RHPv3 Methods

The RHPv3 assay assesses regions in 88 genes (some with full coding sequence and others with only targeted loci) that are recurrently mutated in myeloid neoplasms and in lymphoid neoplasms with a common leukemic presentation. DNA from fresh peripheral blood or bone marrow is submitted to NEBNext Direct chemistry (New England Biolabs, Inc., Ipswich, MA) with dual indices and unique molecular identifiers (UMIs), with an overall analytical sensitivity of 3% variant allele fraction at 325x mean consensus coverage and many loci with far greater coverage resulting in lower limits of detection for those loci at or even below 1% variant allele fraction. The library preparation includes an overnight hybridization step and extension from the baits prior to amplification. Samples are then pooled prior to massively parallel sequencing on a NextSeq 550Dx (150 base pair paired-end sequencing, Illumina, San Diego, CA). The bioinformatic pipeline integrates several publicly available and internally designed modules, including BWA Mem (v0.7.17) for raw data processing, Fgbio (v0.4.0) for UMI correction, Vardict (v1.6.0) for calling of single nucleotide variants (SNVs) and small insertion/deletions (indels) with filtering of variants based upon a panel of normals as well as germline frequency threshold, RobustCNV (internally developed) for copy number variants (CNV), and TsaiITD (internally developed) for the detection of *FLT3* internal tandem duplications (*FLT3*-ITDs) (PMID: 32603763). Additional loci have been targeted specifically for improved CNV analysis, the detection of copy neutral loss of heterozygosity of *JAK2* and *TP53*, and the detection of partial tandem duplications of *KMT2A* (*MLL*-PTDs). Variant annotation employs an internally developed tiering algorithm and knowledgebase.

Detection of internal tandem duplications of *FLT3* (*FLT3*-ITDs)

For the detection of *FLT3*-ITDs, a novel algorithm was utilized to infer the presence of ITDs based upon soft clips. An *in silico* reference sequence using the inferred ITD was then utilized to identify candidate reads supporting the presence of that ITD. This algorithm results in 100% concordance with Polymerase chain reaction/capillary electrophoresis (PCR/CE), the gold standard for the detection of *FLT3*-ITDs in the determination of the size of the ITD and an R squared of 0.96 for the allelic ratio of the ITD. This work has been detailed in our publication (PMID: 32603763).

Detection of partial tandem duplications of *KMT2A* (*MLL*-PTDs)

For the detection of KMT2A, 23 additional sets of probes were included covering all or portions of exons 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 24, 25, 26, 28, 29, and 30. Since PTDs typically involve the duplication of exons 3 through 9, 3 through 10, or 3 through 11, the log2 ratios of these exons are compared to those of the terminal exons 24-30 (with the exclusion of exon 27 due to its size) for normalization and determination of relative gains.

Cut-off for assay

All runs must achieve a minimum Q30 score of 80 and cluster density of >129 to pass. In addition, a minimum of 90% of 30 monitored positive control variants (including numerous low-level variants at the limit of detection) must be detected in order for the run to pass. All samples must achieve a minimum mean consensus (after UMI deduplication) target coverage of 325x and a minimum percent target bases with greater than 100x of 85%.

Limit of Detection

The limit of detection of the overall assay is 3% variant allele fraction (VAF) at 325x mean consensus target coverage. However, the use of UMIs allows much lower detection at areas of higher coverage and should be considered as an absolute number of molecules rather than a % fraction. The established limit of detection on a per nucleotide basis is 5 unique molecules. For loci with greater than 500x coverage, this corresponds to 1% VAF. For loci with 2000x coverage, this corresponds to 0.3% VAF.

Analytical sensitivity

In validation, the current assay was compared to variants detected on an orthogonal NGS assay. The analytical sensitivity for single nucleotide variants and small indels was 99.02% after manual review. The sensitivity for copy number variants was 95.8%.

Analytical specificity

In validation, the current assay was compared to variants detected on an orthogonal NGS assay. The analytical sensitivity for single nucleotide variants and small indels was 100.00% after manual review. The specificity for copy number variants was 100.00%.

Variant tiering

Variants are tiered using a proprietary algorithm for separate pathogenic/likely pathogenic from variants of uncertain significance. This algorithm utilizes the most up-to-date knowledge of pathogenic loci from the literature, OncoKB, PeCan, PHANTM, and many other databases. The algorithm was initially established by Dr. R. Coleman Lindsley and the current version is a collaboration between Dr. R. Coleman Lindsley and Dr. Annette S. Kim with support from a knowledgebase team of hematologists and hematopathologists. This tieiring has been vetted by the American Society of Hematology (ASH) Somatic Variant Working Group and Precision Medicine Committee (Dr. Annette S. Kim is a member of both the working group and committee) and a minority of the results are publicly available on the ASH Gene Table website (https://www.hematology.org/research/gene-table). The algorithm is regularly updated as new variant information becomes available.

Targeted Gene/Exon List (genomic coordinates available upon request):

The BWH Rapid Heme Panel (RHP) Assay is a next generation sequencing assay based on the NEBNext kit from New England Biolabs, Inc. A detailed description of the assay is available upon request from Center for Advanced Molecular Diagnostics, Brigham and Women's Hospital.

ABL1 ENST00000372348 5 alt e1	ABL1 ENST00000318560 5 e1-e10
ASXL1 ENST00000306058 4 e11-e12	ATM ENST00000278616 4 e2-e63
ATRX ENST00000373344 5 e1-e35	BCOR ENST00000378444 4 e2-e15
BCORL1 ENST00000540052 1 e1-e12	BCORL1 NM_001184772 1 alt e8
BRAF ENST00000288602 6 e12-e16	BRCC3 ENST00000369462 1 e1-e11
BTK ENST00000308731 7 e11,e15-e16	CALR ENST00000316448 5 e9
CBL ENST00000264033 4 e7-e9	CCND1 ENST00000227507 2 e1-e5
CD79B ENST00000392795 3 e5-e6	CDKN2A ENST00000498124 1 e1-e4
CDKN2A NM_058195 1 alt e1	CDKN2B ENST00000276925 6 e1-e2
CEBPA ENST00000498907 2 e1	CREBBP ENST00000262367 5 e1-e31
CRLF2 ENST00000381567 3 e5	CSF3R ENST00000373103 1 e14-e17
CSNK1A1 ENST00000377843 2 e1-e10	CSNK1A1 ENST00000515768 2 alt e5
CTCF ENST00000264010 4 e3-e12	CUX1 ENST00000292535 7 e1-e24
CUX1 ENST00000292538 7 alt e1,e15-e23	CXCR4 ENST00000241393 3 e3
DDX41 ENST00000507955 1 e1-e17	DKC1 ENST00000369550 5 e1-e15
DNMT3A ENST00000380746 3 alt e1-e2	DNMT3A ENST00000264709 3 e2-e23
DNMT3A NM_001320893 3 alt e1	EP300 ENST00000263253 7 e1-e31
ERG ENST00000288319 2 alt e1	ERG ENST00000417133 2 e3-e12
ERG NM_001243432 2 alt e12	ETNK1 ENST00000266517 4 e3
ETV6 ENST00000396373 4 e1-e8	EZH2 ENST00000320356 2 e2-e20
FBXW7 ENST00000281708 4 e10-e14	FLT3 ENST00000241453 7 e14,e16-e17,e20
GATA1 ENST00000376670 3 e2-e6	GATA2 ENST00000341105 2 e2-e6
GNAS ENST00000371085 3 e8-e9	GNB1 ENST00000378609 4 e5-e6
IDH1 ENST00000345146 2 e3-e10	IDH2 ENST00000330062 3 e1-e11
IKZF1 ENST00000331340 3 e2-e8	IKZF1 ENST00000413698 3 alt e4
IKZF1 ENST00000492782 3 alt e5	IL7R ENST00000303115 3 e5-e7
JAK1 ENST00000342505 4 e10-e25	JAK2 ENST00000381652 3 e12-e20
JAK3 ENST00000458235 1 e16-e24	KIT ENST00000288135 5 e8-e11,e17
KRAS ENST00000311936 3 e2-e6	KRAS ENST00000256078 3 alt e5

KMT2A ENST00000534358 1 e1-e13,e24-e26 MAP2K1 ENST00000307102 5 e2-e3,e6 e28-e30
MPL ENST00000372470 3 e4,e10 NF1 ENST00000356175 3 e1-e57
NF1 ENST00000358273 3 alt e31 MYC ENST00000377970 2 e1-e3
MYD88 ENST00000396334 3 e5 NOTCH2 ENST00000256646 2 e24-e28,e34
NFE2 ENST00000553070 1 e3-e4 NOTCH1 ENST00000277541 6 e24-e28,e34
NSD2 ENST00000382895 3 e20-e21 NPM1 ENST00000296930 5 e10-e11
NRAS ENST00000369535 4 e2-e5 PIGA ENST00000333590 4 e1-e62
NT5C2 ENST00000343289 5 e10-e18 PHF6 ENST00000394292 1 e1-e9
PRPF8 ENST00000304992 6 e25-e34 PLCG2 ENST00000359376 3 e18-e19,e23 e26,e29
PPM1D ENST00000305921 3 e6 RAD21 ENST00000297338 2 e2-e14
PTEN ENST00000371953 3 e1-e9 PTPN11 ENST00000351677 2 e1-e15
SBDS ENST00000246868 2 e1-e5 RIT1 ENST00000368322 3 alt e1
RIT1 ENST00000368323 3 e2-e6 RUNX1 ENST00000437180 1 e2-e9
RUNX1 ENST00000358356 1 alt e5 SF3B1 ENST00000335508 6 e12-e18
SETBP1 ENST00000282030 5 e4 SETD2 ENST00000409792 3 e1-e21
SMC3 ENST00000361804 4 e1-e29 SH2B3 ENST00000538307 2 alt e1
SH2B3 ENST00000341259 2 e2-e8 SMC1A ENST00000322213 4 e1-e25
SMC1A NM_001281463 4 alt e2 STAT3 ENST00000264657 5 e2-e24
SRSF2 ENST00000359995 5 e1 STAG2 ENST00000218089 9 e3-e35
TERT ENST00000310581 5 e1-e16 STAT5B ENST00000293328 3 e13-e19
TERC ENST00000602385 1 e1 U2AF1 ENST00000291552 4 e2,e6
TET2 ENST00000380013 4 e3-e11 TP53 ENST00000269305 4 e2-e11
TP53 ENST00000420246 4 alt e10 ZRSR2 ENST00000307771 7 e1-e11
WT1 ENST00000379079 3 alt e1 WT1 ENST00000332351 3 e1-e10
XPO1 ENST00000401558 2 e15-e16