NSCLC Driver-Mutation Multiplex ddPCR Panel

KRAS / EGFR / BRAF hotspots + EML4-ALK fusion -- cfDNA, Bio-Rad QX200 / QX600

AI Bioinformatician by Labterna | 29 June 2026 | GRCh38

Panel designed and QC-screened -- borderline items flagged for validation

De novo multiplex ddPCR panel for 6 actionable NSCLC drivers from cfDNA: 20 oligos across 6 assays, all amplicons 86-110 bp. Every primer pair is genome-specific (isPcr, all PASS; BRAFP1 pseudogene designed out). Full-panel multiplex compatibility = WARN: no inter-assay primer-dimer below -5 kcal/mol, but one intra-assay FAIL -- the two competitive EGFR L858R probes cross-dimer (locus-intrinsic GGCC palindrome) -- is flagged for an LNA-shortened probe + empirical validation. A common SNP under the T790M probe (rs1050171) is neutralised with a degenerate base.

1. Targets and Clinical Context

Six clinically actionable driver alterations in non-small-cell lung cancer (NSCLC) are targeted for quantitative detection from circulating cell-free DNA (cfDNA): the KRAS codon-12 mutations G12C (c.34G>T) and G12D (c.35G>A); the EGFR sensitising mutation L858R (exon 21) and the gatekeeper resistance mutation T790M (exon 20); the BRAF V600E mutation (exon 15); and the EML4-ALK fusion variant 1 (EML4 exon 13 fused to ALK exon 20, E13;A20). KRAS G12C is the target of covalent inhibitors (sotorasib, adagrasib); EGFR L858R is a first-line TKI indication and T790M the dominant first/second-generation TKI resistance mechanism; BRAF V600E is targeted by dabrafenib+trametinib; and EML4-ALK confers sensitivity to ALK inhibitors. Tracking these from a liquid biopsy supports non-invasive genotyping and resistance monitoring.

cfDNA constraints. Plasma cfDNA is fragmented (modal ~167 bp), so amplicons are kept short (86-89 bp for the SNV assays) to maximise recoverable template. ddPCR scores every droplet as positive or negative, so a single false-positive droplet on a wild-type-only control is an assay failure -- the QC bar throughout is the binary-droplet bar, not a qPCR log-suppression bar.

Gene	Variant	Region	GRCh38 position (plus strand)	dbSNP
KRAS	p.Gly12Cys	exon 2	chr12:25245351 (C>A)	rs121913530
KRAS	p.Gly12Asp	exon 2	chr12:25245350 (C>T)	rs121913529
EGFR	p.Leu858Arg	exon 21	chr7:55191822 (T>G)	rs121434568
EGFR	p.Thr790Met	exon 20	chr7:55181378 (C>T)	rs121434569
BRAF	p.Val600Glu	exon 15	chr7:140753336 (A>T)	rs113488022
EML4-ALK	EML4(1-13)::ALK(20-29)	E13;A20 junction	chr2 (mRNA junction)	n/a

2. Discrimination Strategy (per target)

Each SNV uses **competitive hydrolysis-probe discrimination**: one primer pair flanks the hotspot, with a mutant-allele probe (FAM) and a wild-type-allele probe (HEX) competing for the same site. This yields the allele fraction MUT/(MUT+WT) directly in one well, the WT probe doubling as the per-locus reference -- the architecture of Bio-Rad's PrimePCR ddPCR mutation assays, run at 55 degC. Allele-specific (ARMS) priming and WT-blocking LNA clamps were evaluated and rejected as the primary chemistry: ARMS gives no co-amplified WT in the same colour (allele fraction needs a separate reference and a mis-primed WT template becomes a false-positive droplet), and a WT clamp destroys the WT reference the brief asks for. Both remain available as orthogonal confirmation / sensitivity boosters.

The two KRAS codon-12 mutations share one amplicon and one WT reference probe (they sit 1 bp apart). The **EML4-ALK fusion is detected by a junction-spanning probe** over the E13;A20 boundary, which exists only in the rearranged transcript. Because the fusion is defined at the mRNA junction and the genomic breakpoints scatter across EML4 intron 13 / ALK intron 19, this assay requires an RNA/cDNA input arm (one-step RT-ddPCR) and is normalised to a single-copy reference gene rather than a WT allele; a DNA-native ALK 3'/5' expression-imbalance assay is the alternative if only cfDNA is available.

EML4-ALK substrate. Variant 1 is an mRNA-junction target. Genomic breakpoints are patient-specific, so a fixed-primer genomic assay will not reliably detect the fusion in cfDNA -- use plasma RNA / one-step RT-ddPCR, or the DNA-native ALK 3'/5' imbalance assay.

3. Panel Design -- Primers and Probes

Primers were designed with Primer3 under the Bio-Rad ddPCR Supermix buffer model (3.8 mM Mg2+, 0.8 mM dNTP, 50 mM monovalent; SantaLucia 1998 nearest-neighbour Tm). Primer Tm is the standard NN oligo Tm; probe Tm is the probe-target duplex Tm (the same NN parameters, evaluated as a heterodimer) -- confirm both on IDT OligoAnalyzer with the matching buffer before ordering. Probe sequences carry their suggested 5' fluorophore / 3' quencher in the workbook; strand was chosen to keep a non-G 5' base and the cleanest 5'-dye neighbourhood (photoinduced-electron-transfer rule). **Amplicon length:** the SNV amplicons are 86-89 bp, above the <60 bp cfDNA sweet spot, because a competitive two-primer + two-probe (WT + MUT) architecture needs that much sequence to seat both primers and the off-centred probe pair; 86 bp is the shortest that keeps a valid probe window. The fusion cDNA amplicon is 110 bp (RNA-derived input, less fragmentation-limited than cfDNA).

3.1 KRAS G12C -- 86 bp amplicon

Oligo	Role	Sequence 5'->3'	nt	Tm	GC%	Mod
KRAS12_F	fwd	ATTAGCTGTATCGTCAAGGC	20	59.5	45.0	-
KRAS12_R	rev	TAAGGCCTGCTGAAAATGAC	20	60.3	45.0	-
KRAS12_WT	wt	AGCTGGTGGCGTAGGC	16	63.9	68.8	-
KRAS_G12C_MUT	mut	AGCTTGTGGCGTAGGC	16	61.6	62.5	-

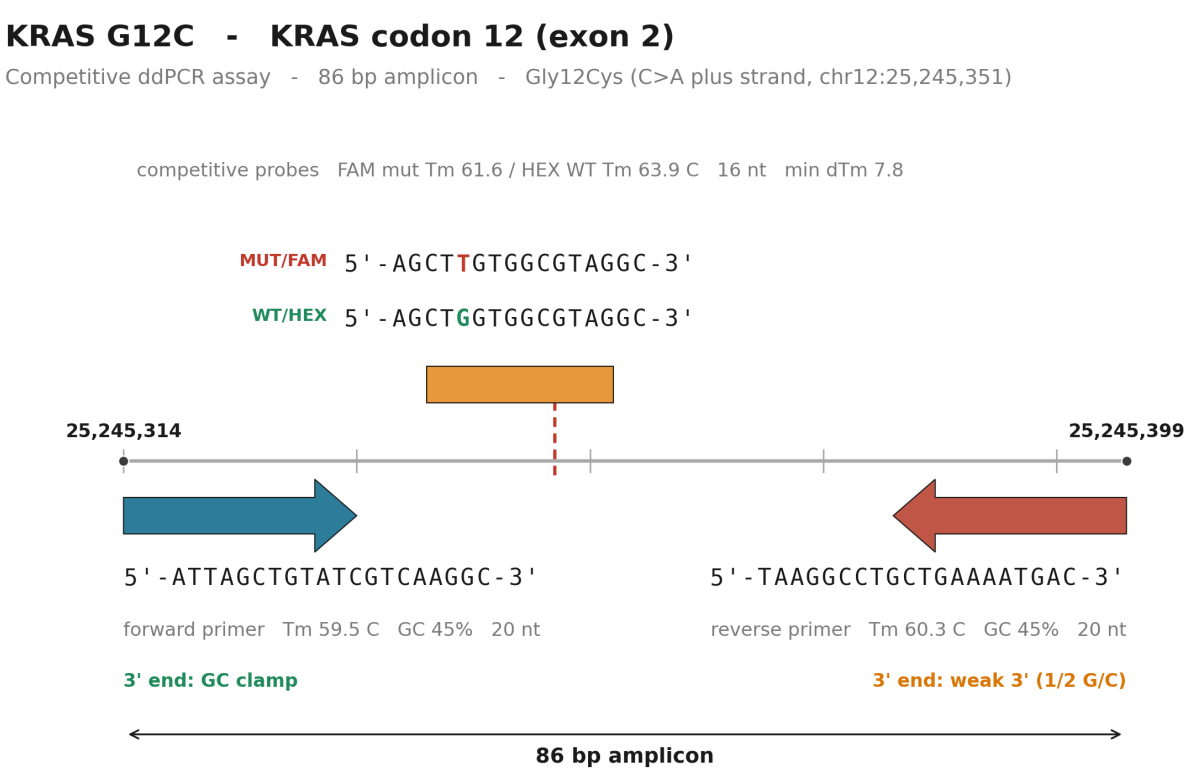

Figure A1. KRAS G12C amplicon -- primer arrows, probe block, monospace sequences, Tm and 3'-end status. Competitive MUT/FAM (red variant base) vs WT/HEX (green variant base) probes.

3.2 KRAS G12D -- 86 bp amplicon

Oligo	Role	Sequence 5'->3'	nt	Tm	GC%	Mod
KRAS12_F	fwd	ATTAGCTGTATCGTCAAGGC	20	59.5	45.0	-
KRAS12_R	rev	TAAGGCCTGCTGAAAATGAC	20	60.3	45.0	-
KRAS12_WT	wt	AGCTGGTGGCGTAGGC	16	63.9	68.8	-
KRAS_G12D_MUT	mut	AGCTGATGGCGTAGGC	16	60.9	62.5	LNA

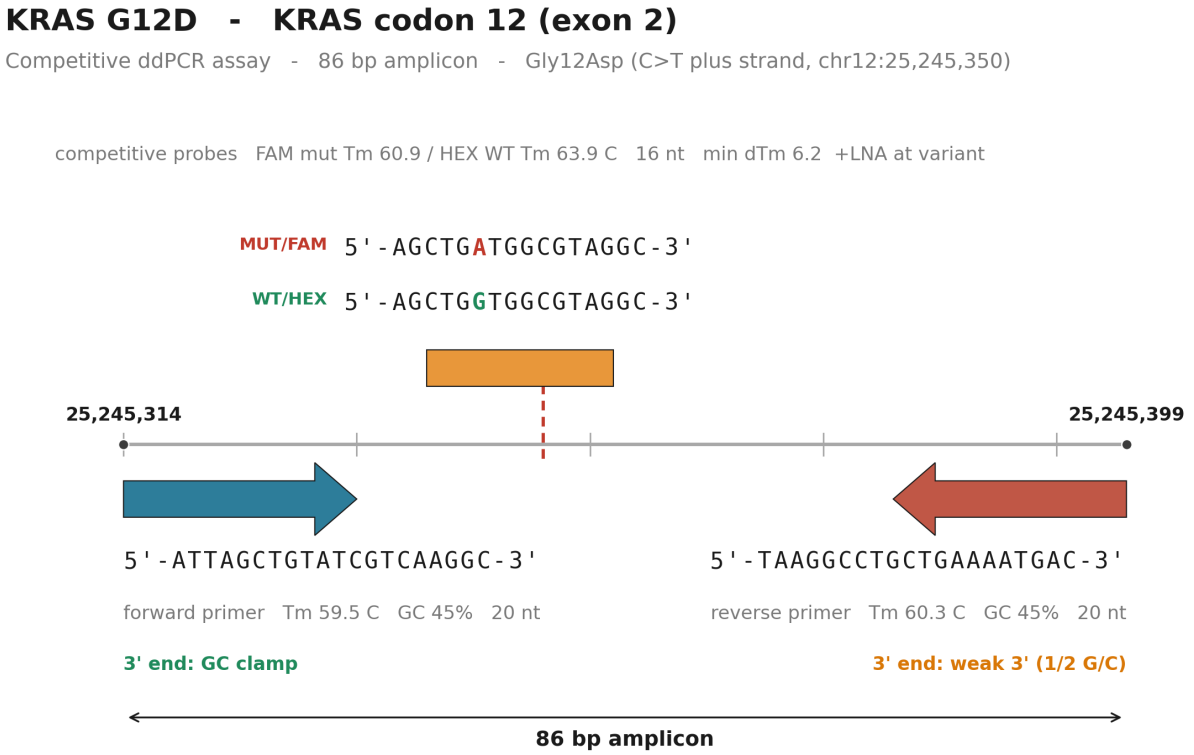

Figure A2. KRAS G12D amplicon -- primer arrows, probe block, monospace sequences, Tm and 3'-end status. Competitive MUT/FAM (red variant base) vs WT/HEX (green variant base) probes.

3.3 EGFR L858R -- 86 bp amplicon

Oligo	Role	Sequence 5'->3'	nt	Tm	GC%	Mod
EGFR858_F	fwd	AGGAACGTACTGGTGAAAAC	20	59.8	45.0	-
EGFR858_R	rev	TATTCTTTCTCTTCCGCACC	20	59.4	45.0	-
EGFR858_WT	wt	TTTTGGGCTGGCCAAA	16	58.8	50.0	-
EGFR858_MUT	mut	TTTTGGGCGGGCCAAA	16	62.1	56.2	-

EGFR L858R - EGFR exon 21

Competitive ddPCR assay - 86 bp amplicon - Leu858Arg (T>G plus strand, chr7:55,191,822)

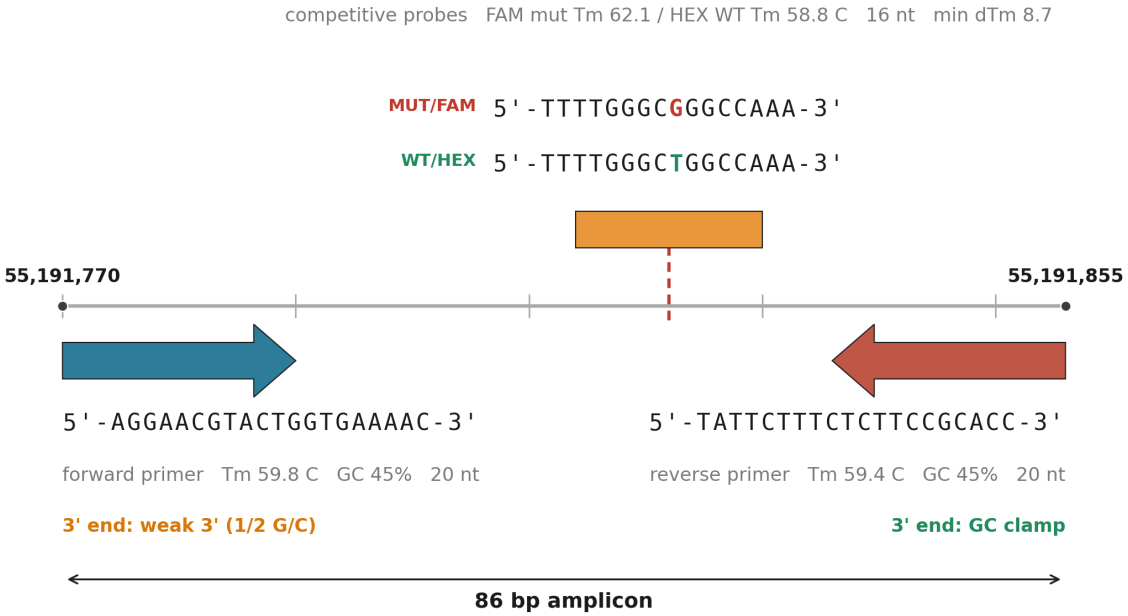

Figure A3. EGFR L858R amplicon -- primer arrows, probe block, monospace sequences, Tm and 3'-end status. Competitive MUT/FAM (red variant base) vs WT/HEX (green variant base) probes.

3.4 EGFR T790M -- 89 bp amplicon

Oligo	Role	Sequence 5'->3'	nt	Tm	GC%	Mod
EGFR790_F	fwd	CATCTGCCTCACCTCCAC	18	61.5	61.1	-
EGFR790_R	rev	TATTGTCTTTGTGTTCCCGG	20	59.9	45.0	-
EGFR790_WT	wt	AGCTGCGTGATGAGYTGC	18	65.5	55.6	degen Y
EGFR790_MUT	mut	AGCTGCATGATGAGYTGC	18	62.8	50.0	LNA,degen Y

EGFR T790M - EGFR exon 20

Competitive ddPCR assay - 89 bp amplicon - Thr790Met (C>T plus strand, chr7:55,181,378)

competitive probes FAM mut Tm 62.8 / HEX WT Tm 65.5 C 18 nt min dTm 5.6 +LNA at variant +degenerate

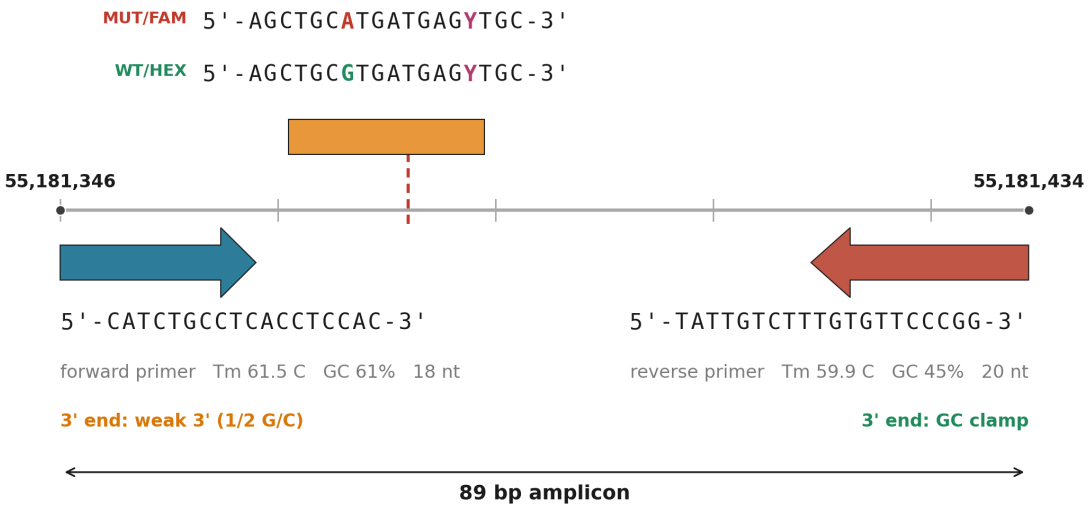

Figure A4. EGFR T790M amplicon -- primer arrows, probe block, monospace sequences, Tm and 3'-end status. Competitive MUT/FAM (red variant base) vs WT/HEX (green variant base) probes.

3.5 BRAF V600E -- 87 bp amplicon

Oligo	Role	Sequence 5'->3'	nt	Tm	GC%	Mod
BRAF600_F	fwd	CTGTTCAAACTGATGGGACC	20	60.6	50.0	-
BRAF600_R	rev	CTTCATGAAGACCTCACAGTA	21	58.9	42.9	-
BRAF600_WT	wt	ACAGTGAAATCTCGATGGAG	20	59.4	45.0	-
BRAF600_MUT	mut	ACAGAGAAATCTCGATGGAG	20	58.8	45.0	LNA

BRAF V600E - BRAF exon 15

Competitive ddPCR assay - 87 bp amplicon - Val600Glu (A>T plus strand, chr7:140,753,336)

competitive probes FAM mut Tm 58.8 / HEX WT Tm 59.4 C 20 nt min dTm 5.5 +LNA at variant

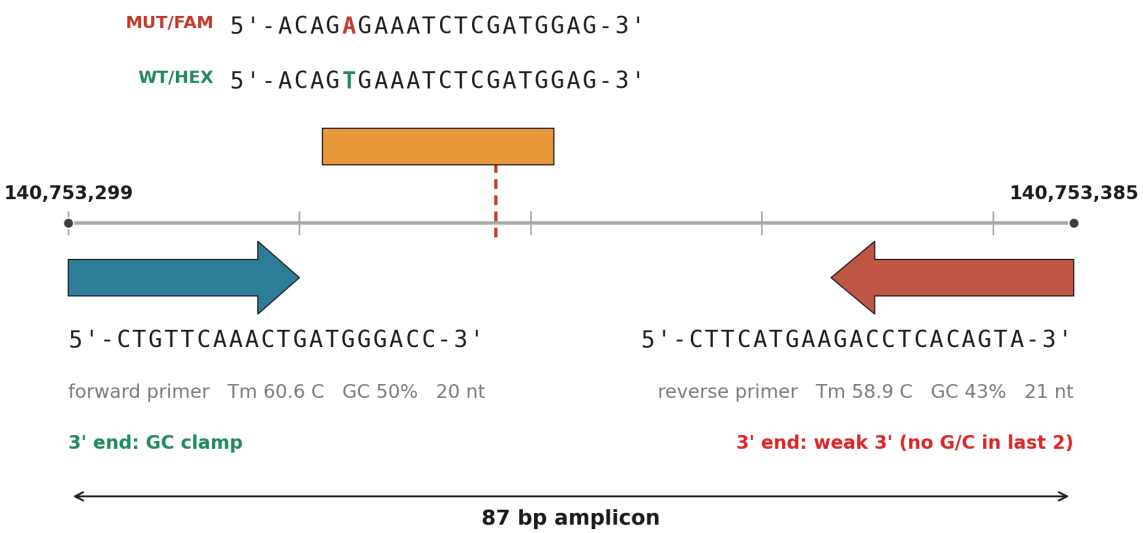

Figure A5. BRAF V600E amplicon -- primer arrows, probe block, monospace sequences, Tm and 3'-end status. Competitive MUT/FAM (red variant base) vs WT/HEX (green variant base) probes.

3.6 EML4-ALK v1 -- 110 bp amplicon

Oligo	Role	Sequence 5'->3'	nt	Tm	GC%	Mod
ALKfus_F	fwd	CTACTGTAGAGCCCACACC	19	60.9	57.9	-
ALKfus_R	rev	CTTGCTCAGCTTGTACTCAG	20	60.3	50.0	-
ALKfus_JUNC	junction	ACCTAAAGTGTACCGCCGGA	20	65.6	55.0	-

EML4-ALK v1 - EML4 exon 13 :: ALK exon 20

Junction-spanning ddPCR assay - 110 bp cDNA amplicon - EML4 exon 13 :: ALK exon 20 (variant 1)

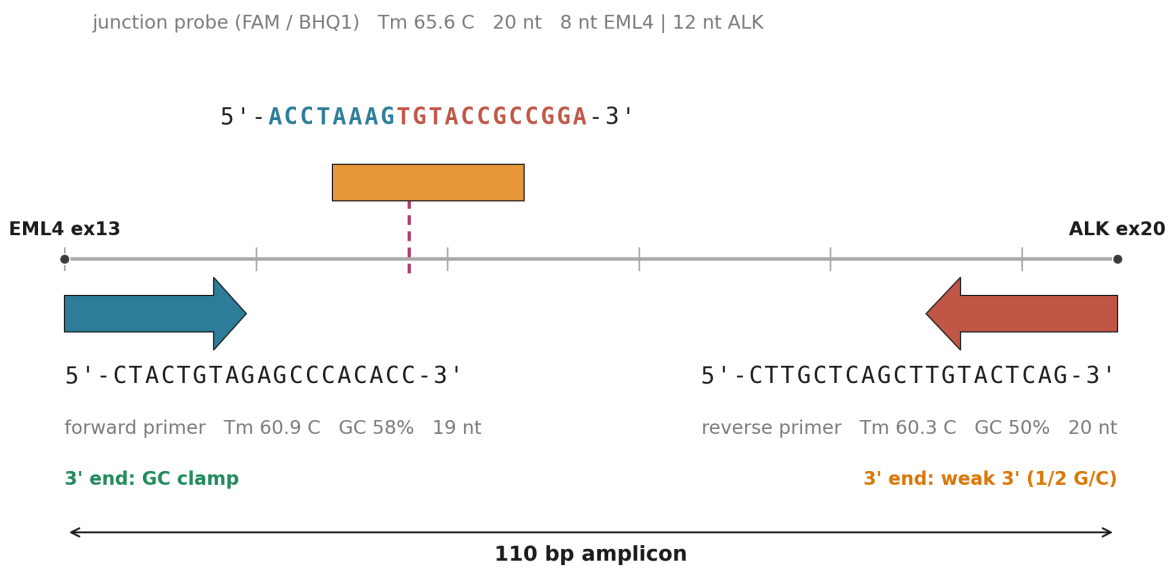

Figure A6. EML4-ALK v1 amplicon -- primer arrows, probe block, monospace sequences, Tm and 3'-end status. Junction probe two-toned: EML4 (blue) | ALK (terra-cotta).

4. Allele Discrimination

Allele discrimination is driven by the melting-temperature gap between the matched and mismatched probe-template duplexes (probe-target duplex Tm by nearest-neighbour under the ddPCR buffer). Mutant probes are 16-20 nt with the variant base placed off-centre; BRAF V600E sits at the long (20 nt) end because its AT-rich locus needs the extra length to reach a usable Tm. Three probes whose unmodified discrimination gap is below 7 degC are flagged for a single LNA at the variant base (KRAS G12D, EGFR T790M, BRAF V600E); KRAS G12C and EGFR L858R clear the threshold unmodified. The discrimination gap (Delta-Tm) is shown for both the mutant and the WT probe -- the controlling value is the smaller of the two.

Assay	Probe strand	nt	Variant 5'-pos	MUT Tm match/mismatch	dTm MUT / WT	Recommendation
KRAS G12C	antisense	16	5	61.6 / 53.6	8.0 / 7.8	unmodified
KRAS G12D	antisense	16	6	60.9 / 54.7	6.2 / 6.3	LNA
EGFR L858R	sense	16	9	62.1 / 52.6	9.5 / 8.7	unmodified
EGFR T790M	antisense	18	7	62.8 / 57.0	5.9 / 5.6	LNA
BRAF V600E	antisense	20	5	58.8 / 53.3	5.5 / 5.9	LNA

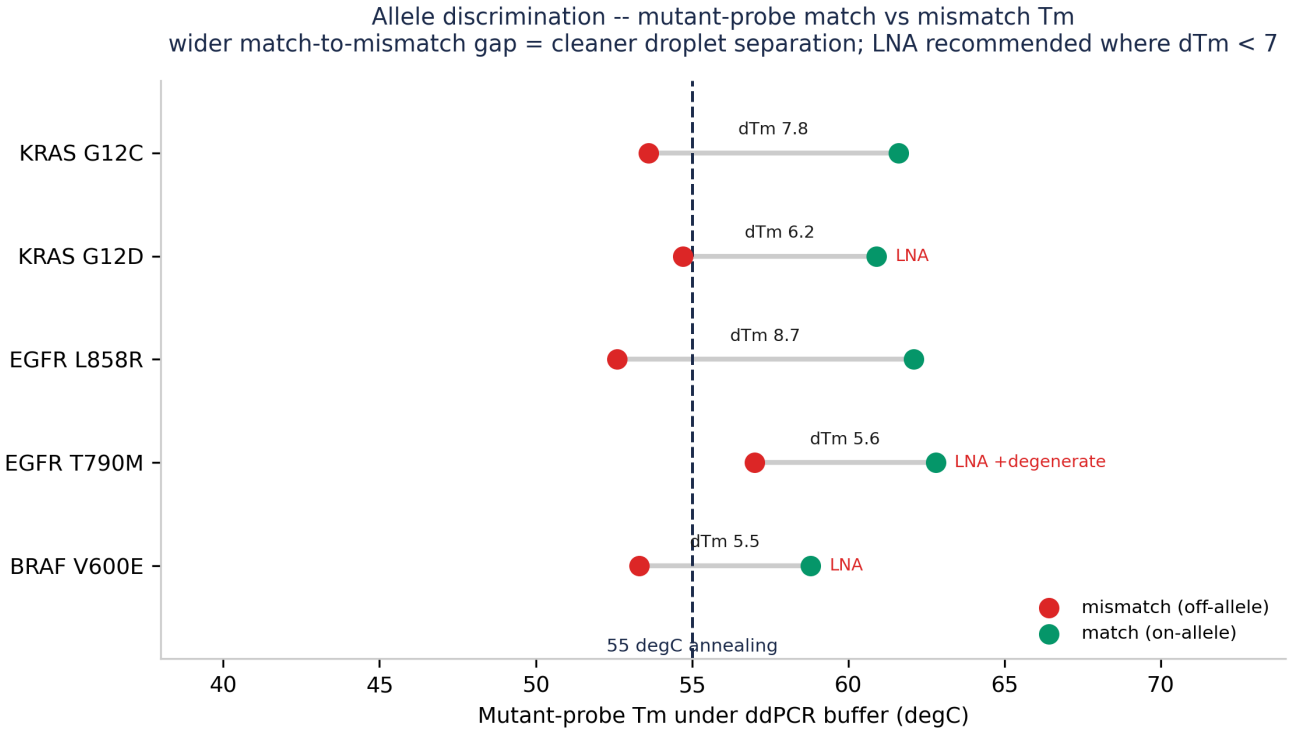

Figure B. Mutant-probe match (green) vs mismatch (red) Tm. A wider gap gives cleaner FAM/HEX cluster separation; the dashed line marks 55 degC annealing.

5. Multiplex Compatibility QC (the make-or-break step)

Every pair among the 20 oligos (190 pairs) was screened for 3'-end-anchored cross-dimer free energy and any-alignment free energy under the ddPCR buffer, plus per-oligo hairpin, self-dimer, Tm spread and homopolymer runs. **Verdict: WARN.** The decisive inter-assay result is that **no inter-assay primer-primer 3'-end dimer is at or below -5 kcal/mol** (worst = -4.8 kcal/mol), so the six primer pairs are mutually safe to co-amplify. Primer Tm spread is 2.6 degC (58.9-61.5). 6 inter-assay interactions are flagged at the WARN level (-5.0 to -5.6 kcal/mol, each involving a probe, not two primers); no inter-assay pair reaches FAIL.

One intra-assay interaction does reach FAIL and must be called out: the two competitive EGFR L858R probes (WT-HEX and MUT-FAM), which are co-resident in the same well by design, form a -12 kcal/mol cross-dimer with each

other (and each self-dimers at ~ -11.5 kcal/mol) -- driven by a GGCC palindrome intrinsic to the L858R locus. This mutual probe sequestration is the panel's only FAIL-severity cross-dimer. It is locus-intrinsic (both strands carry the palindrome, so it cannot be designed away by strand choice) and is addressed in the recommendations: an LNA-shortened L858R probe plus an empirical annealing-gradient check of FAM/HEX cluster separation before the assay is used.

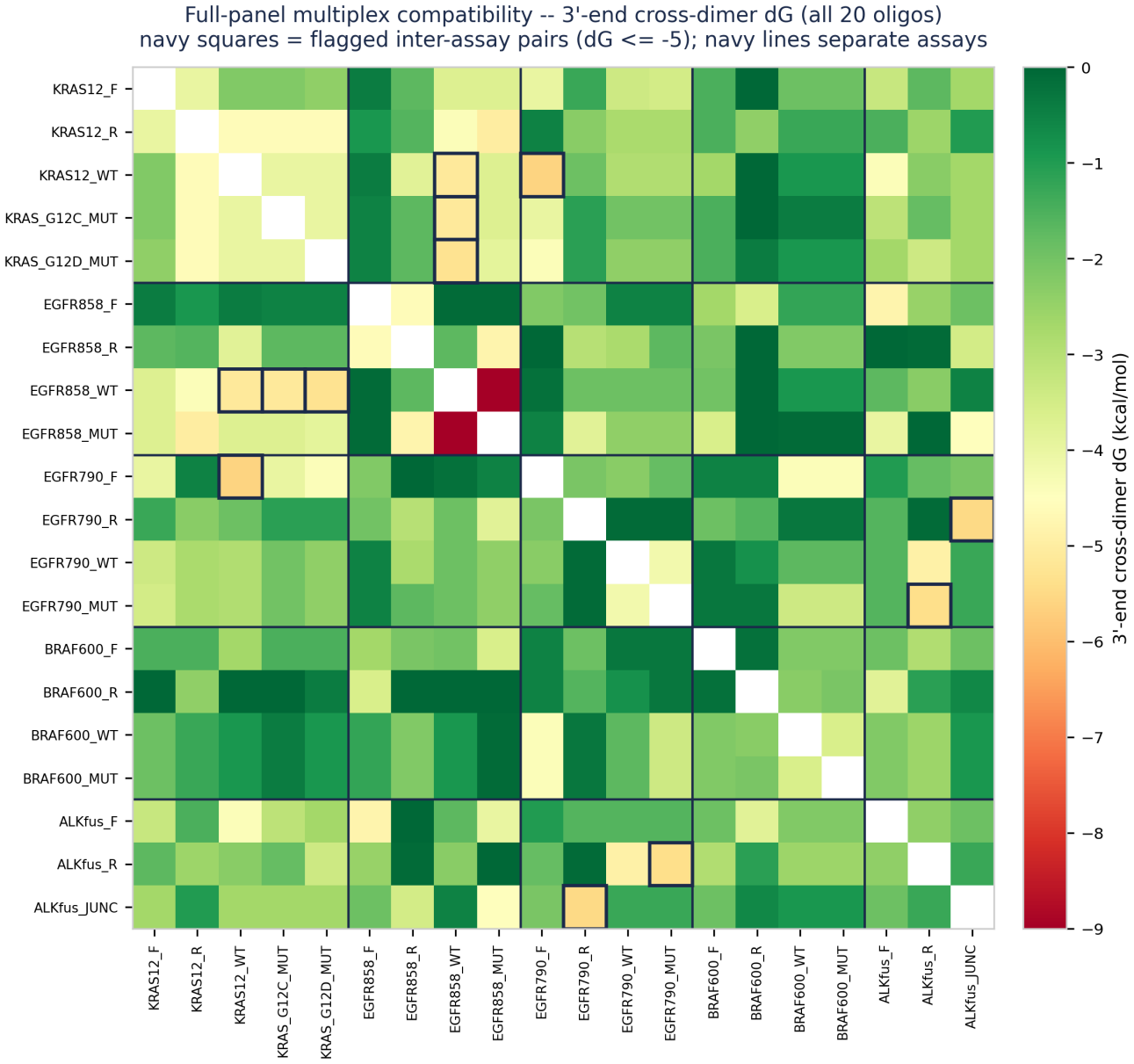

Figure C. Full-panel 3'-end cross-dimer dG (all 20 oligos). Navy squares ring the flagged inter-assay pairs; the dark-red intra-assay cell is the EGFR L858R WT x MUT competitive-probe cross-dimer (-12 kcal/mol), the panel's only FAIL-severity interaction.

Per-oligo flags (surfaced, not forced through)

Oligo	Flag
KRAS12_R	homopolymer=4(borderline); self-dimer dG=-6.8
KRAS12_WT	hairpinTm=60>55; GC=69%>65
KRAS_G12C_MUT	hairpinTm=60>55; GC=62%(>60,Tm-controlled)
KRAS_G12D_MUT	hairpinTm=60>55; GC=62%(>60,Tm-controlled)
EGFR858_F	homopolymer=4(borderline)
EGFR858_WT	homopolymer=4(borderline); self-dimer dG=-11.4
EGFR858_MUT	homopolymer=4(borderline); self-dimer dG=-11.6
BRAF600_R	weak 3' end (no GC in last 2; rain risk); self-dimer dG=-9.4
ALKfus_F	self-dimer dG=-5.6
ALKfus_JUNC	self-dimer dG=-7.3

Other borderline items, per the brief (the L858R probe-pair FAIL is described above). (1) The KRAS codon-12 probes are GC-rich (69% / hairpin Tm ~60 degC) -- workable but validate amplitude. (2) The BRAF reverse primer has a weak (AT) 3' end -- the deliberate cost of BRAFP1 pseudogene discrimination (Section 6); watch for rain and consider a strong-3'-end backup. (3) Four oligos carry a 4-base homopolymer run (borderline, not a violation), concentrated at the T-rich L858R locus.

6. Specificity

Each primer pair was run through genome-wide in-silico PCR (UCSC isPcr) against GRCh38 -- the correct specificity test for genomic cfDNA. All five SNV pairs give a single on-target product with no off-target genomic amplicon; the fusion primers (EML4 exon 13 / ALK exon 20, ~12 Mb apart in opposing genomic orientation) correctly give no genomic product, confirming the on-target product forms only from the rearranged cDNA. A focused paralog-transcriptome BLAST (RAS/RAF/ERBB families + EML4/ALK/LTK) found two benign single-oligo homologies, neither able to generate signal: the EGFR-T790M forward primer vs ERBB2 (89%, 2 mismatches) and the BRAF-V600E WT probe vs ARAF (90%, 2 mismatches). In both cases only one oligo matches the paralog, no primer pair flanks it (genomic isPcr finds no off-target product), and a hydrolysis probe generates no signal without an amplicon -- so neither is amplified or detected. "Transcriptome" specificity here is met by genome-level isPcr (every mRNA exon is also genomic) plus this targeted paralog cDNA screen, not a transcriptome-wide scan; the fusion junction probe spans an exon-exon boundary present in no normal transcript and is intrinsically transcriptome-unique.

Assay	Method	Result
KRAS_codon12	isPcr vs hg38	**PASS**
EGFR_L858R	isPcr vs hg38	**PASS**
EGFR_T790M	isPcr vs hg38	**PASS**
BRAF_V600E	isPcr vs hg38	**PASS**
EML4_ALK_v1	isPcr vs hg38	**PASS**

BRAFP1 pseudogene designed out. Naive BRAF primers co-amplify the BRAFP1 pseudogene (chrX, ~90-95% identical across this region), which would dilute the V600E allele fraction. The reverse primer 3' end was anchored on a BRAF-specific base, giving a single 3'-terminal mismatch vs BRAFP1 (primer A opposite pseudogene G -- an A:G mismatch, among the most extension-abolishing 3' types); isPcr confirms this eliminates the pseudogene product (single chr7 amplicon). Because discrimination rests on one terminal mismatch against a ~95%-identical pseudogene, the validation plan (Section 10) includes a zero-false-positive check on BRAFP1-containing genomic DNA.

7. Common-SNP Screen (gnomAD)

Every primer and probe footprint was screened against gnomAD v4 for common germline SNPs (allele frequency >= 1%). One common variant falls under an oligo: **rs1050171 (EGFR c.2361G>A, Q787Q; gnomAD AF 0.56)** sits under the T790M probe, 8 bp from the mutation. Rather than sacrifice discrimination by shifting the probe off it, a degenerate base (Y) was placed at that probe position so the probe tolerates both alleles. The T790M probe Tm reported in Section 3 and the figures is the reference-allele match Tm (MUT 62.8 / WT 65.5 degC); in rs1050171-alt carriers the degenerate base keeps the worst-case match Tm at 60.5 / 63.1 degC -- still well above the 55 degC annealing, so binding is retained in either genotype. No common SNP sits under any primer 3' end. A T790M control gBlock carrying the rs1050171 alt (A) allele is recommended during validation to confirm the degenerate probe's tolerance.

8. Channel-Layout Plans

QX200 has 2 optical channels (FAM, HEX); QX600 has 6 (FAM, HEX, Cy5, Cy5.5, ROX, ATTO 590). On QX200 the six targets need ~5 wells (4 DNA + 1 RNA): genotyping 5 SNVs plus a fusion cannot collapse into one or two wells on two channels without losing per-locus allele fraction. On QX600 the panel meets the 'one-to-two reaction' goal: a single well can carry all six (each mutant on its own channel, WT references amplitude-multiplexed, fusion via one-step RT), or -- recommended for robustness -- a 2-well split that, guided by the dimer matrix, places no flagged cross-dimer pair in the same well (Well A: KRAS + BRAF + EML4-ALK; Well B: EGFR L858R + T790M). One caveat for QX200: the GC-rich KRAS codon-12 mutant probes carry two G's in their 5' neighbourhood, and on QX200 they must occupy FAM -- the most G-sensitive dye -- with no PET-optimal alternative; validate FAM amplitude there, or prefer QX600 (which moves KRAS onto the G-tolerant red channels Cy5.5/ROX).

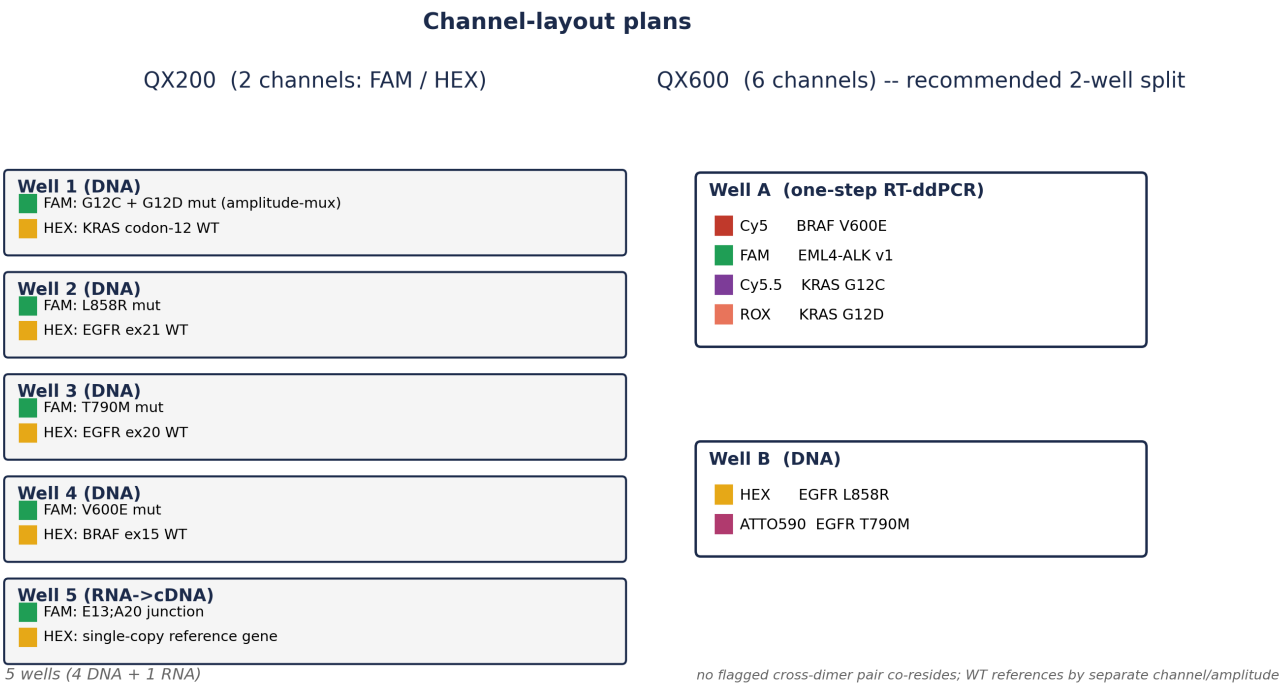

Figure D. QX200 (5 wells, FAM/HEX) and the recommended QX600 2-well split (6 dyes); dye assignment follows the PET-quenching rule (clean 5' neighbourhoods -> FAM/HEX).

9. Synthetic gBlock Controls

10 double-stranded synthetic controls (156-180 bp; amplicon + >= 30 bp flank) are provided ready to order: a mutant control per variant (positive control / LoD spike-in) and a wild-type control per amplicon (the KRAS codon-12 WT is shared). The EML4-ALK control carries the spliced E13;A20 junction and serves as a fusion-positive control directly (dsDNA, no RT needed for the control). Full sequences are in the workbook and FASTA.

Control	Allele	Length	Carries
KRAS_G12C_MUT_gBlock	Mutant	156 bp	p.Gly12Cys
KRAS_codon12_WT_gBlock	Wild-Type	156 bp	KRAS wild-type (exon 2)
KRAS_G12D_MUT_gBlock	Mutant	156 bp	p.Gly12Asp
EGFR_L858R_MUT_gBlock	Mutant	156 bp	p.Leu858Arg
EGFR_L858R_WT_gBlock	Wild-Type	156 bp	EGFR wild-type (exon 21)
EGFR_T790M_MUT_gBlock	Mutant	158 bp	p.Thr790Met
EGFR_T790M_WT_gBlock	Wild-Type	158 bp	EGFR wild-type (exon 20)
BRAF_V600E_MUT_gBlock	Mutant	157 bp	p.Val600Glu
BRAF_V600E_WT_gBlock	Wild-Type	157 bp	BRAF wild-type (exon 15)
EML4_ALK_v1_fusion_gBlock	Fusion	180 bp	EML4 exon13 :: ALK exon20 (variant 1)

10. LoB / LoD Validation Plan

Validation should follow CLSI EP17 in the binary-droplet framework. Recommended steps:

Step	Procedure
Limit of blank (LoB)	>=20 wild-type-only replicates per assay over >=3 days/operators; LoB = mean + 1.645 SD false-positive droplets. ddPCR target: 0-1 false-positive droplet per WT-only well at the locked annealing temp.
Limit of detection (LoD)	Spike each mutant gBlock at 5-7 decreasing copy levels into a constant WT background; >=20 replicates/level; LoD = lowest level detected in >=95% of replicates above LoB (probit/hit-rate). Established NSCLC cfDNA ddPCR assays reach ~0.1-0.5% mutant allele fraction.
Limit of quantitation (LoQ)	Lowest allele fraction meeting a precision target (CV <= 25%); typically at/just above LoD for ddPCR.
Linearity / dynamic range	Serial-dilute the mutant gBlock over >=4 logs; measured-vs-expected R^2 >= 0.98, slope 0.9-1.1; confirm no saturation of high-abundance targets in multiplex wells.
Annealing-temperature gradient	Validate 53-62 degC per assay and per multiplex well; lock the temperature giving zero WT-only false positives with retained mutant amplitude (rain minimised).
Specificity / controls	0 false-positive droplets on WT-only gBlocks, no-template control, and BRAFP1-containing genomic DNA; re-confirm no inter-assay cross-amplification in the final multiplex well.
Input / fragmentation	Validate on fragmented cfDNA (sheared or clinical plasma); the fusion arm needs an RNA/cDNA input (one-step RT-ddPCR).

Restriction-digest compatibility

Pre-PCR restriction digestion is not required for cfDNA (already fragmented), but per design convention every ddPCR amplicon is screened at design time against the five Bio-Rad ddPCR-compatible enzymes so the assay stays usable on long templates and so the dsDNA gBlock controls and the BRAFP1-containing genomic DNA control have a fragmentation option that does not cut the amplicon. Every amplicon has at least three compatible enzymes:

Assay	Amplicon	Compatible enzymes (no cut site)	Recommended
KRAS G12C	86 bp	CviQI, HindIII, MseI	CviQI
KRAS G12D	86 bp	CviQI, HindIII, MseI	CviQI
EGFR L858R	86 bp	AluI, HindIII, MseI	AluI
EGFR T790M	89 bp	CviQI, HaeIII, HindIII, MseI	CviQI
BRAF V600E	87 bp	CviQI, HaeIII, HindIII, MseI	CviQI
EML4-ALK v1	110 bp	HaeIII, HindIII, MseI	HaeIII

11. Recommendations and Caveats

- All Tm values are computed (SantaLucia 1998 NN under the ddPCR buffer); confirm on IDT OligoAnalyzer with the matching buffer before ordering, and treat the match/mismatch dTm values as thermodynamic estimates pending empirical cluster separation.
- Order the three LNA-flagged probes (KRAS G12D, EGFR T790M, BRAF V600E) with one LNA at the variant base if a validation gradient shows insufficient cluster separation unmodified.
- EGFR L858R is the priority risk: its two competitive probes form a -12 kcal/mol WT x MUT cross-dimer (the panel's only FAIL-severity interaction, Section 5) from a locus-intrinsic GGCC palindrome. Before use, validate FAM/HEX cluster separation empirically and adopt an LNA-shortened L858R probe if the unmodified pair under-separates; because the palindrome is on both strands, expect this to need bench optimisation, not just a strand swap.
- The EML4-ALK assay needs an RNA/cDNA input arm; for a DNA-only workflow, substitute an ALK 3'/5' imbalance assay (detects any ALK fusion but does not identify the variant). The E13;A20 junction template was reconstructed from canonical Ensembl exons and confirmed in-frame by translation; verify it against a clinical EML4-ALK variant-1 reference (e.g. a characterised cell line such as NCI-H3122) before deployment.

- This panel was designed and screened in silico; LoB/LoD, gradient, and multiplex cross-talk must be confirmed at the bench (Section 10) before clinical use.

12. Output Files

File	Contents
NSCLC_Hotspot_ddPCR_Panel_Report.pdf	This report.
NSCLC_Hotspot_ddPCR_Panel_Data.xlsx	Oligos, gBlocks, multiplex QC, specificity, SNP screen, channel layout, LoB/LoD plan.
sequences.fasta	All 20 oligos + 10 gBlock controls.
figures/	Figures A-D (PNG 300 dpi + vector PDF).

The *scripts/* folder ships the reproducible numbered pipeline (sequence retrieval through report build).

13. References

[1] Bio-Rad Laboratories. *Droplet Digital PCR Applications Guide* (Bulletin 6407): primer/probe design, supermix buffer, restriction-digest compatibility.

[2] Bio-Rad Laboratories. Mutation Detection Multiplexing Using the QX600 ddPCR System (6-colour amplitude multiplexing).

[3] A rapid, multiplex digital PCR assay for EGFR, KRAS, BRAF, ERBB2 variants and ALK/RET/ROS1/NTRK1 fusions in NSCLC. *bioRxiv* 2023.03.09.531949 / PMC10620117 (amplitude-modulation multiplexing; DNA and RNA wells separated to minimise cross-target amplification; 1% VAF DNA threshold).

[4] Decousus S et al. Development of multiplex drop-off digital PCR assays for KRAS/NRAS/BRAF/PIK3CA hotspots in plasma. *J Mol Diagn* 2023 (LoD 0.08-0.18% mutant allele fraction).

[5] Soda M et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature* 2007;448:561-6.

[6] Sabir SR et al. EML4-ALK variants: biological and molecular properties. *Genes* 2017 (PMC5615333): ALK kinase domain encoded by exons 20-29; variant 1 = E13;A20.

[7] SantaLucia J. A unified view of polymer, dumbbell and oligonucleotide DNA nearest-neighbor thermodynamics. *PNAS* 1998;95:1460-5.

[8] Untergasser A et al. Primer3 -- new capabilities and interfaces. *Nucleic Acids Res* 2012;40:e115.

[9] Stadhouders R et al. The effect of primer-template mismatches on 5'-nuclease assay detection. *J Mol Diagn* 2010;12:109-17.

[10] Chen S et al. A genomic mutational constraint map (gnomAD v4). *Nature* 2024;625:92-100.

[11] CLSI EP17-A2. Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures.

[12] Crockett AO, Wittwer CT. Fluorescein-labeled oligonucleotides for real-time PCR. *Anal Biochem* 2001;290:89-97 (5'-neighbourhood G-quenching of FAM).