Double mismatch interactions in probe design enable improved hybridization-based SNV discrimination

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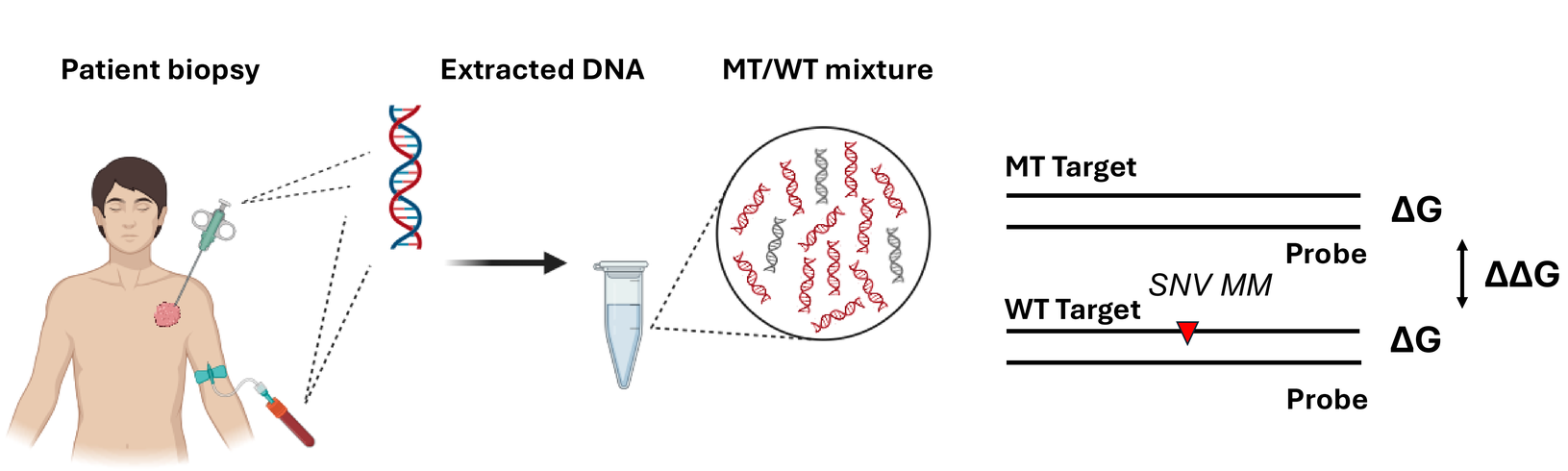
INTRODUCTION

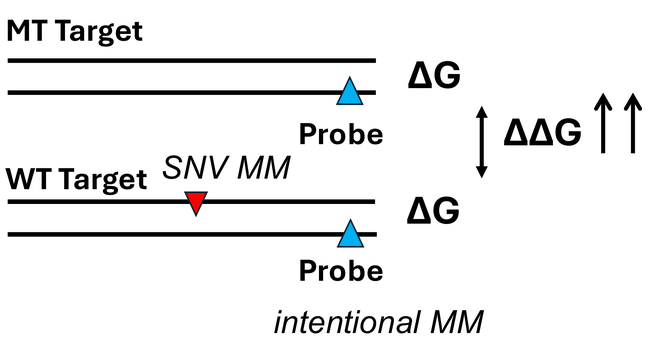
MT and WT sequences differ by only one nucleotide, leading to nearly identical probe binding energy (ΔG)



Single-Nucleotide Variants (SNVs) differ from wild-type (WT) DNA by only one base (**red** triagle), leading to nearly identical probe binding affinities (ΔG). This high homology causes rare mutant (MT) signals to be overwhelmed by WT background. Classical ”equilibrium shifting methods” (e.g., higher hybridization temperature, shorter probes, or LNAs/PNAs) increases stringency but shifts both hybridization curves simultaneously. While this reduces unintented WT binding (**X**), it causes a proportional loss in MT signal (**O**). Because intrinsic thermodynamic gap (ΔΔG) remains constant, gaining selectivity requires a sacrifice in sensitivity.

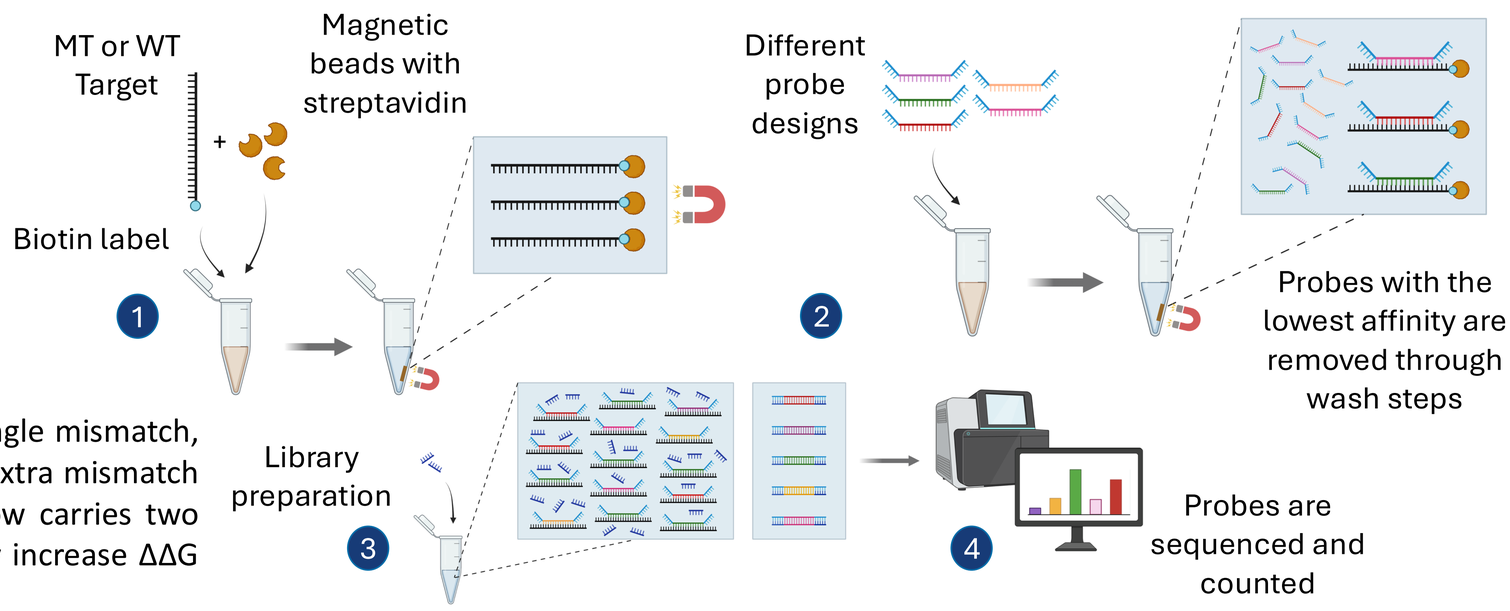
METHODS

Intentional mismatches can destabilize wild-type binding



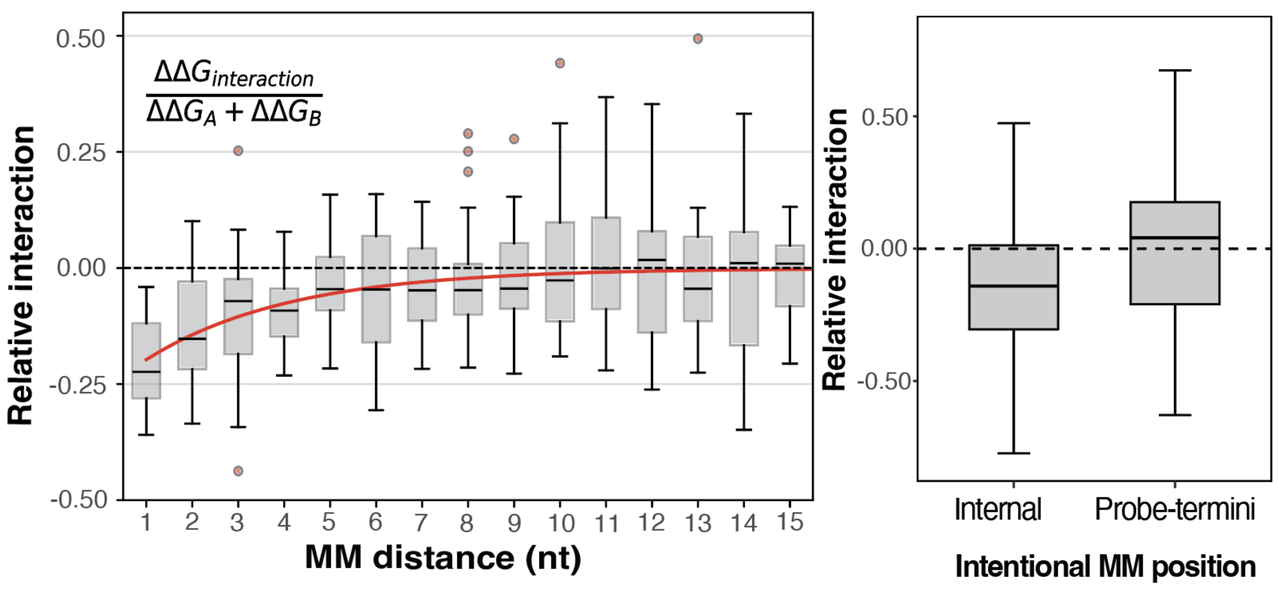
In classic probe design, selectivity is limited by the small ΔΔG of a single mismatch, making it difficult to distinguish MT from excess WT. Introducing an extra mismatch adds an additional free-energy penalty, especially for WT, which now carries two mismatches. If these mismatches interact in a destabilizing way, they increase ΔΔG beyond the single-mismatch limit, leading to higher selectivity.

Probe enrichment platform was developed to investigate probe hybridization



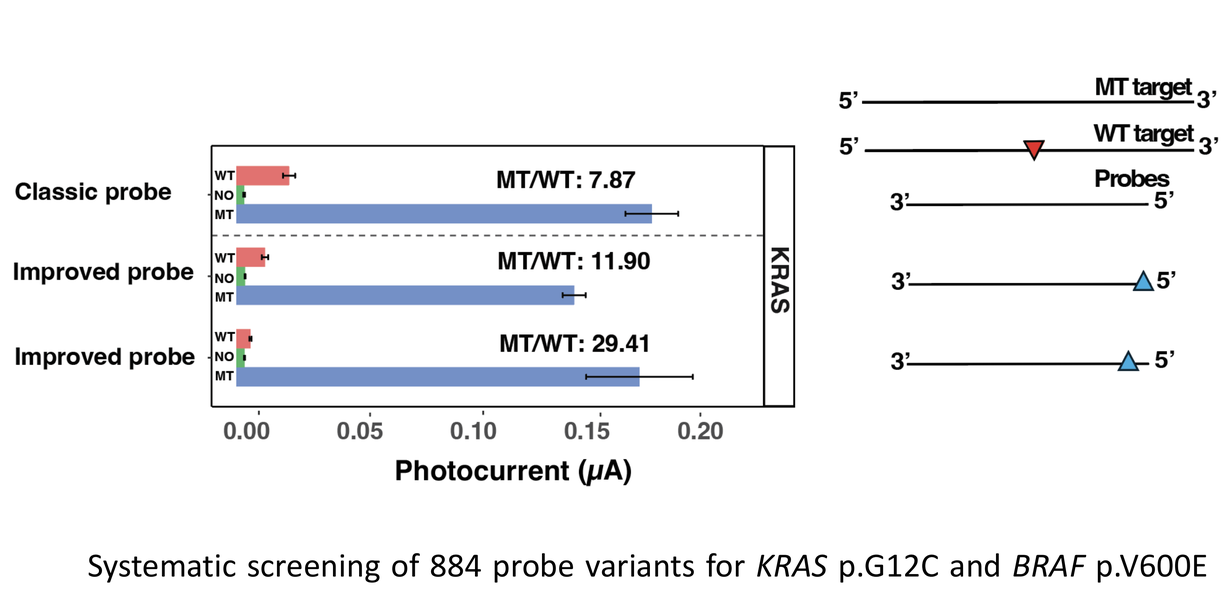
RESULTS

Mismatch position and interaction determine hybridization selectivity



While each mismatch (MM1 ($\Delta\Delta G_A$) or MM2 ($\Delta\Delta G_B$)) contributes a fixed free-energy penalty, combining them introduces an interaction term ($\Delta\Delta G_{int}$) that alters duplex stability beyond simple addition. Closely spaced mismatches tend to stabilize the duplex, whereas more distant mismatches can cause additional destabilization. This increases the total ΔΔG beyond additive effects, improving target discrimination.

Probe screening for *KRAS* p.G12C and *BRAF* p.V600E revealed improved probe designs that increased ΔΔG



Systematic screening of 884 probe variants for *KRAS* p.G12C and *BRAF* p.V600E reveals that “classic” perfeclty matched probes are often outperformed by rationally designed alternatives. By pairing a central variant with an intentional terminal mismatch, the ΔΔG is maximized. When benchmarked on a PEC platform, this design motif achieved a 29.41 fold MT/WT (for KRAS p. G12C) selectivity ratio, a nearly 4-fold improvement over classic designs.

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