Continuously tunable nucleic acid hybridization probes

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In silico–designed nucleic acid probes and primers often do not achieve favorable specificity and sensitivity tradeoffs on the first try, and iterative empirical sequence-based optimization is needed, particularly in multiplexed assays. We present a novel, on-the-fly method of tuning probe affinity and selectivity by adjusting the stoichiometry of auxiliary species, which allows for independent and decoupled adjustment of the hybridization yield for different probes in multiplexed assays. Using this method, we achieved near-continuous tuning of probe effective free energy. To demonstrate our approach, we enforced uniform capture efficiency of 31 DNA molecules (GC content, 0–100%), maximized the signal difference for 11 pairs of single-nucleotide variants and performed tunable hybrid capture of mRNA from total RNA. Using the Nanostring nCounter platform, we applied stoichiometric tuning to simultaneously adjust yields for a 24-plex assay, and we show multiplexed quantitation of RNA sequences and variants from formalin-fixed, paraffin-embedded samples.

Oligonucleotide probes and primers are used for detecting and quantifying nucleic acids in genomics discovery and molecular diagnostics1–3. Methods in molecular biology and analytical biochemistry have evolved, but the use of probes has only increased. In particular, with the advent of high-throughput techniques4–6 there has been an even greater demand for probes that function reliably in a highly multiplexed setting.

Probe design considers binding affinity (sensitivity) and sequence selectivity (specificity) as opposing goals; improvement of one generally leads to deterioration of the other7–9. Molecular assays typically seek a compromise with acceptable levels of both specificity and sensitivity. However, reliable in silico design of probes that achieve this tradeoff has heretofore eluded researchers10,11, particularly in highly multiplexed settings12–13, as a result of imperfect DNA biophysical models and parameters14–16 and the intractability of analyzing complex mixtures. Empirical adjustments of operational conditions and probe sequences are typically used to optimize assay performance, but such methods can be expensive and time-consuming in multiplexed settings.

Here we present a new method for controlling nucleic acid probe specificity and sensitivity on the basis of the stoichiometry of an auxiliary oligonucleotide. The theory underlying the method is as follows. The hybridization of a nucleic acid target T to a probe C can be written as $T + C \rightleftharpoons TC$ and has a reaction standard free energy $\Delta G_0^{\text{rxn}}$. Probes with more negative $\Delta G_0^{\text{rxn}}$ values bind with higher affinity or yield, but they also spuriously bind other nucleic acid sequences8. Conversely, probes with less negative $\Delta G_0^{\text{rxn}}$ values bind their targets with low yield but high selectivity. Probes with intermediate values of $\Delta G_0^{\text{rxn}}$ show reasonable yield and selectivity15–18.

One major challenge in probe design is the inaccuracy and incompleteness of literature-reported nucleic acid thermodynamics parameters and models. For a typical 25-nucleotide (nt) probe, the real $\Delta G_0^{\text{rxn}}$ value may differ from the predicted value by up to 2.4 kcal mol$^{-1}$ (95% confidence interval)15, and the melting temperature $T_m$ similarly may differ from the predicted value by up to 4 °C (refs. 10,15). Sequence tuning is the iterative adjustment of probe length or sequence to achieve a more positive or negative $\Delta G_0^{\text{rxn}}$ (Supplementary Note 1). The ‘granularity’ of such sequence tuning is determined by the change in $\Delta G_0^{\text{rxn}}$ due to a single-base extension or deletion. At 25 °C in 0.15 M Na$^+$ (1× PBS), the granularity ranges between 0.64 and 2.32 kcal mol$^{-1}$, depending on the identities of the bases at the two ends of the probe (Fig. 1a).

Here we present stoichiometric tuning, an on-the-fly method of tuning probe yield and selectivity on the basis of the relative concentration of an auxiliary species. Stoichiometric tuning is possible for any ‘double block’ probe PC, such as the toehold probe7 or the X-probe8, that releases an auxiliary protector molecule P upon hybridization to its target T (Fig. 1b). The protector acts as a molecular competitor species that competes with the target for binding to the complementary probe. The equilibrium yield of the hybridization reaction ($T + PC \rightleftharpoons TC + P$) is defined as

$$\chi \equiv ([T]_0/[P]_0)^{\text{eq}} \equiv ([T]_0/[P]_0)^{\text{eq}}/ ([T]_0/[P]_0)^{\text{eq}},$$

where $[T]_0$ represents the equilibrium concentration of TC, and $[T]_0$ and $[P]_0$ represent the initial concentrations of T and PC, respectively.

To simplify the consideration of reaction behavior, we introduce the effective reaction free energy $\Delta G_\text{eff}$, a nonlinear mapping of reaction yield $\chi$: $\Delta G_\text{eff} = -RT \ln (\chi/(1 - \chi))$, where R is the universal gas constant and $\tau$ is the temperature in Kelvin. $\Delta G_\text{eff} > 0$ means that the reaction is not favorable and $\chi < 50%$. Intuitively, the value of $\Delta G_\text{eff}$ usually tracks that of $\Delta G_0^{\text{rxn}}$, a constant numerical change in $\Delta G_0^{\text{rxn}}$ usually results in a quantitatively similar change in $\Delta G_\text{eff}$ (Supplementary Note 2).
We calculated the analytic solution values of $\chi$ and $\Delta G_{\text{eff}}$ for a toehold probe with $\Delta G_{\text{rxn}}^{0} = -2$ kcal mol$^{-1}$ (Fig. 1c and Supplementary Note 2). Regardless of the relative concentrations of target and probe ($y$-axis), the excess protector stoichiometry $[P]_{0}/[PC]_{0}$ could be modulated to allow hybridization yields between essentially 0% and 100%. As an important case, when there is an excess of probe relative to the target ($[T]_{0}/[PC]_{0} < 1$) and when there is at least a 1× excess of protector ($[P]_{0}/[PC]_{0} > 1$), the hybridization yield is dependent only on the protector stoichiometry $[P]_{0}/[PC]_{0}$ and is unaffected by the target or probe concentration. Consequently, samples with unknown target concentrations can be easily and quantitatively analyzed.

The stoichiometry of $[P]_{0}/[PC]_{0}$ can be adjusted during probe formulation and can be modified in real time by the addition of extra $P$. This results in a continuous achievable $\Delta G_{\text{eff}}$ range of roughly 10 kcal mol$^{-1}$, corresponding to yields between 0.02% and 99.98% (0% $\leq \Delta G_{\text{eff}} \leq 5$ kcal mol$^{-1}$, respectively), with roughly 0.03 kcal mol$^{-1}$ granularity (from 5% total pipetting error).

Unlike traditional probe optimization based on iterative redesign and resynthesis, our method allows on-the-fly adjustment of binding yield and selectivity with the same molecules. Furthermore, because the stoichiometry can be controlled to within 5%, our stoichiometric tuning method allows fine control over $\Delta G_{\text{eff}}$. Finally, unlike assay temperature and salinity adjustment, stoichiometric tuning allows independent modulation of individual probe yields in multiplexed settings. Here we describe proof-of-concept experiments in several applications: the uniform yield capture of 31 DNA targets, differential signal maximization for 11 pairs of single-stranded probes with the protector lengthened by 1 nt. ($[P]_{0}/[PC]_{0}$ + 100 pM) and is unaffected by the target or probe concentration. Consequently, samples with unknown target concentrations can be easily and quantitatively analyzed.

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We designed our first toehold probe to bind its target (a subsequence of the Staphylococcus aureus mecA gene) with $\Delta G_{\text{eff}}^{0} = -2.05$ kcal mol$^{-1}$ (25 °C, 1× PBS); $\Delta G_{\text{eff}} = 0$ is predicted to occur when $[P]_{0}/[PC]_{0} = 46.3$. The probe is functionalized with a TAMRA fluorophore on the C strand and an Iowa Black RQ quencher on the P strand. When the target hybridizes to the initially dark probe, the quencher-labeled protector is displaced, and the solution fluorescence increases.

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Holding $[PC]_{0}$ and $[T]_{0}$ constant, we adjusted the concentration of free protector $[P]_{0}$ to modulate $\Delta G_{\text{eff}}$ and yield (Fig. 1d and Supplementary Note 3). Experimental results were qualitatively consistent with our analytical predictions; inferred experimental yields varied between 12% ($[P]_{0}/[PC]_{0} = 1,000$) and 97% ($[P]_{0}/[PC]_{0} = 1$). Every factor-of-three change in $[P]_{0}/[PC]_{0}$ alters $\Delta G_{\text{eff}}$ by roughly 0.65 kcal mol$^{-1}$, and 4.7% changes in $[P]_{0}/[PC]_{0}$ (changes in $\Delta G_{\text{eff}}$ of 0.027 kcal mol$^{-1}$) resulted in 2% yield changes (Fig. 1d). The observed s.d. values were somewhat high, presumably because of factors such as cuvette variability, nonspecific DNA adsorption, pipetting error and plasticware liquid retention. However, the relative ordering of the mean fluorescence intensities for the six stoichiometries shown in Figure 1d was as expected, corresponding to a $P$ value of 1/720 = 0.0014.

For comparison, we also show experimental results for sequence tuning of the toehold probe via the resynthesis of a new protector molecule (Fig. 1d). A single-nucleotide (G) extension of the protector resulted in a decrease of the yield from 61% to 9.6%, corresponding to a change in $\Delta G_{\text{eff}}$ of 1.59 kcal mol$^{-1}$, due to a similar magnitude change in $\Delta G_{\text{rxn}}^{0}$.
Yield can usually be calculated via a paired control experiment with unprotected probes. In assays where yield cannot be measured accurately, stoichiometric tuning can be applied effectively after the raw signal has been collected at two different \([P]_o/[PC]_0\) ratios. The second \([P]_o/[PC]_0\) data point allows one to additionally fit the scaling-constant mapping signal to the yield (Supplementary Note 4).

### Uniform capture efficiency

For enrichment applications, uniform capture yields for many different target sequences would facilitate relative and absolute quantitation. Commercial enrichment kits often show considerable biases in capture yield, particularly for sequences with high or low GC contents. We designed 31 different target sequences and probes with GC contents that varied uniformly between 0% and 100%. Here we used the X-probe architecture to facilitate economical testing: X-probes utilize universal fluorophores and quencher oligonucleotides, but they are otherwise similar to toehold probes and can likewise be stoichiometrically tuned (Supplementary Note 5).

Initially, we observed yields that varied between 3.1% and 49% (Fig. 2a), even though all probes were designed for 30% yield. We performed one round of sequence tuning on the basis of observed yields to attempt to build a new probe set with uniform 30% yield, but experimental yields varied between 13% and 61%. Through the course of two rounds of stoichiometric tuning (Supplementary Note 5), we were able to dramatically tighten the yield distribution to the range of 27–37%, reducing the high-to-low yield variability by nearly tenfold (Fig. 2a). Notably, predictable and uniform capture yield was achieved even for target sequences at the extremes of GC content (Fig. 2b,c), and uniformity was preserved in the presence of 100 ng of genomic DNA, corresponding to more than a six-times excess over target. Nonspecific capture of sequences similar to the target was minimal (Fig. 2d).

### SNV discrimination

SNVs are clinically important because of their contributions to disease susceptibility. SNV probes exemplify the challenge of balancing yield and selectivity because of the small thermodynamic change (\(\Delta G^0\)) associated with a single-nucleotide mismatch (Fig. 3a). On the basis of a simple reaction analysis (Supplementary Note 6), an optimal yield difference (\(\Delta \chi\)) is achieved when the \(\Delta G_{eff}\) of the correct target satisfies \(\Delta G_{eff} = -\Delta G^0/2\) (Fig. 3b). Because \(\Delta G^0\) values vary between +1 and +6 kcal mol\(^{-1}\) depending on the sequence, different SNV pairs require different \(\Delta G_{eff}\) values for optimal discrimination.

We measured the difference in fluorescence signals produced by a toehold probe when it reacted with its DNA target versus 11 SNVs (Fig. 3c). On the basis of these results, we calculated the \(\Delta G^0_{rxn}\) of the toehold probe with the intended target and with each SNV, from which we numerically calculated the \(\Delta G^0\) of each SNV pair. From this, we calculated the \([P]_o/[PC]_0\) stoichiometry needed to achieve \(\Delta G_{eff} = -\Delta G^0/2\) for each individual SNV. After tuning, the yield difference improved from 17–83% to 47–89% (Fig. 3d). Similar results were obtained for RNA SNV pairs (Supplementary Note 6). Note that the toehold probe used here was already near optimal; a probe less well designed at the outset would have exhibited a larger improvement in yield difference.

Stoichiometric tuning allows high SNV discrimination at a uniform set of temperature and buffer conditions that is difficult to achieve with other molecular probes. We compared the performance of X-probes and molecular beacons in the detection of four cancer driver mutation sequences (Fig. 3e,f and Supplementary Note 7). There was not a single temperature at which all four molecular beacons yielded both high sensitivity (high signal for SNV targets) and high specificity (low signal for the wild type). Stoichiometrically tuned X-probes, despite the design error in \(\Delta G^0_{rxn}\), achieved a uniform yield of ≈50% for all four probes across all three temperatures tested.

### Multiplexed and decoupled tuning

To show decoupled tuning of two different probes, we constructed probes labeled with two spectrally distinct fluorophores, Alexa 488 and Alexa 647. An initial stoichiometry of \([P]_o/[PC]_0 = 50\) resulted in yields of 5% and 64%, respectively (Fig. 4a). When we
Optimizing SNV discrimination using stoichiometric tuning. (a) Two SNV sequences bind to a probe with slightly different \( \Delta G^\text{bind} \); the difference is referred to as the \( \Delta \Delta G^\text{bind} \). (b) The same \( \Delta \Delta G^\text{bind} \) value produces different changes in yield (\( \Delta \gamma \)) depending on the \( \Delta G^\text{off} \) of the intended target; \( \Delta \gamma \) is maximized when \( \Delta G^\text{off} = -\Delta \Delta G^\text{bind}/2 \) (Supplementary Note 6). Open circles and crosses represent binding of intended and mismatched targets, respectively. (c) Observed yields for intended target (top edge of bars) and 11 SNVs (bottom edges of bars) at \([P]_0/[PC]_0 = 1\). All experiments were performed in triplicate in 1× PBS at 37 °C; \([T]_0 = 200 \text{nM} \) and \([PC]_0 = 100 \text{nM}\). (d) Yields after stoichiometric tuning. Refer to c for descriptions of the graph and experimental conditions. (e) SNV selectivity of molecular beacons at 68 °C, 72 °C and 76 °C differing by a single nucleotide. (f) SNV selectivity of stoichiometrically tuned X-probes at 33 °C, 37 °C and 41 °C. Error bars in c–f represent ±s.d.

Adjusted \([P]_0/[PC]_0\) to 0.765 and 208, the yields of both probes reached 40%. The addition of formamide, in contrast, decreased capture yield, as expected. Yields could be tuned over a range of 7.2–97% on the basis of the cycle-threshold quantitation (Fig. 4c and Supplementary Note 8).

Multiplexed RNA analysis

We used the Nanostring nCounter platform and the Elements assay\(^2\) to demonstrate highly multiplexed quantitation and tuning (Fig. 5a and Supplementary Note 9). First, every probe A in the standard Nanostring Elements gene expression panel (192 in total) was modified to include new nonhomologous regions (tilted) to accommodate protectors. Protectors were designed to produce 50% yield at \([P]_0/[PC]_0 = 2\). Observed yields in experiments with RNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue samples were calculated as Counts\(_{\text{toehold}}/\text{Counts}_{\text{unprotected}}\) (Fig. 5b). For roughly 25% of the probes, the toehold probes actually produced higher counts than the unprotected probe A did, resulting in a calculated yield of >100%. We interpret this to mean that the hybridization of the unprotected probe A to its RNA target had not reached completion, and that the toehold probes hybridized to targets more rapidly than single-stranded probes did.

We performed multiplexed sequence tuning for a 60-plex panel for fusion transcripts commonly associated with leukemia (Fig. 5c). Protectors were truncated to produce probes with \( \Delta G^\text{bind} \) values \( 1, 2 \) and \( 3 \text{kcal mol}^{-1} \) more favorable than those of the reference protectors. Although most probes behaved as expected, with more truncated probes yielding higher signals, a small fraction resulted in unpredictable or ineffective yield tuning. These results are unlikely to be due to nonspecific target-probe binding; target-to-probe signals seemed to be orthogonal in a 22-plex subset panel (Fig. 5d).

Next we applied one round of multiplexed stoichiometric tuning on a 24-plex panel (Fig. 5e and Supplementary Note 10), with the goal of achieving a uniform 50% yield. After tuning, yields varied between 25% and 70% (except for one outlier at 127%). The multiplex and pre-equilibrium nature of the assay

![Figure 4](https://example.com/figure4.png) **Figure 4** Independent stoichiometric tuning in multiplex assays. (a) Two-plex stoichiometric tuning assayed using two toehold probes functionalized with the spectrally distinct Alexa 488 (A488) and Alexa 647 (A647) fluorophores. For comparison, adjusting formamide concentration does not allow for decoupled tuning. (b) Multiplex hybrid capture of two mRNA species, ACTB (NM_001101.3–pos. 1673–1709) and EIF4A2 (NM_001967.3–pos. 1640–1675), from total RNA using magnetic beads. (c) RT-qPCR analysis of hybrid-capture efficiency; shown in the table are \( C_t \) values for each of the two targets.
probably decreased the quantitative predictability of the tuning; however, the qualitative improvement indicates that further rounds of tuning could have resulted in improved uniformity.

Finally, we designed a 14-plex panel against cancer driver mutation sequences (Supplementary Note 11). Paired in vitro transcript targets (250 nt containing a local sequence) were used to characterize SNV selectivity (Fig. 5f). One round of stoichiometric tuning was applied to improve specificity. In all but one case (the NFE2L2 p.R34Q mutant), toehold probes and stoichiometric tuning significantly increased the selectivity (Fig. 5g).

**Kinetics and use as PCR primers**
To clarify confusion in the literature, the toehold-probe displacement reaction has a speed comparable to that of direct hybridization, given a sufficiently long (for example, 7 nt) single-stranded toehold. To demonstrate kinetics, we constructed toehold PCR primers and amplified from 2 ng of human genomic DNA (Supplementary Note 12). The observed quantitation cycles $C_\text{q}$ for toehold primers were not significantly different from those for single-stranded primers, indicating that strand displacement proceeds to near completion within 30 s at 200 nM primer concentrations.

**DISCUSSION**
We have introduced and experimentally validated stoichiometric tuning as an effective, predictable and flexible method for modulating the yield and selectivity of hybridization. Unlike traditional sequence-based probe adjustment, stoichiometric tuning does not require the synthesis of new molecules, allowing for on-the-fly modulation of the affinity-selectivity tradeoff. In contrast to empirical adjustment of assay conditions such as temperature and buffer conditions, stoichiometric tuning allows for decoupled adjustment of individual probes in multiplexed settings. As far as we are aware, this is a fundamentally novel and useful capability for multiplexed analysis of nucleic acids.

Our analyses of stoichiometric tuning assume reaction equilibrium, and they may be less quantitatively predictive for applications that require pre-equilibrium readout. Kinetic simulation of the underlying hybridization reactions can inform the probe formulation or design for pre-equilibrium assays, but it might be impractical for highly multiplexed systems. Our Nanostring results show that even in pre-equilibrium assays without perfect quantitative predictability, stoichiometric tuning can be useful in equalizing yields and improving SNV selectivity.

Oligonucleotide synthesis is imperfect, and even with HPLC purification, up to 15% of the molecules may harbor nucleotide deletions and/or truncations. We believe that these impurities are the main cause of the deviation between the curves for the experimental and theoretically predicted yields versus $[P]/[P]_0$ (Fig. 1d and Supplementary Note 13). Nonetheless, our analyses and experiments indicate that, regardless of impurity fractions,
there is a \([P]_0/[PC]_0\) ratio appropriate for the production of any desired \(\Delta G_{\text{eff}}\).

We believe that hybridization probes and primers used for a variety of nucleic acid analytic and diagnostic applications could benefit from stoichiometric tuning. For example, allele-specific PCR primers might benefit from a controllable tradeoff between mutation sensitivity and amplification efficiency (i.e., \(C_q\) delay), and hybrid-capture probes for next-generation-sequencing target enrichment\(^{26}\) could benefit from yield uniformity or allele-specific enrichment. Direct hybridization assays, such as those based on fluorescent barcodes\(^ {22,27}\), label-free detection\(^ {28,29}\) or \textit{in situ} hybridization, might be most easily adapted for stoichiometric tuning.

**METHODS**

Methods and any associated references are available in the online version of the paper.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

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**AUTHOR CONTRIBUTIONS**
L.R.W., J.S.W. and D.Y.Z. conceived the project and performed theoretical analysis on stoichiometric tuning. L.R.W., J.S.W., E.R.E., J.Z.F., A.P., I.P., R.B., C.N., P.J.W. and D.Y.Z. designed and conducted experiments. L.R.W., J.S.W., J.Z.F., A.P., I.P., R.B., C.N., P.J.W., J.B. and D.Y.Z. analyzed the data. L.R.W., J.S.W. and D.Y.Z. wrote the paper with input from all authors.

**COMPETING FINANCIAL INTERESTS**
The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

DNA and RNA oligonucleotides. All DNA oligonucleotides were purchased from Integrated DNA Technologies (IDT). RNA in vitro–transcribed targets for Nanostring experiments were transcribed using T7 RNA polymerase from a gBlock gene fragment synthesized by IDT. Each oligonucleotide was, depending on its length and modifications, PAGE purified, HPLC purified or not purified (Supplementary Note 14). In all cases, oligonucleotides were quality-controlled by IDT via capillary electrophoresis and electrospray-ionization mass spectrometry. Oligonucleotide sequences are listed in tables in Supplementary Note 15.

Standard buffer conditions. Individual DNA oligonucleotides were resuspended and stored in 1× PBS buffer (0.15 M Na+, purchased as 10× stock from Sigma-Aldrich) with 0.1% Tween-20 (Sigma-Aldrich) at 4 °C. RNA oligonucleotides were resuspended and stored in the above-mentioned buffer with additional 0.1% RNaseZap (Life Technologies) at 4 °C. Non–RNA-related experiments were performed in 1× PBS with 0.1% Tween-20, and RNA-related experiments were performed in 1× PBS with 0.1% Tween-20 and 0.1% RNaseZap. Multiplex hybrid-capture experiments used binding and washing buffer, which consisted of TE buffer (Sigma-Aldrich, T9285) supplemented with 0.5 M sodium chloride and 0.1% (vol/vol) Tween-20.

Experimental temperature and buffer conditions. Experimental temperature and buffer conditions varied according to the experiment; these are shown in the Supplementary Note 14. On the basis of both our prior experiments and the experiments described in Figure 3f, toehold probes were temperature and buffer robust.

Protocol. Demonstration of basic stoichiometric tuning. We prepared toehold probes by mixing protector and complement solutions (both ≥10 nM) at the desired stoichiometry and incubating them at room temperature (25 °C) for at least 1 h. Probe was mixed with target and diluted in 1× PBS buffer (the final concentration of the complement was 100 pM), and then the reaction mixture was incubated at room temperature (25 °C) in a dark box for 12–24 h. Kinetics experiments showed that equilibrium was achieved within 4 h at the experimental conditions (25 °C, 1× PBS; Supplementary Note 3). Fluorescence measurements were performed using a Horiba Fluoromax 4 spectrofluorimeter and Hellma Semi-Micro 114F spectrofluorimeter cuvettes. Excitation and emission wavelengths were set at 558 nm and 580 nm, respectively, which generated the maximum fluorescence signal for the TAMRA fluorophore; slit widths were set at 10 nm, and the integration time was 10 s. An external temperature bath maintained the reaction temperature at the desired temperature ±0.2 °C. The fluorescence signal of each sample was calculated as the mean of three reads.

Demonstration of two-point tuning. Blinded experiments were performed; J.S.W. prepared three samples with different target concentrations, and L.R.W., without knowing the concentrations, performed calibration and tuning experiments. Experimental conditions were 1× PBS buffer and room temperature (25 °C). Toehold probes were prepared using the methods described above; probe was then mixed with target and incubated for at least 1 h with no exposure to light. The resulting final concentration of the probe ([PC]0) was 10 nM. The fluorescence of each sample was calculated as the mean of three measurements on a Qubit 3.0 fluorometer. Background signal was calculated as the mean fluorescence of [P]0/[PC]0 = 1 and [P]0/[PC]0 = 100 dark probes in the absence of target. A detailed description can be found in Supplementary Note 4.

Uniformity of capture for targets of varying GC content. We formulated X-probes in 5× PBS by thermal annealing using one of three Eppendorf MasterCycler Personal PCR machines, following a process of initial heating to 95 °C for 5 min and subsequent uniform cooling to 20 °C over the course of 75 min. Formulated X-probe solutions were then stored at 4 °C until use. Then sample solutions of probe with or without target were pipetted into 96-well PCR plates (Bio-Rad) at 10 μL per well and sealed. Experiments were performed in a Bio-Rad CFX96 machine. 20 continuous data points were collected in each well after 20 min of incubation at the designated temperature; all experiments were performed in triplicate. All fluorescence signals collected were corrected for position and subsequently background-subtracted; subsequently, the equilibrium binding yield was inferred from the corrected, background-subtracted signal relative to that of a maximally unquenched signal. A detailed description of the procedures is provided in Supplementary Note 5. 100 ng of human genomic DNA (NA18562, Coriell) was added in each experiment to serve as nonspecific background.

Optimizing yield differences for detection of single-nucleotide polymorphisms. Probe was mixed with target in 1× PBS at room temperature, resulting in a final concentration of 100 nM of complement; then the samples were added to 96-well PCR plates (Bio-Rad) at 25 μL per well and sealed. A CFX96 Real-Time PCR detection system (Bio-Rad) was used to incubate the samples at 37 °C for 20 min and then measure fluorescence multiple times with channel 1 (excitation at 450–490 nm and emission at 510–530 nm), which was consistent with the properties of the Alexa 488 fluorophore. The fluorescence signal of each sample was calculated as the mean of four reads.

Performance comparison with molecular beacons. We formulated X-probes in 5× PBS by thermal annealing using one of three Eppendorf MasterCycler Personal PCR machines, following a process of initial heating to 95 °C for 5 min and subsequent uniform cooling to 20 °C over the course of 75 min. Formulated X-probe solutions were then stored at 4 °C until use. Then sample solutions of probe with or without target were pipetted into 96-well PCR plates (Bio-Rad) at 20 μL per well and sealed. Experiments were performed in a Bio-Rad CFX96 machine. 20 continuous data points were collected in each well after 10 min of incubation at the designated temperature; all experiments were performed in triplicate. All fluorescence signals collected were background-subtracted; subsequently, the equilibrium binding yield was inferred from the background-subtracted signal relative to that of a maximal signal. A more detailed description of the procedures is provided in Supplementary Note 7.

Demonstration of multiplexed tuning. Experiments were performed in 1× PBS buffer at room temperature (25 °C). Toehold probes were prepared and reacted with targets using the methods described above; here the two probes were pre-hybridized separately and subsequently mixed together to react with the sample containing two target species. The resulting final concentration of each probe ([PC]0) was 2 nM, and that of each target ([T]0) was 3 nM. The fluorescence of each sample was calculated
as the mean of three measurements on a Qubit 3.0 fluorometer. We measured the fluorescence of the dark probe corresponding to each sample as the background and the fluorescence of non-protected complement (C) as the maximum signal. The yield was calculated as (Sample fluorescence − Background)/(Maximum fluorescence − Background). Calculation methods for multi-round stoichiometric tuning can be found in Supplementary Note 5. Formamide tuning samples contained 20% formamide in the total volume.

**Multiplexed total RNA hybrid-capture experiments.** 100 ng of human brain total RNA (Thermo Fisher Scientific, AM7962) were dissolved in a 70-µL final volume of binding and washing buffer in the presence of 7.5 pmol of probes against ACTB and 7.5 pmol of probes against EIF4A2. The samples were denatured at 75 °C for 5 min and cooled down (1 °C per minute) to 60 °C for 30 min of incubation under gentle shaking. After the incubation, 30 µL of streptavidin-coated magnetic beads (Dynabeads MyOne C1, Thermo Fisher Scientific, 65001), previously washed twice in 100 mM NaOH solution and three times in binding and washing buffer, were added to the sample at room temperature. Subsequently the samples were further incubated at 60 °C for 15 min, again under gentle shaking. At the end of this incubation, the samples were incubated for 5 min at room temperature in a DynaMag 2 magnetic rack for recovery of the magnetic beads and conjugated nucleic acids. The solution supernatant was decanted, and the particles were washed three times in 200 µL of binding and washing buffer and resuspended in 100 µL diethylpyrocarbonate-treated water. Afterward the samples were incubated for 5 min at 95 °C and then for 2 min at room temperature in the magnetic rack to release captured RNA molecules. The supernatant from this elution was recovered, and 1 µL was used as a template in RT-qPCR reactions. One-step RT-qPCR was performed using the iTaq Universal SYBR Green One-Step kit (Bio-Rad, 1725150) according to the manufacturer’s instructions. A 10-µL final volume reaction contained 0.2 µL forward and reverse primers for either the ACTB or the EIF4A2 target. The Bio-Rad CFX96 Real-time PCR was set as follows: 10 min at 50 °C, 1 min at 95 °C, and 40 cycles of 10 s denaturation at 95 °C and an annealing and extension step at 60 °C.

**FFPE RNA extraction.** FFPE breast tumor (infiltrating ductal carcinoma) tissue samples ORO-2965, ORO-2869 and ORO-2820 were purchased from Asterand with donor I.D.s 62234, 62729 and 66280, respectively. FFPE tissue sample WCP-ONYF was a normal kidney tissue sample. RNA was purified from all FFPE samples using a commercial FFPE RNA isolation kit (Roche).

**Nanostring experiments.** For each multiplexed target, the corresponding probe A and protector strand were pooled together at a predetermined stoichiometric ratio and diluted down to 120 pM. This diluted pool was then annealed for 75 min. The target strands of interest were also pooled together and diluted down to 1.54 pM. To prepare the hybridization reaction, we filled each microtube well with 10 µL of buffer and 5 µL each of probe A or protector pool, probe B, Tagset, and target pool (Tagset strands and probe B strands are available from Nanostring). The reaction samples were then placed in a temperature bath at 67 °C for 18 h.

Once the 18-h reactions were finished, the samples were loaded into the Nanostring nCounter Prep Station. The standard protocol for operating the nCounter was followed until data were obtained in the form of molecular counts. Instructions for operating the nCounter Prep Station are available on Nanostring’s website. (http://www.nanostring.com/support/request_info)

Because individual molecular counts scale linearly with concentration, it was possible to calculate the yield by comparing protected probe A and nonprotected probe A versions of this experiment on the same target. For each individual target species, the yield could then be used to back-calculate the stoichiometric ratio of protector to probe A required to bring the yield as close to 50% as possible. This entire procedure could then be repeated for additional rounds of tuning. A more detailed description of the procedures is provided in Supplementary Notes 9–11.

For determination of limits of detection in **Figure 5**, experiments were performed with 256 fM of in vitro–transcribed RNA (256 fM each). Because background counts varied between 0 and 40 counts, 100 counts was used as a conservative estimate for a minimum definitive positive signal.

**Toehold primers for allele-specific amplification.** Primer and protector oligonucleotides were purchased from IDT with standard desalting. Repository human genomic DNA samples (NA18562 and NA18537) were purchased from Coriell Cell Repositories. Oligonucleotides were suspended in 1× TE buffer (diluted from 100× TE; Sigma-Aldrich) and stored at 4 °C. Genomic DNA samples were stored at −20 °C and were diluted to 2 ng/µL working solution before use in 1× TE buffer with 0.1% Tween-20 (Sigma-Aldrich). The primer concentration was 200 nM for both forward and reverse primers, and the genomic DNA concentration was 0.2 ng/µL; Bio-Rad iTQ SYBR Green Supermix was used for amplification. The total volume was 10 µL per well in Bio-Rad 96-well PCR plates; each experiment was done in triplicate. A fast two-step PCR cycling protocol was used on a CFX96 Touch real-time PCR detection system (Bio-Rad): 5 min initiation at 95 °C, then 10 s at 95 °C and 30 s at 60 °C; the cycle number was at least 60. Background subtraction and Cq calculation were performed using a Matlab code; background was calculated as the average fluorescence of 15–20 cycles. The quantitation threshold was set at 20 relative fluorescence units above baseline, and a linear interpolation was used.