS_5F	NTRK1	10,78	NoCall	8.35E-4		NTRK1.5p.eNST00000392302.e2e3,NTRK1.3p.eNS	71.911922,560.912994
Unclassified	chr2:29551347, chr2:29430138	ASSAYS_5F	ALK	0,1949	Present	0.023933		ALK.5p_NM_004304.4.e5e6,ALK.3p_NM_004304.4	0.0,14015.633652
Unclassified	chr6:117711009, chr6:117632280	ASSAYS_5F	ROS1	924,18849	NoCall	0.220114		ROS1.5p_NM_002944.2.e11e12,ROS1.3p_NM_002	6644.661618,135546.782301

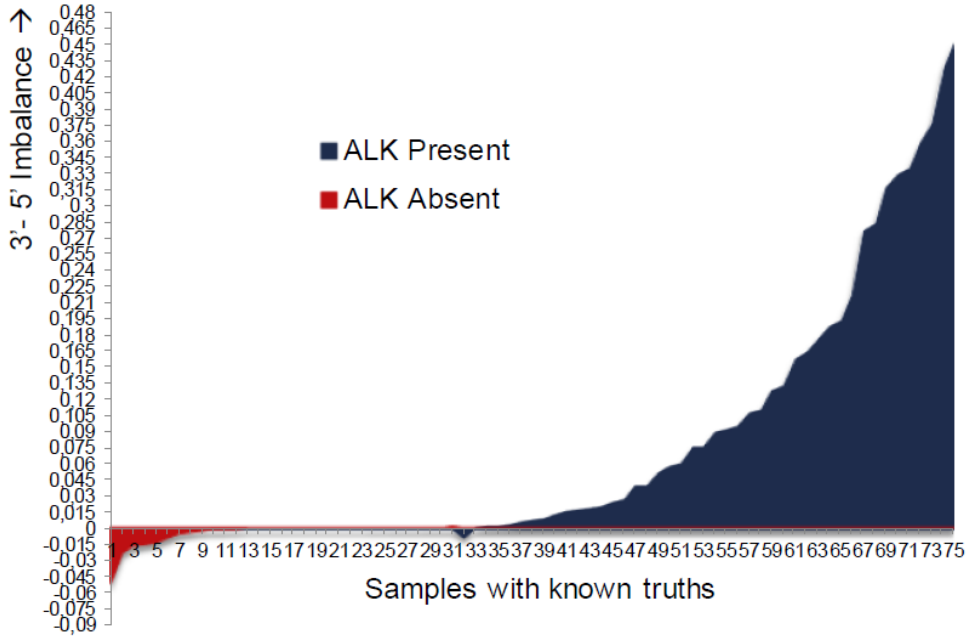
1 | 20 items per page

1 - 13 of 13 items

Expression Imbalance

ALK 3'-5' Imbalance defined as:
[(3' ALK count -5' ALK count) /Sum of Expression Control Genes counts]





Gene	No Evidence of a Fusion	Uncertain	Strong Evidence of a Fusion
ALK	≤0.001	0.001-0.025	≥0.025
RET	≤0.03	0.03-0.045	≥0.045
ROS1	≤0.2	0.2-0.5	≥0.5

Expression Imbalance

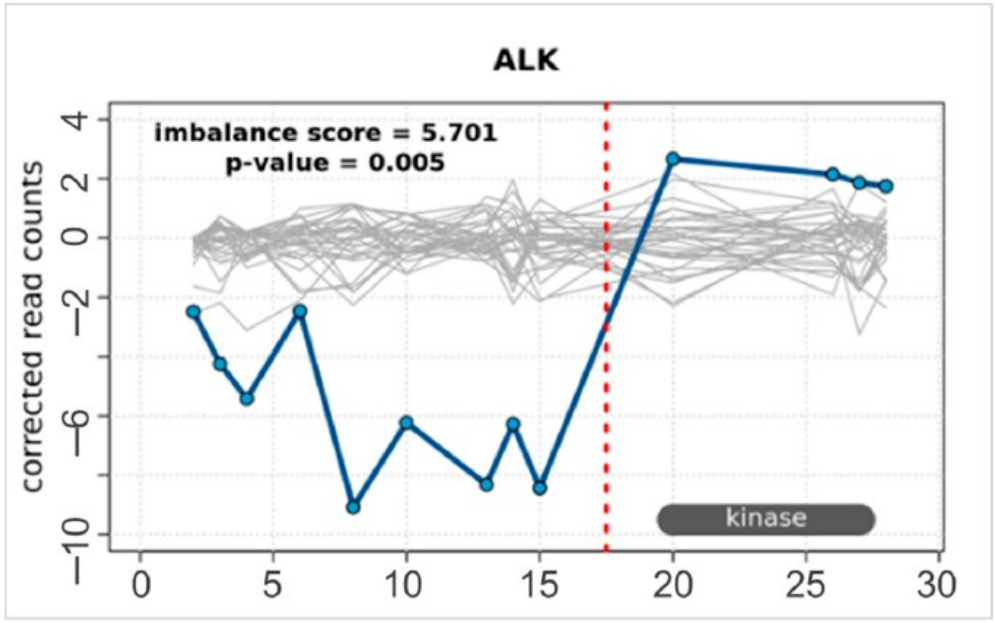


Figure 4. Detection of a novel ALK fusion. Exon-tiling imbalance in ODxET was able to detect a novel *ALK* fusion in a NSCLC sample pre-characterized as *ALK*-positive in Valencia using the Oncomine Precision Assay.

Normanno et al., Int J Mol Sciences 2023.

Amplicon-based approach: AmpliSeq™

- AmpliSeq Lung Fusion Panel
  - Sensitivity/specificity >90%

Table 1 Targeted Partners for *ALK*, *RET*, *ROS1*, and *NTRK1*

ALK	RET	ROS1	NTRK1
EML4	KIF5B	CD74	CEL
KIF5B	CCDC6	SDC4	NFASC
KLC1	CUX1	SLC34A2	IRF2BP2
HIP1		EZR	TFG
TPR		TPM3	SQSTM1
		LRIG3	SSBP2
		GOPC	CD74
			DYNC2H1
			MPRIP

50 FFPE NSCLC samples
(10ng of RNA – minimum 2,5ng)

	Concordance with Ref Meth	sensitivity	Specificity
ALK	95%	93% (26/28)	100% (15/15)
ROS1	100%	100% (7/7)	100% (15/15)

Pfarr et al., Genes, Chromosomes & cancers 2016

138 clinical samples (10ng of RNA)

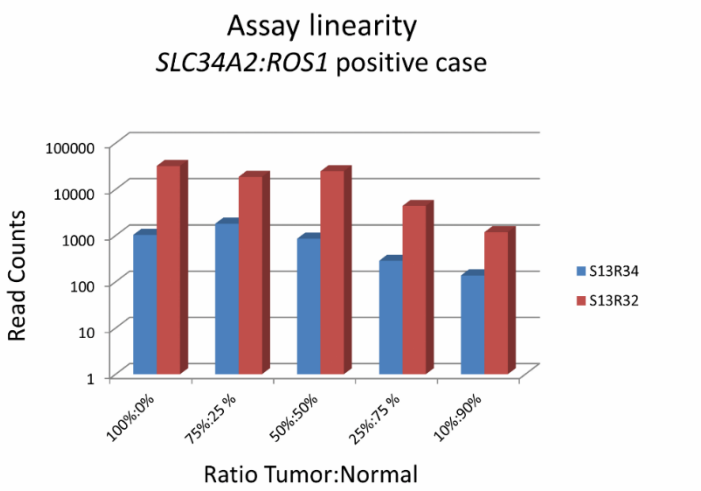
	Concordance with FISH	sensitivity	Specificity
ALK	97%	93,3% (24+4/30)*	98,6% (69/70)
ROS1	95%	75% (3/4)	100% (18/18)
RET	93%	100% (1/1)	93% (13/14)*

* From 3'/5' imbalance results

Vaughn et al., BMC Cancer 2018

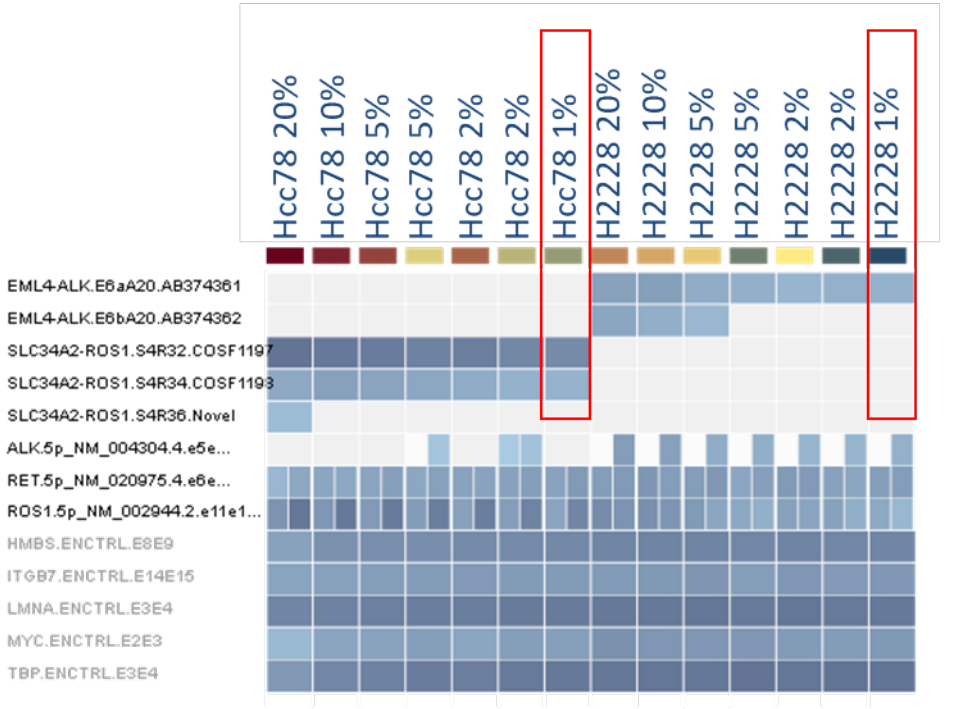
Amplicon-based approach: AmpliSeq™

- Serial dilution of a positive sample with 50% tumor cells in normal control tissue (50% - 5%)



		SLC34A2:ROS1				
Ratio Tumor:Normal	Tumor Cell Content	S13R34 Reads	S13R32 Reads	Total Reads	3p5p	3p5p comp.
100%:0%	50%	1033	31965	357150	5.026	15.027
75%:25 %	37,5%	1800	18602	395231	0.243	1.845
50%:50%	25.0%	854	24592	380026	0.162	1.358
25%:75 %	12.5%	284	4365	250985	0.054	0.907
10%:90%	5.0%	137	1181	331860	0.007	0.493

- Dilution of positives cell lines
  - H2228 : ALK positive cell line
  - Hcc78 : ROS1 positive cell line



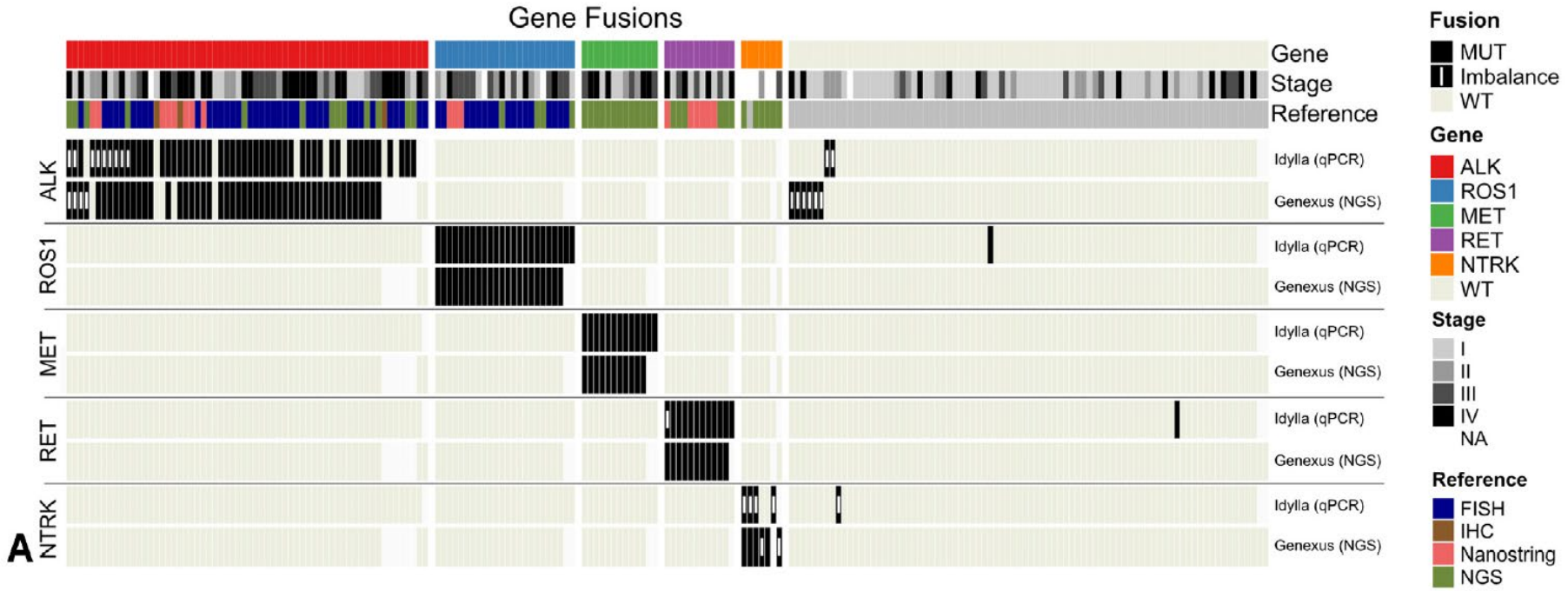
Pfarr et al., Genes, Chromosomes & cancers 2016

Amplicon-based approach: Oncomine on Genexus

- Fully Automated from library prep to analysis
- TAT 24h
- Several Oncomine panels (some with CE/IVD label)

Amplicon-based approach: Oncomine on Genexus

- Oncomine Precision Assay on Genexus
  - 188 NSCLC from five academic hospitals
  - Comparison of NGS with the standard procedures



Hofman et al., JTO clinical and Research reports 2022

Amplicon-based approach: Oncomine on Genexus

- Oncomine Precision Assay on Genexus

Table 2. Diagnostic Performance Summary

Performance According to Biomarkers	Idylla	Idylla (No Imbalance)	Genexus	Genexus (No Imbalance)
Accuracy (95% CI)	0.923 (0.88-0.96)	0.867 (0.81-0.91)	0.931 (0.89-0.96)	0.931 (0.89-0.96)
Sensitivity (95% CI)	0.914 (0.86-0.97)	0.793 (0.72-0.87)	0.934 (0.89-0.98)	0.877 (0.82-0.94)
ALK	0.87	0.72	0.88	0.80
ROS1	1.00	1.00	1.00	1.00
MET	1.00	1.00	1.00	1.00
RET	1.00	0.92	1.00	1.00
NTRK	0.67	0.000	1.00	0.67
Specificity (95% CI)	0.951 (0.91-1.0)	0.988 (0.96-1.0)	0.927 (0.87-0.98)	1.0 (1.0-1.0)
ALK	0.99	1.00	0.95	1.00
ROS1	0.99	0.99	1.00	1.00
MET	1.00	1.00	1.00	1.00
RET	0.99	0.99	1.00	1.00
NTRK	0.99	1.00	1.00	1.00

Hofman et al., JTO clinical and Research reports 2022

=> Imbalance analysis: improve sensitivity but decrease specificity !

Amplicon-based approach: Oncomine on Genexus

- Oncomine Dx Expert panel on Genexus (CE/IVD)
  - Full automatization - Average TAT 18,3h

Table 1. Total run time for ODxET, including library preparation, templating, sequencing, and analysis for six samples and controls at each of the study centers.

Study Center	Total Run Time	
	Run #1 (h:min)	Run #2 (h:min)
Basel	18:06	18:03
Naples	18:22	18:15
Nice	18:00	17:56
Porto	18:16	18:01
Rome	18:38	18:34
Valencia	19:01	18:34

Normanno et al., Int J Mol Sciences 2023.

Amplicon-based approach: AmpliSeq™ on Illumina

- AmpliSeq™ for Illumina Childhood Cancer Panel
  - 100ng RNA
  - Sensitivity 94,4%
  - Specificity 100%
  - Limit of detection 10^{-2} (RNA from SeraSeq Fusion RNA Mix diluted in IVS-0035 negative control (10^{-2} – 10^{-5}))

TABLE 2 | Obtained reads, mean SD, and %CV for undiluted RNA and 10^{-2} dilution for each of the fusion genes analyzed. Libraries were performed by two operators (A and B).

Gene Id	Hgvs	Operator A		Operator B		Mean		SD		% CV	
		Undiluted RNA reads	10^{-2} dilution reads	Undiluted RNA reads	10^{-2} dilution reads	Undiluted RNA reads	10^{-2} dilution reads	Undiluted RNA reads	10^{-2} dilution reads	Undiluted RNA reads	10^{-2} dilution reads
BCR::ABL1	BCR(NM_004327.3):r.1_3378 ABL (NM_005157.3):r.83_5384	40,916	2014	40,376	1168	40,646	1,591	381.8	598.2	1	38
ETV6::ABL1 (transcript 1)	ETV6(NM_001987.4):r.1_737 ABL1(NM_007313.2):r.576-5881	40,128	672	15,024	678	27,576	675	17,751.2	4.2	64	1
ETV6::ABL1 (transcript 2)	ETV6(NM_001987.4):r.1_1283 ABL1(NM_007313.2):r.576-5881	15,728	1052	20,890	676	18,309	864	3,650.1	265.9	20	31
FIP1L1::>PDGFRA	FIP1L1(NM_030917.3):r.1_1109 PDGFRA(NM_006206.5):r.2037_6590	54,960	4104	44,730	1938	49,845	3021	7,233.7	1,531.6	15	51
MYST3::>CREBBP	MYST3(NM_006766.4):r.1_3803 CREBBP(NM_004380.2):r.290_10197	20,532	1066	26,814	680	23,673	873	4,442.0	272.9	19	31
PCM1::JAK2	PCM1(NM_006197.3):r.1_4365 JAK2(NM_004972.3):r.2008_5285	17,866	974	26,152	736	22,009	855	5,859.1	168.3	27	20
PML::RARA	PML (NM_033238.2):r.1_1786_ ins134bp RARA (NM_000964.3): r.657_3,301	Not detected	Not detected	9,396	Not detected	9,396[†]	–	–	–	–	–
RUNX1::>RUNX1T1	RUNX1 (NM_001754.4): r.1-803 RUNX1T1 (NM_004349.3):r.419-7420	11,028	856	12,968	462	11,998	659	1,371.8	278.6	11	42
TCF3::PBX1	TCF3(NM_003200.3):r.1_1519 PBX1(NM_002585.3):r.729_6918	21,434	1842	23,426	868	22,430	1,355	1,408.6	688.7	6	51

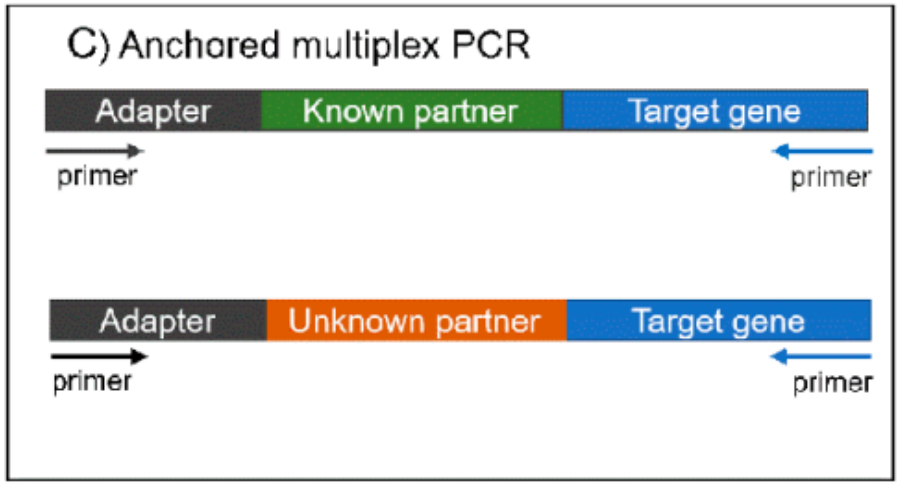
(Vincente-Garcés et al., Frontiers in Molecular Biosciences 2022)

Amplicon-based approach: AmpliSeq™

- Only for Known fusion
- Sensitivity/specificity

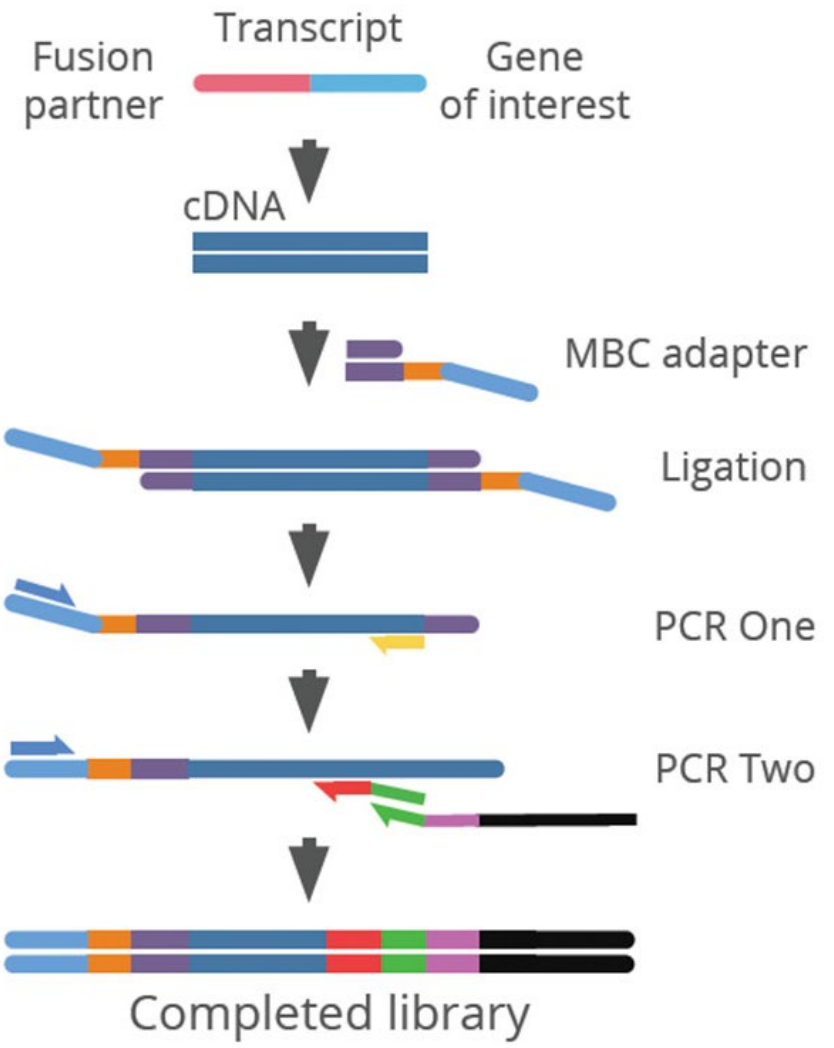
- Low RNA input
- Easy analysis
- ThermoFisher & Illumina
- 5’/3’ imbalance can increase sensitivity
- low limit of detection
- TAT

Anchored multiplex PCR

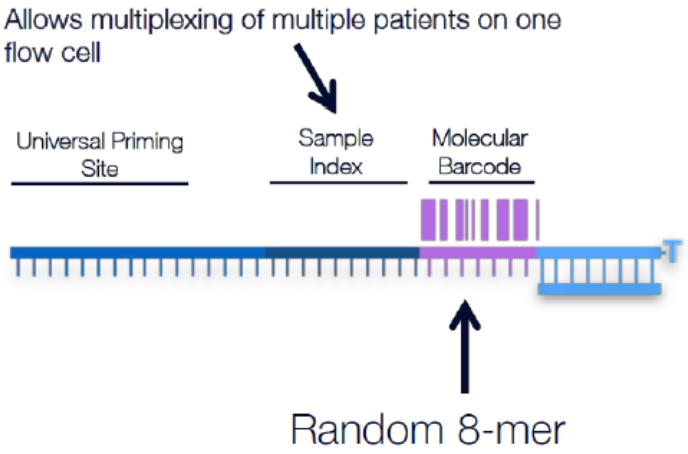


- Based on multiplex RT-PCR
- Primer in the target genes and an universal primer in adapter
- Detections of **known and Unknown** fusion transcripts
- Commercial & Custom panels

Anchored multiplex PCR: Archer Fusion Plex panels



+ Molecular Barcodes (MBC)

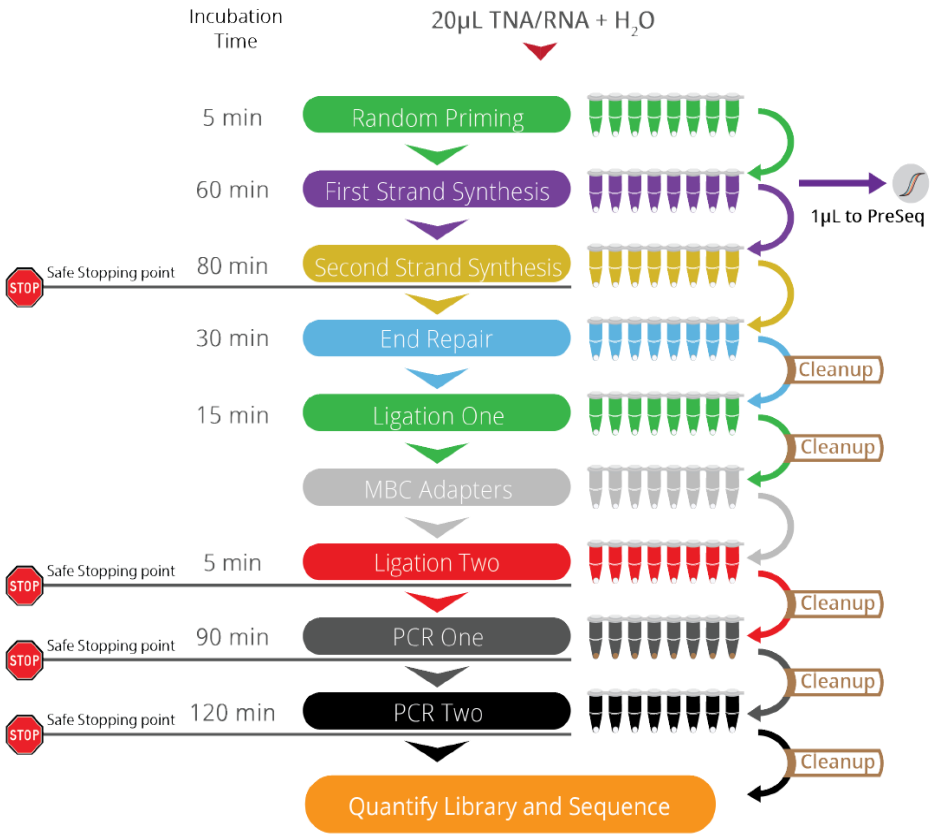


- Allows higher sensitivity
- Beter read quality

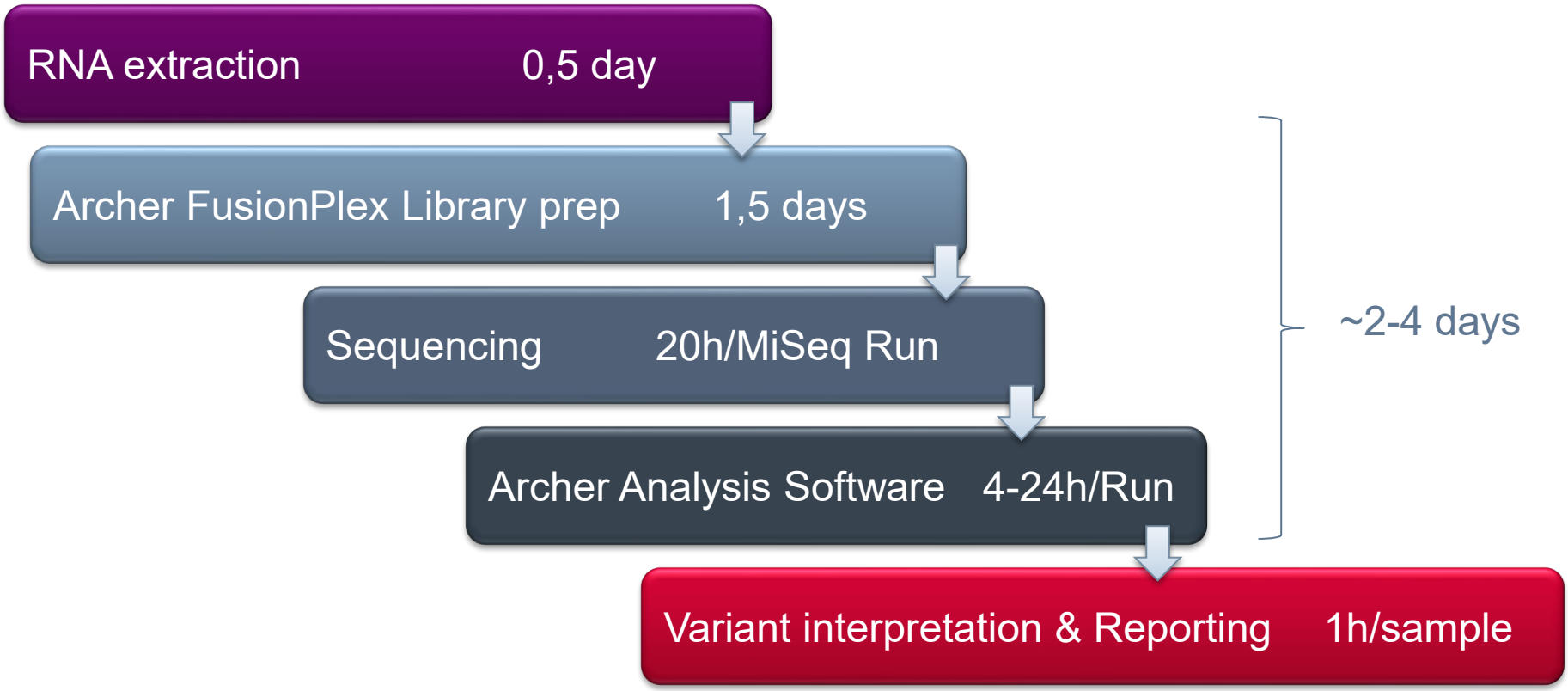
Anchored multiplex PCR: Archer Fusion Plex panels

- Single-use, Lyophilized reagents
  - No master mixes
  - Minimizes user error and contamination
  - Stable at RT
  - Strips are color Coded
  - Include a QC for RNA quality
- Input
  - 50-200ng RNA

- Variants and gene expression Analysis?



Anchored multiplex PCR: Archer Fusion Plex panels



Anchored multiplex PCR: Archer Fusion Plex panels

Molecular Barcode Statistics

Total Fragments	Fragments with Complete Adapter	Number of Reads After Trimming Adapters
1,380,786	1,312,743	1,070,721

Export Data (tsv)

QC Statistics

Avg. Unique DNA And Ambiguous Start Sites Per GSP2	Avg. Unique RNA Start Sites Per GSP2 Control
55.24	234.12

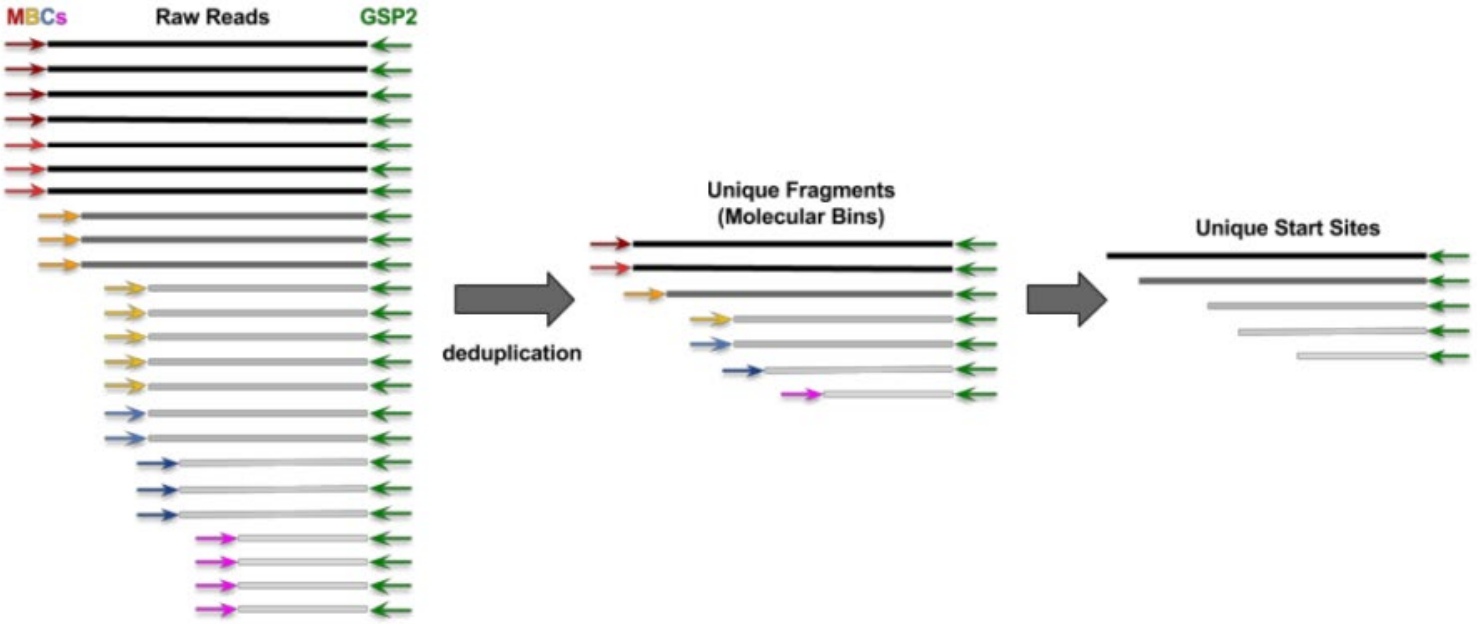
Search:

Strong Fusions & Oncogenic Isoforms | Low Confidence Fusions | All Results | Novel Isoform | New | Edit Columns | Save Grid | Reset

Actions	Classification	Report	Artifact	Genes	SS	Reads	%Reads	Strong	Brkpt	Cat	Type	InFrame	ITD Length	TO	Rept
		☑	☐	TCF3 → PBX1	321	3660	37.93	True	chr19:1619110,chr1:164761731	Fusion		True	N/A	4	2
		☐	☐	TCF3 → PBX1	15	18	0.11	True	chr19:1619809,chr1:164761773	Fusion		False	N/A	3	0
		☐	☐	IKZF1	8	12	0.27	True	chr7:50367353,chr7:50467616	Oncogenic Isoform	Exon(s) Skipped	True	N/A	66	7

Anchored multiplex PCR: Archer Fusion Plex panels

- Criteria
  - minimum 5 unique reads covering the breakpoint
  - min 3 unique start sites covering the breakpoint
  - > 2% of the unique reads covering the breakpoint compared to the total number of unique reads that span either breakpoint
  - Fusion is In Frame



Anchored multiplex PCR: Archer Fusion Plex panels

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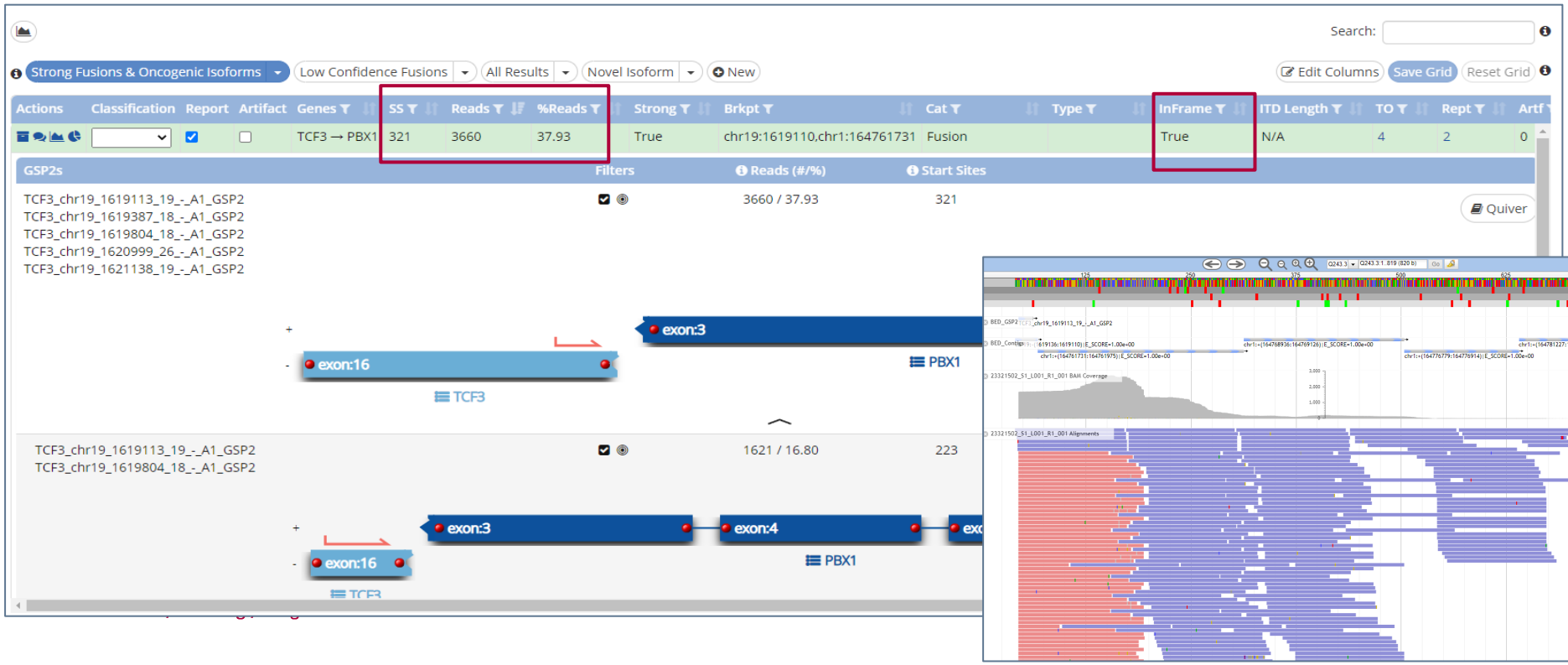
1,380,786	1,312,743	1,070,721

Export Data (tsv)

QC Statistics

Avg. Unique DNA And Ambiguous Start Sites Per GSP2	Avg. Unique RNA Start Sites Per GSP2 Control
55.24	234.12



Anchored multiplex PCR: Archer Fusion Plex panels

- Archer FusionPlex Heme v2 sequenced on a MiSeq
  - Starting with 200ng of RNA extracted from blood of bone marrow)
  - All sample passed QC
  - **Specificity: 100%** (8 “normal” sample)
  - **Sensitivity: 100%** (20 samples or cell lines with known fusions)

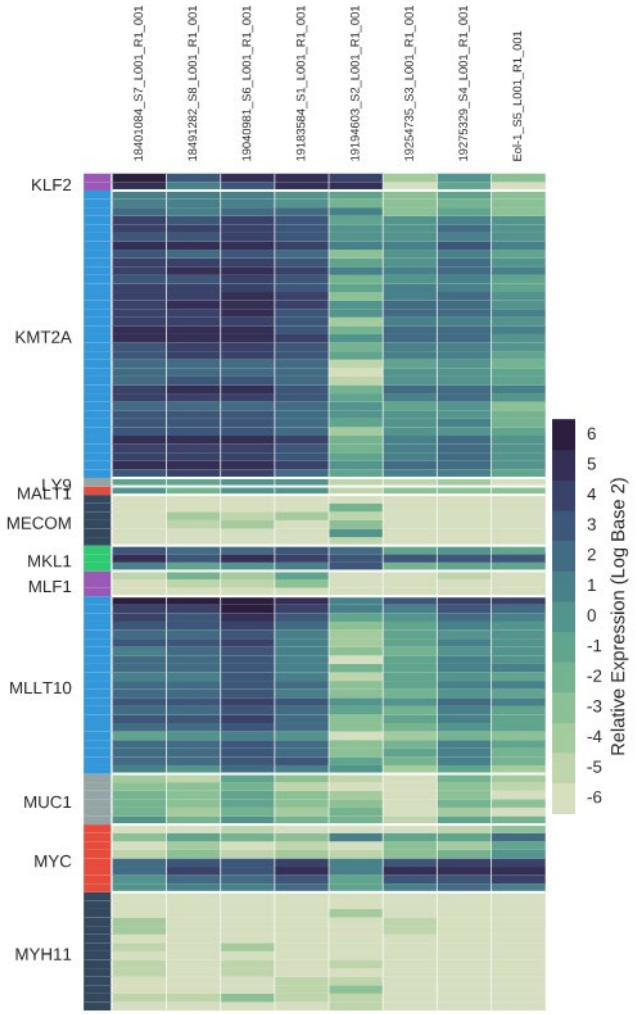
ABL1	ABL2	ALK	BCL11B	BCL2	BCL3	BCL6	BCR	BIRC3
CBFB	CCND1	CCND2	CCND3	CD274	CDK6	CDKN2A	CEBPA	CEBPD
CEBPE	CEBPG	CHD1	CHIC2	CIITA	CREBBP	CRLF2	CSF1R	CTLA4
DEK	DUSP22	EBF1	EIF4A1	EPOR	ERG	ETV6	FGFR1	FOXP1
GLIS2	ID4	IKZF1	IKZF2	IKZF3	IRF4	IRF8	JAK2	KAT6A
KLF2	KMT2A	MALT1	MECOM	MKL1	MLF1	MLLT10	MLLT4	MUC1
MYC	MYH11	NF1	NFKB2	NOTCH1	NTRK3	NUP214	NUP98	P2RY8
PAG1	PAX5	PDCD1	PDCD1LG2	PDGFRA	PDGFRB	PICALM	PML	PRDM16
PTK2B	RARA	RBM15	ROS1	RUNX1	RUNX1T1	SEMA6A	SETD2	STIL
TAL1	TCF3	TFG	TP63	TYK2	ZCCHC7			

Anchored multiplex PCR: Archer Fusion Plex panels

- Archer FusionPlex Heme v2 sequenced on a MiSeq
  - Limit of detection: Dilution of 2 cell lines

	Results Ref test		Results Archer Heme v2 panel			
		Exon (brkpt)	SS (>3)	Reads (>5)	%Reads (>2%)
Eol-1_undiluted	FIP1L1-PDGFRA	E12E12	148	637	90,23
Eol-1_10-1	FIP1L1-PDGFRA	E12E12	37	80	74,77
Eol-1_10-2	FIP1L1-PDGFRA	ND			
Eol-1_10-3	FIP1L1-PDGFRA	ND			
Eol-1_10-4	FIP1L1-PDGFRA	ND			
K562-undiluted	BCR-ABL p210 (86,86 /100 cp ABL)	E14E2	798	18308	75,82
K562-10-2	BCR-ABL p210 (3,27 /100 cp ABL)	E14E2	66	187	2,42
K562-10-3	BCR-ABL p210 (0,327 / 100 Cp ABL)	E14E2	12	16	0,28
K562-10-4	BCR-ABL p210 (0,022 / 100 cp ABL)	ND			
19194603	KMT2A-ELL zwak (Cp=36,95)	E8E2	7	8	13,11
17400926	RUNX1-RUNX1T1 Zwak (Cp=35,47)	ND			

- RNA expression



- RNA variants

RNA Variant | RNA Variant with Outlier | All Results | New

Actions	Classification	Report	Artifact	Symbol	HGVSp	HGVSc	Depth	AO	AF	C
				ABL2	p.Leu460Phe	c.1380G>T	177	177	1.0000	6
				BCR	p.Ser488LysfsTer2	c.1461_1461+1insA	1220	544	0.4459	2
				CCND1	p.Lys72Arg	c.210_219delinsGCAGCGCTGT	21	19	0.9048	7
				CCND1	p.Phe88Tyr	c.263T>A	442	442	1.0000	1
				CCND1	p.Val67=	c.201C>A	21	16	0.7619	5
				CCND1	p.Lys72Arg	c.214_216delinsCGC	20	19	0.9500	6
				CCND3	p.Pro134Ser	c.400C>T	9659	4782	0.4951	1
				FOXP1		c.-71dup	1147	150	0.1308	5
				IKZF2	p.Lys111Glu	c.331A>G	183	129	0.7049	4
				IKZF3	p.Cys123Tyr	c.368G>A	251	23	0.0916	8
				KAT6A	p.Val813CysfsTer8	c.2437-1_2437insT	15	7	0.4667	2

Not Validated

Anchored multiplex PCR: Archer Fusion Plex panels

- Archer Custom FusionPlex Panel sequenced on Miseq
  - **Accuracy 97,6% (80/82)** by comparing with other methods on 82 FFPE case -> 3 extra discordant cases if RNA input <100ng
  - LOD measures via dilution of Ref material (10%)

Table 6. Limit of detection.

Specimen	Fusion detected	Calculated transcript copies	Unique start sites ≥3	Unique reads ≥5	Percentage of reads supporting fusion ≥10	Average unique RNA start sites per GSP2 control ≥10
HD796	TPM3(6)-NTRK1(9)	2940	109	290	85	85
	ETV6(5)-NTRK3(14)	2400	77	189	86	85.9
	SLC34A2(4)-ROS1(32)	840	79	186	94	93.5
	EML4(12)-ALK(20)	780	29	60	92	92.3
HD783	No Fusion Detected	0	na	na	na	249
20% Dilution	TPM3(6)-NTRK1(9)	588	52	88	56	283
	ETV6(5)-NTRK3(14)	480	43	61	79	283
	SLC34A2(4)-ROS1(32)	168	36	53	23	283
	EML4(12)-ALK(20)	156	16	24	80	283
10% Dilution	TPM3(6)-NTRK1(9)	294	25	28	32	249
	ETV6(5)-NTRK3(14)	240	24	31	91	249
	SLC34A2(4)-ROS1(32)	84	21	24	96	249
	EML4(12)-ALK(20)	78	5	5	71	249
5% Dilution	TPM3(6)-NTRK1(9)	147	15	19	26	241
	ETV6(5)-NTRK3(14)	120	11	11	65	241
	SLC34A2(4)-ROS1(32)	42	14	17	100	241
	EML4(12)-ALK(20)	39	not detectable	not detectable	not detectable	241
2.5% Dilution	TPM3(6)-NTRK1(9)	74	6	7	13	241
	ETV6(5)-NTRK3(14)	60	5	6	75	241
	SLC34A2(4)-ROS1(32)	21	5	5	100	241
	EML4(12)-ALK(20)	19	not detectable	not detectable	not detectable	241

Cheng et al., JALM 2021

Anchored multiplex PCR: Archer Fusion Plex panels

- Custom Archer FusionPlex Panel sequenced on NextSeq
  - Specificity and sensitivity of 100% on 72 FFPE cases (starting with 125ng of RNA)
  - LOD (>12,5% tumor cells)

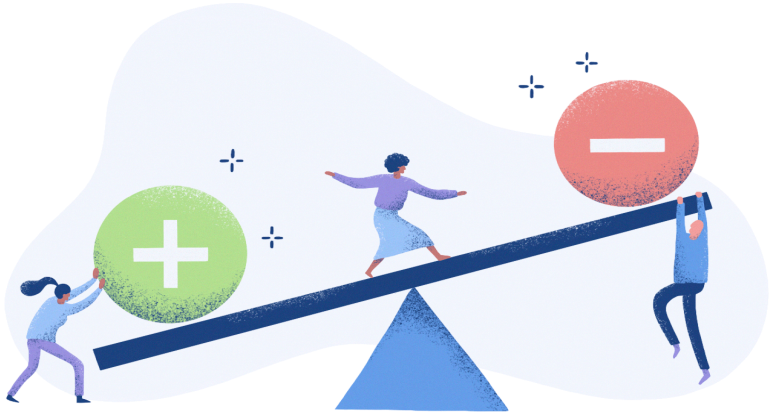
Table 2
Summary of the experiments for lower limit of tumor % detection.

Sample/Tumor %	# of Detected Fusions	RNA input	# Missed fusions
SERACARE100%	16	125 ng	0
SERACARE 50% IVS35 50%	16	125 ng	0
SERACARE 50% IVS35 50%	16	125 ng	0
SERACARE 25% IVS35 75%	16	125 ng	0
SERACARE 25% IVS35 75%	16	125 ng	0
SERACARE 12.5% IVS35 87.5%	16	125 ng	0
SERACARE 12.5% IVS35 87.5%	15	125 ng	1
SERACARE 5% IVS35 95%	12	125 ng	4
SERACARE 5% IVS35 95%	11	125 ng	5
SERACARE 2.5% IVS35 97.5%	8	125 ng	8
SERACARE 2.5% IVS35 97.5%	8	125 ng	8

Hindi et al., Exp Mol Pathol 2020

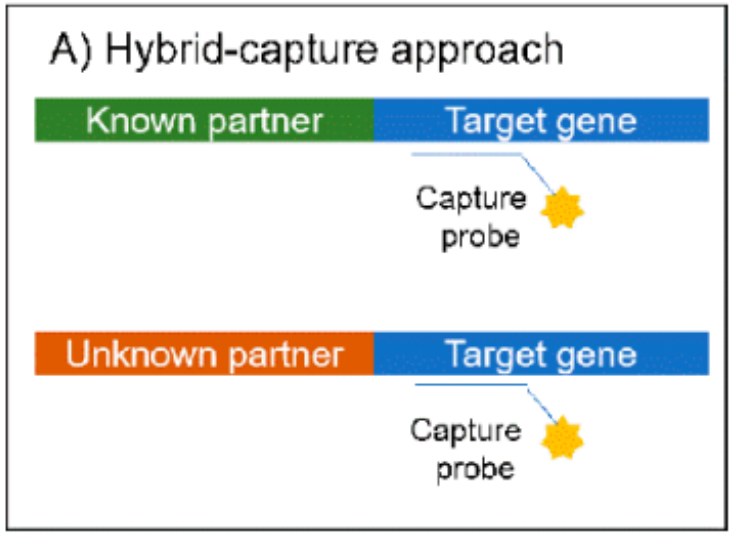
Anchored multiplex PCR: Archer Fusion Plex panels

- Higher RNA input
- Cost
- TAT



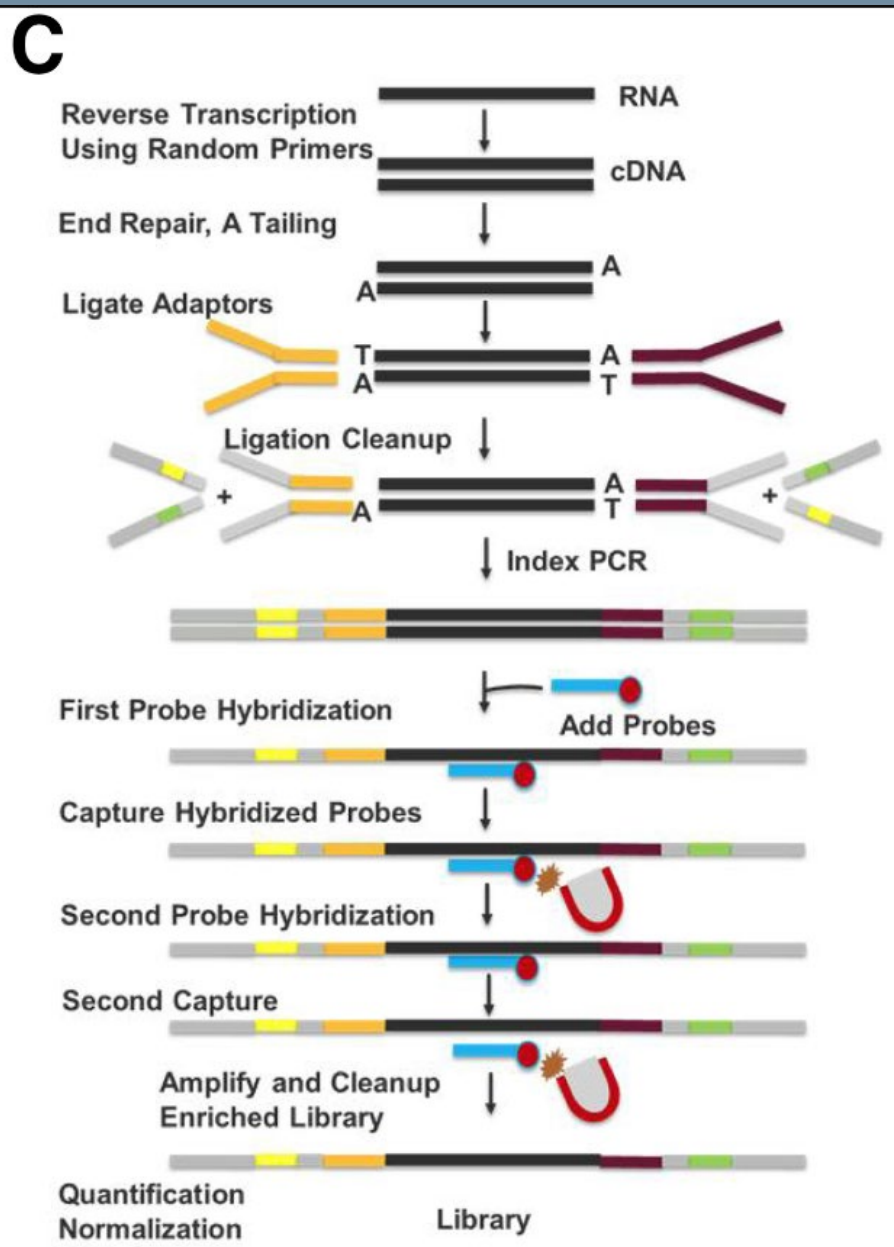
- User friendly library prep
- Easy analysis
- Detection of Unknown fusion transcript
- Compatible with Illumina and Thermofisher
- Very good Accuracy

Hybrid-capture approach



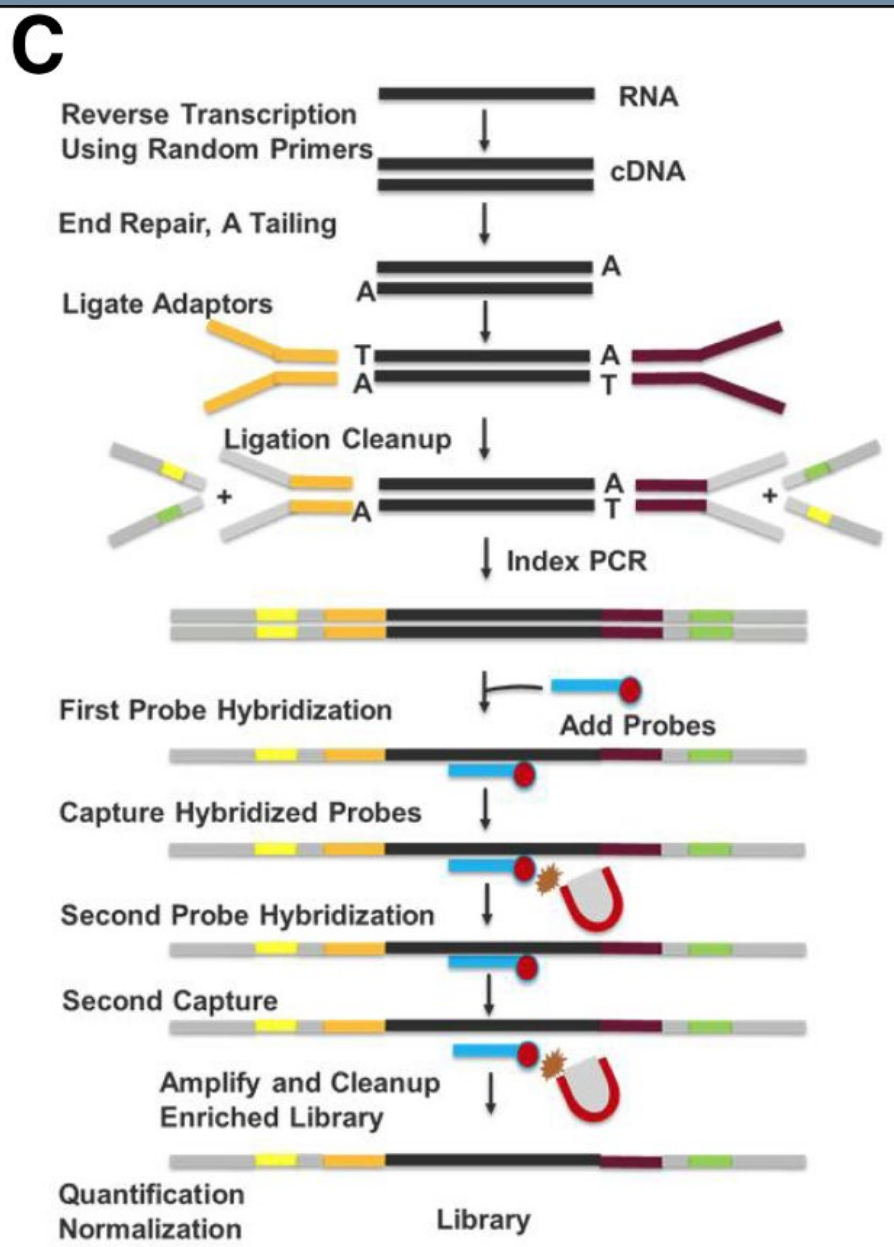
- Gene-specific enrichment by hybridization with specific DNA or RNA probes
- Starting from DNA or RNA
- Detections of known and Unknown fusion transcripts
- Commercial & Custom panels

Hybrid-capture Approach – Trusight Illumina



- Sequencing on Illumina platforms
- Several commercial panels, some CE/IVD
- input (from 40 ng RNA)

Hybrid-capture Approach – Trusight Illumina



Day 1

1. **Denataure and Anneal RNA**
   Hands-on: 20 minutes
   Total: 40 minutes
   Reagents: EPH3
2. **Synthesize First Strand cDNA**
   Hands-on: 10 minutes
   Total: 50 minutes
   Reagents: FSM, RVT
3. **Synthesize Second Strand cDNA**
   Hands-on: 10 minutes
   Total: 30 minutes
   Reagents: SSM
4. **Clean Up cDNA**
   Hands-on: 30 minutes
   Total: 40 minutes
   Reagents: SPB, RSB, 80% EtOH

Safe Stopping Point

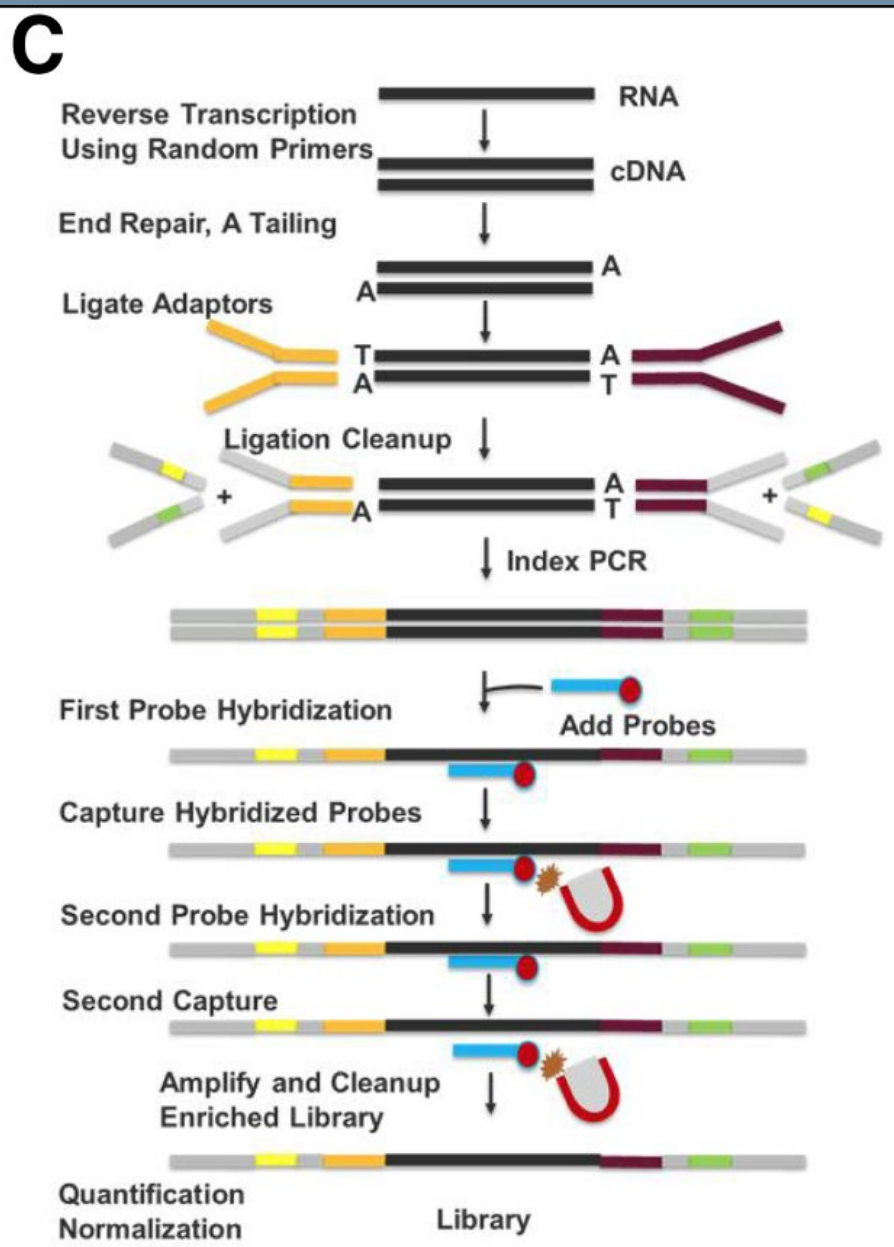
5. **Perform End Repair and A-Tailing**
   Hands-on: 10 minutes
   Total: 70 minutes
   Reagents: ERA1-A, ERA1-B
6. **Ligate Adapters**
   Hands-on: 15 minutes
   Total: 50 minutes
   Reagents: ALB1, LIG3, SUA1, STL
7. **Clean Up Ligation**
   Hands-on: 40 minutes
   Total: 50 minutes
   Reagents: SPB, RSB, 80% EtOH
8. **Index PCR**
   Hands-on: 15 minutes
   Total: 60 minutes
   Reagents: EPM, UPxx

Day 1 (continued)

1. **Set Up First Hybridization**
   Hands-on: 15 minutes
   Total: overnight
   Reagents: TCA1, TCB1, OPR1

Overnight Hybridization

Hybrid-capture Approach – Trusight Illumina



Day 2

2 **Capture Targets One**
Hands-on: 60 minutes
Total: 100 minutes
Reagents: SMB, EEW, EE2, HP3, ET2

3 **Set Up Second Hybridization**
Hands-on: 10 minutes
Total: 1.5–4 hours
Reagents: TCA1, TCB1, OPR1

4 **Capture Targets Two**
Hands-on: 25 minutes
Total: 60 minutes
Reagents: SMB, RSB, EE2, HP3, ET2

Safe Stopping Point

5 **Amplify Enriched Library**
Hands-on: 5 minutes
Total: 60 minutes
Reagents: PPC3, EPM

6 **Clean Up Amplified Enriched Library**
Hands-on: 30 minutes
Total: 40 minutes
Reagents: SPB, RSB, 80% EtOH

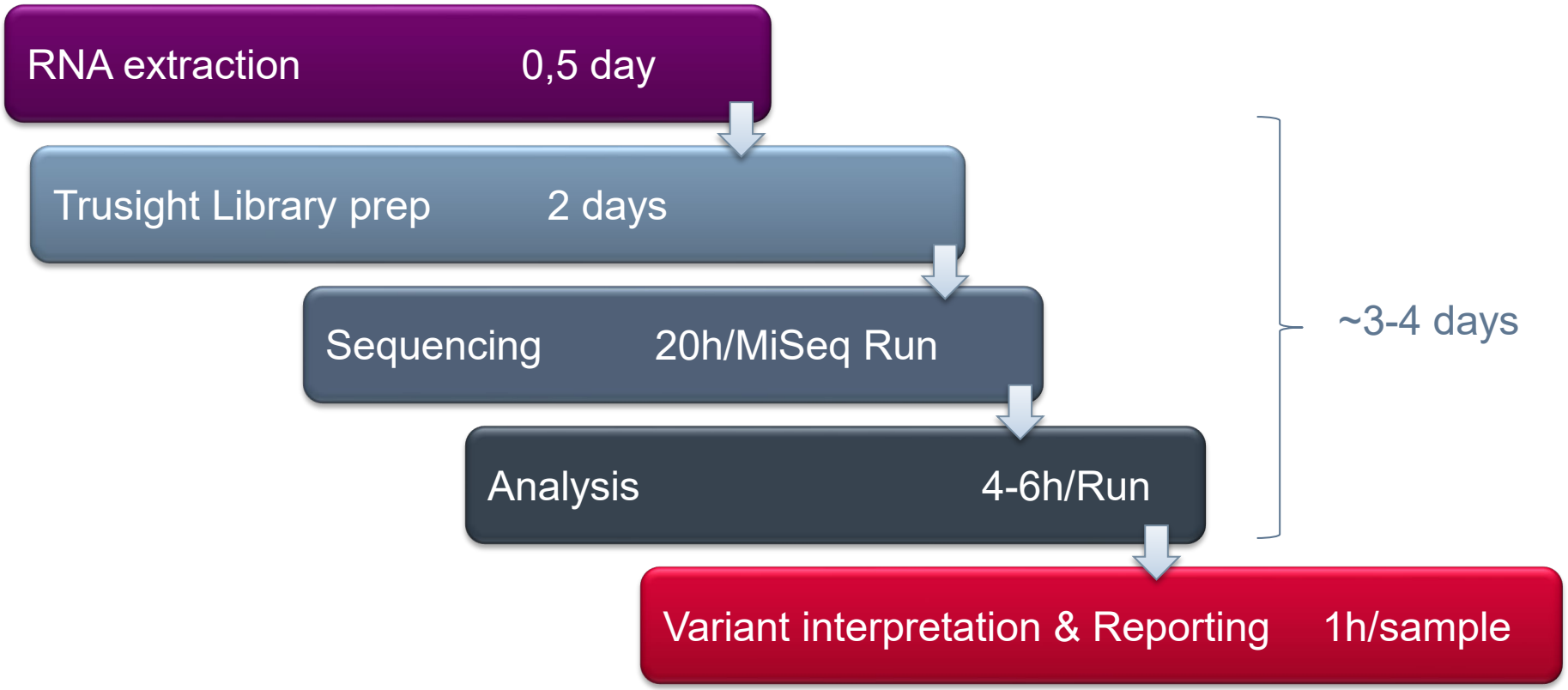
Safe Stopping Point

7 **Quantify Libraries (Optional)**

8 **Normalize Libraries**
Hands-on: 40 minutes
Total: 50 minutes
Reagents: LNA1, LNB1, LNW1, HP3, LNS1, EE2

Safe Stopping Point

Hybrid-capture Approach – Trusight Illumina



Hybrid-capture Approach – Trusight Illumina

- Illumina TSO 500

Confirmed structural variant	supp reads
EGFR vIII	2144
MET ex14	6885
CCDC6::RET	4550
CD74::ROS1	250
EML4::ALK var1	162
EML4::ALK var3a	24
ETV6::NTRK3	1170
EWSR1::ERG	3376
EWSR1::FLI1	1963
EWSR1::NFATC2	7287
FGFR3::BAIAP2L1	1474
FGFR3::TACC3	140
HIP1::ALK	460
KIF5B::RET	795
LMNA::NTRK1	2082
NCOA4::RET	1992
NPM1::ALK	40
PAX3::FOXO1	5593
PAX8::PPARG	1419
SDC4::ROS1	2162
SLC34A2::ROS1	272
SLC45A3::BRAF	1559
TMPRSS2::ERG	1804
TPM3::NTRK1	565

Froyen et al., Cancers 2022

- Illumina Trusight Tumor 170 (TST170)

A: 44 samples + 2 Commercial controls
B: 173 patient samples + 5 controls
Input: 40-85ng RNA

TABLE 2 | Accuracy analysis.

Variant type	Read depth; filter	TP	TN	FP	FN	PPA (%)	NPA (%)	PPV (%)	NPV (%)
Laboratory A, control specimens									
Substitutions	≥100×; VAF ≥2.6%	10652	7349439	303	73	99.3	99.9	97.2	99.9
Indels	≥250×; VAF ≥5%	594	7359678	180	75	88.8	99.9	76.7	99.9
Fusions/splice variants	"high confidence"	301		15	67	81.8		95.2	
Laboratory A, clinical specimens									
Substitutions	≥100×; VAF ≥2.6%	41^			0	100			
Indels	≥250×; VAF ≥5%	5†			0	100			
Copy number variants		14			2	87.5			
Fusions/splice variants		17‡			1§	94.4			
Laboratory B, combined data for control and clinical specimens									
Substitutions	≥250×; VAF ≥5%					99.87	100	100	98.33
Indels	≥250×; VAF ≥5%					97.56	100	100	97.43
Copy number variants	Filter pass, > 7 copies					96.87	100	100	97.67
Fusions/splice variants	"high confidence"					97.87	100	100	98.36

Boyle et al., Frontiers in Genetics 2021

79 samples + 1 Commercial control (SeraCare):
35 known fusions and 5 splicing events: all confirmed (100%)
Input: 40ng (QC ok from 1-38ng)

Hybrid-capture Approach – Trusight Illumina

- Illumina TSO 500

Sample	TC	fusion	Contribution to the mixture 100%	75%	25%
F1	70%	KIF5B::RET	2878	1398	322
F2	35%	EML4::ALK	427	520	82
F3	90%	SLC45A2::ERG	126	67	16
F4	50%	none	0	0	0

fusion	*undiluted*	*5x diluted*	*15x diluted*
EGFR vIII	61	22	12
MET ex14 splice	32	*nd*	*nd*
SLC45A3-BRAF	54	14	*nd*
FGFR3-TACC3	174	39	16
FGFR3-BAIAP2L1	79	27	8
KIF5B-RET	94	19	8
NCOA4-RET	118	19	7
TMPRSS2-ERG	65	18	13
EML4-ALK	70	8	5
CD74-ROS1	8	*nd*	*nd*
ETV6-NTRK3	67	6	8
LMNA-NTRK1	71	20	8
TPM3-NRTK1	93	8	*nd*
PAX8-PPARG	56	10	7
SLC34A2-ROS1	18	*nd*	*nd*
EGFR-SEPT14	64	13	*nd*

nd: not detected

Froyen et al., Cancers 2022

Hybrid-capture Approach – Trusight Illumina

- Hands on time
- TAT
- Only on Illumina

- Detection of Unknown fusion transcript
- Commercial and Custom panels
- Very good Accuracy
- Relatively low RNA input

Comparison of the different methods in the literature

Sensitivity

- Comparison **Oncomine Focus Assay, Oncomine Precision Assay, Trusight Oncology 500** & **Archer FusionPlex Lung panel** for NTRK gene fusions detection
  - RNA input: 200ng (Archer), 40ng (TSO500), 10ng (OFA, OPA)
  - Cell lines and commercial reference material

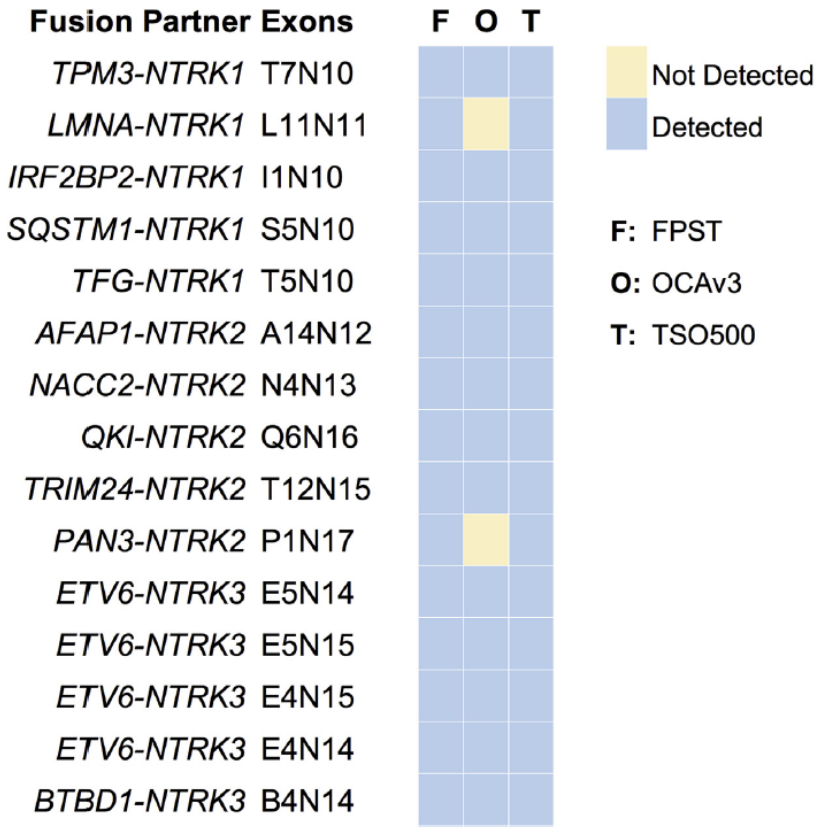
Table 4 Results of the Pilot Study Evaluating the Ability of the AFL, TSO500, OPA, and OFA Assays to Detect *NTRK* Fusions in Cell Line Samples and Reference Materials

Sample ID	Sample type	Expected fusion	AFL	TSO500	OPA	OFA
KM-12	Cell line	*TPM3:NTRK1*	Detected	Detected	Detected	Detected
BaF3-AFAP1-NTRK2	Cell line	*AFAP1:NTRK2*	Detected	Detected	Detected	Detected
IMS-M2	Cell line	*ETV6:NTRK3*	Detected	Detected	Detected	Detected
ML-2	Cell line	None	*No NTRK fusion detected*			
SeraSeq *NTRK*	Reference material	*IRF2BP2:NTRK1*	Detected	Detected	Detected	Detected
		LMNA:NTRK1	Detected	Detected	Detected	*Not detected (missing from panel)*
		SQSTM1:NTRK1	Detected	Detected	Detected	Detected
		TFG:NTRK1	Detected	Detected	Detected	*Not detected (missing from panel)*
		TPM3:NTRK1	Detected	Detected	Detected	Detected
		AFAP1:NTRK2	Detected	Detected	Detected	Detected
		NACC2:NTRK2	Detected	Detected	Detected	Detected
		PAN3:NTRK2	Detected	Detected	Detected	*Not detected (missing from panel)*
		QKI:NTRK2	Detected	Detected	Detected	Detected
		TRIM24:NTRK2	Detected	Detected	Detected	Detected
		BTBD1:NTRK3	Detected	Detected	Detected	Detected
		ETV6:NTRK3 (E4N14)	Detected	Detected	Detected	Detected
		ETV6:NTRK3 (E4N15)	Detected	Detected	Detected	Detected
		ETV6:NTRK3 (E5N14)	Detected	*Not reported*	Detected	Detected
		ETV6:NTRK3 (E5N15)	Detected	*Not reported*	Detected	Detected
SeraSeq WT	Reference material	None	*No NTRK fusion detected*			

Bormann Chung et al., J Mol Diag 2022

Sensitivity

- Comparison **Oncomine Comprehensive Assay v3, Trusight Oncology 500 & Archer FusionPlex Solid Tumor** for NTRK gene fusions detection
  - RNA input: 20ng (FPST and OCAv3), 40ng (TSO500)
  - 39 FFPE + 10 FNA samples + SeraSeq FFPE NTRK fusion reference material



Park et al., J Mol Diag 2021

Sensitivity

- Comparison **Oncomine Comprehensive Assay v3, Trusight Oncology 500 & Archer FusionPlex Solid Tumor** for NTRK gene fusions detection

PID	*NTRK* Fusions	MAPK Driver Alterations	Other Fusions	F	O	T
1	*TPM3-NTRK1* (T7N10)		ND			
2	*KANK1-NTRK3* (K2N14)	*KIT, PDGFRA* Amplification	ND			
3	*EML4-NTRK3* (E2N14)		ND			
4	*RBPMS-NTRK3* (R5M14)		ND			
5	*TFG-NTRK1* (T4N10)		ND			
6	*ETV6-NTRK3* (E4N14)		ND			
7	*ETV6-NTRK3* (E4N14)	ND	ND			
8	*ETV6-NTRK3* (E4N14)		ND			
9	*TPR-NTRK1* (T21N10)		ND			
10	*TPR-NTRK1* (T21N10)		ND			
11	*ETV6-NTRK3* (E4N14)		ND			
12	*ETV6-NTRK3* (E4N14)		ND			
13	*EML4-NTRK3* (E2N14)		ND			
14	*EML4-NTRK3* (E2N14)		ND			
15	*ETV6-NTRK3* (E4N14)	ND	ND			
16	*ETV6-NTRK2* (E4N16)	*IDH1* R132H	ND			
17	*ETV6-NTRK3* (E4N14)	ND	ND			

In raw data but filtered out because not targeted

- NTRK Fusion Detected
- Nontargeted Fusion Detected
- Not Tested
- Other Fusion Detected
- No Fusion Detected

F FPST
O OCAv3
T TSO500

Park et al., J Mol Diag 2021

Sensitivity

- Comparison **Oncomine Comprehensive Assay v3, Trusight Oncology 500 & Archer FusionPlex Solid Tumor** for NTRK gene fusions detection

PID	*NTRK* Fusions	MAPK Driver Alterations	Other Fusions	F	O	T
18	ND	ND	*AFAP1L2-RET* (A6R12)			
19	ND	ND	*EML4-ALK* (E2A20)			
20	ND		ND			
21	ND		ND			
22	ND		*CCDC6-RET* (C1R12)			
23	ND		*NCOA4-RET* (N7R12)			
24	ND	ND	ND			
25	ND	ND	*CTTNBP2-BRAF* (C3B11)			
26	ND	ND	*KIAA1549-BRAF* (K14B9)			
27	ND		*EML4-ALK* (E2A20)			
28	ND		*NCOA4-RET* (N9R12)			
29	ND	ND	*EML4-ALK* (E13A20)			
30	ND	ND	*EML4-ALK* (E14A20)			
31	ND	ND	*EML4-ALK* (E13A20)			
32	ND	ND	*PAX8-PPARG* (P9P2)			
33	ND	ND	*FGFR3-TACC3* (F17T6)			
34	ND		*FGFR1-TACC1* (F17T6)			
35	ND	*EGFR* Amplification	ND			
36	ND	*BRAF* V600E	ND			
37	ND	*BRAF* G466V	ND			
38	ND	*BRAF* V600E	ND			
39	ND	*BRAF* G464V	ND			
40	ND	ND	*CD74-ROS1* (C6R34)			
41	ND	ND	*SLC34A2-ROS1* (S13R34)			
42	ND	ND	*TMPRSS2-ERG* (T2E4)			
43	ND	ND	ND			
44	ND	ND	*KIAA1549-BRAF* (K15B9)			
45	ND		*PAX8-PPARG* (P8P2)			
46	ND		*PAX8-PPARG* (P8P2)			
47	ND	ND	*KIAA1549-BRAF* (K16B9)			
48	ND	ND	*CCDC6-RET* (C1R12)			
49	ND		*CCDC6-RET* (C1R12)			

Park et al., J Mol Diag 2021

Limit of detection

- Comparison **Trusight RNA fusion Panel** & **Archer Pan-Heme Kit**
  - 100 ng RNA
  - 24 patients' samples with known fusion => **all detected with both methods**
  - Limit of detection: Dilution of samples with BCR::ABL1 e1a2 transcript

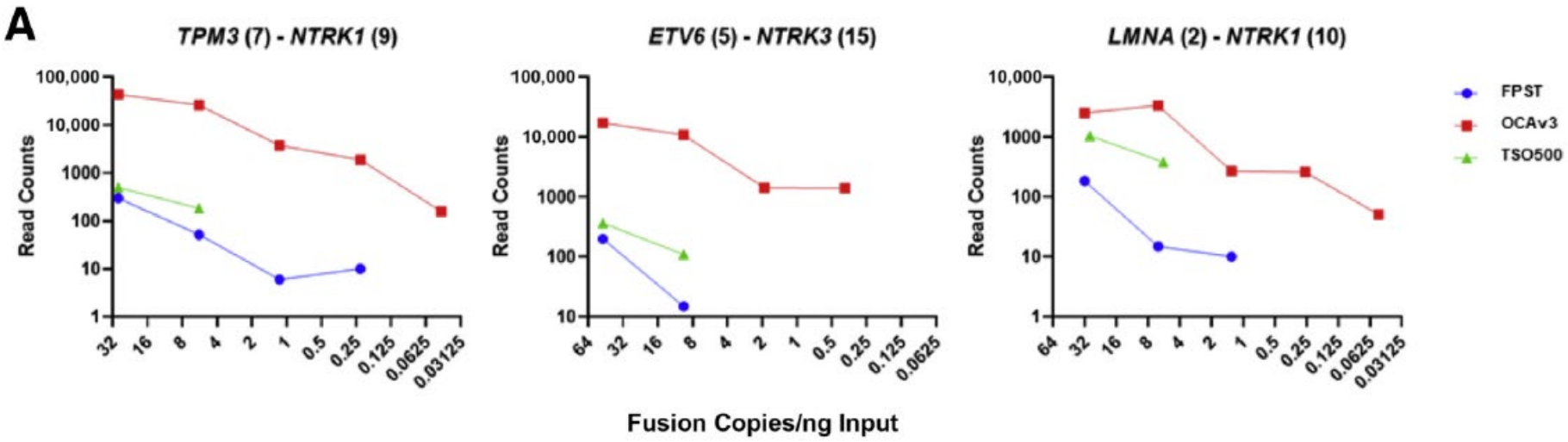
Table 4 Detection Limits of Two Next-Generation Sequencing Assays

	TruSight RNA fusion			FusionPlex Pan-Heme		
Variable	Results	Total no. of reads	No. of fusion supporting reads	Results	Total no. of reads	No. (%) of fusion supporting reads
BCR-ABL1 e1a2 control material						
10^{-1}	Not detected	4,282,660	—	Detected	2,471,217	131 (16.4)
10^{-2}	Not detected	4,356,800	—	Detected	2,409,933	11 (15)
10^{-3}	Not detected	4,313,057	—	Not detected	2,471,016	—
10^{-4}	Not detected	4,555,943	—	Not detected	2,498,515	—
Diluted patient samples						
1:1 dilution	Detected	11,085,226	10 (score 0.738)	Detected	2,712,284	53 (9.2)
1:4 dilution	Not detected	10,634,659	—	Detected	2,703,858	024 (4.4)

Kim et al., J Mol Diag 2019

Limit of detection

- Comparison **Oncomine Comprehensive Assay v3, Trusight Oncology 500 & Archer FusionPlex Solid Tumor** for NTRK gene fusions detection
  - RNA input: 20ng (FPST and OCAv3), 40ng (TSO500)
  - Limit of detection: Dilution of SeraSeq Fusion Mix v3 in Wild-type RNA



Park et al., J Mol Diag 2021

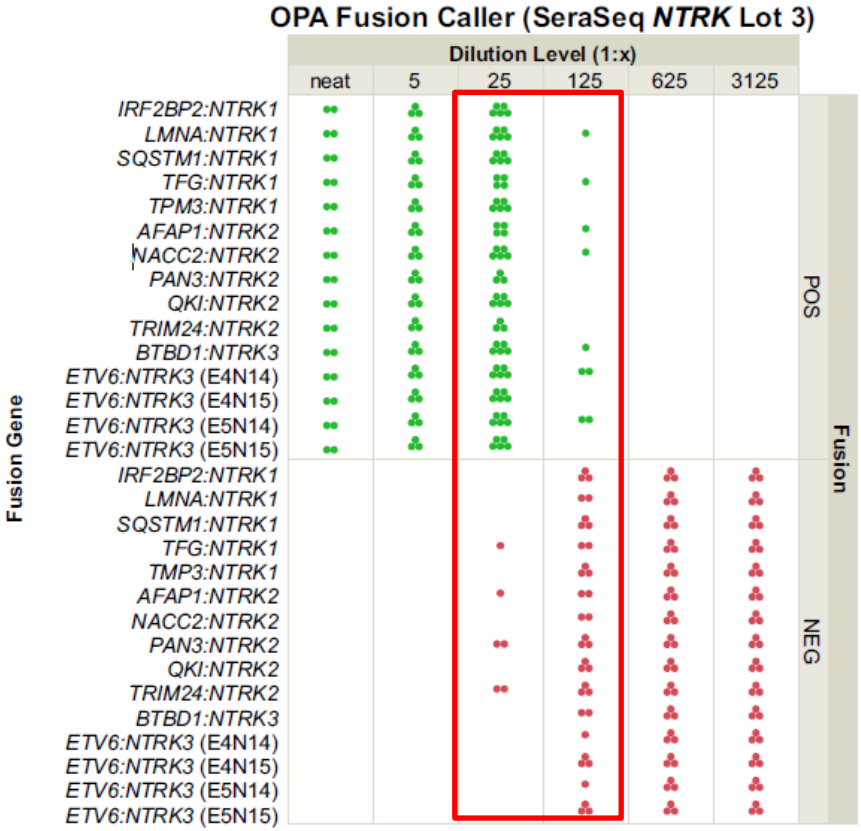
Limit of detection

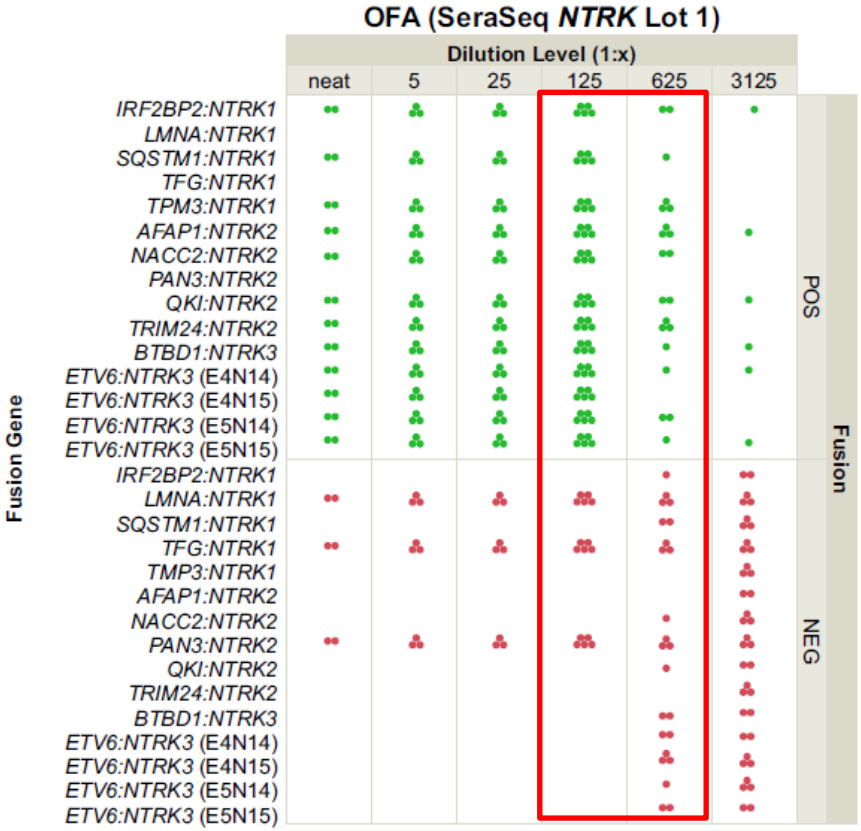
- Comparison **Oncomine Focus Assay, Oncomine Precision Assay, Trusight Oncology 500** & **Archer FusionPlex Lung panel** for NTRK gene fusions detection
  - RNA input: 200ng (Archer), 40ng (TSO500), 10ng (OFA, OPA)
  - Limit of detection: Dilution of SeraSeq Fusion Mix v3

Bormann Chung et al., J Mol Diag 2022

Limit of detection

- Comparison **Oncomine Focus Assay, Oncomine Precision Assay, Trusight Oncology 500** & **Archer FusionPlex Lung panel** for NTRK gene fusions detection
  - RNA input: 200ng (Archer), 40ng (TSO500), 10ng (OFA, OPA)
  - Limit of detection: Dilution of SeraSeq Fusion Mix v3





Bormann Chung et al., J Mol Diag 2022

TAT

- Comparison **Trusight RNA fusion Panel** & **Archer Pan-Heme Kit**

Table 5 Comparison of Characteristics of Two Next-Generation Sequencing Assays

Variable	TruSight RNA fusion	FusionPlex Pan-Heme
Read length, bp	2 × 75	2 × 150
Turnaround time, days	5	3
Hands-on time, hours	7	2.5
Data analysis	Either by Local Run Manager or on web-based analysis	On the Archer Analysis web
No. of target genes	507	199
Pan-cancer application	Yes	No
Sensitivity	Slightly low but sufficient for diagnostic samples	Sufficient for diagnostic samples
For disease monitoring	Not recommended	Not recommended

Kim et al., J Mol Diag 2019

TAT

- Comparison **Oncomine Focus Assay, Oncomine Precision Assay, Trusight Oncology 500** & **Archer FusionPlex Lung panel** for NTRK gene fusions detection

Variable	AFL	TSO500	OPA	OFA
Technology	Anchored multiplex PCR	Hybrid capture	Amplicon-based enrichment	Amplicon-based enrichment
RNA sample input, ng	20—250 (200 used for this study)	40	10	10
Turnaround time, days	~5 Manual library preparation	~5 Manual library preparation Automated option available	~1 Fully automated with minimal hands-on time	~5 Manual library preparation Automated option available
Samples per run, *n*	48 (MiSeq)	8 DNA + 8 RNA (NextSeq High Output)	4—16	24 DNA + 24 RNA (530 chip)
Sequencing system compatibility	Illumina and Ion Torrent	Illumina only	Ion Torrent only	Ion Torrent only
Pilot study				
Cell lines	All *NTRK* fusions detected	All *NTRK* fusions detected	All *NTRK* fusions detected	All *NTRK* fusions detected
Reference materials	All *NTRK* fusions detected	16/18 *NTRK* fusions reported: 2 *ETV6:NTRK3* variants not reported, although they were present in sequencing data	All *NTRK* fusions detected	15/18 *NTRK* fusions detected: 3 missing were not included in panel
NTRK fusion-negative samples	No false-positive result	No false-positive result	No false-positive result	No false-positive result
Sensitivity				
Total fusion copies at estimated LoD	Cell lines: 30—620 Reference materials: 710—5200	All samples: ~30—290	Fusion Caller, all samples: ~1—28 Imbalance Caller, all samples: 12—28	All samples: ~1—28
Reproducibility at estimated LoD	Good: 3/5 to 5/5	Very good: 4/5 to 5/5	Very good: 4/5 to 5/5	Very good: 4/5 to 5/5

Kenni

Bormann Chung et al., J Mol Diag 2022

Conclusion

	RNA input	Sensitivity/ specificity	Limit of detection	TAT (days)	Hands on Time
Ampliseq/ Oncomine	Low	++	+++	1-3	-/+
Archer	Middle to high	+++	++	3-5	++
Trusight	Low to Middle	+++	++	4-6	+++

RNA extraction and QC

RNA Extraction and QC

- RNA extraction
  - Many kit for RNA extraction
  - Depend on starting material (Tissue, FFPE, Blood, Bone marrow, cells, fresh/frozen)
- QC for RNA quality
  - Yield (concentration measurement via **fluorescent dye-based assay** (Qubit) of spectrophotometry (Nanodrop)
  - RNA integrity
    - Tape station / Bioanalyzer (RIN (RNA integrity numbers) of DV200 value (percentage of RNA fragments larger than 200 nt)
    - qPCR (included in some library prep kit such as Archer)

- RNA quality is more of an issue for FFPE samples

RNA Extraction and QC

- QC for RNA quality
  - RNA integrity
    - Tape station / Bioanalyzer (RIN (RNA integrity numbers) of DV200 value

RIN

28S/18S RNA ratio

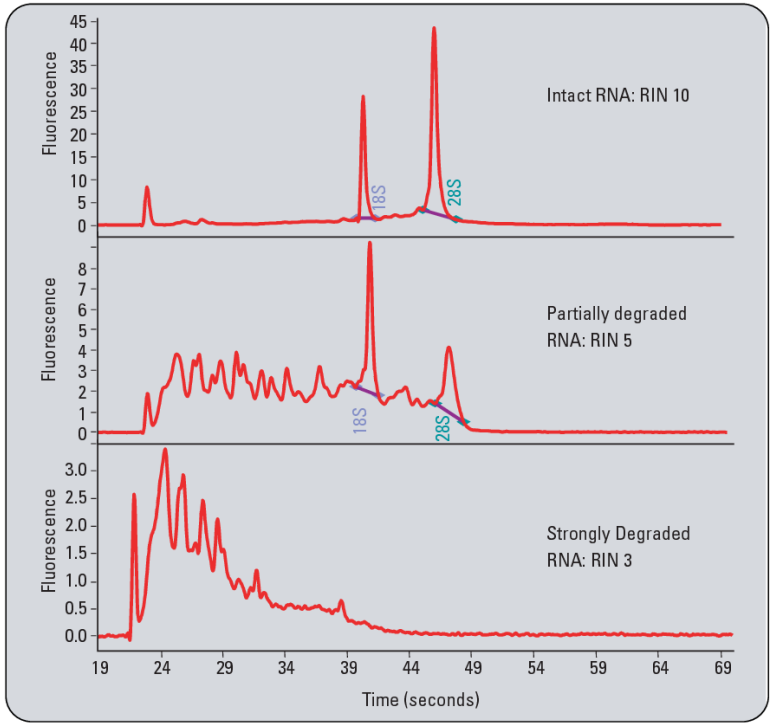
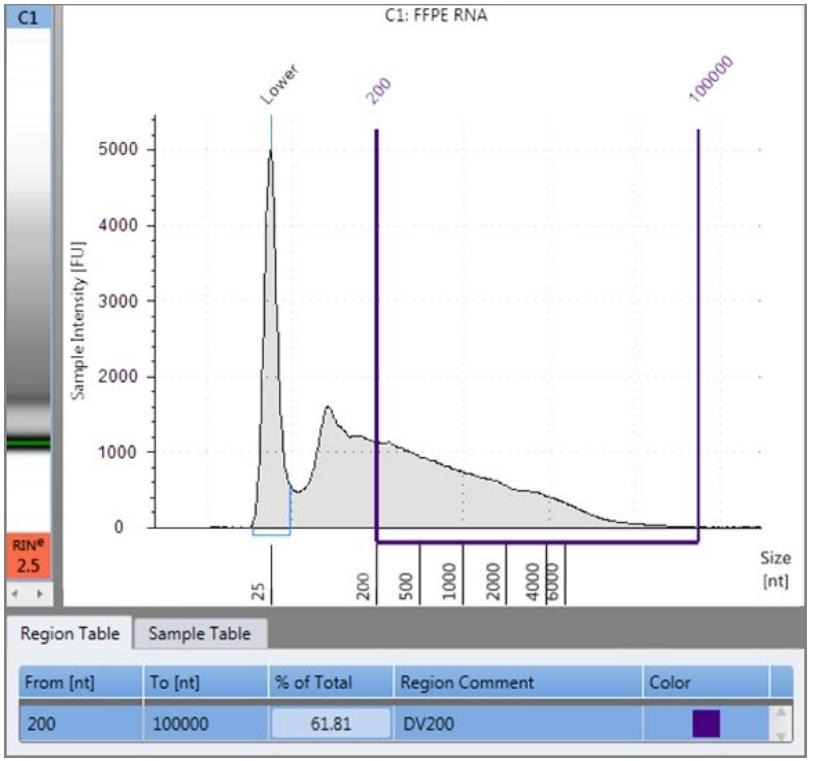


Figure 6

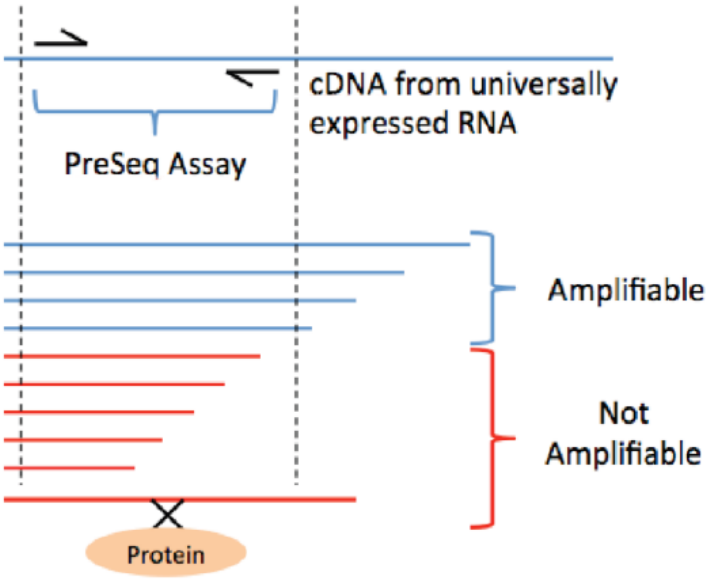
DV200

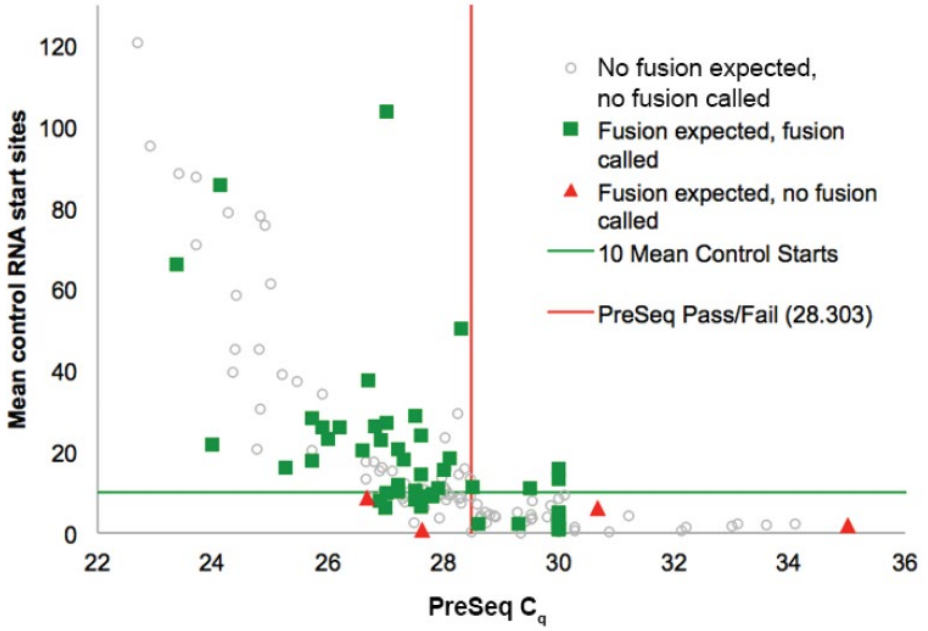
% of RNA fragments larger than 200 nt



RNA Extraction and QC

- QC for RNA quality
  - RNA integrity
    - qPCR (included in some library prep kit such as Archer = PreSeq QC)
      - Concentration
      - Length: fragments greater than 100bp in lengths
      - Crosslinking

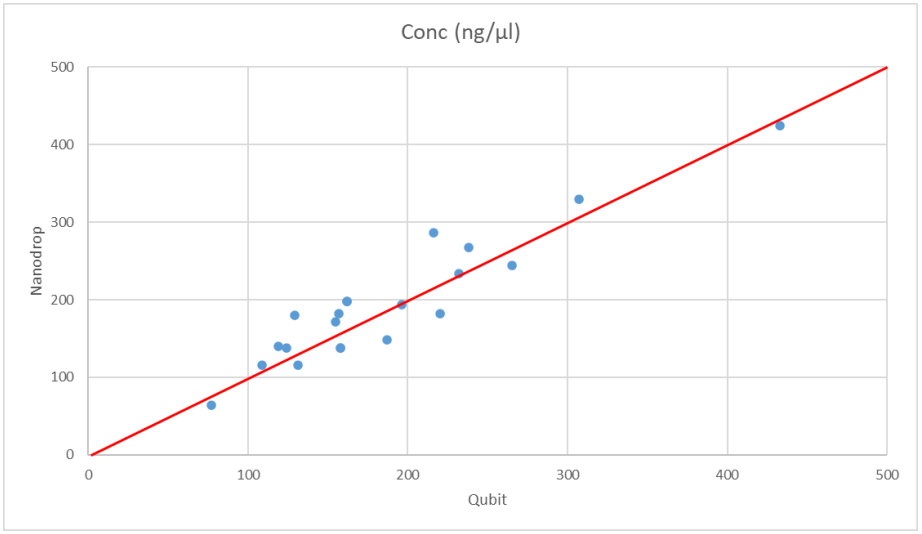




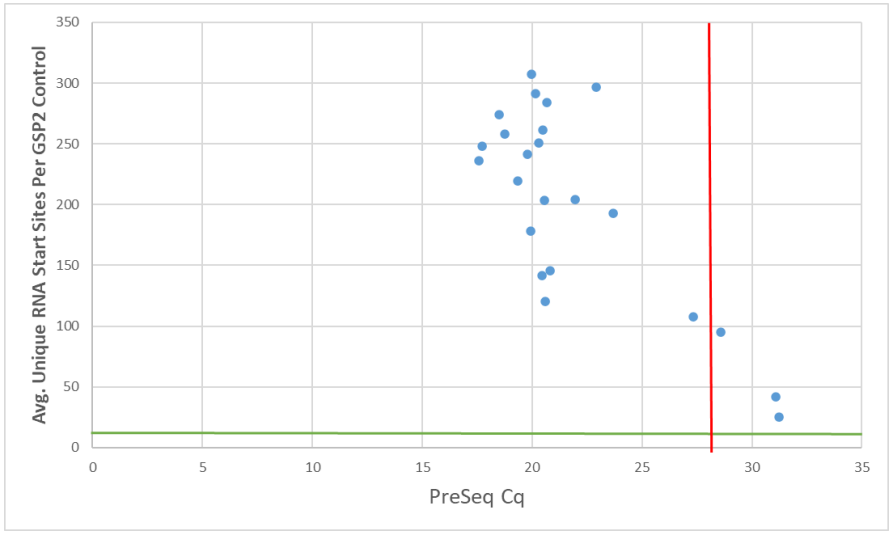
RNA Extraction and QC

- RNA quality is more of an issue for FFPE samples
  - 33 retrospective samples
  - RNA extracted from Blood or Bone marrow samples (fresh)

Concentration: Qubit vs Nanodrop



>90% samples above cut-off

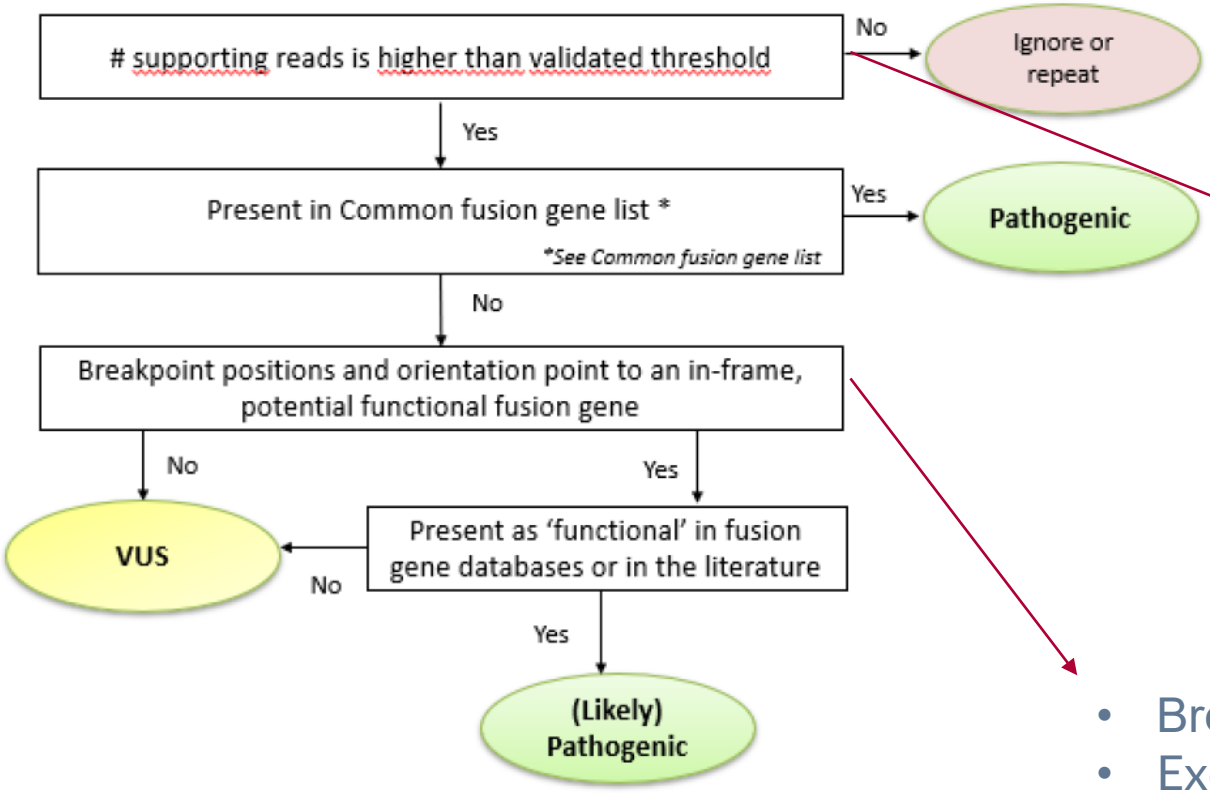


Tertiary analysis

Tertiary analysis

- BELAC NGS Guidelines:
  - Tertiary analysis is composed of two different step:
    - Annotation and biological classification
      - annotates each variant in relation to its position in the gene => software
      - classification into 5 biological classes
    - Clinical classification
      - classification into 4 clinical classes
      - Annotation with their clinical utility (diagnostic, prognostic or therapeutic)

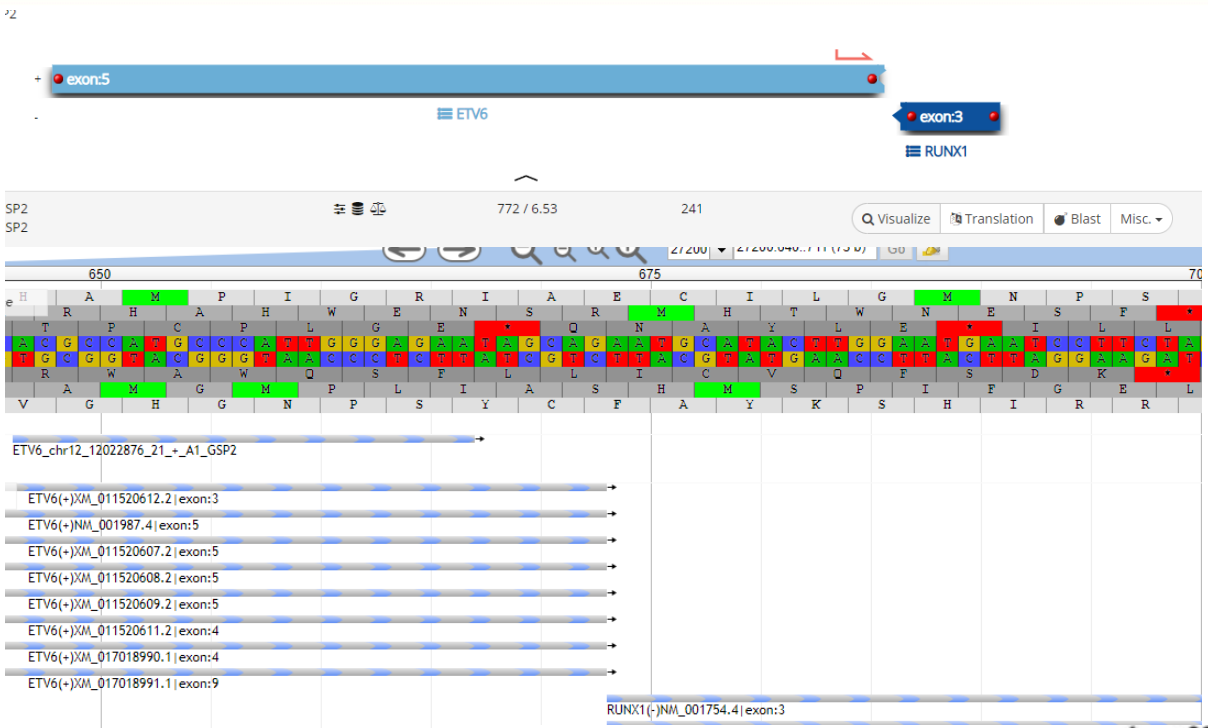
Biological Classification

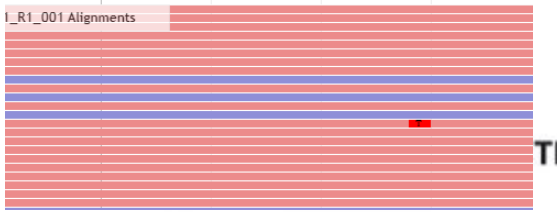


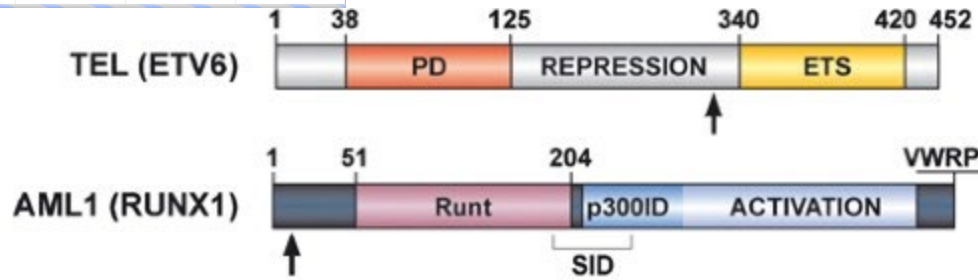
- Method dependent
- Cf. Validation file

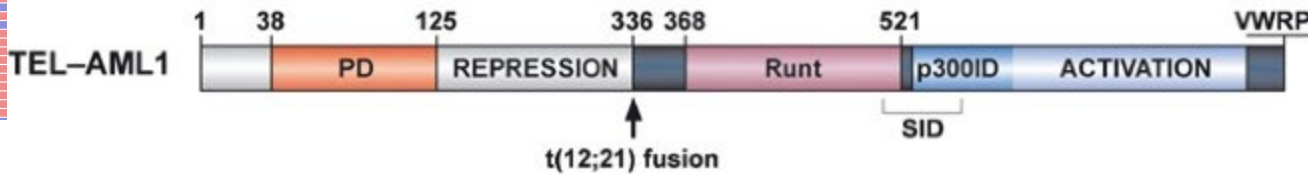
- Breakpoint position/visualization
- Exon boundary
- In Frame fusion
- Retention of relevant domains

Biological Classification

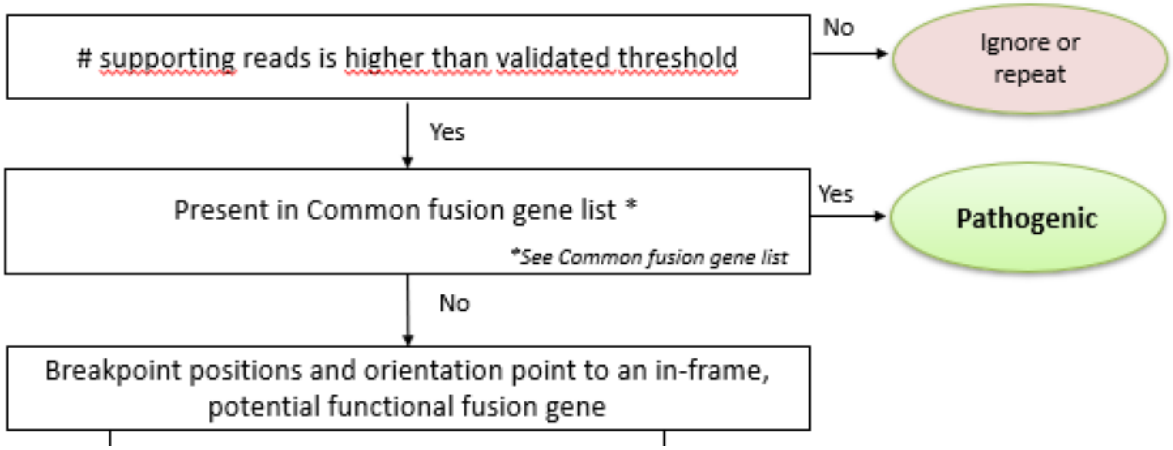








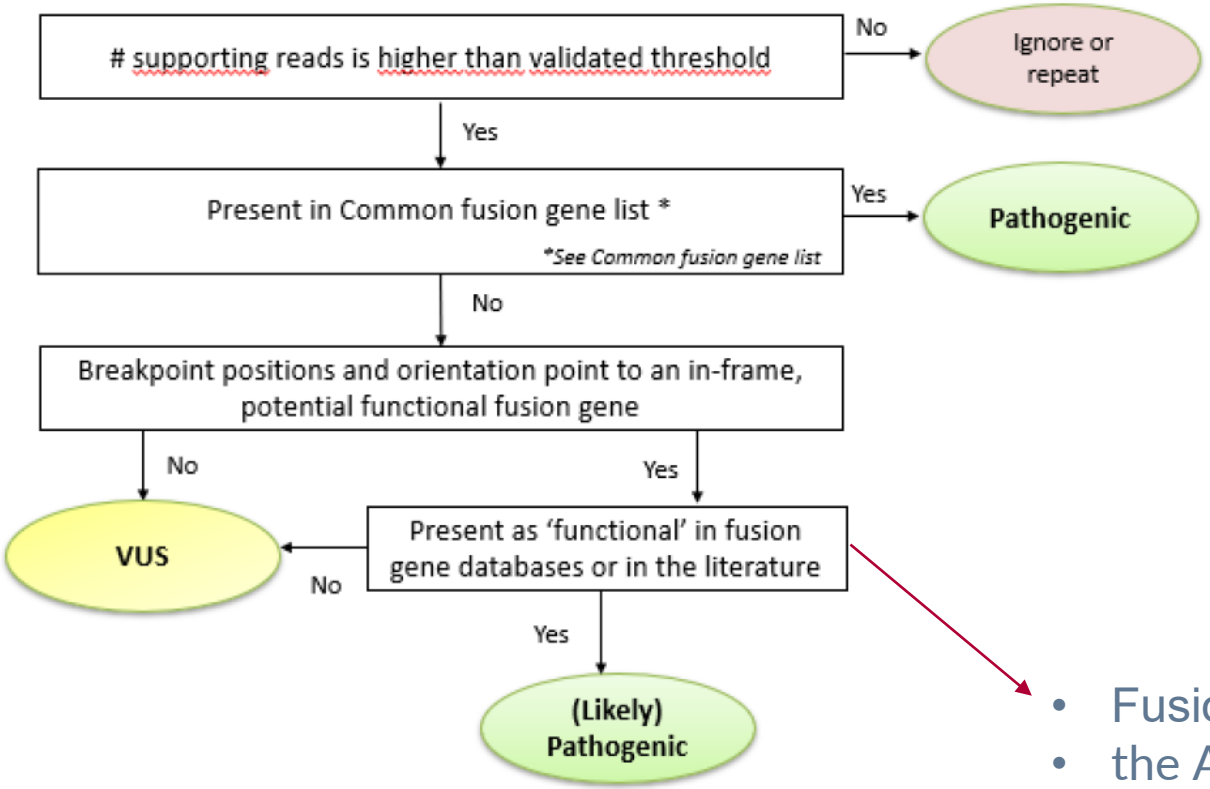
Biological Classification



Annex 3 Common fusion gene list (v1)

Fusion gene	Gene A	Gene B	Transcript ID A	Transcript ID B	Exon A	Exon B	Breakpoint A	Breakpoint B	remark	Found in
ADCY9-PRKCB	ADCY9	PRKCB								Lung
AFAP1-NTRK2	AFAP1	NTRK2		NM_006180.3						Glioma
AGBL4-NTRK2	AGBL4	NTRK2		NM_006180.3						Glioma
AGGF1-RAF1	AGGF1	RAF1		NM_002880.3						Prostate
AGGF1-RAF1	AGGF1	RAF1		NM_002880.3						Prostate
AGK-BRAF	AGK	BRAF		NM_004333.4						Skin; thyroid
AKAP13-RET	AKAP13	RET		NM_020975.4						Thyroid
ANK1-FGFR1	ANK1	FGFR1		NM_023110.2						Breast
ANXA4-PKN1	ANXA4	PKN1								Liver
AP3B1-BRAF	AP3B1	BRAF		NM_004333.4						Thyroid
ARHGEF18-INSR	ARHGEF18	INSR		NM_000208.2						Ovarian
ARHGEF2-NTRK1	ARHGEF2	NTRK1		NM_002529.3						Glioblastoma
ATG7-BRAF	ATG7	BRAF		NM_004333.4						Skin
AXL-MBIP	AXL	MBIP	NM_021913.4							
BAG4-FGFR1	BAG4	FGFR1		NM_023110.2						Lung

Biological Classification



- FusionGDB
- the Atlas of Genetics and Cytogenetics in Oncology and Haematology
- Mitelman Database
- literature

Biological Classification

Biological class	Reporting
Pathogenic	must be reported
Likely pathogenic	must be reported
VUS	must be reported clearly separated from pathogenic and likely pathogenic variants, but should not be clinically discussed
Likely benign	should not be reported
Benign	should not be reported

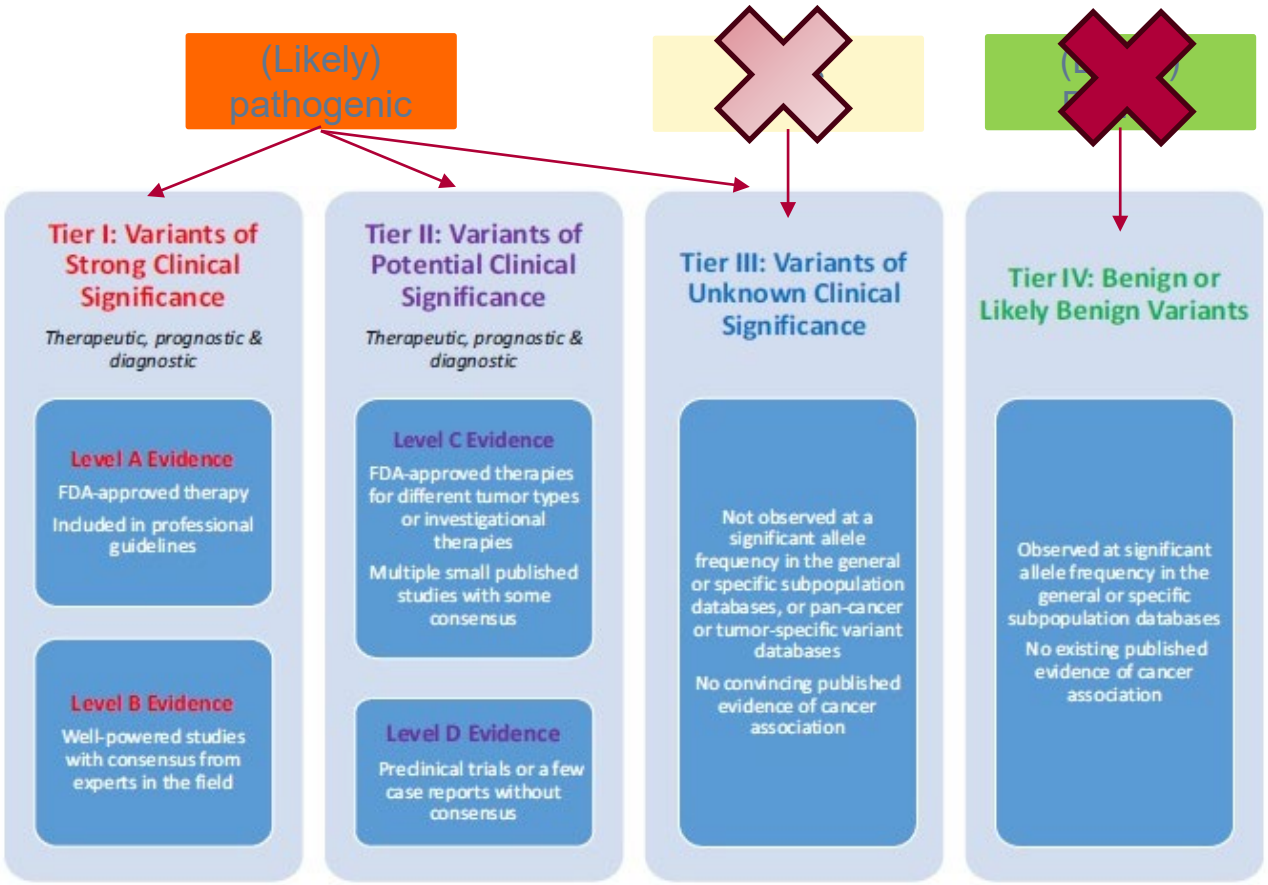
For DNA: common variant in the population MAF>0,1%

=> Database for fusion transcript in the “normal population” ??

Clinical Classification

- Clinical Interpretation: Impact on Prognosis? Therapy?

  -> Literature review, guidelines



Biological class

VUS and (likely) Benign variants are not clinically discussed

Li, MM *et al. J Mol Diagn 2017*

Sukhai, M. A. *et al. Genet. Med.* 2015

Questions?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