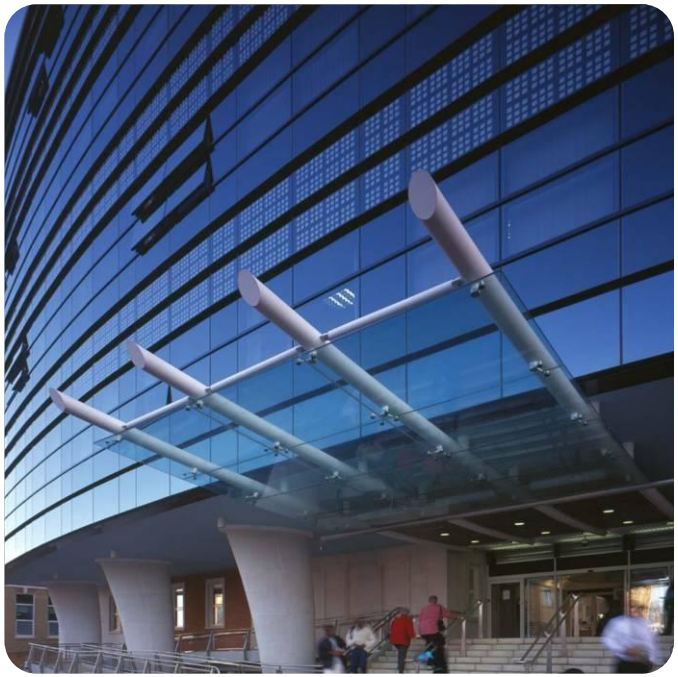
King's College Hospital NHS
NHS Foundation Trust

Next generation sequencing analysis - A UK perspective

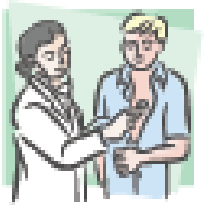
Nicholas Lea

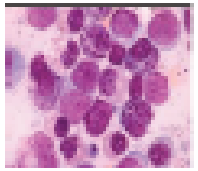
King's

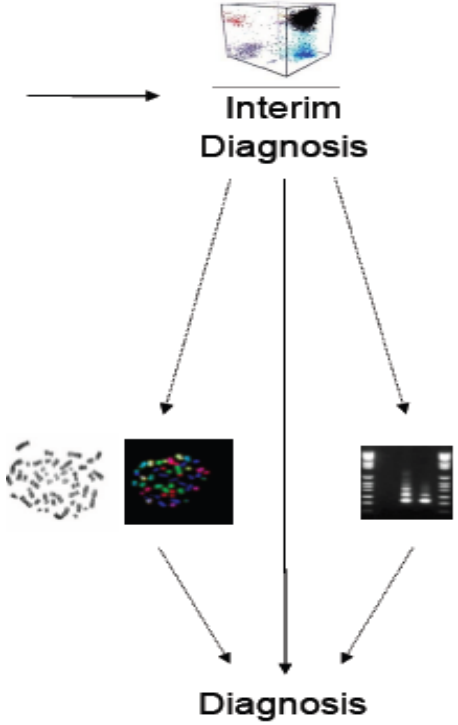


King's HMDC

LMH is part of an integrated pathology service at King's Haematological Malignancy Diagnostic Centre (HMDC)







HMDC serves population of >5 million

Around 20,000 samples per annum

> 8000 molecular diagnostic tests per annum

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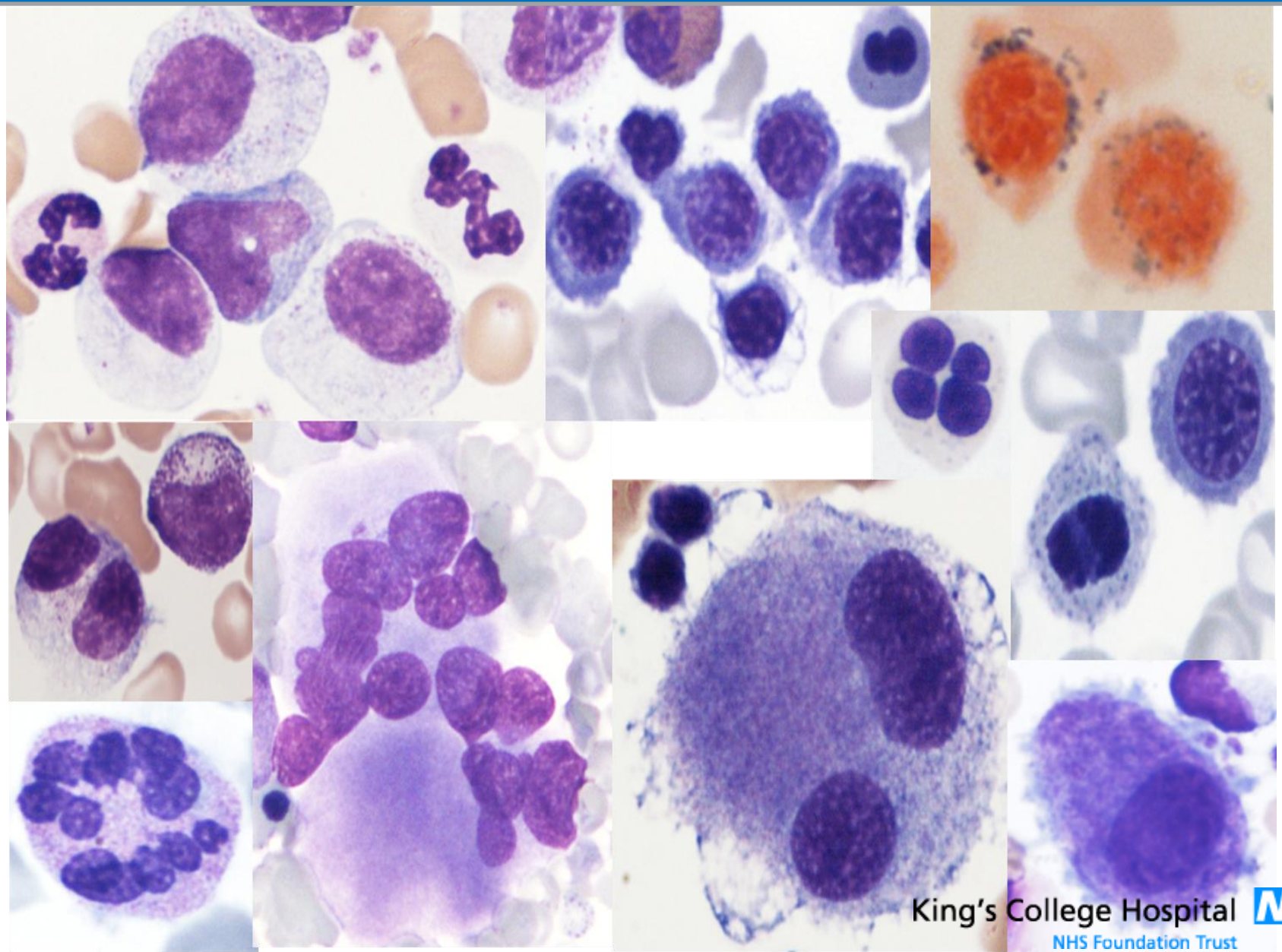
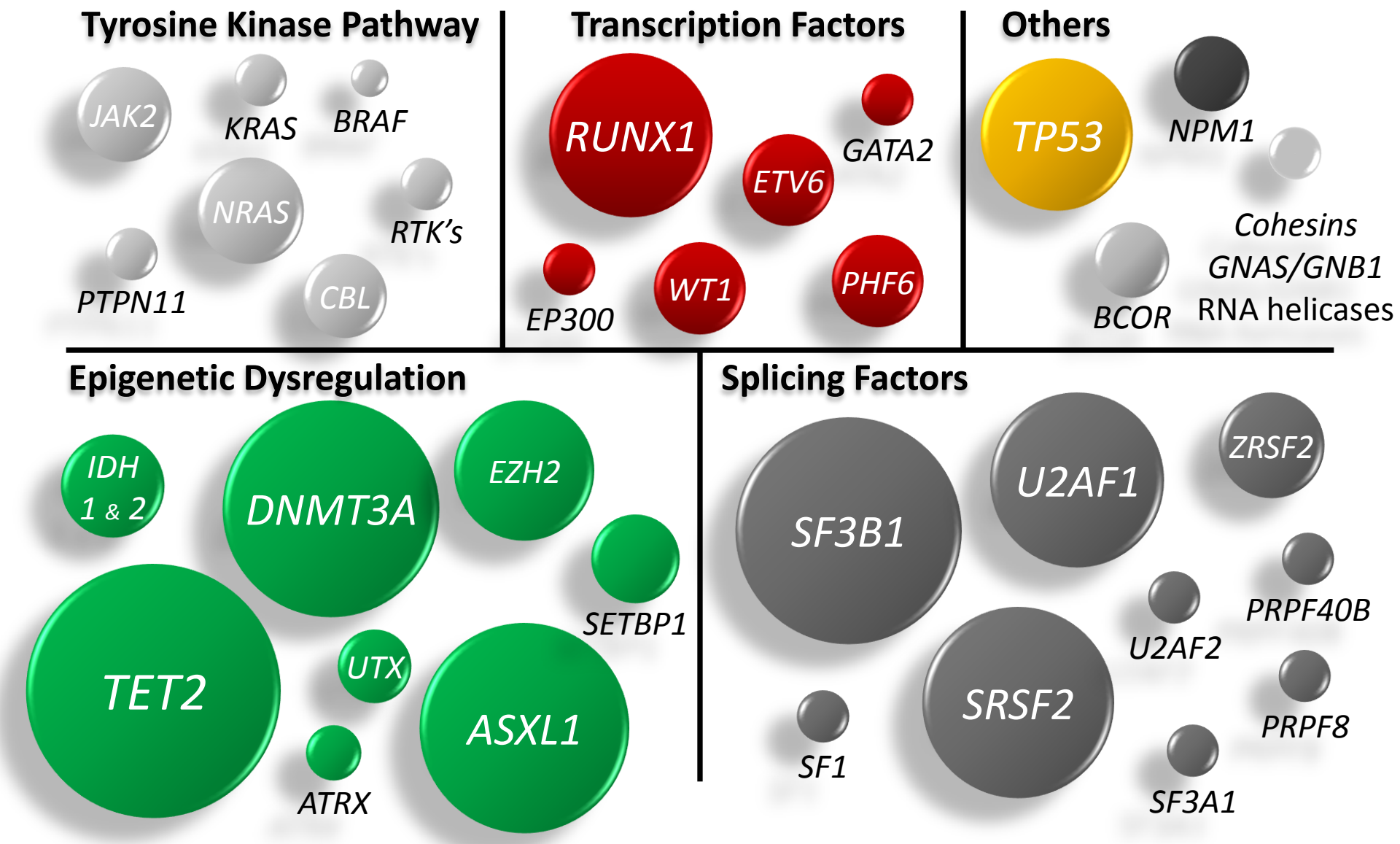
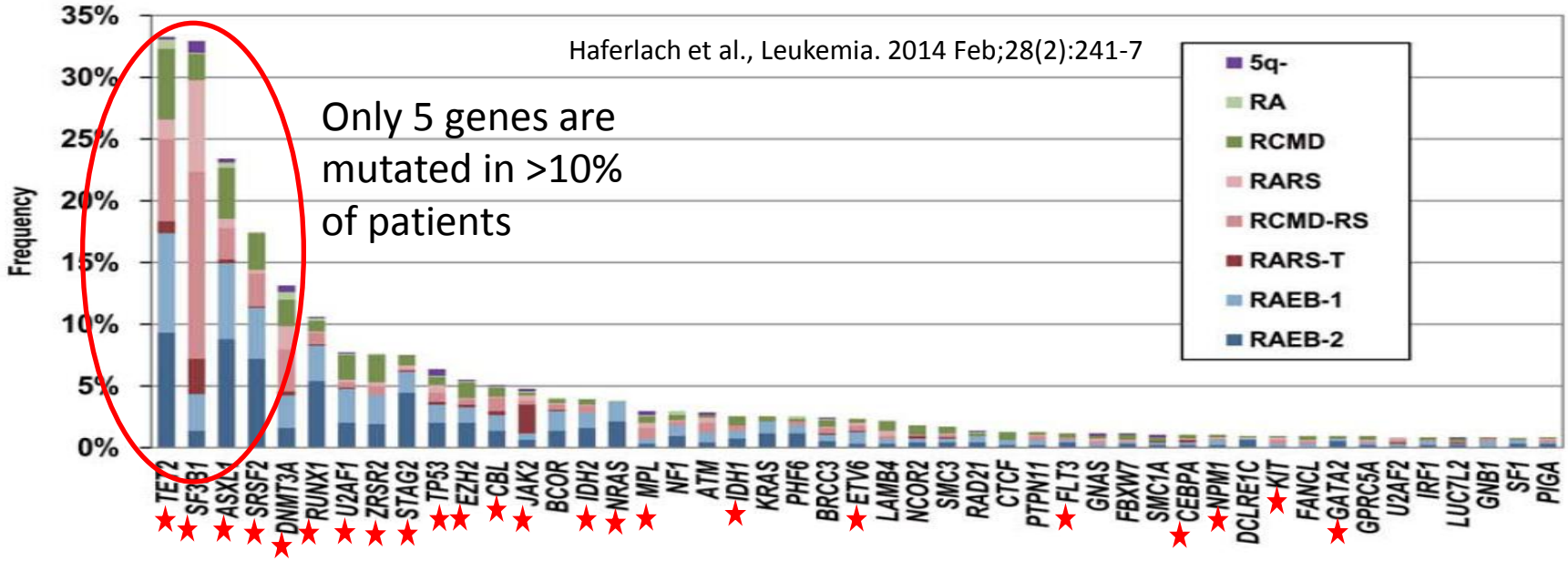
MDS: a heterogeneous disease



Point Mutations in MDS



Many mutations are very rare



★ Target present on the KCH panel

King's Myeloid Panel development

- Targets selected to aid in the diagnosis and prognosis of MDS and AML patients.
- DNA sources mostly from BM aspirates and PB using the Qiagen mini blood kit with 200 ul blood. Also from bone marrow films or other sources provided.
- Decided to use the Illumina Tru-Seq Custom Amplicon workflow, with the MiSeq.

Myeloid NGS Panel Workflow

- Quantify DNA
- Preparation of NGS sequencing libraries
  (annealing primers, extension, indexing, PCR)
- Verify, purify and normalise libraries using magnetic bead system
- Load onto MiSeq instrument
- 2 days of data collection
- Data and variant analysis

KCH: Myeloid Gene Panel (MGP)

24 genes mutation panel:

Transcription factors and cell cycle regulators

RUNX1
TP53
GATA2
ETV6
CEBPA
NPM1*

Spliceosome component

SF3B1*
U2AF1
SRSF2
ZRSR2

Epigenetic modifications

TET2
IDH1*
IDH2*
DNMT3A
KDM6A
ASXL1*
EZH2

Signaling

NRAS*
KRAS*
FLT3*
CBL
JAK2*
KIT*

Cohesin complex

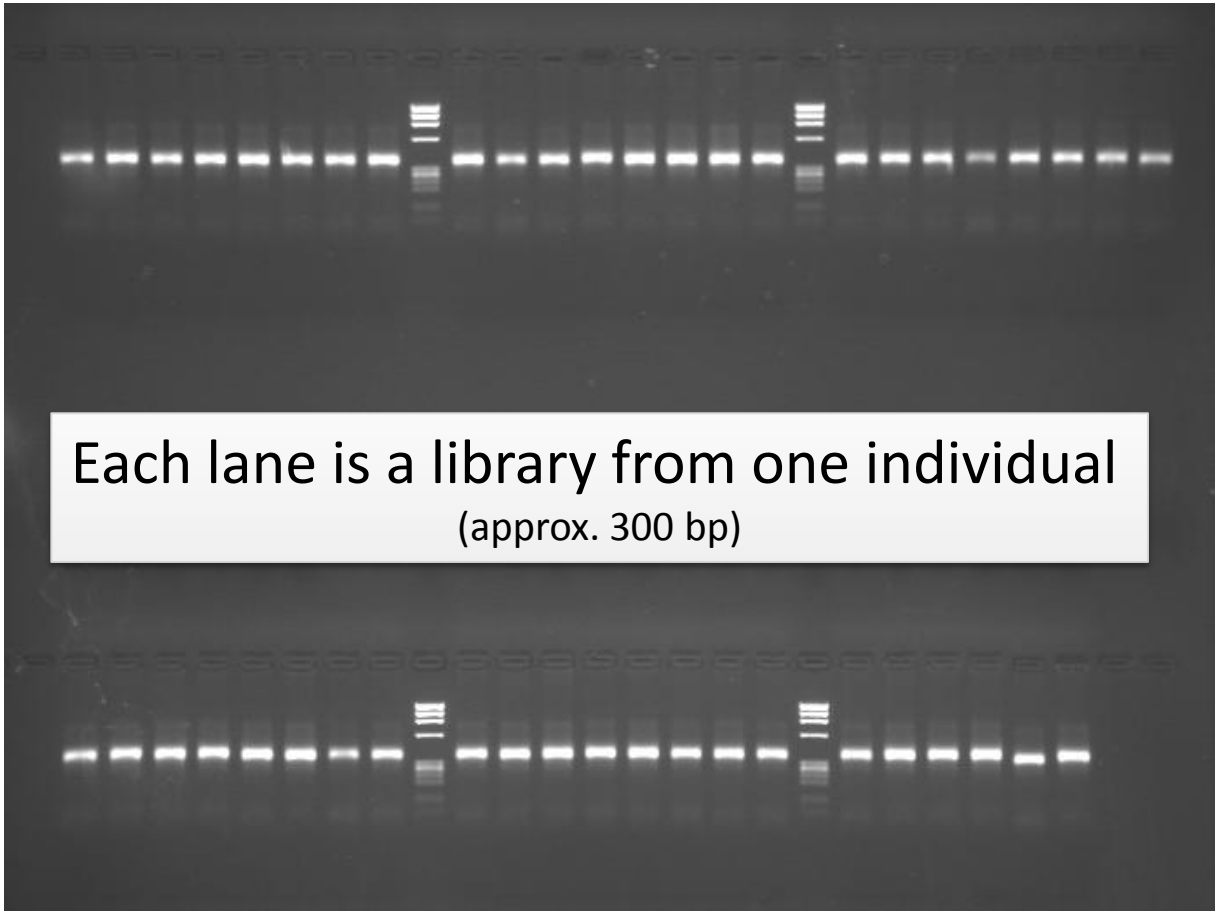
STAG2

Designed to target 24 genes or *gene mutation hotspots

MGP library QC

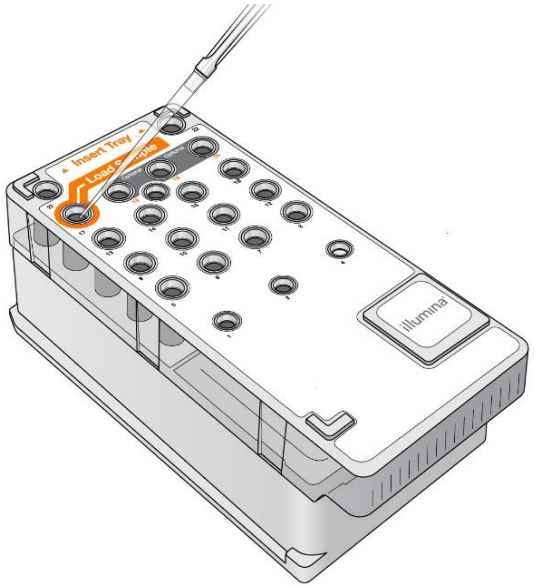
Quantity of DNA used = approx. 200 ng *

- 24-32 samples per run *



MiSeq-Sequencing

- Magnetic bead clean-up and normalisation
- Pool 5 µl of each library into 1 tube
- Take 10-20 µl into loading buffer
- Denature and load into MiSeq cartridge



Some design issues

- Not all amplicons work, even if predicted to be 100% in DesignStudio.
- Problem with software refusing to design a TP53 amplicon ……
- Several TP53 amplicons were spiked into the oligo pool, but didn't appear to work.
- With Ian Lewis's help, a new primer pool was manufactured, with a new TP53 oligo added to the mix……..good result.

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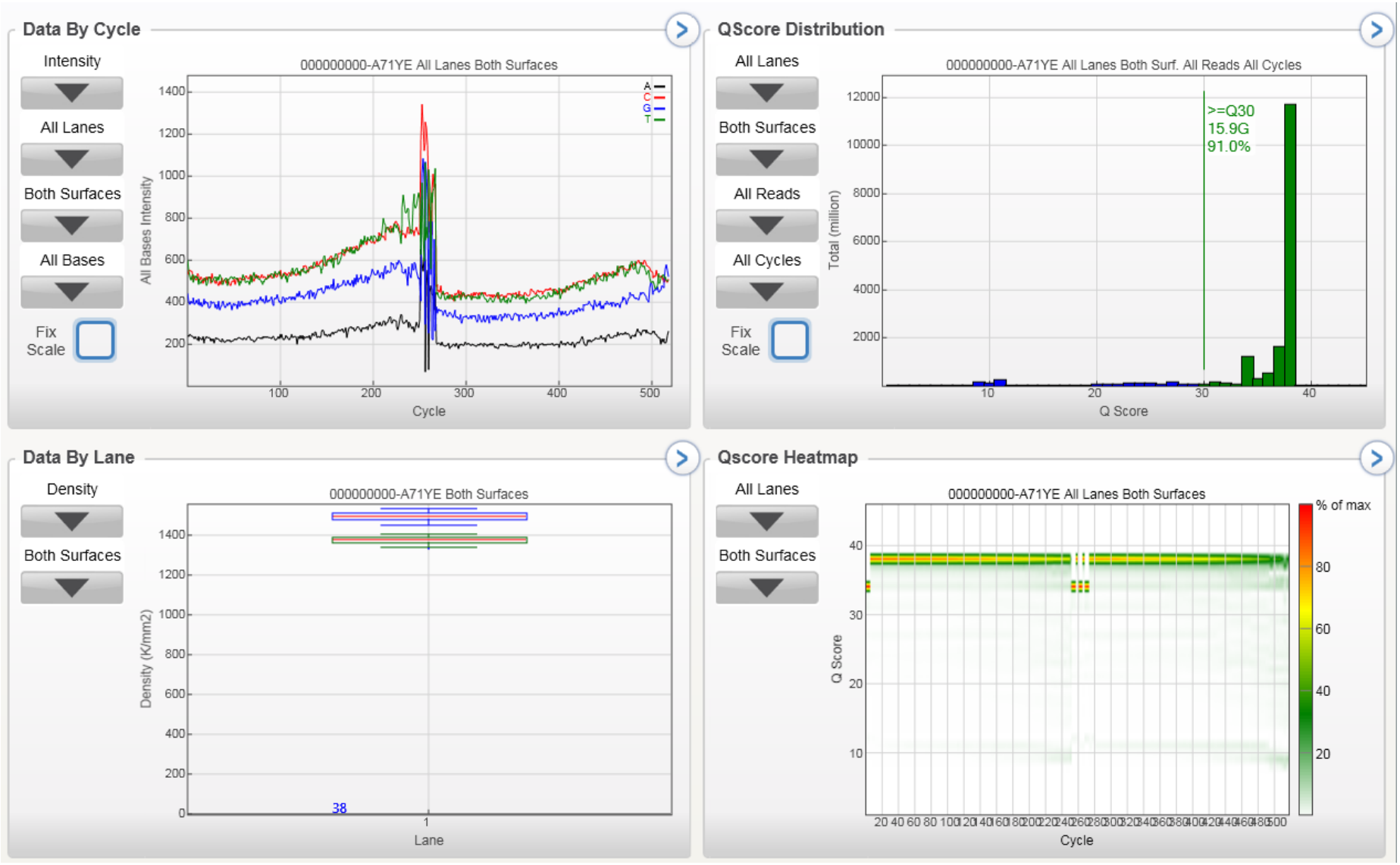
Version 3 kit (2 x 250)



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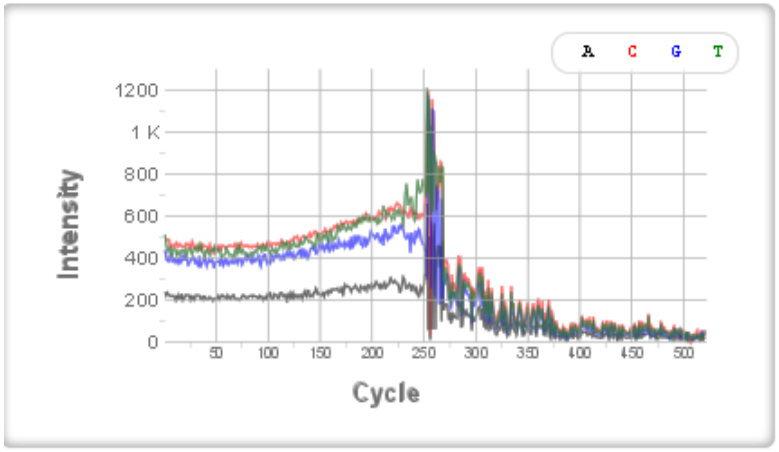
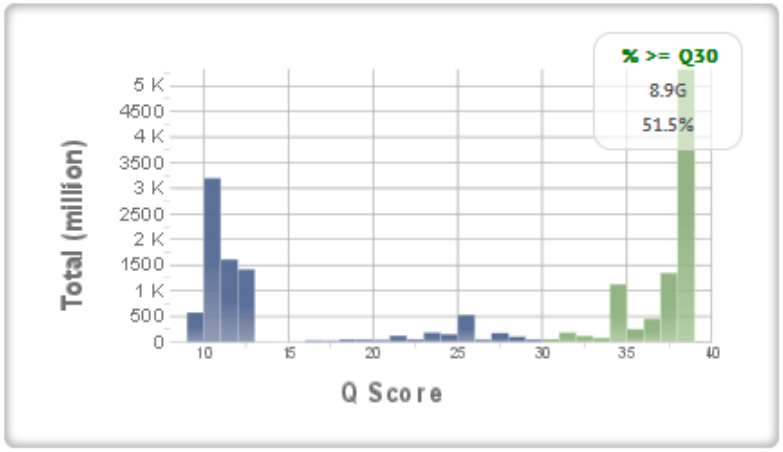
Version 3 kit (2 x 250)

000000000-A6H9E All Lanes All Channels

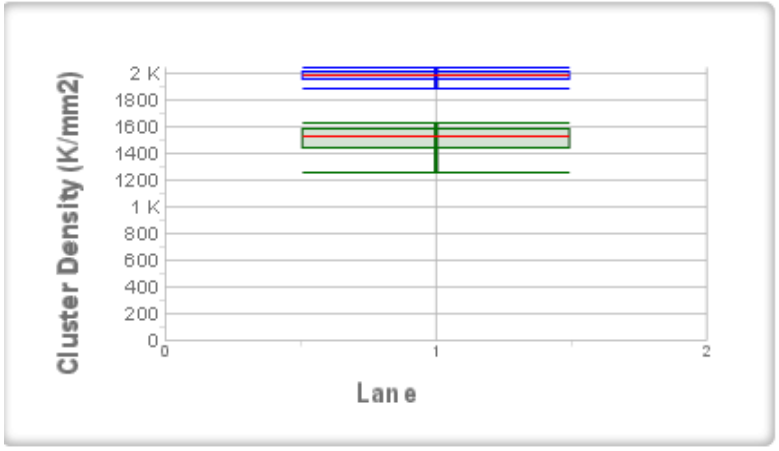


QScore Distribution 000000000-A6H9E All Lanes All Reads All Cycles

Surface
Both Surfaces
Lane
All Lanes
Read
All Reads
Cycle Up To
All Cycles

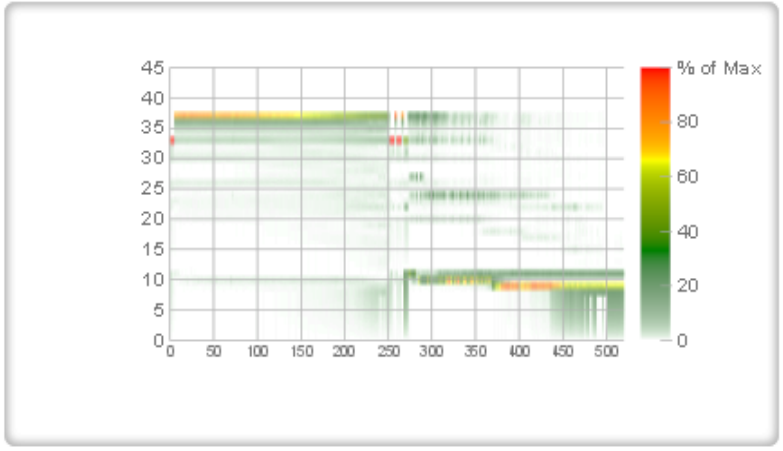


000000000-A6H9E Read 1



QScore Heatmap 000000000-A6H9E All Lanes

Surface
Both Surfaces
Lane
All Lanes



Variant Studio: filtering

Align on the instrument > import vcf files > filters results

Illumina VariantStudio

Home | Annotation & Classification | Reports | Help

New | Open | Close | Save | Save As | Import VCF | Add Variants to Sample | Import Folder | Current Sample: 53431_S4 | Remove Sample | Current: AML 2 | Manage Favorites

Project | Samples | Filter Favorites

Filters

Variant

- [x] Pass Filter
- [ ] Quality > 0
- [x] Read Depth > 150
- [x] Alt Variant Freq > 9 %
- [x] Show only variants:
  - [ ] Inside genes
  - [x] Inside coding regions
  - [ ] In conserved regions
- [ ] Only variants without dbSNP ID

Gene View

Chr 12 — 25,358,180 — KRAS

Gene	Variant	Chr	Coordinate	Type	Alt Variant Freq	Classification	dbSNP ID	COSMIC ID	Read Depth
DNMT3A	C>C/G	2	25457242	snv	40.45	...	rs147001...	COSM442676	8735
TET2	C>C/T	4	106156187	snv	49.12	Unknown Signi...	rs17253672		9622
TET2	T>T/G	4	106196829	snv	49.86	Unknown Signi...	rs34402524	COSM87176	17662
TET2	A>A/G	4	106196951	snv	49.91	High freq SNP	rs2454206		9391
NPM1	C>C/CTCTG	5	170837543	insertion	35.5	...		COSM1319222	3380
IKZF1	C>C/T	7	50467941	snv	48.07	...	rs61731356		3360
CUX1	G>G/A	7	101917521	snv	47.15	...	rs803064		8252
EZH2	C>C/G	7	148525904	snv	48.3	...	rs2302427		4783
KRAS	T>T/G	12	25380275	snv	31.65	...	rs17851045	COSM1135364	3081
ASXL1	T>C/C	20	31022959	snv	99.64	High freq SNP	rs6058694		12345
STAG2	G>G/T	X	123195666	snv	23.05	...			334

Variant Studio: identify variants

Gene View

Chr 12 — KRAS — 45.7Kb

25,358,180 — 25,403,642

Gene	Variant	Chr	Coordinate	Type	Alt Variant Freq	Classification	dbSNP ID	COSMIC ID	Read Depth	Protein Position	Amino Acids	Sift	PolyPhen
DNMT3A	C>C/G	2	25457242	snv	40.45	...	rs147001...	COSM442676	8735	882	R/P	deleterious(0)	probably_dam...
TET2	C>C/T	4	106156187	snv	49.12	Unknown Signi...	rs17253672		9622	363	P/L	deleterious(0.01)	benign(0.161)
TET2	T>T/G	4	106196829	snv	49.86	Unknown Signi...	rs34402524	COSM87176	17662	1721	L/W	deleterious(0.01)	possibly_dama...
TET2	A>A/G	4	106196951	snv	49.91	High freq SNP	rs2454206		9391	1762	I/V	tolerated(0.32)	benign(0.029)
NPM1	C>C/CTCTG	5	170837543	insertion	35.5	...		COSM1319222	3380	287			
IKZF1	C>C/T	7	50467941	snv	48.07	...	rs61731356		3360	243	Q/*		
CUX1	G>G/A	7	101917521	snv	47.15	...	rs803064		8252	464	A/T	tolerated(0.18)	benign(0.006)
EZH2	C>C/G	7	148525904	snv	48.3	...	rs2302427		4783	185	D/H	deleterious(0.03)	possibly_dama...
KRAS	T>T/G	12	25380275	snv	31.65	...	rs17851045	COSM1135364	3081	61	Q/H	deleterious(0)	possibly_dama...
ASXL1	T>C/C	20	31022959	snv	99.64	High freq SNP	rs6058694		12345	815	L/P	tolerated(0.17)	benign(0)
STAG2	G>G/T	X	123195666	snv	23.05	...			334	527	C/F	deleterious(0)	possibly_dama...
BCORL1	T>C/C	X	129147079	snv	99.86	...	rs4830173		10770	111	F/L	tolerated(1)	unknown(0)

Variant 9 of 12

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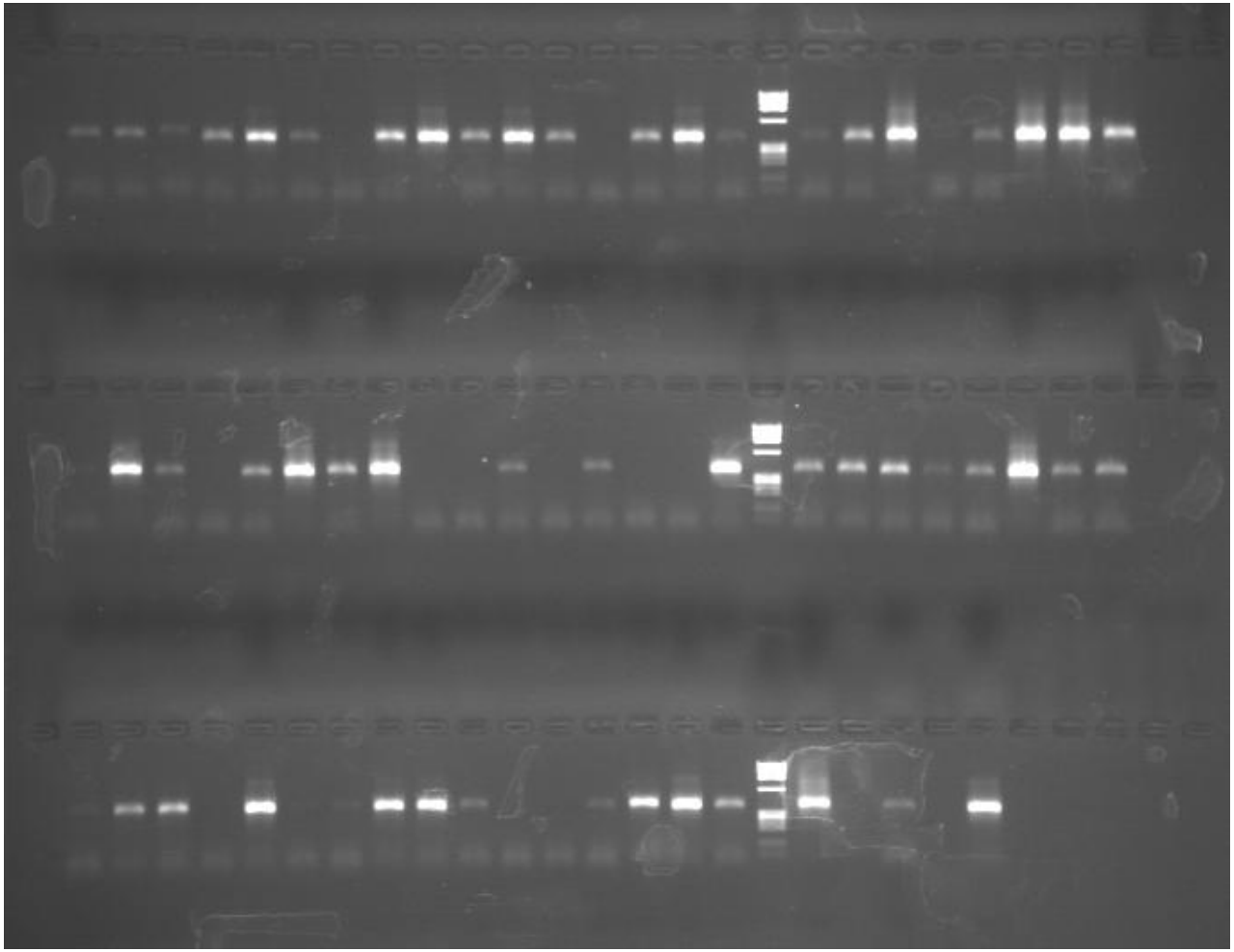
Suboptimal DNA

Problems with very

small amounts of DNA

Suboptimal DNA

These TSCA library preps are from old BM aspirate slides



Suboptimal DNA: WGA

If we use Whole Genome Amplification first.....

Unamplified DNA

Gene	Variant	Chr	Type	Alt Variant Freq	dbSNP	COSMIC	Consequence	Read Depth	Alt Read Depth	Protein Position	Amino Acids
DNMT3A	G>G/A	2	snv	**51**			missense_variant	2466	1248	368	A/V
SF3B1	T>T/C	2	snv	**35**		COSM84677	missense_variant	5727	2027	700	K/E
TET2	C>C/G	4	snv	**49**	rs12498609		missense_variant	9363	4593	29	P/R
TET2	C>C/T	4	snv	**30**		COSM43446	stop_gained	17728	5387	810	Q/*
TET2	C>C/CT	4	insertion	**32**			fs_variant	13571	4384	879	
EZH2	C>C/G	7	snv	**49**	rs2302427		missense_variant	9431	4614	185	D/H

WGA

DNMT3A	G>G/A	2	snv	**66**			missense_variant	602	398	368	A/V
DNMT3A	G>G/T	2	snv	**13**			stop_gained	868	112	337	S/*
SF3B1	T>T/C	2	snv	**31**		COSM84677	missense_variant	2126	656	700	K/E
TET2	C>C/G	4	snv	**46**	rs12498609		missense_variant	2250	1044	29	P/R
TET2	C>C/T	4	snv	**43**		COSM43446	stop_gained	19307	8317	810	Q/*
TET2	C>C/CT	4	insertion	**30**			fs_variant	11306	3416	879	
EZH2	C>C/G	7	snv	**46**	rs2302427		missense_variant	7943	3658	185	D/H
TP53	A>A/C	17	snv	**11**		COSM10788	missense_variant	1045	113	255	I/S
ZRSR2	C>C/T	X	snv	**12**			missense_variant	1187	148	385	P/L

Reporting

KingsHMDC KingsPath

Combined Pathology Report on KHMDC Referral No: IOG-14-6066041

Patient Details

Name
DOB
NHS No
KCH No
KHMDC No

Source

Clinician McLornan, Dr Dr
Location King's College Hospital NHS FTs
Date Collected 05/03/2014 00:00
Date Received 05/03/2014 13:15

Clinical Details

Additional Clinical Information

FINAL REPORT CONCLUSION

Diagnosis

CALR mutation was DETECTED

Results

JAK2 (V617F) Assay Result: NOT DETECTED

JAK2 V617F Assay Sensitivity: 0.2%

Mutation Report Comment: The V617F mutation is highly associated with primary myeloproliferative disorders. The result is reported as "Not Detected" (if undetectable), "Detected but Clinically Equivocal" <1% or "Detected" >1%. At a level of <1%, the clinical diagnostic relevance is unclear and results must be interpreted in the context of other clinical and laboratory findings. If equivocal, we suggest a repeat sample in 3-6 months. (Beech et al BJH 2013;160(1):25-34). Serial quantification has utility in monitoring therapy especially post-transplant.

The incidence of JAK2 mutation is 97%, 57%, and 50% for PV, ET and MF respectively.

Most JAK2 V617F negative MPD cases harbour a mutation in either JAK2 exon 12, CALR or MPL. JAK2

Calreticulin (CALR) V617F negative cases of PV should be screened for JAK2 exon 12 mutations, whereas negative cases of ET and MF should be screened sequentially for CALR exon 9 and MPL exon 10 mutations. Please contact the laboratory if you require additional tests (no additional sample is required).

Performed by Dr N Tabata, authorised by Dr N Lea 10-Mar-14

Mutation Report: CALR exon 9 MUTATION was DETECTED, as shown by PCR fragment length analysis (Assay Sensitivity 2%).

Mutation Report Comment: The sample was analysed for mutations in exon 9 of the CALR gene by fluorescent PCR fragment analysis followed by capillary electrophoresis. These analyses are capable of detecting all known variants reported in this gene associated with myeloproliferative disease with a sensitivity estimated to be <=2%.
It has recently been reported that most patients with essential thrombocythaemia or primary myelofibrosis not associated with a JAK2 or MPL mutation carry a somatic mutation in CALR exon 9. The clinical course of patients with a CALR mutation is thought to be more indolent than that in patients with the JAK2 V617F mutation. N Engl J Med 2013; 369 Dec 19; 2379-2390; N Engl J Med 2013;369:2390-2405.
Please note this test does not currently form part of the LMH laboratories CPA accredited test repertoire. Performed by authorised by Dr. N Lea 24-Mar-14

MPL (W515) Assay

Mutation Report: MPL W515 MUTATION was NOT DETECTED, as shown by Allele specific PCR (Assay Sensitivity 1%).
The sample was analysed for c-MPL W515 mutations by allele specific PCR analysis. This assay is capable of detecting mutations leading to the substitution of codon W515 with amino acids L or K. Other rare mutations have also been detected in the c-MPL

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KingsHMDC KingsPath

gene but are not detected in this assay and are not commonly screened for. The W515 c-MPL mutation has been reported in several large studies to be highly correlated with ET (3%) and PMF (5%). C-MPL W515 mutations are not a feature of polycythaemia vera, and are absent in normal and reactive controls.

Most JAK2 V617F negative / MPL negative ET and MF cases harbor a mutation in either CALR. JAK2 MPL will be accounted cases of ET and MF should be screened sequentially for CALR exon 9 mutations. Please contact the laboratory if you require additional tests (no additional sample is required CALR analysis).
ref: Tefferi A, et al Blood 2006 108 no 10 pp3472-3476, N Engl J Med 2013; 369 Dec 19; 2379-2390; N Engl J Med 2013;369 Dec 19; 2391-2405

AML panel (AML-P) Assay

Mutation Report: King's Myeloid Gene Panel Results

Sample Information
NAME
Hospital no
Date of birth
Sample I.D: LMH 55255 Sample type: PB Sample date: 05/05/2014

RESULTS

gene | amino acid | allele burden

TIER 1 (Known published diagnostic / pathogenic mutations)

gene	amino acid	allele burden
TET2	Ser1548Ter	25%
TET2	Asp1549Tyr	25%

TIER 3 (Mutations of unknown significance / polymorphisms / research interest only)

gene	amino acid	allele burden
TET2	Ala1919Pro	16%
ZRSR2	Ser420SerArg	41%

Genes in the Panel
ASXL exons 1-12, CBL exons 7-9, CEBPA all coding exons, DNMT3A all coding exons, ETV6 TEL all coding exons, EZH2 all coding exons, FLT3 exons 14+20, GATA2 all coding exons, IDH1 exon 4, IDH2 exon 4, JAK2 exons 12+14, KDM6A all coding exons, KIT exons 17, KRAS exons 2+3, NPM1 exon 12, NRAS exons 2+3, RUNX1 all exons except 1+2, SF3B1 exons 12 to 16, SRSF2 exon 1, STAG2 all coding exons, TET2 all coding exons, TP53 all coding exons, U2AF1 exons 2+6, ZRSR2 all coding exons

Mutations in gene regions below 10% frequency are NOT reported. Mutations may be present in regions which failed read depth thresholds identified below, yet

Regions excluded in this sample due to low coverage
CEBPA: 33792459-33792858, 33792833-33792854, 33792833-33795106, RUNX1: 36164341-36164569

Test Overview
24 genes or gene mutation hotspots, sequenced on Illumina Miseq. Results filtered by: minimum read depth of 200 and variant call at least 10% frequency. The sensitivity of this test is undetermined and may not detect all mutations. NGS is unable to identify the majority of large copy number variations, though it is possible that small insertions/deletions < ~50 bp will be detected.

Authorised by: Dr R. Ireland Position: Consultant Date 26.06.2014
Performed by: Dr S Best Position: Scientist Date 24.04.2014

Please note that this test does not currently form part of the LMH laboratory's CPA accredited test repertoire. It is offered on a research basis only and the results should be interpreted in the overall clinical and pathological context.

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FNB Test (FNB) Assay

Sample Type: Peripheral Blood

Granulocyte FNB clone: 0.000 [%]
Monocyte FNB clone: 0.000 [%]
Red cell FNB clone: 0.000 [%]
Type II FNB clone: 0.000 [%]
Type III FNB clone: 0.000 [%]

Conclusion: No myeloproliferative evidence of a type II or type III FNB abnormality

Mutation (MCL) Assay

Mutation Report: FLT3-ITD MUTATION was NOT DETECTED, as shown by PCR fragment length analysis (Assay Sensitivity 2%).

NPM1 MUTATION was NOT DETECTED, as shown by PCR fragment length analysis (Assay Sensitivity 2%).

Mutation Report Comment: This sample tested NORMAL (wild type) for both Nucleophosmin 1 (NPM1) insertion and FMS-like Kinase3 internal tandem duplication (FLT3 ITD). These mutations were NOT DETECTED.

AML patients can be stratified into 3 prognostic groups based on their FLT3 and NPM1 mutation status (Good FLT3 ITD- NPM1+, Intermediate FLT3- NPM1+ or FLT3 ITD+ NPM1+, Poor FLT3+ NPM1-). In particular 3 groups of patients are identified as being at particularly high risk of early relapse within 1 year of achieving remission: those with either high FLT3 ITD mutant level consistent with loss of WT alleles or those carrying an FLT3 ITD in the absence of an NPM1 mutation (Ref: Gale et al Blood 2008 111 2776-2784). Although rare in MDS FLT3 ITD is associated with a high probability of evolution to AML.

For AML patients, the FLT3 ITD and NPM1 analysis of this sample are associated with the INTERMEDIATE RISK GROUP
Performed by Dr S Best, authorised by Dr N Lea 16-May-14

Addendum - Authorised and Released

Authorised by:[illegible] 21/05/2014 08:47:39z

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A combined final report is issued, which includes
Myeloid panel results and others tests and an overall diagnosis.

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Reporting

King's Myeloid Gene Panel Results

NAME:
Hospital no.
Date of birth:
Sample I.D. LMH 53255. Sample type: PB. Sample date: 05/03/2014

RESULTS:

[gene] [amino acid] [allele burden]

DNMT3A [Trp860Ter] [12%]
SF3B1 [Lys700Glu] [12%]

"Well-known" pathogenic variants, Stops and frame shifts

Mutations / variants below 10% frequency are NOT reported. All variants of unknown significance have been excluded. Mutations may be present in regions which failed read depth thresholds: identified below:

Regions excluded in this sample due to low coverage:
CEBPA: 33792459-33792686, 33792633-33792884, 33792833-33793106. RUNX1: 36164341-36164569.

Reporting

Test Overview
24 genes or gene mutation hotspots, sequenced on Illumina MiSeq. Results filtered by: minimum read depth of 200 and variant call of at least 10% frequency. The sensitivity of this test is undetermined and may not detect all mutations. NGS is unable to identify the majority of large indels, though it is possible that small indels < ~30 bp will be detected.

Genes in panel: ASXL exons 1 12, CBL exons 7-9, CEBPA all coding exons, DNMT3A all coding exons, ETV6/TEL all coding exons, EZH2 all coding exons, FLT3 exons 14+20, GATA2 all coding exons, IDH1 exon 4, IDH2 exon 4, JAK2 exons 12+14, KDM6A all coding exons, KIT exons 17, KRAS exons 2+3, NPM1 exon 12, NRAS exons 2+3, RUNX1 all exons except 1+2, SF3B1 exons 12 to 16, SRSF2 exon 1, STAG2 all coding exons, TET2 all coding exons, TP53 all coding exons, U2AF1 exons 2+6, ZRSR2 all coding exons.

Authorised by: Dr R. Ireland Position: Consultant Date:24.04.2014
Performed by: Dr S Best Position: Scientist Date:24.04.2014

Please note that this test does not currently form part of the LMH laboratory's CPA-accredited test repertoire. It is offered on a research-basis only and the results should be interpreted in the overall clinical and pathological context.

Problems still unresolved...

- NEQAS schemes/ISO15189
- Data storage issues.
  Local/network storage
  BaseSpace
- Which data to keep?
  vcf, FastQ, BAM,
  whole analysis folder for
  re-queuing in MSR?

Assays in development

Amplicon-specific NGS using NexteraXT:

- TP53
- SF3B1
- RUNX1
- BCR-ABL TKD

More disease-focused NGS panels for:

- Aplastic anaemia
- Lymphoid malignancies
- Myeloproliferative disorders

Case studies:

1. Elderly lady 91 years presenting with shoulder pain and tiredness
- Macrocytosis (MCV=105)
- Investigated by KCH haematologists - no anaemia or thrombocytopenia
- Peripheral blood MGP shows TET2 [Ile750ArgTer62] 13%
- ? Clonal disorder/ ? evidence of MDS / ? normal finding in 91 year old

2. Gentleman of 66 years persistent neutropenia (1.4-1.9)
- 3 bone marrow aspirates performed none consistent with MDS referred to KCH
- Additional testing rules out other malignancies
- MGP shows TET2[H786EfsTer], TET2[E1191Ter] and ZRSR2[K406NfsTer]
- Not diagnostic but good evidence of MDS and a candidate for transplantation if symptoms worsen.

3. Gentleman diagnosed with RARS-T at 43 years old now 60 years becoming transfusion dependant and “looking more proliferative” spleen slightly enlarged.
- Marrow still looks like RARS-T
- BM MGP shows SF3B1[K700E], TET2[R1467KfsTer11], JAK2[V617F], TP53[R282G]
- Mutations all fit with clinical picture disease maybe progressing but with TP53 mutation we know that transplant outcome is poor.???

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