Supplementary Materials

An All-In-One Transcriptome-Based Assay to Identify Therapy-Guiding Genomic Aberrations in Nonsmall Cell Lung Cancer Patients

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Afbeelding met schermafbeelding

Automatisch gegenereerde beschrijving

**Figure S1.** IGV screenshot of EGFR exon 19 with some correctly aligned reads with additional soft-clipping reads as a result of exon 19 deletion in P22. The total coverage was 38, with 2 reads with a deletion showing the correct alignment and 11 reads with an exon 19 deletion for which the reads were miss-aligned.

Afbeelding met schermafbeelding

Automatisch gegenereerde beschrijving

Forward adapter sequence

5’*AATGATACGGCGACCACCGAGATCTACAC*AGAGCACACGTCTGAACTCCAGTCACX8N6 TCTTTCCCTACACGACGCTCTTCCGATCT(N40)

Reversed adapter sequence  
5’-(N40) AGATCGGAAGAGCACACGTC *ATCTCGTATGCCGTCTTCTGCTTG* 3’

Italic: PCR primers

X8: sample specific barcode

N6: unique molecular identifier

N40: mutation hotspot-specific landing-probe

……..: genomic segment to be sequenced

**Figure S2.** Schematic presentation of the final cDNA fragments of the sequence libraries. Various components of the adapter sequences are indicated, with the full sequence of the adapters given below the figure.

**Table S1.** QC data of our all-in-one transcriptome-based NGS assay.

| **Sample ID** | **Design** | **Origin** | **Year of FFPE** | **RNA Input (ng)** | **DV 200** | **Ct Value** | **Ampl. Cycles** | **Tapestation Average Size (bp)** | **Kapa qPCR on Library Molarity (pmol/l)** | **Panel Version** | **Clean Reads** | **Q20** | **Unique Reads on Target** | **U%** |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| A549 | 1 | cell line | na | 200 | nd | 28 | 28 | 508 | nd | D1 | 3,101,968 | 99 | 284,952 | 9% |
| H1299 | 1 | cell line | na | 121 | nd | 25 | 25 | 429 | nd | D1 | 2,927,998 | 99 | 321,756 | 11% |
| H1650 | 1 | cell line | na | 200\* | nd | 26 | 27 | 415 | nd | D1 | 1,235,674 | 97 | 97,845 | 8% |
| H1975 | 1 | cell line | na | 200 | nd | 27 | 28 | 508 | nd | D1 | 1,440,484 | 99 | 235,397 | 16% |
| H2228 | 1 | cell line | na | 200\* | nd | 21 | 22 | 436 | nd | D1 | 2,640,582 | 99 | 222,701 | 8% |
| H596 | 2 | cell line | na | 200 | 98 | 28 | 29 | 370 | 449 | D2 | 2,548,806 | 98 | 1,262,964 | 50% |
| H820 | 2 | cell line | na | 200 | 99 | 22 | 24 | 451 | 3,699 | D2 | 1,993,700 | 96 | 618,358 | 31% |
| HCT116 | 1 | cell line | na | 188 | nd | 21 | 22 | 487 | nd | D1 | 1,908,568 | 99 | 289,423 | 15% |
| Hs746T | 2 | cell line | na | 200 | 97 | 22 | 24 | 466 | 3,932 | D2 | 1,852,642 | 95 | 639,210 | 35% |
| KM12 | 2 | cell line | na | 200 | 94 | 23 | 24 | 491 | 2,946 | D2 | 2,394,708 | 98 | 792,640 | 33% |
| KOPN-8 | 2 | cell line | na | 200 | 99 | 21 | 24 | 457 | 6,548 | D2 | 1,855,904 | 99 | 201,459 | 11% |
| P01 | 2 | PE | na | 137 | 90 | 25 | 26 | 458 | 1,032 | D2 | 2,502,766 | 98 | 199,647 | 8% |
| P02 | 2 | FFPE | 2015 | 500 | 51 | 27 | 30 | 328 | 3,865 | D2 | 1,747,596 | 98 | 36,593 | 2% |
| P03 | 1 | FFPE | 2015 | 500 | nd | 29 | 32 | 356 | nd | D1 | 3,765,136 | 97 | 67,381 | 2% |
| P04\_S1 | 3 | FFPE | 2017 | 500 | 26 | 24 | 25 | 372 | 693 | D3 | 3,193,166 | 98 | 221,944 | 7% |
| P04\_S2 | 3 | PE | na | 200 | 88 | 22 | 23 | 459 | 2,976 | D3 | 3,896,308 | 99 | 1,331,315 | 34% |
| P05 | 2 | PE | na | 200 | 17 | 26 | 29 | 364 | 4,450 | D2 | 1,960,866 | 98 | 167,871 | 9% |
| P06 | 1 | FFPE | 2016 | 500 | 37 | 29 | 32 | 338 | nd | D1 | 1,606,174 | 97 | 23,351 | 1% |
| P07 | 2 | FFPE | 2016 | 500 | 65 | 25 | 26 | 379 | 1,270 | D2 | 2,219,310 | 98 | 41,696 | 2% |
| P08 | 1 | FFPE | 2016 | 500 | 67 | 25 | 26 | 359 | nd | D1 | 4,665,964 | 97 | 178,860 | 4% |
| P09 | 3 | FFPE | 2017 | 500 | 38 | 23 | 25 | 324 | 1,104 | D3 | 2,802,770 | 98 | 308,991 | 11% |
| P10 | 3 | FFPE | 2017 | 500 | 36 | 24 | 25 | 349 | 234 | D3 | 2,408,928 | 98 | 76,990 | 3% |
| P11 | 2 | FFPE | 2017 | 500 | 81 | 27 | 29 | 346 | 1,396 | D2 | 2,318,920 | 98 | 79,747 | 3% |
| P13 | 3 | PE | na | 200 | 89 | 21 | 22 | 476 | 2,763 | D3 | 2,605,532 | 98 | 1,297,907 | 50% |
| P14 | 2 | PE | na | 200 | 86 | 22 | 24 | 462 | 2,176 | D2 | 2,197,688 | 98 | 600,434 | 27% |
| P15 | 1 | FFPE | 2014 | 304 | 40 | 28 | 32 | 358 | nd | D1 | 2,827,802 | 97 | 79,865 | 3% |
| P16 | 3 | FFPE | 2017 | 25 | 50 | 24 | 25 | 411 | 87 | D3 | 16,938 | 98 | 1,223 | 7% |
| P17 | 1 | FFPE | 2014 | 195 | 57 | 25 | 25 | 369 | nd | D1 | 315,038 | 97 | 79,759 | 25% |
| P18 | 2 | Frozen | na | 200 | 86 | 23 | 25 | 447 | 2,681 | D2 | 4,500,346 | 98 | 466,598 | 10% |
| P19 | 3 | FFPE | 2017 | 200 | 47 | 24 | 25 | 372 | 309 | D3 | 2,796,480 | 98 | 81,208 | 3% |
| P21 | 2 | FFPE | 2017 | 432 | 34 | 27 | 29 | 337 | 1,403 | D2 | 2,261,980 | 98 | 80,234 | 4% |
| P22 | 2 | FFPE | 2014 | 500 | 69 | 24 | 26 | 353 | 2,648 | D2 | 1,638,530 | 98 | 86,309 | 5% |
| P23 | 2 | FFPE | 2015 | 500 | 44 | 28 | 30 | 343 | 478 | D2 | 2,544,046 | 98 | 19,638 | 1% |
| P25 | 1 | FFPE | 2015 | 500 | 71 | 26 | 29 | 364 | nd | D1 | 3,346,274 | 96 | 139,373 | 4% |
| P26 | 1 | FFPE | 2014 | 500 | nd | 29 | 32 | 341 | nd | D1 | 3,134,408 | 96 | 59,613 | 2% |
| P28 | 3 | FFPE | 2017 | 380 | 38 | 24 | 25 | 344 | 309 | D3 | 1,990,944 | 98 | 88,560 | 4% |
| P29 | 1 | FFPE | 2014 | 500 | nd | 29 | 32 | 343 | nd | D1 | 2,811,104 | 97 | 75,307 | 3% |
| P31 | 1 | FFPE | 2014 | 500 | 66 | 25 | 28 | 356 | nd | D1 | 2,009,752 | 97 | 211,390 | 11% |
| P32 | 2 | FFPE | 2011 | 500 | 32 | 28 | 29 | 394 | 135 | D2 | 812,936 | 99 | 4,915 | 1% |
| P33 | 2 | FFPE | 2012 | 500 | 58 | 28 | 30 | 361 | 990 | D2 | 1,856,548 | 98 | 21,902 | 1% |
| P34\_S1 | 2 | FFPE | 2013 | 500 | 76 | 25 | 27 | 351 | 2,729 | D2 | 2,117,588 | 98 | 78,515 | 4% |
| P34\_S1 | 1 | Frozen | na | 160 | 82 | 20 | 21 | 489 | nd | D1 | 5,066,278 | 96 | 455,287 | 9% |
| P34\_S2 | 2 | FFPE | 2013 | 500 | 70 | 24 | 25 | 350 | 2,633 | D2 | 2,347,452 | 97 | 145,018 | 6% |
| P35 | 3 | PE | na | 200 | 81 | 21 | 22 | 425 | 3,333 | D3 | 3,312,072 | 97 | 1,276,594 | 39% |
| P36 | 2 | PE | na | 200 | 93 | 21 | 22 | 461 | 3,320 | D2 | 2,353,046 | 98 | 1,037,592 | 44% |
| P37 | 2 | PE | na | 200 | 21 | 25 | 26 | 432 | 1,002 | D2 | 2,407,286 | 97 | 232,418 | 10% |
| P38 | 2 | FFPE | 2014 | 500 | 55 | 24 | 26 | 340 | 4,444 | D2 | 1,873,690 | 98 | 107,167 | 6% |
| P39 | 2 | FFPE | 2014 | 500 | 65 | 27 | 28 | 359 | 1,551 | D2 | 1,847,672 | 98 | 57,088 | 3% |
| P40 | 2 | FFPE | 2014 | 500 | 68 | 27 | 28 | 376 | 571 | D2 | 2,233,814 | 98 | 42,301 | 2% |
| P41 | 1 | FFPE | 2015 | 380 | nd | 27 | 30 | 333 | nd | D1 | 1,617,424 | 97 | 67,491 | 4% |
| P42 | 2 | PE | na | 200 | 53 | 24 | 26 | 401 | 2,352 | D2 | 2,769,332 | 98 | 599,272 | 22% |
| Control Samples | | | | | | | | | | | | | | |
| KM12a | 2 | cell line | na | 50 | 96 | 24 | 26 | 487 | 1,978 | D2 | 2,155,384 | 96 | 253,719 | 12% |
| KM12a | 2 | cell line | na | 10 | 96 | 26 | 28 | 460 | 2,625 | D2 | 2,329,356 | 96 | 148,920 | 6% |
| P34\_S1a | 2 | Frozen | na | 50 | 80 | 23 | 24 | 436 | 3,338 | D2 | 1,925,044 | 96 | 482,731 | 25% |
| P34\_S1a | 2 | Frozen | na | 10 | 81 | 25 | 28 | 426 | 6,751 | D2 | 1,979,056 | 96 | 188,064 | 10% |
| P42a | 2 | PE | na | 50 | 51 | 26 | 28 | 404 | 2,814 | D2 | 1,952,360 | 96 | 139,801 | 7% |
| P42a | 2 | PE | na | 10 | 45 | 27 | 28 | 462 | 474 | D2 | 2,052,066 | 96 | 49,665 | 2% |
| P31b | 2 | FFPE | 2014 | 500 | 66 | 26 | 28 | 387 | 1,229 | D2 | 2,120,348 | 98 | 66,964 | 3% |
| P15b | 2 | FFPE | 2014 | 500 | 43 | 25 | 27 | 372 | 1,051 | D2 | 1,731,948 | 98 | 73,397 | 4% |
| P34\_S1b | 2 | Frozen | na | 200 | 82 | 26 | 28 | 407 | 2,611 | D2 | 1,697,218 | 98 | 90,148 | 5% |
| P25C | 1 | FFPE | 2015 | na | na | na | na | na | na | D1 | 532,946 | 98 | 86,792 | 16% |
| P03C | 1 | FFPE | 2015 | na | na | na | na | na | na | D1 | 332,448 | 98 | 22,639 | 7% |
| P17C | 1 | FFPE | 2014 | na | na | na | na | na | na | D1 | 315,038 | 98 | 79,759 | 25% |
| P29C | 1 | FFPE | 2014 | na | na | na | na | na | na | D1 | 252,738 | 98 | 26,561 | 11% |
| P15C | 1 | FFPE | 2014 | na | na | na | na | na | na | D1 | 250,314 | 98 | 29,124 | 12% |
| P34\_S1C | 1 | Frozen | na | na | na | na | na | na | na | D1 | 274,416 | 98 | 57,478 | 21% |

\* samples of which we lost 1/3 to 1/4 of the RNA accidently during library preparation that were excluded from linear regression analysis by SPSS, control samples were not analyzed in linear regression analysis by SPSS; a samples analyzed with lower input; b samples analyzed both in design one and design two; c samples of which 1/8 of the required library input was pooled and sequenced; U%: percentage of unique reads; PE: pleural effusion; FFPE: formalin-fixed paraffin-embedded tissue; na: not applicable; nd: not done.

**Table S2.** Overview of RNA-based ddPCR results.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Sample ID** | **Origin** | **DV 200** | **MD Variant** | | **NGS Result** | | | **ddPCR Validation** | | | | |
| **Gene** | **Amino Acid Change** | **Mutant Reads** | **Total Reads** | **VAF** | **Input RNA (ng)** | **Mutant Copies** | **Wild Type Copies** | **Fractional Abundance** | **Wells** |
| Variants Detected by Our All-In-One Assay | | | | | | | | | | | | | |
| A549 | cell line | na | *KRAS* | p.G12S | 512 | 513 | 100% | 1 | 380 | 0 | 100% | 2 |
| H1650 | cell line | na | *EGFR* | E19 DEL | 72 | 98 | 73% | 1 | 2350 | 1110 | 68% | 2 |
| H1975 | cell line | na | *EGFR* | p.L858R | 564 | 684 | 82% | 1 | 1988 | 402 | 83% | 2 |
| H1975 | cell line | na | *EGFR* | p.T790M | 347 | 425 | 82% | 2 | 4831 | 996 | 83% | 1 |
| HCT116 | cell line | na | *KRAS* | p.G13D | 223 | 456 | 49% | 1 | 259 | 217 | 54% | 2 |
| KOPN-8 | cell line | 99 | *KRAS* | p.G12D | 99 | 177 | 56% | 1 | 268 | 283 | 49% | 2 |
| P01 | PE | 90 | *KRAS* | p.G12D | 14 | 111 | 13% | 8 | 64 | 364 | 15% | 1 |
| P03 | FFPE | na | *KRAS* | p.G12A | 8 | 8 | 100% | 8 | 10 | 0 | 100% | 1 |
| P06 | FFPE | 37 | *EGFR* | E19 DEL | 20 | 28 | 71% | 5 | 527 | 55 | 91% | 3 |
| P15 | FFPE | 40 | *EGFR* | E19 DEL | 119 | 143 | 83% | 5 | 629 | 165 | 79% | 3 |
| P17 | FFPE | 57 | *EGFR* | E19 DEL\* | 311 | 373 | 83% | 8 | 1540 | 203 | 88% | 2 |
| P17 | FFPE | 57 | *EGFR* | p.T790M | 62 | 127 | 49% | 6 | 1420 | 614 | 70% | 1 |
| P22 | FFPE | 69 | *EGFR* | E19 deletion | 13 | 38 | 34% | 8 | 186 | 167 | 53% | 2 |
| P39 | FFPE | 65 | *KRAS* | p.G12D | 8 | 12 | 67% | 8 | 25 | 23 | 53% | 1 |
| Variants Not Detected by Our All-In-One Assay | | | | | | | | | | | | | |
| P05 | PE | 17 | *EGFR* | E19 DEL | 0 | 0 | na | 100 | 12 | 9 | 57% | 4 |
| P06 | FFPE | 37 | *EGFR* | p.T790M | 0 | 0 | na | 324 | 21 | 188 | 10% | 3 |
| P23 | FFPE | 44 | *KRAS* | p.G12C | 0 | 0 | na | 25 | 19 | 8 | 71% | 2 |
| P26 | FFPE | na | *EGFR* | p.L858R | 0 | 0 | na | 370 | 177 | 422 | 30% | 4 |
| P32 | FFPE | 32 | *KRAS* | p.G12D | 0 | 0 | na | 497 | 0 | 8 | 0% | 3 |
| P40 | FFPE | 68 | *KRAS* | p.G12F | 0 | 0 | na | 30 | 95 | 68 | 59% | 1 |

\* Read counts for EGFR exon 19 are based on IGV; MD: molecular diagnostic; VAF: Variant allele frequency; PE: pleural effusion; FFPE: formalin-fixed paraffin-embedded tissue; na: not analyzed; E19 DEL: exon 19 deletion**.**

**Table S3.** Overview of NanoString results on samples with expected fusions.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sample ID** | **Origin** | **MD\*** | **Fusion Transcript  by RNA-Based Assay** | **Nanostring  Validation** |
|
| P07 | FFPE | *ALK* | not detected | Positive |
| P08 | FFPE | *ALK* | KIF5B\_E24-ALK\_E20 | Positive |
| P08 | FFPE | *RET* | not detected | Negative |
| P11 | FFPE | *RET* | KIF5B\_E15-RET\_E12 | Positive |
| P18 | FFPE | *ALK* | EML4\_E6-ALK\_E20 | Positive |
| P34\_S1 | FFPE | *ALK* | EML4\_E6-ALK\_E20 | Positive |
| P38 | FFPE | *ROS1* | EZR\_E10-ROS1\_E34 | Positive |

\* FISH break and/or IHC positive cases as reported by the molecular diagnostics.

**Table S4.** Progression free survival of ALK positive patients.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Patient ID** | **FISH** | **IHC** | **Fusion Transcript** | **TKI** | **PFS** |
| P07 | + | + | EML4\_E6-ALK\_E20\* | crizotinib | 14 |
| P08 | + | + | KIF5B\_E24-ALK\_E20 | ceritinib | 19 |
| P13 | + | nd | EML4\_E6-ALK\_E20 | crizotinib | 8 |
| P14 | + | nd | DCTN1\_E26-ALK\_E20 | crizotinib /alectinib | no response |
| P18 | + | + | EML4\_E6-ALK\_E20 | crizotinib | 9 |
| P33 | + | + | EML4\_E6-ALK\_E20 | crizotinib | 6 |
| P34\_S1 | + | + | EML4\_E6-ALK\_E20 | crizotinib | 15 |
| P34\_S2 | + | + | EML4\_E6-ALK\_E20 | ceritinib | 12 |
| P36 | + | + | EML4\_E6-ALK\_E18 | crizotinib | 24 |
| P42 | + | + | MPRIP\_E21-ALK\_E20 | crizotinib | 8 |

\* fusion gene detected by NanoString; PFS: progression free survival; TKI: Tyrosine Kinase Inhibitor; nd: not done

**Table S5.** Overview of variant calling for samples sequenced with low and normal library input.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Sample ID** | **Gene** | **Variant** | **Standard Library Input** | | | **1/8 Library Input** | | |
| **Altered Reads/ Splitting Reads** | **Total Reads/ Spanning Reads** | **MAF** | **Altered Reads/ Splitting Reads** | **Total Reads/ Spanning Reads** | **MAF** |
| P03 | *KRAS* | p.G12A | 8 | 8 | 100% | 4 | 4 | 100% |
| P15 | *EGFR* | E19 deletion | 81 | 110 | 74% | 49 | 58 | 84% |
| P15 | *EGFR* | p.T790M | 22 | 88 | 25% | 13 | 50 | 26% |
| P17 | *EGFR* | E19 deletion | 123 | 143 | 86% | 175 | 198 | 88% |
| P17 | *EGFR* | p.T790M | 62 | 127 | 49% | 92 | 141 | 65% |
| P25 | *BRAF* | p.V600E | 33 | 46 | 72% | 9 | 18 | 50% |
| P34\_S1 | *ALK* | fusion | 62 | 41 |  | 7 | 3 |  |

**Table S6.** Overview of variant calling for samples with normal and low RNA input.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sample ID** | **Gene** | **Input** | **Fusion (5’ gene-3’ gene)** | **5' Gene Count-3' Gene Count** |
| KM12 | *NTRK1* | 200 | TPM3\_E7-NTRK1\_E9 | 188, 87 |
| KM12 | *NTRK1* | 50 | TPM3\_E7-NTRK1\_E9 | 132, 22 |
| KM12 | *NTRK1* | 10 | TPM3\_E7-NTRK1\_E9 | 88, 26 |
| P34\_S1 | *ALK* | 200 | EML4\_E6-ALK\_E20 | 6, 2 |
| P34\_S1 | *ALK* | 50 | EML4\_E6-ALK\_E20 | 16, 23 |
| P34\_S1 | *ALK* | 10 | EML4\_E6-ALK\_E20 | 9, 10 |
| P42 | *ALK* | 200 | MRPIP\_E21-ALK\_E20 | 8, 20 |
| P42 | *ALK* | 50 | MRPIP\_E21-ALK\_E20 | 1, 6 |
| P42 | *ALK* | 10 | MRPIP\_E21-ALK\_E20 | 0, 6 |

**Table S7.** Overview of variant calling results in relation to QC data.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Sample ID** | **Variant 1 (\*)** | **Variant 2** | **Variant 3** | **Unique reads** | **RNA input (ng)** | **Origin** | **Year of FFPE** | **DV200** | **Pass QC \*\*** |
| A549 | *KRAS* G12S:+ |  |  | 284,952 | 200 | cell line | na | nd | na |
| H1299 | *NRAS* Q61K:+ |  |  | 321,756 | 121 | cell line | na | nd | na |
| H1650 | *EGFR* E746\_A750del:+ |  |  | 97,845 | 200 | cell line | na | nd | na |
| H1975 | *EGFR* L858R :+ | *EGFR* T790M :+ |  | 235,397 | 200 | cell line | na | nd | na |
| H820 | *EGFR* E746\_A750del:+ | *EGFR* T790M :+ |  | 618,358 | 200 | cell line | na | 99 | Yes |
| KOPN-8 | *KRAS* G12D:+ |  |  | 201,459 | 200 | cell line | na | 99 | Yes |
| HCT116 | *KRAS* G13D:+ | *PIK3CA* H1047R:+ |  | 289,423 | 188 | cell line | na | nd | na |
| H596 | *PIK3CA* E545K:+ | *MET* E14 skipping:+ |  | 1,262,964 | 200 | cell line | na | 98 | Yes |
| Hs746T | *MET* E14 skipping:+ |  |  | 639,210 | 200 | cell line | na | 97 | Yes |
| H2228 | *ALK* fusion:+ |  |  | 222,701 | 200 | cell line | na | nd | na |
| KM12 | *NTRK1* fusion:+ |  |  | 792,640 | 200 | cell line | na | 94 | Yes |
| P18 | *ALK* fusion:+ |  |  | 466,598 | 200 | Frozen | na | 86 | Yes |
| P34\_S1 | *ALK* fusion:+ |  |  | 455,287 | 160 | Frozen | na | 82 | Yes |
| P35 | *AKT1* E17K:+ | *BRAF* V600E :+ |  | 1,276,594 | 200 | PE | na | 81 | Yes |
| P05 | *EGFR* E746\_A750del:- |  |  | 167,871 | 200 | PE | na | 17 | No |
| P04\_S2 | *EGFR* L858R:+ |  |  | 1,331,315 | 200 | PE | na | 88 | Yes |
| P01 | *KRAS* G12D:+ |  |  | 199,647 | 137 | PE | na | 90 | Yes |
| P14 | *ALK* fusion:+ |  |  | 600,434 | 200 | PE | na | 86 | Yes |
| P36 | *ALK* fusion:+ |  |  | 1,037,592 | 200 | PE | na | 93 | Yes |
| P37 | *ROS1* D2033N:+ | *ROS1* fusion:+ |  | 232,418 | 200 | PE | na | 21 | No |
| P42 | *ALK* fusion:+ |  |  | 599,272 | 200 | PE | na | 53 | Yes |
| P13 | *ALK* I1171N:+ | *ALK* G1269A :+ | *ALK* fusion:+ | 1,297,907 | 200 | PE | na | 89 | Yes |
| P25 | *BRAF* V600E:+ |  |  | 139,373 | 500 | FFPE | 2015 | 71 | Yes |
| P15 | *EGFR* E746\_A750del:+ | *EGFR* T790M:+ |  | 79,865 | 304 | FFPE | 2014 | 40 | Yes |
| P17 | *EGFR* E746\_A750del:+ | *EGFR* T790M:+ |  | 79,759 | 195 | FFPE | 2014 | 57 | Yes |
| P22 | *EGFR* E746\_A750del:+ |  |  | 86,309 | 500 | FFPE | 2014 | 69 | Yes |
| P06 | *EGFR* L747\_P753delinsS:+ | *EGFR* T790M:- |  | 23,351 | 500 | FFPE | 2016 | 37 | No |
| P26 | *EGFR* L858R:- | *PIK3CA* E542K:- |  | 59,613 | 500 | FFPE | 2014 | nd | na |
| P04\_S1 | *EGFR* L858R:+ |  |  | 221,944 | 500 | FFPE | 2017 | 26 | No |
| P03 | *KRAS* G12A:+ |  |  | 67,381 | 500 | FFPE | 2015 | nd | na |
| P28 | *KRAS* G12A:+ |  |  | 88,560 | 380 | FFPE | 2017 | 38 | Yes |
| P23 | *KRAS* G12C:- |  |  | 19,638 | 500 | FFPE | 2015 | 44 | No |
| P32 | *KRAS* G12D:- |  |  | 4,915 | 500 | FFPE | 2011 | 32 | No |
| P39 | *KRAS* G12D:+ |  |  | 57,088 | 500 | FFPE | 2014 | 65 | Yes |
| P40 | *KRAS* G12F:- |  |  | 42,301 | 500 | FFPE | 2014 | 68 | No |
| P31 | *KRAS* Q61H:+ |  |  | 211,390 | 500 | FFPE | 2014 | 66 | Yes |
| P02 | *PIK3CA* H1047L:+ |  |  | 36,593 | 500 | FFPE | 2015 | 51 | No |
| P21 | *MET* E14 skipping:+ |  |  | 80,234 | 432 | FFPE | 2017 | 34 | Yes |
| P07 | *ALK* L1196M:+ | *ALK* Fusion: - |  | 41,696 | 500 | FFPE | 2016 | 65 | No |
| P08 | *ALK* fusion:+ | (*RET* fusion) |  | 178,860 | 500 | FFPE | 2016 | 67 | Yes |
| P33 | *ALK* fusion:+ |  |  | 21,902 | 500 | FFPE | 2012 | 58 | No |
| P34\_S1 | *ALK* fusion:+ |  |  | 78,515 | 500 | FFPE | 2013 | 76 | Yes |
| P34\_S2 | *ALK* fusion:+ |  |  | 145,018 | 500 | FFPE | 2013 | 70 | Yes |
| P11 | *RET* fusion:+ |  |  | 79,747 | 500 | FFPE | 2017 | 81 | Yes |
| P38 | *ROS1* fusion:+ |  |  | 107,167 | 500 | FFPE | 2014 | 55 | yes |
| P41 | *ROS1* fusion:+ |  |  | 67,491 | 380 | FFPE | 2015 | nd | na |

\* variants observed are indicated in black and marked with +; variants not observed are indicated in red and marked with - ; \*\*Pass QC: passed assay quality control; Variants between brackets are true negatives. PE: pleural effusion; FFPE: formalin-fixed paraffin-embedded; na: not applicable; nd: not determined**.**

**Table S8.** Linear Regression analysis to identify variables associated with percentage of unique reads.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Variable** | **Univariable Analysis** | | **Multivariable Analysis** | |
|  | **ß (95% Confidence)** | ***p* Value** | **ß (95% Confidence)** | ***p* Value** |
| Design Version | 0.063 (0.003, 0.123) | 0.039 | 0.037 (−0.022, 0.096) | 0.215 |
| Material Type | 0.213 (0.15, 0.275) | 0.000 | 0.133 (0.016, 0.249) | 0.027 |
| RNA Input | −0.001 (−0.001, 0) | 0.000 | −0.0001 (−0.0004, 0.0003) | 0.604 |
| DV200 | 0.004 (0.003, 0.006) | 0.000 | 0.002 (0, 0.004) | 0.019 |
| PCR Cycles | −0.03 (−0.041, −0.019) | 0.000 | −0.006 (−0.023, 0.011) | 0.488 |

**Table S9.** Number of landing probes per gene in the three designs.

| **Category** | **Gene** | **Landing Probe Count** | | |
| --- | --- | --- | --- | --- |
| **Design 1** | **Design 2** | **Design 3** |
| mutation | *AKT1* |  | 2 | 1 |
| mutation | *BRAF* | 7 | 4 | 7 |
| mutation | *DDR2* |  | 5 | 2 |
| mutation | *EGFR* | 10 | 11 | 10 |
| mutation | *ERBB2* |  | 5 | 5 |
| mutation | *HRAS* |  | 6 | 7 |
| mutation | *JAK2* |  | 2 | 17 |
| mutation | *KIT* |  | 15 | 14 |
| mutation | *KRAS* | 4 | 6 | 7 |
| mutation | *MAP2K1* |  | 13 | 13 |
| mutation | *NRAS* | 4 | 11 | 8 |
| mutation | *PDGFRA* |  | 8 | 8 |
| mutation | *PIK3CA* | 6 | 8 | 9 |
| mutation | *POLE* |  | 5 | 12 |
| mutation | *STK11* | 5 |  |  |
| mutation | *STAT3* |  |  | 12 |
| mutation | *TP53* |  |  | 27 |
| exon skipping | *MET* | 111 | 14 | 11 |
| fusion | *ALK* | 70 | 105 | 70 |
| fusion | *RET* | 60 | 98 | 58 |
| fusion | *ROS1* | 92 | 140 | 140 |
| fusion | *KMT2A* |  | 14 |  |
| fusion | *MLLT1* |  | 14 |  |
| fusion | *NRG1* |  |  | 34 |
| fusion | *NTRK1* |  | 20 | 46 |
| fusion partner | *ATP1B1* |  |  | 34 |
| fusion partner | *CCDC6* | 48 | 86 | 24 |
| fusion partner | *CD74* | 24 | 34 | 26 |
| fusion partner | *EML4* | 62 | 86 | 50 |
| fusion partner | *EZR* | 36 | 54 | 38 |
| fusion partner | *HIP1* | 87 | 116 | 66 |
| fusion partner | *KIF5B* | 70 | 100 | 62 |
| fusion partner | *SDC4* | 22 | 38 | 12 |
| fusion partner | *SLC34A2* | 44 | 65 | 32 |
| expression | *BRCA1* |  |  | 8 |
| expression | *BRCA2* |  |  | 8 |
| expression | *HPRT1* |  | 6 |  |
| expression | *MPRIP* |  | 10 | 10 |
| expression | *MYC* | 18 | 4 | 4 |
| expression | *MYCN* | 21 | 4 |  |
| expression | *POLR2A* |  | 4 | 12 |
| expression | *PXDNL* |  |  | 8 |
| expression | *RYR1* |  |  | 8 |
| expression | *SCN8A* |  |  | 6 |
| expression | *SLIT3* |  |  | 12 |
| expression | *FGFR1* | 60 | 6 | 4 |
| expression | *FGFR3* | 47 | 6 | 6 |
| expression | *RICTOR* |  |  | 12 |
| gender control | *AMELY* |  | 2 | 2 |
| housekeeping | *B2M* |  |  | 14 |
| housekeeping | *CD274* |  |  | 8 |
| housekeeping | *GAPDH* | 8 |  |  |
| housekeeping | *HRPT1* | 59 |  |  |
| housekeeping | *GP9* |  | 4 | 4 |
| housekeeping | *GUSB* |  |  | 9 |
| housekeeping | *HAVCR2* |  |  | 6 |
| housekeeping | *HMBS* |  |  | 4 |
| housekeeping | *IPO8* |  | 4 | 6 |
| housekeeping | *PAPPA2* |  |  | 22 |
| housekeeping | *PPIA* |  |  | 4 |
| housekeeping | *PTPRC* |  | 8 | 6 |
| housekeeping | *RP2* | 42 | 6 | 6 |
| housekeeping | *RPS9* |  |  | 8 |
| housekeeping | *TBP* |  |  | 11 |
| housekeeping | *UBC* |  |  | 2 |
| housekeeping | *YWHAZ* |  |  | 10 |

|  |  |
| --- | --- |
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