Multiplexing With MicroRNAs: Mismatch Probes and Temperature Compensation for Increased Specificity and Reduced Variability in Hybridization Assays

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Multiplexing with MicroRNAs: Mismatch Probes and Temperature Compensation for Increased Specificity and Reduced Variability in Hybridization Assays.

Conor Stefan Rafferty

A Thesis in the Field of Biotechnology
for the Degree of Master of Liberal Arts in Extension Studies

Harvard University
May 2019
Abstract

MicroRNAs constitute a very interesting class of circulating biomarkers. They are present in many body fluids, and appear to avoid degradation by enzymes or the immune system. Organs shed microRNAs into the blood stream, offering the exciting possibility of detecting solid tumors or other hidden pathologies without invasive tissue biopsy.

Multiplex microRNA assays offer superior specificity and differential diagnosis relative to quantitation of single analytes. A variety of multiplex assay technologies exists, including RNA sequencing, quantitative polymerase chain reaction, and hybridization, with different tradeoffs of cost, coverage, throughput and sample volume.

The focus of this work is on improving reproducibility and specificity of a hybridization-based multiplex microRNA assay. The assay binds microRNA targets in solution to complementary DNA probes conjugated in a hydrogel particle matrix. The hydrogel environment differs from a classic surface-bound or bead-bound hybridization assay and has advantages for binding nucleic assay from complex biological samples, for instance tissue lysates and serum.

Non-specific binding combined with variable well temperature across a plate is shown here to be a major source of assay variability. We describe the development of novel probes to reduce non-specific binding is described, and the development of a methodology to correct for temperature. An over 10-fold reduction in non-specific
binding is achieved, and well-to-well variability is reduced from ~30% in raw data to single-digit percentages in most cases.
Author’s Biographical Sketch

Conor Rafferty did his undergraduate training at Trinity College Dublin, where he graduated with a Bachelor of Arts in Mathematics and Bachelor of Science in Physics. He went on to do a Master of Science in Electrical Engineering at Trinity College before earning a Ph.D. in Electrical Engineering at Stanford University, California. He researched solid-state diffusion and wrote software to model silicon processing, as well as software to design integrated circuits at Bell Laboratories, Murray Hill, NJ from 1989 to 2003, starting first as an individual contributor and later serving as Director of Silicon Processing Research and then Director of Computer-Aided Design Research. In 2003 he co-founded NoblePeak Vision, a silicon camera chip company with a wavelength window twice as wide as a conventional silicon chip by virtue of in-pixel germanium crystals. He served as Chief Technical Officer, raised $25M in three rounds of venture capital, recruited the engineering team and led technical activities until 2010, when the company was acquired by Infrared Labs. He took the opportunity to pursue a long-held interest in biology and started coursework at Harvard Extension School. In late 2011 he joined MC10, a flexible medical electronics company and since late 2013 has worked at Firefly Bioworks, a multiplex assay company. Firefly was acquired in 2015 by Abcam, PLC.
Acknowledgments

I would like to thank Abcam, PLC and particularly Dr. Daniel Pregibon, founder of Firefly Bioworks, for allowing me the use of Abcam facilities to complete the experimental parts of this thesis. I also appreciate the patience of my co-workers during the writing of this thesis and my colleague Dr. Long To for preserving momentum in the software department while I focused on experiments and analysis.

I especially would like to thank Dr. Graeme Doran for teaching an electrical engineer how to navigate a biotechnology lab. This thesis would be a much weaker work without his extensive knowledge of molecular biology techniques and literature, numerous experimental and organizational suggestions, critical reading of the text and most of all his inexhaustible supply of probing questions, discussions and ideas.

Lastly, I would like to thank Marjorie Serrano for her support and companionship in adventure over the last year, and her patient understanding while I was lost to the outside world through the final months of this thesis. I look forward to more epics on rock, snow and ice and happy returns by headlight!
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Chapter I
Introduction

Multiplex microRNA assays hold promise for the development of sophisticated diagnostic signatures. In the first part of this chapter, we review the possibilities a multiplex assay can offer over assaying a single analyte. MicroRNAs are particularly interesting molecules to assay, being present in many body fluids, and appearing to avoid degradation by enzymes or the immune system. The focus on this work is on microRNA detection via hybridization. The thermodynamics of hybridization is reviewed in order to set the stage for the discussion of results. We then review one of the challenges encountered in any assay, which is normalizing between samples in order to make comparison between samples meaningful. Variability between samples can arise from a variety of sources, both biological and technical. Removing the unwanted technical variation to expose the biological variation is the goal of normalization. A number of well-established techniques are described, and shown to have limited utility when different probes are systematically affected differently by technical factors. Subsequent chapters will describe new methods to mitigate such systematic variability.

Multiplex Assays

The simplest diagnostic assays rely on a single analyte to monitor a condition. For instance, glucose is the key analyte for diabetes, hepatitis C antibody as a marker for hepatitis C, prostate specific antigen for prostate cancer. Other common diagnostic
assays use just a pair of analytes, such as high-density lipoprotein / low-density lipoprotein (HDL/LDL) for cardiovascular risk or aspartate transaminase / alanine transaminase (AST/ALT) for liver disease [Etheridge2010].

A multiplex assay measuring dozens or even hundreds of analytes simultaneously offers the possibility of much more refined diagnostic methods, with the possibility of discriminating between different stages or variants of a condition, based on an entire pattern of over- or under-expression. Better yet, multiplex assays offer the promise of a “liquid biopsy” for conditions where the target tissue is difficult to access or assess directly, such as cancers of unknown primary or neurodegenerative disease. For instance, there is presently no diagnostic test for Parkinson’s disease (PD), and at the onset of symptoms, little possibility of distinguishing, on the basis of clinical signs and symptoms, between PD itself or one of several other Parkinsonian conditions such as progressive supranuclear palsy (PSP), multiple system atrophy (MSA) or corticobasal degeneration (CBD). Many other conditions such as fibromyalgia, chronic fatigue syndrome, or complex regional pain syndrome (CPRS) lack any diagnostic tools. At present, these conditions are mainly diagnosed by exclusion of other conditions, and treatment is limited to symptomatic relief.

A striking example of a multiplex assay is Exact Science’s Cologuard stool test for colon cancer [Cologuard2014]. The test combines three separate analyze categories, examining methylation of promoter sites on two cancer-related genes, mutations in seven cancer-related genes, and occult hemoglobin. The test has similar specificity and sensitivity to colonoscopy without the need for dietary preparation or the risks of the
procedure; it was approved by the FDA in 2014. Multiplex diagnostic assay based on microRNAs are in development for numerous conditions, reviewed below.

In addition to novel diagnostics, a considerable benefit of a multiplex assay is that it extracts the maximum information from a small sample volume, as low as 10-20 µL, in a short amount of time and usually at substantially lower cost relative to running multiple single-plex assays. Small sample volume is particularly critical in research, where the fluid may be derived from a small animal and may already be in low abundance. A further advantage of a multiplex assay is that numerous other analytes within the same sample can serve as a control for the target analyte, facilitating comparison of samples potentially originating from different sources, processed under different conditions, or possibly taken and stored under less than ideal conditions, for instance, formalin-fixed paraffin-embedded (FFPE) samples or dried blood samples, collected in the field in a third world country.

Multiplex assays generally fall into three categories, solution assays such as bead-based assays, planar assays such as microarrays, and for DNA/RNA, direct sequencing. Sequencing offers the ultimate in coverage, since it can find all possible transcripts, including variant alleles and previously unknown mutations. However, preparation of nucleic acids for sequencing remains labor intensive, and high-quality sequencing often requires more material than is readily accessible from limiting biological samples. Microarrays offer the ability to assay hundreds of thousands of known targets, but not all possible targets can be anticipated in the chip design. The workflows for sample preparation on microarrays are labor intensive and binding at the microarray plane is thermodynamically unfavorable to binding in solution, leading to slower binding and
lower capture rates and lower sensitivity relative to solution-based assays [Pregibon2008, Pregibon2009, Chapin2011]. Bead-based assays in solution typically have modest multiplex (10-100 analytes, due to the coding capacity of optical bead systems), but they have excellent binding, high throughput and low sample volume requirements. They offer the possibility of running 96 or 384 samples simultaneously on one plate. Using a plate stacker, a single scientist can process thousands of samples in a day. The search for a novel diagnostic, therefore, often starts with next-generation sequencing (NGS) or microarray assays for discovery of dysregulated transcripts, then validation of the diagnostic signature on a larger cohort of samples using a bead-based assay, before proceeding to clinical work. The landscape of multiplex assays is summarized schematically in Figure 1.

Figure 1. Landscape of multiple assays (schematic)
There is an intrinsic tension between the desire for high throughput and high target coverage. Methods that provide blanket coverage of all or most targets (sequencing, microarray) become costly when applied to hundreds of samples. Methods that allow an assay in a single well of a 96-well or 384-well plate typically have lower plex, of order 25-100 targets. With a limited number of targets, the researcher will often choose targets most likely to be dysregulated between case and control samples in the study, so that the overall expression profile might be markedly different between case and control samples. With microarray or sequencing, a huge number of targets is detected, of which only a handful may be dysregulated. The implications for discerning differential expression of targets are important and will be discussed further below.

MicroRNA assays

Multiplex microRNA assays are particularly interesting for diagnostic purposes, and an intense hunt for diagnostic signatures of many conditions is under way [Chen2008, Etheridge2010, Scholer2010, Ajit2012, Creemers2012, Moldovan2013Detassis2017, Wang2017, Pordzik2018]. In most cases there is an emphasis on a pattern of dysregulation for diagnosis, requiring a multiplex assay. For instance, each of the eight “hallmarks of cancer” has several associated dysregulated microRNAs [Detassis2017]. For systemic lupus erythematosus, dysregulation of 9 microRNAs has been linked to active lupus T-cells [Wang2017]. In cardiovascular disease, miR-223, together with miR-126, miR-140, and miR-26b have diagnostic and prognostic value for endothelial dysfunction [Pordzik2018]. Specific microRNAs have been measured in the blood after ischemic stroke and are under investigation as potential prognostics after cardiac arrest [Devaux2015].
With protein diagnostics, a specialized antibody is needed. If a catalog antibody for each analyte is not available or is insufficiently specific or sensitive, custom antibodies need to be developed, a process that usually takes 6-12 months per target at substantial development cost. In contrast, the development of probes for microRNAs is relatively straightforward, using the reverse complement of the target strand. Furthermore, microRNAs can be amplified by polymerase chain reaction (PCR), allowing the detection of very low levels in biofluids with little or no pre-processing. MicroRNAs are present in many body fluids and appear to avoid degradation by enzymes or the immune system [Chen2008, Etheridge2010]. Organs shed microRNAs into the bloodstream [Chen2008], offering the exciting possibility of detecting solid tumors or other hidden pathologies without biopsy [Chen2008, Etheridge2010, Creemers2012].

MicroRNA diagnostics are relatively late to market compared to immunoassays due to the historically late discovery of microRNAs. Mello and Fire discovered in 1998 [Fire1998] that many if not all genes in animals were potentially subject to suppression by introducing exogenous double-stranded RNA (dsRNA) into the cell, a phenomenon they termed RNA interference (RNAi). This work built on earlier work in plants and indicated a universal importance of small RNAs in gene expression, and earned them a Nobel prize in 2006. Earlier, Ambros and coworkers had discovered [Lee1993] that a key developmental gene in C. elegans was suppressed by a short endogenous RNA transcript and found that the suppressing gene had a complementary sequence to part of the 3’ untranslated region (3’UTR) of the target gene. However, the significance of endogenous RNA silencing did not really come into focus until 2000, when Ruvkun
discovered another short endogenous repressor gene, let-7, in C. elegans that had a complementary sequence to the 3’ UTR of its target [Ruvkun2000], and found that the gene was conserved across many species [Ruvkun2001]. RNA silencing was therefore not limited to exogenous dsRNA or a single endogenous gene in a single species but appeared to be widespread. In the following half decade, hundreds of new short suppressing non-coding RNA transcripts were discovered and came to be known as microRNAs. By 2009, it was predicted that most mammalian messenger RNAs (mRNAs) are conserved targets of microRNAs [Bartel2009], establishing the significance of microRNAs in all aspects of cell function. MicroRNAs in mammals primarily act to reduce and regulate protein levels (as opposed to complete suppression) and play a particularly important role in organism development and cell differentiation [Bartel2004]. They are among the most abundant classes of gene-regulatory molecules [Bartel2004], with hundreds of family members and as many as 50,000 copies in a cell.

Curiously, microRNAs typically have hundreds of targets, often unrelated and expressed in different tissues. Conversely, a target mRNA may have binding sites for numerous microRNAs. As a result, it is likely that a successful microRNA diagnostic will be a multiplex assay, with the coordinated behavior of several different microRNAs providing the required specificity and statistical power.

Structurally, microRNAs are 21- to 23- long oligonucleotides with sequence homology to the 3’ UTR of their target [Bartel2004]. They originate as longer transcripts, known as pri-miRNAs. A single pri-miRNA may contain several microRNA precursors. Inside the nucleus, the enzyme Drosha cleaves a precursor of ~80 nucleotides (nt) from the pri-miRNA, known as a pre-miRNA. The pre-miRNA binds to
itself, forming a characteristic hairpin structure with a long arm and a short arm. It is actively transported to the cytoplasm where it is cleaved by the enzyme Dicer, yielding two ~22-nt single strands of RNA. Either or both single-stranded RNA (ssRNA) strands may be active in gene suppression; they are distinguished by the suffices -3p or -5p depending on whether they originated from the 3’ or 5’ branch of the hairpin. The full nomenclature scheme for microRNAs is of the form hsa-miR-195b-5p where the first three letters indicate the species, the next three denote that it is a microRNA, the first set of digits indicate the pre-miRNA from which the microRNA derives from, the letter suffix indicates which of several possible homologous versions is involved, and the numerical suffix indicates from which end of the pre-miRNA it is derived [Ambros2003].

A microRNA suppresses its target in the cytoplasm, through the action of the RNA-induced silencing complex (RISC), whose active component is the Argonaute enzyme. The microRNA associates with Argonaute and guides it to its target by sequence complementarity. The target is repressed either by inhibiting its translation, or by destabilizing the transcript, or a combination of both mechanisms [Bartel2004]. The surprising stability of the 22-nt single-stranded RNA transcripts in blood may be due to their association with Argonaute, protecting them from RNAse enzymes. In addition, microRNAs may be released from the cell in vesicles [Chen2008, Etheridge2010].

Hybridization Assays

In a nucleotide hybridization assay, probes designed with a sequence complementary and reversed to the desired targets [Figure 2] are incubated with samples in a specialized buffer. The buffer usually has high cation concentration to stabilize
probe-target binding, especially Mg$^+$ to stabilize the double helix [Every2008]. An intercalating agent is sometimes added to increase specificity, such as tetramethylammonium chloride (TMAC) and an agent to reduce secondary structure formation, such as dimethyl sulfoxide (DMSO) [Mok2016].

![Complementary probe for DNA target](image)

**Figure 2.** Complementary probe for DNA target

The probes are bound and labeled in some way to identify them. The probes might be bound to a surface and identified by position in a microarray. In a bead-based or particle-based assay, the probes are bound to a bead or particle which is fluorescently labeled for identification. A hydrogel-bound probe has significant advantages over a surface-bound probe, providing solution-like capture kinetics and lower electrostatic barriers [Chapin2011, Vainrub2003]. Fold-change compression can result in a microarray, and signal intensity is found to vary as much as 30-fold with GC content [Affymetrix2018]. In the hydrogel assay, little or no dependence of signal intensity on GC content is found.

A key concept in hybridization is the melt curve. At low temperatures, a probe and its target will remain firmly bound. At high temperatures, the target will be released into solution. At the melting temperature ($T_m$) half of the target is bound and half is in solution. 0 shows schematic hybridization efficiency curves as a function of temperature
for three probe-target duplexes with different melting temperatures. The fraction of bound target decreases when the assay temperature exceeds the melting temperature. Three curves are shown, corresponding to different possibilities for the melting temperature relative to plate temperature. A probe with $T_m$ significantly above the assay temperature (“high $T_m$ probe”) will have a signal that is consistent from well to well. A probe with $T_m$ close to the assay temperature (“low $T_m$ probe”) will have significant variation in signal between replicate wells depending on the temperature of the individual well. A probe that binds to targets besides its intended target with a $T_m$ that is close to or above the assay temperature will be non-specific, as it will bind to both intended and unintended targets. Like a low $T_m$ probe, it will have variable signals between replicates.

![Theoretical melt curves for probe-target duplexes](image)

Non-specific binding is of potential concern in microRNA assays because many microRNAs belong to families, with close sequence homology between family members. If a microRNA of diagnostic interest is present at low abundance, and another family

---

10
member is present in high abundance, the signal of the low-abundance target might be
obscured by non-specific binding of the high abundance target to the probe for the low-
abundance target. For brevity, such non-specific binding will often be referred to as
cross-talk, and the non-specific binding between a probe and an off-target relative to the
probe to its own target is the cross-talk coefficient. A cross-talk coefficient under 10% would be considered good, while 1% would be excellent. An ideal probe would have $T_m$
for its intended target substantially above the plate temperature and $T_m$ for all other
targets substantially below the plate temperature.

Hybridization thermodynamics

The thermodynamics of binding are frequently modeled in the context of the
nearest-neighbor (NN) model. The nearest neighbor model posits that the binding
enthalpy of two DNA strands can be calculated by adding the binding enthalpies of the
neighboring pairs in the strand, and similarly for the entropy of binding
[SantaLucia2004]. The consideration of neighbor pairs allows the model to include not
just the Watson-Crick binding energy between base pairs, but also the base stacking
energy in the double helix. Figure 3 shows the model schematically. The total enthalpy
and entropy is the sum of the neighbor enthalpies and entropies, with an extra
contribution from initiation and a potential symmetry correction if the sequence is
palindromic. The Gibbs free energy at any temperature is then calculated as $\Delta G = \Delta H - T \Delta S$, where $\Delta G$ is the free energy for binding (negative for a forward reaction), $\Delta H$
is the change in enthalpy due to binding (negative) and $\Delta S$ is the change in entropy for binding, also negative because entropy is reduced by binding.

$$\begin{array}{c}
\text{5'} & \text{C} & \text{G} & \text{T} & \text{T} & \text{G} & \text{A} & \text{3'} \\
\text{3'} & \text{G} & \text{C} & \text{A} & \text{A} & \text{C} & \text{T} & \text{5'}
\end{array}$$

Figure 3. Nearest-neighbor model of DNA duplex binding energy

The individual enthalpy and entropy values for NN pairs have been estimated by SantaLucia and colleagues [SantaLucia2004] in an extensive series of experiments spanning a decade, by measuring the thermodynamics of numerous oligomer combinations. The measurements are made using microcalorimetry or absorbance melting curves [SantaLucia1997]. Values for Watson-Crick pairs are listed in Table 1, under 1M NaCl conditions. CG and GC dinucleotides are found to be the most stable, while GG and CC dinucleotides are only marginally more energetically favorable than pairs containing T or A. Notably, all pairs are predicted to have similar entropy values.
Table 1. Nearest neighbor binding enthalpies and entropies

<table>
<thead>
<tr>
<th>Propagation Sequence</th>
<th>( \Delta H^\circ ) (kcal/mole)</th>
<th>( \Delta S^\circ ) (cal/mole/K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA/TT</td>
<td>-7.6</td>
<td>-21.3</td>
</tr>
<tr>
<td>AT/TA</td>
<td>-7.2</td>
<td>-20.4</td>
</tr>
<tr>
<td>TA/AT</td>
<td>-7.2</td>
<td>-21.3</td>
</tr>
<tr>
<td>CA/GT</td>
<td>-8.5</td>
<td>-22.7</td>
</tr>
<tr>
<td>GT/CA</td>
<td>-8.4</td>
<td>-22.4</td>
</tr>
<tr>
<td>CT/GA</td>
<td>-7.8</td>
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<td>-22.2</td>
</tr>
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<td>CG/GC</td>
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<td>-27.2</td>
</tr>
<tr>
<td>GC/CG</td>
<td>-9.8</td>
<td>-24.4</td>
</tr>
<tr>
<td>GG/CC</td>
<td>-8.0</td>
<td>-19.9</td>
</tr>
</tbody>
</table>

\( \Delta H^\circ \) and \( \Delta S^\circ \) values for the nearest neighbor binding are presented in the table. The values are calculated as a function of cation concentration and strand concentration. The symmetry correction is also included. The table shows that the enthalpy changes are relatively small, with values ranging from -7.2 to -10.6 kcal/mole, while the entropy changes are more significant, ranging from -21.3 to -27.2 cal/mole/K.

Corrections factors for the energetics as a function of cation concentration and strand concentration have also been calculated [SantaLucia2004].

Signal Corrections for Multiplex Assays

In a multiplex assay, variability between targets within a sample is controlled because the different targets in a single sample are all detected simultaneously in the same volume, that is, at the same time and in the same place. Imagine the alternative of running multiple single-plex assays sequentially, where the possibilities arise of equipment drift or malfunction, changing reagent batches, or temperature and humidity variations over the course of the experiment. Variability between one target and another could easily creep in, unlike a multiplex assay. Some residual bias may remain, for instance due to differential binding of GC-rich oligonucleotides. Such biases are often difficult if not impossible to eradicate, and tend to be characteristic of the method.
employed, e.g. sequencing vs hybridization, and can lead to imperfect agreement between different methods applied to the same sample [Mestdagh2014]. However, if the scientist focuses on differential expression, comparing each target between a case sample and a control sample, then the fact that target A is systematically under-reported relative to target B becomes irrelevant, as the comparison is always between target A in one sample vs target A in another sample, and similarly for target B. The key is that such biases are systematic and reproducible, rendering them relatively harmless [Affymetrix2018].

Unlike the relatively happy situation for targets, many other sources of variability arise between samples. The case samples and control samples may have been gathered by different personnel on different dates, using different procedures, stored under different conditions, and in rare cases possibly digested or otherwise treated using different protocols. Even if experimental technique is perfect and all samples are collected and prepared identically, further sample to sample variability can arise during the assay due to, for instance, temperature variation across the plate, variations in enzymatic efficiency from well to well, stochastic variation in PCR amplification, manual pipetting variability, and other technical factors, both general and assay-specific.

In the face of such technical variations, extracting the biological variation can be a challenge, and some serious mistakes can ensue. As an example, a now-notorious 2002 study published in the Lancet [Petricoin2002] detected a sensitive and specific diagnostic of ovarian cancer using mass spectrometry of the entire proteome in the samples. The NIH launched a proteomics initiative in the wake of the discovery, and several companies were founded to bring the discovery to market. Ultimately the paper had to be
withdrawn when it was found that the case and control samples had been run separately, and the instrument calibration had changed between the runs [Crowley2012].

Normalization can help mitigate such sample to sample variations. Several authors have identified normalization as a key step for microRNA assays [Etheridge2010, Creemers2012, Meyer2012].

Mean Adjustment – Naïve approaches

To control for sample to sample variability, some form of normalization between samples is required. The most common forms of normalization seek out samples where the overall expression of genes in a sample is elevated or depressed, respectively. Each sample is then raised or lowered to bring it in line with the others. Another perspective on the same process is that each target (protein, mRNA or microRNA depending on the assay) is expressed relative to a reference target or set of targets. After such adjustment, if a target is higher than the reference target in a case sample, and less than the reference target in a control sample, it does not matter that the reference itself has changed between case and control, since we are only looking at the ratio of the target of interest relative to the reference.

While the concept is straightforward, implementation is more challenging than it might appear. A few thought experiments serve to illustrate the problems that can arise. Suppose for instance that gene A, B, C, … are expressed at the same level in “case” and “control” samples, but that in the “case” samples another set of genes α, β, γ, δ, ε are also expressed, for instance, growth factors in a cancer cell. If one equalized the total amount of mRNA transcripts (or proteins, or microRNAs) between samples, then the genes A, B, C, … are each a smaller fraction of the total in the “case” samples, and one would draw
the erroneous conclusion that genes A, B, C were under-expressed in case relative to control. Alternatively, one might consider equalizing the average or geometric mean of all the probes between samples. In the scenario just described, the genes $\alpha$, $\beta$, $\gamma$, $\delta$, $\varepsilon$ might all be expressed at a low level, and would pull down the average of the “case” samples, leading one to believe that the “case” samples needed to be adjusted upwards, leading to the erroneous conclusion that the targets A, B, C, … were overexpressed in “case” vs “control”.

Even setting aside the possibility of many extra genes being turned on in “case” samples, it might well be that several of the targets A, B, C, … are overexpressed, for instance if the “case” samples are cancer cells growing out of control. Normalizing out the overall higher expression in “case” samples would mask the very message the assay is sending to the scientist.

Mean adjustment – reference targets

In the case of mRNA profiling, a common approach is to assume a set of “housekeeping” genes that are expressed at approximately the same level in all the samples [Eisenberg2003]. For instance, it might be reasonable to assume that the genes supporting the cellular cytoskeleton are unchanged in mouse liver before treatment and at 4 hours, 24 hours and a week after treatment. If the assumption is valid, then any other gene can be expressed relative to the average of the housekeeping genes.

In many instances, however, the assumption that some particular set of targets is unchanged by the condition, or by the treatment, takes a leap of faith, and may not be justified. To put it another way, it is frequently unclear what set of targets is unaffected by condition / treatment. The assumption of a conserved set of targets is particularly
challenging for microRNA assays, where for many sample types no “housekeeping” microRNAs are known, or even their existence established.

To avoid the obvious pitfalls described above, some ingenious and subtle normalization approaches have been developed over the last two decades. A well-known method called trimmed mean of M values (TMM) is included in the DeSeq package of Bioconductor [Anders2010]. It works from the assumption that the condition or the treatment changes only a few of the targets, so most targets should be constitutively expressed. If one either chooses a reference sample (say, the controls) or synthesizes a reference sample by averaging all the samples, then one can look at the ratio of gene A in each sample relative to gene A in the reference, and the corresponding ratios for gene B, C, D... If a sample is similar to reference then some of the ratios will be above 1 and some ratios will be below 1, but the average of all ratios will come out to 1. If all the ratios average to a number above 1, then the sample needs to be de-rated by the average ratio; conversely if the ratio is below 1, the sample needs to be boosted to bring it in line with the other samples. The method is very reasonable as long as there is a large set of unperturbed targets, so that the presence of a few differentially expressed targets between case and control do not appreciably change the average. The method is most suitable for techniques with blanket coverage, such as microarray data or sequencing data. In a modest multiplex assay with several dozen measured targets, there may not be enough unperturbed targets to justify the assumption that only a small subset of targets has changed.

The geNorm method developed by Vandesompele [Vandesompele2002] et al also assumes the existence of a set of conserved targets but makes no assumptions as to what
they are. Instead, it takes advantage of the observation that if the targets are indeed conserved, then they will all be high in some samples and all be low in other samples, that is, they will all have a similar pattern of expression from sample to sample.

Meanwhile targets that are genuinely different between various cases and control samples will not fit the common pattern. Therefore, the idea is to look for a common pattern of expression and the targets that follow it. Mathematically, the method measures the distance between one target and another, say target A and target B, by collecting the ratios log (A/B) in every sample and taking the standard deviation of the collection. If A and B rise and fall in lock-step with every sample, then even if targets A and B are expressed at very different levels, the ratio will be the same in every sample, so the standard deviation of the ratios is zero, that is the “distance” between target A and B is zero, as would be the case for constitutively expressed genes. The distance of every target to every other target is measured, and for each target its average distance from other targets is calculated. Targets with low average distance are collected and considered to be the constitutively expressed targets. One might say that the targets with the most friends win. The method is very popular; an Excel implementation has been downloaded over 15,000 times from the source [Vandesompele2002]. TMM and geNorm are among the most widely used mean-adjusting normalization methods.

Distribution adjustment

A different class of methods assumes that the distribution of targets between samples should be the same, apart from a few differentially expressed targets. The distribution of target expression levels in a single sample will often have a simple form, such as a log-normal distribution, where the expression values have a bell-curved
expression around an average, or perhaps a Poisson distribution, or a negative binomial
distribution. Irrespective of the particular distribution chosen, as long as it is a
reasonable match to the data in the control samples, the case samples can then be
constrained to have the same distribution. A particularly elegant implementation of the
method is quantile normalization [Amaratunga2001], which makes no assumptions about
the actual distribution in the reference. Instead, it directly forces a match between the
quantiles of each sample to the quantiles of the reference sample. The reference sample
can be an average of the controls, or an average of all the samples. Unlike the mean-
adjusting methods described above, it has the possibility of changing different targets in
the same sample by different amounts. For instance, if a control sample has the same
mean as the reference samples, but its highly expressed targets are on average more
highly expressed than the reference and its low expressed targets are on average more
under-expressed than reference, then quantile normalization would bring the high targets
down and the low targets up, in the same sample. Quantile normalization is most
appropriate for assays with many more probes than samples, such as sequencing or
microarrays. In the final step of quantile normalization, one of $N_P$ probe values is
assigned to each probe in each sample, where $N_P$ is the number of probes. As long as the
number of probes is much larger than the number of samples, there is minimal constraint
on the value of the probes. For instance, $N_P = 100,000$ and $N_S = 20$ might be typical of a
microarray assay; each of the twenty samples has one of 100,000 possible probe values.
In contrast, in a bead-based or particle-based assay, the panel might have 20 probes and
the plate might have 96 or 384 wells. There are only 20 distinct probe values to be
distributed among the more numerous samples, causing significant discretization error.
Limitation of existing methods

All the above methods just described work directly with the numerical values of the targets, without no reference to the biology of the samples or the chemistry of the assay. While mathematically convenient, such approaches fail to take advantage of other information known to the scientist. For instance, in a hybridization assay where the temperature is variable across a plate, the targets with low melting temperature ($T_m$) may remain partly in solution at the assay temperature, while high $T_m$ targets are firmly bound to their probes. The measured concentration of low $T_m$ probes is likely to be more variable than that of high $T_m$ probes in that situation. The goal of this work is to incorporate such biological information to improve assay performance.

Work outline

The first part of the work quantifies the variability of a particle-based hybridization assay. Several hypotheses as to the cause of variability are examined and experiments carried out in order to verify or refute the hypotheses. Well-to-well temperature variation is shown to be the proximal source of variability. A deeper inspection shows that in many cases high variability results from non-specific binding between probes; the probe for one target can bind a different target, with an efficiency that varies across the plate due to temperature variations. As a result, the binding between target and off-target probe is highly variable.

New probes are designed, with deliberate mismatches to reduce probe binding to off-targets, without compromising on-target performance. Variability is further reduced by developing a numerical method to compensate well-to-well temperature variation.
Chapter II
Materials and Methods

The research in this thesis comprised a combination of experiments and calculations, including 1) hybridization assays, 2) probe-target melting temperature experiments, 3) melting temperature calculations, 4) data visualization and analysis. The assay used is commercially available and is described first. A technique to measure duplex melting temperatures is described next, followed by numerical methods to estimate duplex melting temperatures. Finally the software used to analyze the data is described.

Hybridization assay using FirePlex particles

Abcam PLC offers the FirePlex multiplex microRNA assay [Abcam2018] to measure up to 65 distinct microRNAs per well on a 96-well titer plate. The full assay is described first, then an abbreviated assay used in the experiments here is described.

Full assay

The workflow of the full assay is illustrated schematically in Figure 4. Sample input can be either purified RNA or crude biofluids. Samples are combined on a 96-well titer plate with hydrogel particles. The hydrogel particles bear single-stranded DNA probes at their center, each with a sequence complementary to a desired target. The probe strand also has flanking regions to support subsequent amplification. The particles
are fluorescently barcoded at their ends to identify the probe. Up to 65 microRNAs can be measured in a single well and distinguished by their barcodes.

Figure 4. Schematic of workflow for FirePlex microRNA assay.

The particles are hybridized in hybridization buffer with the samples on a 96-well filter plate for 60 minutes in a shaker heated to 37 °C, to allow target microRNAs to bind to their probes. The hybridization buffer has high cation content to stabilize probe-target binding and contains an intercalating agent to reduce probe-target melting temperature and improve specificity. The plate is rinsed to remove unbound materials, leaving only bound targets on the probes. A labeling mix containing universal adaptors and ligation enzymes is mixed with the particles and incubated on a shaker at room temperature for 60 minutes, resulting in the ligation of adaptors on either side of the target miRNA to
generate a fusion DNA-RNA-DNA molecule. The particles are rinsed again to remove excess un-ligated adapters. The plate is heated at 55 °C for 30 minutes to release the fusion products, which are eluted into a catch plate. The catch plate now contains only the targets that were bound by the assay. Polymerase chain reaction (PCR) program is run on the catch plate for 60 minutes, with universal primers specific to the ligated ends of the fusion products. The reverse primers are pre-conjugated to biotin. The PCR is asymmetric, with an excess of forward primers, resulting in predominantly single stranded biotinylated cDNA.

The mixture containing the amplified products (amplicon) is then transferred back to the filter plate by pipette, and re-hybridized to the particles on a heated shaker for 30 minutes at 37 °C, using the same hybridization buffer as before. The plate is rinsed, and a fluorescent reporter molecule conjugated to streptavidin is incubated with the samples for 15 minutes at room temperature. The reporter binds to the biotin on the reverse primers. Excess reporter is removed in a final rinse, and the plate is read out either on a cytometer or a high content imager. The sensitivity of the assay is driven by the PCR amplification, while specificity is driven by the double capture step, once from the original biological sample and once from the PCR amplicon. Note that the assay is doubly specific against targets that are not in the panel, but singly specific between targets that are in the panel. That is, for a target not in the panel to register a signal, it would have to bind non-specifically before PCR, and then it would have to bind non-specifically again at the re-capture step. If the non-specific binding were 25% at either step, the non-specific binding for that target through the entire assay is 6.25%. For a target that is in the panel, it will be captured on its own probe before PCR, and then might
bind non-specifically to a different probe post-PCR, so specificity for such a target is only as good as specificity in the re-capture step.

Abbreviated assay

In the work here, the focus is on variability and cross-talk introduced during hybridization. To that end, an abbreviated assay was carried out, including only the final hybridization, rinsing and reporting steps from the full assay. Sample input concentrations were titrated to be consistent with concentrations after PCR amplification of biological samples, of the order of 10-40 pM. Particles were read out on a Millipore Guava cytometer using a blue laser to excite a phycoerythrin-cyanide (PE-Cy5) reporter molecule conjugated via streptavidin to the biotinylated ends of the amplicon re-captured on the particles. Excitation is at 488 nm and the reporter has maximum fluorescence at 667 nm, while the particle barcodes have maximum fluorescence at 578 nm. The spatial separation of probe and barcode regions as well as the wavelength separation between reporter and barcode or excitation means that background fluorescence is minimal and the assay has a very high dynamic range, at least 10,000 : 1.

Probe and Target preparation

Probes are DNA strands covalently conjugated to FirePlex particles. The strands have a region complementary to their intended target. They also have flanking regions to support conjugation to the particle as well as to match the PCR flanking regions of the targets.
Targets are DNA oligomers in solution. Two kinds of targets were used, synthetic targets and biological targets. Synthetic targets were synthesized \textit{de novo} chemically. Biological targets originated as cellular RNA, followed by polymerase chain reaction which converted them to ssDNA strands.

The next two subsections detail the preparation of probes and targets.

Probes

Custom probe sequences were designed either to contain a perfect match to their intended target ("matched probes") or with deliberate substitutions, insertions or deletions relative to their intended target ("mismatch probes"). Custom probe sequences were synthesized by IDT Corporation in 25 nmole lyophilized tubes. All probes fabricated had flanking sequences included, complementary to the PCR primer flanking sequences on the PCR amplicon, with 5’ phosphorylation to facilitate attachment to hydrogel particles. The probes were first diluted to 0.1 nmole / µL (100 µM) in nuclease-free water. Aliquots of 20 µL were added to 980 µL of a 3:2 ratio of nuclease-free water : PCR buffer to form 1:50 dilutions (2 µM concentration). A second 1:50 dilution in the same water / buffer mix brought the concentration to 40 nM, in the same range as amplified microRNA concentrations after PCR. Standard dilutions are listed for later reference in Table 2. In some cases, a further dilution to 10 nM was carried out (4,000,000 copy number / µL).

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration (moles/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock solution</td>
<td>100 µM</td>
</tr>
<tr>
<td>1:50</td>
<td>2 µM</td>
</tr>
</tbody>
</table>

Table 2. Standard probe dilutions used in this work
Custom particle panels containing mixtures of probes of interest were ordered from Abcam PLC, courtesy of the FirePlex manufacturing group. One panel, called the Benchmark panel, is listed in Table 3. There are five matched probes to members of the hsa-miR-17-5p family. There are also five mismatch probes, each differing from the corresponding matched probe by the deletion of the 8th base from the 5’ end of the reverse complement microRNA sequence. Three matched probes to members of the hsa-let-7a-5p family are also included, along with a positive control and negative control.

<table>
<thead>
<tr>
<th>hsa-miR-20b-5p</th>
<th>hsa-miR-17-5p</th>
<th>hsa-miR-106b-5p</th>
<th>hsa-miR-18a-5p</th>
<th>hsa-miR-93-5p</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmm-20b-5p</td>
<td>mmm-17-5p</td>
<td>mmm-106b-5p</td>
<td>mmm-18a-5p</td>
<td>mmm-93-5p</td>
</tr>
<tr>
<td>hsa-let7a-5p</td>
<td>hsa-let7d-5p</td>
<td>hsa-let7g-5p</td>
<td>x-control</td>
<td>blank</td>
</tr>
</tbody>
</table>

A second panel, the extended mismatch panel, is listed in Table 4. The sequences for these probes will be described in the Results section.
Table 4. Extended mismatch panel, a custom microRNA panel

<table>
<thead>
<tr>
<th>match17</th>
<th>g3pg</th>
<th>g3pc</th>
<th>gap1m17</th>
<th>match93</th>
<th>gap1m93</th>
<th>g3pc93</th>
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<tbody>
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<td>match20</td>
<td>gap1m20</td>
<td>xc1m20</td>
<td>g3pc20</td>
<td>match15</td>
<td>min4-15b</td>
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<td>gc1-15b</td>
<td>gc2-15b</td>
<td>gc3-15b</td>
<td>match16</td>
<td>gc1-16</td>
<td>gc2-16</td>
<td>match195</td>
</tr>
<tr>
<td>gx2-195</td>
<td>gx1-195</td>
<td>match29a</td>
<td>gt2p-29a</td>
<td>gx3-29a</td>
<td>gt3p-29a</td>
<td>match29b</td>
</tr>
<tr>
<td>gt2p-29b</td>
<td>gx3-29b</td>
<td>gt3p-29b</td>
<td>hsa-miR-let-7a-5p</td>
<td>hsa-miR-let-7d-5p</td>
<td>hsa-miR-let-7g-5p</td>
<td>x-control</td>
</tr>
<tr>
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</tbody>
</table>

A pre-constructed panel was also used, FirePlex V2 Cardiology panel. The cardiology panel has the microRNAs listed in Table 5. In addition to a range of microRNAs relevant to human cardiology, the panel contains a positive control, a negative control, and three off-species probes for background estimation. All probes are conventional matched probes, containing the full reverse complement of their intended target. All the target microRNAs listed are the human homologs, denoted by the hsa-prefix. The prefix is usually dropped in the text for brevity.
Table 5. FirePlex V2 Cardiology panel

<table>
<thead>
<tr>
<th>Target microRNAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-let-7d-5p</td>
</tr>
<tr>
<td>hsa-let-7e-5p</td>
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<td>hsa-let-7g-5p</td>
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<td>hsa-let-7i-5p</td>
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<td>hsa-miR-25-3p</td>
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<tr>
<td>blank</td>
</tr>
</tbody>
</table>

Target microRNAs

Two primary target types were used, synthetic target sequences manufactured chemically, and selectively purified animal RNA.

Synthetic targets: Synthetic microRNA sequences were purchased from IDT Corporate in 25 nmole lyophilized tubes, as single-stranded DNA. All microRNA sequences were fabricated with the PCR primer flanking sequences pre-attached, with biotinylated ends to bind reporter conjugated to streptavidin. Similar to probe sequences, the targets were first diluted to 0.1 nmole / µL in nuclease-free water (100 uM). Aliquots of 20 µL were added to 980 µL of a 3:2 ratio of water / PCR buffer to form 1:50 dilutions (2 µM concentration). A second 1:50 dilution in the same mix brought the concentration to 40 nM.
Cardiology amplicon: A biological amplicon was prepared by combining 5ng each of purified total RNA from brain, lung and liver tissue. The mixture was hybridized with particles from the FirePlex V2 Cardiology panel, following the first steps in the full assay. The post-PCR product was pooled from all the wells, resulting in a tissue amplicon enriched for cardiology-relevant microRNA targets. This sample material will be referred to as the cardiology amplicon.

Probe-target melting temperature experiments

Hybridization and melting of oligomer pairs can be measured optically using a SYBR dye assay. A SYBR assay introduces an intercalating dye in solution with DNA oligomer pairs. The SYBR molecule has high affinity double-stranded DNA (dsDNA), and low affinity to ssDNA, and its fluorescence is greatly enhanced when bound to dsDNA. The bound molecule fluoresces with a maximum excitation at 497 nm, and maximum emission at 520 nm. By monitoring the fluorescence as a function of temperature, the melting temperature ($T_m$) of a probe-target pair can be measured. The assay is shown schematically in Figure 5.

Figure 5. Schematic of SYBR assay.
The melting temperature of probe-target combinations was measured using an Applied Biosystems OneStepPlus Real-Time PCR system. Applied Biosystems SYBR Green Master Mix was combined with water, amplicon and probe in wells of a PCR plate. For each pair of wells, aliquots of 10 µL water, 10 µL probe at 2 nM concentration, 10 µL amplicon at 2 nM concentration, and 10 µL SYBR mix were vortexed in a 0.5 ml tube, for a total volume of 40 µL. Aliquots of 20 µL were then pipetted into each of two replicate wells on the PCR plate. The OneStepPlus program for melting curves was executed, using a temperature range of 45 °C to 85 °C. The manufacturer’s software carried out melt curve analysis to determine melting temperature.

Probe-target melting temperature simulation

Melting temperatures for hybridization between probes and targets were computed using the UNAFOLD web server of SUNY Albany. The DINAMELT two-state melting (hybridization) program was used.

Many $T_m$ estimators exist, but are often intended for calculating the melting temperature of PCR primers with perfect Watson-Crick pairing to their targets, and have tacit limitations such as calculating only the hybridization between targets and probes that are perfectly matched, or allowing only a single isolated substitution, or rejecting any duplexes with gaps or bulges, or rejecting duplexes with end mismatches. For the purpose of designing mismatch probes and calculating non-specific binding between probes and unintentional targets which might have gaps, bulges and multiple adjacent substitutions, a more general tool was needed.
The UNAFOLD family of tools, developed by Zucker and colleagues, is the most advanced of its kind, and considers all possible base pairings between a strand to itself (folding) or between two different strands (hybridization). The key innovation in the suite is a computationally practical way to explore the combinatorial explosion of all possible base pairings.

The tools compute the energetics of different folding configurations based on the nearest neighbor model (NN model) and find the configuration with minimum free energy. The available controls are shown in Table 6.
Table 6. Inputs and outputs of DINAMELT $T_m$ simulator

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st sequence</td>
<td>DNA or RNA base sequence, listed 5’ to 3’</td>
</tr>
<tr>
<td>2nd sequence</td>
<td>DNA or RNA base sequence, listed 5’ to 3’</td>
</tr>
<tr>
<td>Energy rules</td>
<td>Choice between DNA/DNA or RNA/RNA</td>
</tr>
<tr>
<td>Temperature</td>
<td>°C</td>
</tr>
<tr>
<td>[Na$^+$] concentration</td>
<td>M</td>
</tr>
<tr>
<td>[Mg$^+$] concentration</td>
<td>M</td>
</tr>
<tr>
<td>Strand concentration</td>
<td>M</td>
</tr>
<tr>
<td>Gibbs free energy $\Delta G$ for binding</td>
<td>Kcal/mole</td>
</tr>
<tr>
<td>Enthalpy $\Delta H$ for binding</td>
<td>Kcal/mole</td>
</tr>
<tr>
<td>Entropy $\Delta S$ for binding</td>
<td>Cal/mole/K</td>
</tr>
<tr>
<td>Melting temperature $T_m$</td>
<td>°C</td>
</tr>
</tbody>
</table>

Sodium concentration was set to 50 mM and Mg concentration to 2 mM, except where otherwise noted. Simulations were performed for duplexes including the flanking sequences in all cases.
Data visualization

Raw cytometer data was decoded by FirePlex Analysis Workbench, a desktop software application from Abcam PLC distributed freely with the assay. The program interprets the green, yellow and red fluorescence from analyte passing through the cytometer flow cell, and identifies particles, decodes their barcodes, and measures the probe intensities. The data is visualized as bar charts and heatmaps. A Python-language scripting and charting facility built into the program was used for analysis beyond the primary charts presented by the program.
This chapter will begin by illustrating the variability of a hybridization assay. The causes of the variability will be explored. The discussion then moves to a focus on the importance of temperature and non-specific binding (“cross-talk”) between targets. Methods to re-design probes to minimize cross-talk are then described. Finally, a probe-specific normalization method to compensate for temperature variation is developed and the resulting reductions in probe variability are demonstrated.

Probe Variability

The variability in the raw signal of a hybridization assay was illustrated by the following experiment. Particles from the 70-member cardiology panel described under Materials and Methods were hybridized with identical aliquots of the cardiology amplicon described in Materials and Methods, in 24 replicate wells on a plate. The abbreviated assay and readout were performed as described in Materials and Methods, at a nominal hybridization temperature of 38.5 °C. The nominal hybridization temperature is slightly higher than the nominal assay used in the assay because this work used a non-production shaker which ran cold relative to its specification. The wells were distributed in a checkerboard pattern to cover the plate area, as shown in Figure 6.
With the same quantity of the same sample in each well, one might expect to obtain the same signal in every well. In reality, the assay was quite variable from well to well. An example is shown in Figure 7. Each bar represents the average signal level for one probe (hsa-miR-195-5p) in a well, while the dots show individual particle values. The error bar represents the 95% confidence interval for the mean signal in that well, based on the distribution of particle values. Reproducibility is excellent for a target within a well, with all the particles for an analyte being tightly clustered around the mean for that analyte in that well. From well to well, however, the signal is quite variable for this probe, ranging almost 20-fold from a minimum of 455 median fluorescent units (MFI) to a maximum of 8050 MFI. The coefficient of variation (CoV = mean / standard deviation) is 67%.

Figure 6. Layout of wells for hybridization experiment
Variation of signal for target hsa-miR-328-3p across wells of a plate.

Figure 7. Variation of signal for target hsa-miR-195-5p across wells of a plate.

Not all probes are equally variable, as shown for probe hsa-miR-328-3p in Figure 8. The signal is much more consistent well to well, with a CoV of 19%. Intriguingly, although the variation is much less for this probe, the pattern of variation is very similar to that in Figure 7, with wells B11 and H07 being the highest, and wells C01, E01 and G01 being particularly low. High variability probes will sometimes be referred to as “noisy” probes, for brevity.

Figure 8. Variation of signal for target hsa-miR-328-3p across wells of a plate.
Spatial correlation of variation within plate

To highlight the similarity in variation, the signal of a noisy probe is correlated with the signal for a quiet probe, shown in a scatterplot in Figure 9. Each dot in the scatterplot represents one well, with the abscissa being the signal of the noisy probe and the ordinate being the signal of the quite probe. Although the magnitude of the variation is considerably smaller in probe miR-21-5p than probe miR-195-5p, there is a strong correlation between the probes, with the high signal wells in one probe also being the high signal wells in the other. The Pearson correlation coefficient $R^2$ is 87.7%.

![Scatterplot of hsa-miR-21-5p signal vs hsa-miR-195-5p signal](image)

**Figure 9.** Scatterplot of hsa-miR-21-5p signal vs hsa-miR-195-5p signal

To further bring out the consistency of variability, five unrelated probes are compared (Figure 10). Each group of bars represents one probe, each bar inside a group is one well. In certain wells (e.g. D03), all probes are high, in other wells all probes are low.
Correlated variation of five unrelated probes across plate.

Run to run consistency

Variability is not only consistent between probes on a plate, but also from run to run. Two plates were run at 38.5 °C a week apart, both using particles from the same cardiology panel. The variability of each probe that was expressed above background in the two runs is compared in Figure 11. Probes that had high variability one week also had high variability the next, and the same is true of the low variability probes. The same data is shown as a scatter plot in Figure 12. The Pearson correlation coefficient between runs is \( R^2 = 87\% \).

The consistency of the variation pattern in each probe has an un-escapable implication; the variation seen is by no means random but results from some systematic
and reproducible difference between wells on a plate that affects all the probes in a well, albeit to different degrees.

Figure 11. CoV of all probes, two runs at 38.5 °C compared

Figure 12. Scatterplot showing CoV correlation between runs at 38.5 °C
The spatial pattern of variation, shown above to be consistent within a run, is not repeated run to run. The signal for probe hsa-miR-195-5p is shown as a function of position on the same two plates as used in Figure 13. The pattern of variability suggests that it is a feature of the probe and not of the sample or of spatial position.

Figure 13. Spatial comparison of signals from two plates hybridized at 38.5 °C
Sources of variation

To explore the causes of the variability, several experiments were carried out. One potential source of variability is the manual process of pipetting. Assay volumes were varied in order to explore whether accidental pipetting variation could give rise to the variability seen. Another potential source of variability is non-uniform temperature on a plate. To investigate this possibility the assay temperature was varied.

Pipetting variation

One hypothesis as to the source of assay variation was that differences in pipetting between one well to another could account for the variation. The ratio of hybridization buffer volume to sample volume is significant to the assay because the buffer contains an intercalating agent which reduces binding between target and probe. A higher ratio of buffer relative to sample will result in reduced binding, while a lower ratio of buffer will result in enhanced binding. An experiment was carried out where the ratio of hybridization buffer to sample volume was purposefully varied between wells on a plate. The ratio was varied both by changing the volume of sample added, or by changing the volume of hybridization buffer. The assay normally uses a ratio of 60 µL of buffer to 20 µL of sample. In the experiment, the sample volume was varied from 16 µL to 24 µL and the hybridization volume was varied between 40 µL and 80 µL. Four wells of each condition were measured, and the impact on two different probes is shown in Figure 14, a quiet probe in Figure 14 (a) and a noisy probe in Figure 14 (b). Each bar represents the average of four wells for that probe. The dots denote individual well
values (which are in turn the average of all the particles in that well). The left side of each graph shows the effect of varying sample volume, while the right side shows the effect of varying hybridization buffer volume.

![Graphs showing variation of signal with volume of sample or hybridization buffer](image)

(a) Probe hsa-miR-15b-5p  
(b) Probe hsa-let-7a-5p

Figure 14. Variation of signal with volume of sample or hybridization buffer.

Changes in sample volume from 16 to 24 µL changed even the noisy probe by less than a factor of three (Figure 14(b)), which is not consistent with the 20-fold variation previously found across a plate. Reducing the hybridization buffer volume to 40 µL gave at most a 50% increase in signal. Increasing the buffer volume to 70 µL caused only a small decrease in signal. A buffer volume of 80 µL did reduce signal significantly. While manual pipetting is subject to human variability, excursions of more
than a few percent seem unlikely, and an accidental 33% increase in buffer volume from 60 µL to 80 µL seems quite implausible. As a result, it was concluded that pipetting differences could not account for the large variations in signal observed for some probes.

Temperature variation

A second hypothesis as to the source of probe variability is temperature differences between different wells on the plate. To test this hypothesis, an experiment was performed where the hybridization was performed with the shaker heated to a nominal 40.5 °C instead of 38.5 °C. The cardiology panel with the cardiology amplicon was used. Virtually all probes had reduced signal to some degree (Figure 15). Strikingly, many of the probes with most reduced signal are also the probes with most variability in Figure 12.

![Figure 15. Ratio of probe signal for plate temperature of 40.5 °C and 38.5 °C](image-url)
The strong trend that the more consistent the signal between plate temperatures, the lower the variability between wells on the same plate, is consistent with the hypothesis that temperature variation between wells on a plate could give rise to the variation in signal between wells. Variability within a well is not subject to temperature effects, because any temperature gradients inside a well are eliminated by the prolonged shaking step, as evidenced by the tight clustering of particle values within a well.

Referring back to the theoretical melt curve of 0, the correlation of well to well variability with reduced signal at high temperature is easily explained. Raising the plate temperature, corresponding to a shift to the right in the figure, will depress the signal of the low $T_m$ probes, with little effect on the high $T_m$ probes. Thus, the correlation observed between signal decrease at higher temperature and higher variability is consistent with a temperature-dependent model of variation.

Non-specific binding ("cross-talk")

A close examination of the noisy probes in Figure 11 reveals a striking pattern. Noisy probes often appear in groups. One large group is the let-7 family, represented by let-7d-5p, let-7e-5p, let-7g-5p and let-7i-5p. These microRNAs have considerable sequence homology to one another. Other noisy probes are miR-29 a/b, and hsa-miR-26 a/b, which also have high sequence homology.

Further investigation reveals that other noisy probes with unrelated names also have sequence homology. In particular, the family miR-15b-5p, miR-16-5p and miR-195-5p have strong homology, shown in Figure 16.
Another family surrounds hsa-miR-17-5p, with strong correspondence between three members of the family (17, 20b and 93) and weaker correspondence with the other two members.

The finding of homology between noisy probes raises the possibility that the probe variability might be related to cross-talk between probes. Even without a perfect match between two homologous target microRNAs miR-X and miR-Y, the particle-bound probe for miR-X (which contains the reverse complement of X) might hybridize to the sequence of target miR-Y in solution, and vice-versa.

To investigate whether cross-talk was present, a hybridization experiment at 38.5 °C were performed using the same cardiology panel as before. However, in each well
only a single oligomer is introduced, or “spiked in”, rather than adding a complex biological sample. The layout of one such experiment is shown below. In each column of the plate, a different oligomer is spiked-in. The top row has sample at concentration 100 pM/µL, the second row 10 pM/µL, the third 1 pM/µL, and the fourth 0.1 pM/µL. The lower half of the plate contains replicates of the upper half. Different dilutions were used to ensure that non-specific binding did not occur simply due to a massive overload of the target.

![Layout of spike-in experiment to test for non-specific binding](image)

**Figure 18. Layout of spike-in experiment to test for non-specific binding**

Representative data is shown in Figure 19, for the spike-in of oligomer miR-20b-5p. Only one bar in the barchart is expected to be elevated, but three are seen. The particles intended to bind miR-20b-5p fluoresce with high intensity; however, particles intended for targets miR-17-5p and miR-93-5p also fluoresce at high intensity. The level of cross-talk is almost 100%; the signal for the off-target probes is almost as high as the signal for the on-target probe. The cross-talk occurs in spite of the double and triple
substitution mismatch between the target miR-20b-5p and probes miR-17-5p or miR-93-5p respectively [Figure 17], even for the most diluted level of miR-20b-5p.

Figure 19. Assay response to 0.1 pM / µL spike-in of amplicon hsa-miR-20b-5p.

A well with miR-93 spike-in at the lowest concentration is shown next. On a linear scale, little cross-talk is visible. The data is also shown on a logarithmic scale to better show the relatively low levels of fluorescence for probes miR-17 and miR-20b in the presence of target miR-93. The data show that there is reciprocal cross-talk from miR-93 to miR-20b. However, the cross-talk is not symmetrical, that is, the binding of target miR-93 cross-talk to probe miR-20b is not as strong as the binding of target miR-20b to probe miR-93. The asymmetry most likely results from the different lengths of the the 5’ and 3’ linkers attached to sequence when it is present in a probe compared to when it is present in an amplified target.
The overall pattern of cross-talk can be visualized by examining the clustered heatmap of the probes, with probes and samples clustered according to similar patterns of expression (Figure 21). In the image, wells are on the vertical axis, probes are on the horizontal axis. Signal intensity is shown as $\log_{10}$ of MFI. Samples with the same spike-in cluster together. Probes with similar response to a spike-in cluster together. The heatmap has been zoomed to the subset of probes which respond to the spike-ins. The

Figure 20. Crosstalk from hsa-miR-93-5p to hsa-miR-20b-5p
experimental clustering of probes is in concordance with the sequence homology identified above.

Figure 21. Clustered heatmap of cardiology probes from spike-in experiment

Combined temperature variation and non-specific binding

The variability of the probes at 38.5 °C is charted against the guanine / cytosine content of probe sequences in Figure 22. Probes had a substantial spread in GC content,
ranging from 7 bases to 15 bases (out of a total of 23 bases in a typical microRNA). The bar at each GC value is the average over all probes with that GC content, while the dots are individual probe values. Red dots are high-homology probes, defined as probes that have at least one other probe sharing 19 or more bases in common.

There is a strong trend that higher GC content leads to lower variability. The most variable probes are probes that not only have low GC content, but also have a closely homologous target. The melting temperature of a duplex correlates strongly with GC content, so the data provide further reason to suspect temperature variation combined with non-specific binding as a leading source of variability.

Figure 22. Probe variability correlated with GC content of probe sequence
Model of variability

The observation of high variability correlating with homology and GC content, combined with the observation of cross-talk, inspires the following model for probe variability in the assay. The probes in the assay generally have a melting temperature to their own targets which is above the assay temperature, leading to reproducible readings. However, some probes bind to cDNA from off-targets related by homology, with a melting temperature which is lower than on-target binding. For closely related targets, the $T_m$ of the probe to off-target combination is within a few degrees of the plate temperature, shifting the melt curve into a highly variable zone (Figure 23).

![Variability model](image)

Figure 23. Conceptual model for probe variability.
Non-specific binding is exacerbated when a sample contains closely related targets with different abundances. A probe with a low abundance target will also bind some fraction of the high-abundance near-homologous target. When temperature varies across the plate, the contribution of the near-homologous target can vary substantially, leading to variability in the total signal for the low-abundance probe, illustrated in Figure 24.

(a) Binding of high abundance off-target analyte and low abundance on-target analyte to probe. Shaded area is range of temperatures across plate. Circles correspond to three different wells at different temperatures.

(b) Measured signal for three replicate wells at different temperatures on same plate. The total signal in each well is the sum of on-target and off-target contributions.

Figure 24. Variability for samples containing closely homologous targets
Probes that have high GC content have a melting temperature so far above the hybridization temperature that even the off-target binding has $T_m$ above the assay target, explaining the low variability seen in Figure 22 for high GC-content probes.

Probes with improved specificity

The observation of significant cross-talk between probes motivated a search for more selective probes. The preceding discussion suggests that probes with lower cross-talk would bring two advantages. The assay would be both more specific, and it would also be more reproducible.

Probes in the cardiology panel are all perfect matches to their targets, that is, the probe sequence contains the reverse complement of the target microRNA, maximizing Watson-Crick pairing. It would be desirable to have probes that bind sufficiently well to the intended target but have less binding to unintended targets. Modified probes that are imperfect matches to their intended target might satisfy this need. Such probes are likely to have reduced $T_m$ to both their intended target as well as to homologous off-targets. If the unmodified $T_m$ of the matched probe is sufficiently far above the assay temperature, then there may be a window of opportunity where an imperfect ("mismatch") probe still has a $T_m$ above the assay temperature, but the $T_m$ to unintended targets is well below the assay temperature.

A variety of methods to design and test such probes was pursued. The acid test of any probe is how it performs in the particle-based assay, and a particle-based assay experiment measuring the hybridization of a matrix of probes to targets was first carried out to benchmark subsequent probe design techniques. Particle-bound probes are time consuming to build, however, so other techniques were also pursued. Simulation tools
were used to predict the melting temperature of oligomer duplexes. A SYBR dye assay, described in the Materials and Methods chapter, offers a direct measure of duplex $T_m$. The results of mismatch probe design and test are described in the following sections.

Hybridization matrix benchmark experiment

In the first experiment, the miR-17-5p family was selected for improvement. As shown in Figure 17, the sequences are homologous outside bases 10-13. For each matched probe, a corresponding mismatch probe was generated by removing the eighth base from the 5’ end of the reverse complement. The resulting particle panel is listed in Table 3.

A hybridization experiment was performed using this panel at a nominal assay temperature of 38.5 °C. The plate layout is shown below. The full matrix of how strongly each probe bound each target, either intentional or un-intentional, was measured by spiking-in a different oligomer into one column of the plate at a concentration of 50 fM/µL. The pattern repeated after four columns as replicates, to increase statistical power. Particles bearing matched and mismatch probes were used in alternating rows.

Figure 25. Plate layout for hybridization matrix benchmark experiment
Data for matched probes is shown for the samples that were spiked with oligomer miR-20b-5p in Figure 26. Each bar in a multi-bar is the average signal for all the particles of that probe in one well. The highest signal is for the particles with miR-20b-5p probe, as expected. However, particles with miR-17-5p and miR-93-5p have signals that are nearly as high, even though their targets are not present. The off-target signals are less consistent than the on-target signal. The inconsistency has the same pattern for each off-target probe. Similar results were obtained for wells spiked with miR-93-5p. Off-target probes miR-20b-5p and miR-17-5p both had similar intensity to the on-target probe miR-93-5p.

Figure 26. Hybridization of matched probes to target miR-20b-5p.
Data for mismatch probes is shown next for the samples that were spiked with oligomer miR-20b-5p. Cross-talk is still present; however, it is reduced relative to the matched probes shown in Figure 27.

![Graph showing hybridization of mismatch probes to target miR-20b-5p.](image)

Figure 27. Hybridization of mismatch probes to target miR-20b-5p.

Reciprocal cross-talk is seen when the oligomer miR-93-5p is spiked into the wells. Match and mismatch data for miR-93-5p are compared in Figure 28. The average cross-talk signal is very substantially improved in Figure 28(b). Note that there are still a few wells with high off-target signal (presumably cold wells). Such wells would be an important confounding factor when comparing biological samples. Without the temperature compensation techniques to be described later, there would be no way to tell
whether the difference arose from technical variation (well temperature) or biological variation.

(a) Matched probes  
(b) Mismatch probes

Figure 28. Match vs mismatch probe response to target miR-93-5p

The cross-talk was quantified for match and mismatch probes in the miR-17-5p family by taking the geometric mean of all the wells of each type. Matched and mismatch probe results are compared in Table 7 and Table 8, respectively. Cross-talk exceeding 25% is highlighted in red, while on-target binding is indicated in bold.

Table 7. Measured cross-hybridization for matched probes

<table>
<thead>
<tr>
<th>MATCH</th>
<th>miR-17-5p</th>
<th>MiR-20b-5p</th>
<th>MiR-93-5p</th>
<th>MiR-106-5p</th>
</tr>
</thead>
<tbody>
<tr>
<td>MiR-20b-5p</td>
<td>79%</td>
<td>100%</td>
<td>71%</td>
<td>1%</td>
</tr>
<tr>
<td>MiR-93-5p</td>
<td>69%</td>
<td>57%</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>MiR-106-5p</td>
<td>7%</td>
<td>1%</td>
<td>1%</td>
<td>100%</td>
</tr>
</tbody>
</table>
Table 8. Measured cross-hybridization for mismatch probes

<table>
<thead>
<tr>
<th>MISMATCH</th>
<th>miR-17-5p</th>
<th>MiR-20b-5p</th>
<th>MiR-93-5p</th>
<th>MiR-106-5p</th>
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<tbody>
<tr>
<td>MiR-20b-5p</td>
<td>10%</td>
<td>100%</td>
<td>10%</td>
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<tr>
<td>MiR-93-5p</td>
<td>15%</td>
<td>12%</td>
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<td>0%</td>
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<tr>
<td>MiR-106-5p</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
<td>100%</td>
</tr>
</tbody>
</table>

The results gathered in this first particle-based hybridization experiment were used to benchmark other methods of probe screening, simulation and SYBR assays.

Simulating probe-target melting temperatures with DINAMELT

The fastest and most cost-effective way to design probes would be if it were possible to simulate the performance of probes prior to fabricating and testing them. The DINAMELT web server described in Materials and Methods was used to predict melting temperatures for probe-target combinations.

A first step was to investigate the sensitivity of the simulator’s estimates to its input parameters. Sixteen microRNA – target combinations were chosen, comprised of all sixteen combinations of the four probes miR-17-5p, miR-20b-5p, miR-93-5p and miR-106b-5p, paired with the targets of the same probes. This choice coincides with the probes and targets used in the benchmark matrix hybridization study described above.

The results of the simulation are shown in Figure 29, as scatterplots of probe $T_m$ before and after a change. Changing the concentration of sodium from 0 to 50 mM resulted in essentially a rigid shift in $T_m$ of 17.5 °C with a standard deviation of 1.5 °C. Changing magnesium concentration from 0 to 2 mM (with sodium held at 50 mM) resulted in a rigid shift of all the probes of 8.0 °C ± 0.6 °C.
An important consequence of such rigid shifts in temperature with cation concentration is that $T_m$ differences between microRNAs remain unchanged with salt conditions. Conclusions about relative $T_m$ values under one salt condition remain valid under different salt conditions; conversely a relative $T_m$ error at one salt condition cannot be rescued by changing salt concentrations.

The discussion will have occasion to refer to this data, so the values for $[\text{Na}^+] = 50$ mM and $[\text{Mg}^+] = 2$ mM are listed below. The top row denotes the target, the left column denotes the probe. The diagonal elements correspond to the binding of matched pairs and are highlighted with bold text. The off-target combinations are all predicted to have $T_m$ at least $5 \, ^\circ \text{C}$ if not $10 \, ^\circ \text{C}$ or more below the on-target combinations, although the benchmark matrix showed that measured hybridization cross-talk was over $50\%$ for four of those combinations, highlighted in blue in the table.
Table 9. Predicted $T_m$ of probe-target combinations from miR-17-5p family.

<table>
<thead>
<tr>
<th></th>
<th>miR-17-5p</th>
<th>MiR-20b-5p</th>
<th>MiR-93-5p</th>
<th>MiR-106-5p</th>
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<tbody>
<tr>
<td>MiR-17-5p</td>
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<td>69.9 °C</td>
<td>60.9 °C</td>
<td>52.7 °C</td>
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<tr>
<td>MiR-93-5p</td>
<td>61.2 °C</td>
<td>61.1 °C</td>
<td>71.9 °C</td>
<td>54.4 °C</td>
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<tr>
<td>MiR-106-5p</td>
<td>57.9 °C</td>
<td>53.2 °C</td>
<td>51.2 °C</td>
<td>68.1 °C</td>
</tr>
</tbody>
</table>

A similar matrix was calculated for members of the let-7a family, shown below.

The top row denotes the target, the left column denotes the probe. The diagonal elements correspond to the binding of matched pairs (bold text). The elements where hybridization is known to be strong are highlighted in blue; here the simulator correctly predicts that the probe to off-target $T_m$ is dangerously high.

Table 10. Predicted $T_m$ of probe-target combinations from hsa-let-7x-5p family

<table>
<thead>
<tr>
<th></th>
<th>Let-7a-5p</th>
<th>Let-7d-5p</th>
<th>Let-7g-5p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Let-7a-5p</td>
<td>62.3 °C</td>
<td>58.2 °C</td>
<td>51.1 °C</td>
</tr>
<tr>
<td>Let-7d-5p</td>
<td>59.7 °C</td>
<td>65.6 °C</td>
<td>47.0 °C</td>
</tr>
<tr>
<td>Let-7g-5p</td>
<td>56.3 °C</td>
<td>50.8 °C</td>
<td>63.1 °C</td>
</tr>
</tbody>
</table>

The predicted melting temperatures are compared with observed hybridization efficiencies (Figure 25) reported in the hybridization matrix experiment. The comparison is illustrated in Figure 30. There is a definite trend that lower predicted melting temperatures result in lower hybridization intensity. However, the correlation is rather irregular, with two sudden jumps. The irregular correlation led to the pursuit of a second method to estimate hybridization efficiency in supplement of simulations.
Melting temperature assays using SYBR dye were carried out as described under Materials and Methods. Mimicking the benchmark experiment, each combination of four probes and four targets from the miR-17-5p family were hybridized. The targets were the microRNAs miR-17-5p, miR-20b-5p, miR-93-5p and miR-106b-5p, while the probes were their reverse complements. Probes and targets were prepared with flanking sequences, as described under Materials and Methods, and the SYBR assay performed using the protocol described there. A representative set of melt curves are shown in Figure 31. The sharp decline in fluorescence over a narrow temperature range is unmistakable and corresponds to the melting temperature of the probe-target combination. The width of the transition region is of order 3 °C. A useful rule of thumb
observed in many such charts is that in the steepest part of the curve, the hybridization intensity decreases approximately 2-fold for every 1 °C rise in temperature.

![SYBR Normalized Fluorescence Curve](image)

**Figure 31.** Melt curves using SYBR assay for three probes.

To quantify the melting temperature, the derivative of each curve is taken, and the temperature at its peak is calculated. The data manipulation is performed by the instrument manufacturer’s software. The derivative curves for the data above are shown below in Figure 32. Note that the absolute melting temperatures in the SYBR assay are quite different from those in the particle hybridization assay, because the two assays used
different buffers with different salts and intercalating agents. We are primarily interested in relative $T_m$, so the absolute shift is not of concern.

![SYBR Normalized Derivative Curve](image)

Figure 32. Derivative melt curves of SYBR assay for three probes

SYBR Assay for miR-17-5p family and let-7x-5p family: A matrix of probe-target melt temperatures was measured, corresponding to the calculations performed previously. The results are shown in Table 11. The top row gives the target identity, the left column gives the probe identity. As usual, matched probe-target combinations are on the diagonal and highlighted with bold text. The SYBR assay indicates that many of the matched probes have high $T_m$ to homologous targets, consistent with the particle results.
Table 11. Measured $T_m$ of members of the miR-17 family using a SYBR assay

<table>
<thead>
<tr>
<th></th>
<th>miR-17-5p</th>
<th>MiR-20b-5p</th>
<th>MiR-93-5p</th>
<th>MiR-106-5p</th>
</tr>
</thead>
<tbody>
<tr>
<td>MiR-17-5p</td>
<td>77.1 °C</td>
<td>73.5 °C</td>
<td>73.9 °C</td>
<td>71.9 °C</td>
</tr>
<tr>
<td>MiR-20b-5p</td>
<td>74.2 °C</td>
<td>76.9 °C</td>
<td>73.9 °C</td>
<td>70.6 °C</td>
</tr>
<tr>
<td>MiR-93-5p</td>
<td>73.4 °C</td>
<td>76.1 °C</td>
<td>78.1 °C</td>
<td>71.1 °C</td>
</tr>
<tr>
<td>MiR-106-5p</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>76.5 °C</td>
</tr>
</tbody>
</table>

Data was also gathered for probes from the let-7 family, binding to let-7a target. One disappointment is that the SYBR melting temperatures were not entirely predictive of hybridization efficiency. In the data of Table 12, the melting temperature of let-7d-5p probe to let-7a-5p amplicon was measured to be below that of let-7g-5p to let-7a-5p, but in particle hybridization assays, the latter consistently has lower hybridization efficiency.

Table 12. Measured $T_m$ using SYBR assay of members of the let-7 family

<table>
<thead>
<tr>
<th>Hsa-let-7a-5p target sequence</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe sequence for Let-7a-5p</td>
<td>73.2 °C</td>
</tr>
<tr>
<td>Probe sequence for Let-7d-5p</td>
<td>70.5 °C</td>
</tr>
<tr>
<td>Probe sequence for Let-7g-5p</td>
<td>71.4 °C</td>
</tr>
</tbody>
</table>

SYBR $T_m$ compared to predicted $T_m$: The measured $T_m$ is compared with the predicted $T_m$ for the same matrix given in Table 9. First consider the Watson-Crick matched pairs (diagonal entries in the matrix). The values are plotted in Figure 40. The ranking of $T_m$ is identical for simulation and measurement, and the predicted values closely follow a trendline. The offset between predicted and measured melting temperatures is of little concern, as the cation concentrations in the SYBR reagents are not known exactly, and cation concentrations have already been shown to result in constant shifts in $T_m$. 

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Intriguingly, the slope of the line is 47%, meaning that the measured differences in melting temperature are only half as large as those predicted. Since cation changes in the simulator lead to constant shifts of predicted $T_m$, no change in salt conditions would remedy the slope discrepancy between prediction and measurement. The discussion will return later to observed discrepancies between prediction and measurement.

![Predicted vs measured Tm](image)

**Figure 33.** $T_m$ measured by SYBR assay vs predicted $T_m$, matched probes

Comparing the full matrix of predicted $T_m$ to measured $T_m$ still results in a satisfactory trend line but there is a larger scatter of values around the trend line (Figure 34), while the slope has decreased to 30%. The range of measured values is 7.5 °C while the range of predicted values is 22.7 °C. The scatter in predicted $T_m$ is materially significant for probe design. Consider the 2 °C spread in measured $T_m$ at a predicted temperature of 57 °C. In the melt curves of Figure 31, we see that the width of the transition region from near full binding to near zero binding is about 3 °C. Thus a 2 °C
error could mean the difference between unacceptable cross-talk and acceptable cross-talk.

![Graph showing Predicted vs measured Tm](image)

$$y = 0.30x + 55.79$$

Figure 34. Predicted $T_m$ vs measured $T_m$, matched and unmatched duplexes

**SYBR $T_m$ compared to hybridization benchmark:** Next, the measured matrix of melting temperatures was compared to the hybridization efficiencies in the benchmark experiment described previously. For each combination of spiked-in oligomer and probe, the melt temperature measured by the SYBR assay was compared to the hybridization intensity in the particle assay (Figure 35). Data from both matched probes and mismatch probes are combined.
There is a strong trendline of hybridization intensity vs melting temperature measured in the SYBR assay. The correlation is not perfect (two combinations around 74.5 °C had hybridization intensities differing 10-fold) but the data provide a good guideline that SYBR $T_m$ below 72 °C results in little hybridization while $T_m$ above 74 °C results in strong hybridization.

The uncertain relation between predicted $T_m$ and measured $T_m$, as well as the strong trend in hybridization intensity with SYBR $T_m$, led to the adoption of the SYBR assay as a screening tool. The assay itself is very simple (typically 15-30 minutes of bench time combined with 45 minutes of PCR instrument time) and does not require conjugation of oligomers to particles. The melt curves are extremely reproducible, with
run to run variation around 0.25 °C. New probe sequences can be ordered and received within two days, allowing rapid iteration and learning.

**SYBR screen of abasic probes:** The SYBR assay was first used to assess the impact of different types of base modifications. Three mismatch probes were designed for each of two targets, miR-17-5p and miR-93-5p. A substitution, a gap, and an abasic site were compared, all at the same site on the probe, 8 locations from the 5’ end of the reverse complement of the microRNA sequence (Figure 36).

![Mismatch probes](image)

(a) Matched probe  
(b) Substitution probe  
(c) Abasic probe  
(d) Gap probe

Figure 36. Different types of mismatch probes
Each type of probe was hybridized with either its intended target (e.g., modified miR-17-5p to oligomer miR-17-5p) or an off-target (modified miR-17-5p to oligomer miR-93-5p), and the melting temperatures measured. The results are shown in Figure 37. The right side of the figure presents each probe in a scatterplot with the abscissa equal to the reduction in on-target binding and an ordinate of the reduction in off-target binding. The matched probes lie on the vertical axis.

![Graph showing melting temperatures of match and mismatch probes](image)

(a) Measured melting temperatures of match and mismatch probes  
(b) Relative melting temperatures of match and mismatch probes

Figure 37. Comparison of $T_m$ for different mismatch probe types

The results in Figure 37 suggest that the different types of modifications result in rather similar changes to probe binding to intended targets as well as off-targets, quantitatively as well as qualitatively. All three changes dropped the on-target binding temperature by ~2 °C and the off-target binding temperature by ~0.5 °C. There are slight differences between the different types of probe, but they are not consistent from
target to target, and for both targets the three different types of mismatch probes lie along a tradeoff line where more reduction in off-target binding comes at the expense of on-target binding. Since abasic oligomers are considerably more expensive (~10X) relative to substitutions and deletions, they were not pursued further.

**SYBR screen for miR-17-5p**: An ideal mismatch probe would have little change in on-target binding combined with a large drop in off-target binding. The mismatch probes measured so far have had more impact on the on-target binding than the off-target binding, the reverse of what one would like. The SYBR assay was used to cast a wider net to identify probes for miR-17-5p which might have a better window between on-target and off-target binding.

Two primary probe modifications were considered, base deletions, and base insertions. Referring to the sequences listed in Figure 17, the members of the miR-17-5p family have sequence identity at their ends, with a “bubble” between bases 10-13 when a probe hybridizes to an off-target member of the family. In order to reduce the binding to off-target oligomers, mismatches (insertions and deletions) were introduced into the common area of the probes. Gaps were introduced at the edge of the bubble with the hope of extending the bubble and perhaps destabilizing the duplex when bound to an off-target oligomer, while creating just a single mismatch when bound to the on-target oligomer. Other probes introduced an extra cytosine or thymine close to the bubble with the same intention. If a mismatch were found to be useful for knocking down oligomer miR-93-5p binding to probe miR-17-5p, it was hypothesized that the same change made
to the probe for miR-93-5p might also be useful for knocking down unwanted miR-17-5p
target binding. A list of the probes screened for target miR-17-5p is given in Figure 38.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>match</td>
<td>C T A C C T G C A C T G T A A G C A C T T T G</td>
</tr>
<tr>
<td>gap1</td>
<td>C T A C C T G _ A C T G T A A G C A C T T T G</td>
</tr>
<tr>
<td>dgap</td>
<td>C T A C C T _ _ A C T G T A A G C A C T T T G</td>
</tr>
<tr>
<td>gbb3</td>
<td>C T A C C _ _ _ A C T G T A A G C A C T T T G</td>
</tr>
<tr>
<td>gbb4</td>
<td>C T A C _ _ _ _ A C T G T A A G C A C T T T G</td>
</tr>
<tr>
<td>geb2</td>
<td>C T A C C T G C _ _ T G T A A G C A C T T T G</td>
</tr>
<tr>
<td>geb3</td>
<td>C T A C C T G _ _ _ T G T A A G C A C T T T G</td>
</tr>
<tr>
<td>geb4</td>
<td>C T A C C T _ _ _ _ T G T A A G C A C T T T G</td>
</tr>
<tr>
<td>xc1</td>
<td>C T A C C T G C A C C T G T A A G C A C T T T G</td>
</tr>
<tr>
<td>xc2</td>
<td>C T A C C T G C A C T G T A A G C A C T T T G</td>
</tr>
<tr>
<td>xt1</td>
<td>C T A C C T G C A C T G T A A G C A C T T T G</td>
</tr>
<tr>
<td>xt2</td>
<td>C T A C C T G C T A C T G T A A G C A C T T T G</td>
</tr>
<tr>
<td>xcc1</td>
<td>C T A C C T G C A C C C T G T A A G C A C T T T G</td>
</tr>
<tr>
<td>gp5c</td>
<td>C T A C C T G C A _ _ _ T G T A A G C A C T T T G</td>
</tr>
<tr>
<td>g3pc</td>
<td>C T A C C T G C A C T G T A A G C A _ _ _ T T T G</td>
</tr>
<tr>
<td>g5pg</td>
<td>C T A C C T _ _ _ C A C T G T A A G C A C T T T G</td>
</tr>
<tr>
<td>g3pg</td>
<td>C T A C C T G C A C T G T A A _ _ _ C A C T T T G</td>
</tr>
</tbody>
</table>

Figure 38. miR-17-5p probes screened by SYBR assay
To test the efficacy of the probes, each was hybridized in a SYBR assay with either its intended target (miR-17-5p) or a target showing strong cross-talk (miR-93-5p). The melting temperatures of the resulting combinations are shown in Figure 39. The shaded band represents the temperature range over which hybridization was previously found to change rapidly. An ideal probe for miR-17-5p would have $T_m$ to miR-17-5p above the band and a $T_m$ to miR-93-5p below the band.

![Probe screen for target mir-17-5p](image)

**Figure 39.** On-target $T_m$ vs off-target $T_m$ in SYBR assay screen

The probe-target bindings are all found to fall in a narrow temperature range, from 70 °C to 76.5 °C. The matched probe has a 2.2 °C difference between its on-target and off-target binding. The results in Figure 39 suggest a number of interesting
candidates to pursue. Probes xt1 and xt2 have particularly large gaps between on-target
and off-target melting temperatures, closely followed by g3pc and gbb4. These
candidates were noted for later conjugation to particles.

**SYBR screen compared to simulation screen:** As a further test of predicted binding
temperatures from DINAMELT, the melting temperatures measured above were
simulated. Each of the probe sequences was introduced into DINAMELT and simulated
against the two potential targets miR-17-5p (on-target) and miR-93-5p (off-target). The
measured and simulated data are compared in Figure 40. The correlation between
simulation and measurement is low, with weak trends in measured $T_m$ vs predicted $T_m$.

![Figure 40. Simulated $T_m$ vs SYBR $T_m$, all probes, on-target and off-target](image)

Figure 40. Simulated $T_m$ vs SYBR $T_m$, all probes, on-target and off-target
One might hope that even if the absolute melting temperatures did not have good correlation, at least the difference between on-target and off-target binding could be predicted. Figure 41 shows the comparison between predicted difference of on-target and off-target binding vs measured differences of on-target to off-target binding. The correlation remains weak, with a Pearson coefficient $R^2$ of 13%.

![Figure 41. Scatterplot of simulated $T_m$ differences vs measured $T_m$ differences](chart.png)

**Extended panel hybridization experiment:**

The SYBR probe screen just described identified a new set of promising targets. A panel [Table 4] was built with a number of the most promising candidates and tested with both single target and multiple target amplicons.

**Single-target amplicons:** A set of particles with probes based on the $g3pc$ pattern in Figure 38 were hybridized with spike-in oligomers each of miR-17-5p, miR-20b-5p,
miR-93-5p, miR-106-5p. Four wells of each spike-in were measured. The performance of a conventional matched probe, the previously tested gap probe, and the g3pc pattern are shown in Figure 42. Each group of 16 bars corresponds to 4 wells each of 4 different spiked-in target. As seen in the benchmark experiment, the specificity of the matched probe for mirR-17-5p to miR-20b-5p and miR-93-5p is poor, with cross-talk of 75% and 71%, respectively, comparing four-well averages. For the previously tested gap1 probe, the average cross-talk in this experiment is 3.6% and 2.9%. For the g3pc probe, the cross-talk is further reduced to 2.3% and 1.3%, excellent values.

Figure 42. Assay performance of SYBR-screened probes for miR-17-5p
Similar improvements are found the g3pc variant of the miR-20b-5p and the miR-93-5p probe (Figure 43). For probe match-20, the cross-talk from miR-17 and miR-93 is 32% and 56%. The g3pc variant has cross-talk of 0.7% and 0.6%, respectively. For probe match-93, the cross-talk from miR-17 and miR-20b is 36% and 65%. With the g3pc variant, the cross-talk improves to 1.2% and 1.0%. The expectation that finding a useful change in the miR-17-5p probe would also result in a useful change for the miR-20b-5p and miR-93-5p probes was confirmed.

![Graphs](image)

(a) MiR-20b-5p probes  
(b) MiR-93-5p probes

Figure 43. Assay performance of improved miR-20b-5p and miR-93-5p probes

**Multi-target amplicons:** The utility of cross-talk probes was investigated using an experiment with a mixed amplicon of miR-17-5p and miR-93-5p. The experiment is illustrated in Figure 44 where the layout is shown in (a) and the measured signals for matched probes to miR-17-5p and miR-93-5p are shown in (b). The bottom well in each column had technical issues and was omitted from the analysis. A synthetic sample was
used with different combinations of miR-17-5p and miR-93-5p in different groups of wells.

(a) Plate layout  
(b) Signal for probes match-miR-17-5p and match-miR-93-5p

Figure 44. Mixed analyte, signal for matched probes

If these were biological samples and one had no prior information as to the concentrations of either target, one would be hard pressed to ascertain the relative concentrations of each analyte was in each well. The signal for miR-93 is higher than for miR-17 in every well. In most wells, the signals are quite close, with the biggest ratios in the last two wells. One might be able to deduce that the first column has replicates of one sample, call it X, then there are four replicates of another sample Y, followed by two replicates of a different sample Z, but the relative mixtures of miR-17 and miR-93 in samples X, Y and Z are challenging to discern. Without further information, the best guess might be that the first six wells (sample X) have a roughly 40:60 ratio of miR-17 to
miR-93, the next four (sample Y) have lower amounts of both but in the same ratio, and the last two wells (sample Z) has a 50:50 ratio.

Such an assignment is in fact far from of the mark. A much clearer picture appears if the same experiment is viewed with the aid of mismatch probes (Figure 45). The first column (Sample X) has high values of miR-93 throughout, and little miR-17. Sample Y has somewhat higher miR-93 than miR-17, while sample Z has at least a 3:1 ratio of miR-93 to miR-17. The actual ratios were pipetted as 100:0, 66:33 and 80:20. The re-designed probes with intentional mismatches give a much closer representation of the reality of the situation than the matched probes did.

![Figure 45. Mixed analyte, signal for designed mismatch probes](image-url)
Correcting for temperature variation

In the discussion of assay variability, it was found that all probes vary with plate temperature, some to a lesser degree and some to a greater degree. Furthermore, strong circumstantial evidence was presented to show that a large component of probe variability likely originates in temperature variations across a plate. If there were a way to correct for temperature variation across a plate, assay variability might be reduced.

The theoretical basis for one method of temperature correction is developed in the next section. After the derivation, the application of the method to simple and more complex datasets is discussed.

Hybridization theory

The Gibbs free energy for hybridization between two strands of DNA is

\[ \Delta G = \Delta H - T \Delta S \]

\( \Delta H \) is the change in enthalpy for hybridization (negative), \( \Delta S \) is the change in entropy for hybridization (also negative, since entropy is reduced by hybridization) and \( T \) is absolute temperature. At the melting temperature between a probe and a target, \( \Delta G \) is zero so \( \Delta H = T_m \Delta S \). Thus, we can write

\[ \Delta G = (T_m - T) \Delta S \]

For any probe binding to a target, the bound fraction \( \phi \) depends on temperature according to the general formula for the equilibrium in a reversible first-order chemical reaction

\[ \phi = \frac{1}{1 + e^{\Delta G/RT}} \]
where \( R \) is the universal gas constant. At \( \Delta G = 0 \) and the bound fraction is 50%. Using the alternative formulation for \( \Delta G \), and writing \( \Delta T = T - T_m \), the bound fraction can be written

\[
\phi = \frac{1}{1 + \exp \frac{\Delta T(\Delta S)}{RT}}
\]

Note that at temperatures above the melting point, the curve is approximately an exponential function of temperature with a slope of \( \Delta S/RT \). That is, the slope of the logarithmic melt curve is defined by the entropy of the duplex, not by enthalpy or free energy. (For excursions of a few degrees around the melting temperature, the absolute temperature \( T \) can be treated as a constant in the above equation).

We can now think of the same relation as defining well temperature relative to the probe – target melting temperature. Inverting the equation results in

\[
\Delta T = \frac{RT}{\Delta S} \log(1/\phi - 1)
\]

If the bound fraction for a probe is known, the temperature of a well can be inferred, relative to that probe’s melting temperature. To estimate bound fraction, consider a matched probe \( \hat{A} \) and a mismatch probe \( \hat{a} \) for target A. The fraction of bound \( \hat{a} \) can be approximated as \( \hat{a} / \hat{A} \). Thus

\[
\Delta T = \frac{RT}{\Delta S} \log(\frac{\hat{A}}{\hat{a}}) - 1)
\]

The quantities on the right hand side are all experimentally measured quantities, with the exception of \( \Delta S \) which can be estimated from literature. Temperature measured in this way, using the ratio of a matched to mismatch probe, will be referred to as synthetic temperature. The entropy term in the denominator was estimated from the
thermodynamic data of Table 1. The binding entropy change of nearest-neighbor elements is approximately the same for all elements at -23 cal/mole/K, so a 23-base microRNA has an entropy change in binding of about -500 cal/mole/K.

Measuring the slope of the logarithmic SYBR melt curve between oligomer miR-17-5p and its matched probe shown in Figure 31 gives a value of -0.76 K^{-1}. The entropy corresponding to slope $s$ is $sRT$ and evaluates to -471 cal/mole/K, in conspicuous agreement with the entropy estimate based on literature thermodynamics. The SYBR curves for the various probe-target combinations all have similar slope, supporting the literature prediction that the entropy does not vary widely from target to target.

**Synthetic temperature**

To validate the notion of a synthetic temperature, oligomers of miR-17-5p and let-7a-p at a concentration of 40 pM were hybridized with particles of the Family17 panel which contains matched and mismatch probes to several analytes in the miR-17-5p family (Table 3). 12 replicate wells were run at a nominal shaker temperature of 39 °C. The spatial variation of the members of the let-7x-5p family shows no discernable pattern (Figure 46 (a)), but the ratio of let-7d to let-7a is highly correlated to the ratio of let-7g to let-7a (Figure 46 (b)).
Either let-7d-5p or let-7g-5p could be considered mismatch probes to the matched probe let-7a-5p, so either ratio could be used to define a synthetic temperature. For this experiment, the let-7d-5p / let-7a-5p ratio (7d:a ratio for short) was used to define a synthetic temperature. When the three members of the family are plotted as a function of the synthetic temperature (Figure 47 (a)), a clear pattern emerges. Each family member lies on a melt curve. Even the matched probe shows evidence of a signal decline at higher temperature, whereas in the spatial plot the variation in the matched probe could easily be mistaken for noise. When plotted on a logarithmic scale (Figure 47 (b)), the intensity of the mismatch probes decreases linearly with temperature, consistent with Boltzmann statistics. The range of temperatures on this plate is about 5 °C, from -2 °C.
below the reference temperature to +3 °C above it. (The reference temperature is the temperature at which the 7a:d ratio is 50%.) The width of the transition zone defined by synthetic temperature is consistent with the width of the transition zone measured in SYBR melting temperature experiments, where temperature is measured directly by the thermocouples of the PCR instrument.

![Graphs showing variation of let-7-5p family members with synthetic temperature](image)

Figure 47. Variation of let-7-5p family members with synthetic temperature

To show that the synthetic temperature applies not to just members of this family but also to other probe-target combinations, matched and mismatch probes from the panel are plotted against the same synthetic temperature in Figure 48. Probes for miR-17-5p and miR-93-5p also show melt curve trends, with the matched probes having a $T_m$ above
that of the 7d:a combination, while the mismatch probe has a $T_m$ below the 7d:a combination.

To further validate the concept of a synthetic temperature, three plates with the same panel and amplicon just described were hybridized at a nominal shaker temperature of 39 °C, and one hybridized at 41 °C. Two plates had 12 replicates and two had 8 replicates. When a low $T_m$ probe (gap1-miR-93) is plotted as a function of well location (Figure 49 (a)) the pattern is different on each plate. The 41 °C plate shows overall lower intensities relative to 39 °C, but no other pattern is obvious in the data. When the data is plotted instead against synthetic temperature derived from the 7d:a ratio, the data
from all the wells on all the plates fall on a single curve (Figure 49 (b)). The vertical axis is the logarithm base 10 of the measured signal.

(a) Four plates, signal plotted vs well position
(b) Four plates, signal plotted vs synthetic temperature

Figure 49. Data for probe mismatch-miR-93 from plates at different temperatures

These data suggest that the synthetic temperature, defined using a matched / mismatch probe combination, appears to provide a satisfactory estimate of well temperature.

Temperature corrected data

If the melt curve for each probe were known, and the temperature in each well were known, then a probe measurement at one temperature could be converted to a measurement at a different temperature by sliding up or down the melt curve. The wells
of a plate, which have a variety of temperatures, could all be translated to a single
temperature, mitigating the temperature contribution to variability. The procedure is
illustrated schematically in Figure 50. Two wells at different temperatures are translated
to a common temperature. The variability that arises from each well being at a different
temperature is thus eliminated, because all can be translated to a single target
temperature. Mathematically, the correction formula from a particular well temperature
to a target temperature is

$$S_t = S_w \frac{1 + exp(-T_w \Delta S)}{1 + exp(-T_t \Delta S)}$$

where $S_t$ is the desired signal at the target temperature $T_t$, $S_w$ is the measured
signal at the well temperature and $\Delta S$ is the entropy change of hybridization. Both
temperatures are measured relative to the reference temperature, defined by the 50% ratio
between the match and mismatch probe. The target temperature is not critical; it can be
chosen as the average well temperature on the plate, or the hottest well on the plate. The
variability will be the same in both cases. The hottest well on the plate has the advantage
that off-target binding is reduced to the lowest value on the plate.
For the approach just described, a melt curve for each probe is needed. It might seem that generating such curves for each probe imposes a huge burden. However, a natural way to generate such curves presents itself. If one hybridizes replicates of an amplicon on multiple wells on a plate, along with a matched/unmatch probe pair to provide a temperature estimate in each well, the natural temperature variation across a plate generates a spread of temperatures and hybridization efficiencies; in short just the data that is needed for a melt curve. A curve-fitting procedure can then be used to match the curve to the data. The procedure for fitting a melt curve is illustrated in Figure 51. The vertical axis is the logarithm base 10 of the hybridization signal. The horizontal axis is the synthetic temperature defined a match / mismatch probe pair. For the probe shown, a melt temperature is estimated that best matches the melt curve equation to the data. The accuracy of the $T_m$ estimate is about ±0.5 °C, as illustrated by the poorer fits of curves with a lower and higher $T_m$ relative to the best-fit value. Since each well contains
as many probes as there are probes on the panel (NP probes), a series of 8 or 12 wells generates NP curves, each with 8-12 points per probe, providing a relatively high throughput way to estimate probe melting temperatures.

![Fitting a melt curve to probe data](image)

**Figure 51. Fitting a melt curve to probe data**

To demonstrate the benefit of temperature correction on a biological dataset, 16 replicates of cardiology amplicon were hybridized with particles from the FirePlex cardiology panel (Table 5). With 70 probes, it is not practical to show data for all probes. Selected probes are shown in Figure 52, before and after correction. Each group of bars corresponds to a probe, each bar within a group corresponds to a single well. The probes before correction show the usual wide range of values. After correction, the data are much more uniform.
Data from replicates of a biological sample

To quantify the improvement in uniformity, the coefficient of variation (CoV) is calculated for each probe, before and after correction. The CoV before and after is
shown in Figure 53. The median CoV before correction is 21%, while after correction the median CoV is 4% for this dataset.

![Cardiology probe CoV for 16 replicates of cardiology amplicon](image)

**Figure 53.** Cardiology probe CoV for 16 replicates of cardiology amplicon

Allocating two columns of replicates out of twelve columns on every plate for temperature correction is a high cost and unlikely to be embraced by assay users. However, the $T_m$ of a probe is expected to be a constant from run to run, as long as assay buffers remain unchanged, so that historical values could be used. To investigate whether the $T_m$ from one plate could be usefully applied to another, the plate described above, measured after a 38.5 °C hybridization in August 2018, was used to temperature correct a plate hybridized at 36.5 °C in October 2017. The median raw probe CoV was 20%, while after correction using correction factors from the August 2018 experiment, the median probe CoV was 11%. While the correction from a different plate at a
different temperature does not provide as a good a CoV reduction as wells on the same plate, the variability reduction is still substantial.

Figure 54. Probe CoV of data measured in October 2017, corrected using temperature data from an August 2018 plate.

Stacking corrections

The temperature correction developed above is a particular form of normalization, one that provides a probe specific normalization to compensate for temperature variations between wells. The use of temperature correction does not preclude using other normalization methods. After temperature correction, a conventional normalization approach such as geometric mean normalization, GeNorm, or TMM as described in the introduction, can be applied to normalize other sources of variability, such as variation in sample collection, sample digestion, and other technical factors. Using the last example in the previous section, adding a geometric mean normalization following the temperature correction reduced the remaining CoV from 11% to 4.8%.
Off-species probes for in-situ temperature estimation

The temperature correction technique described above needs a match / mismatch pair of probes to estimate temperature of each well from the ratio of mismatch to match signal. The requirements on using such a probe pair are

- The target of the probe should not appear endogenously in samples, nor should there be a homologous target in expected samples
- The matched probe should have reproducible signal across a plate, which translates to having a $T_m$ to its target at least 4 °C above the average plate temperature
- The mismatch probe should have a $T_m$ to the same target close to the average plate temperature
- There should be a simple protocol for including the match / mismatch probe pair in an assay

In the examples to this point, the probe pair hsa-let-7a-5p and hsa-let-7d-5p was used. For human and rodent samples, this would not be a suitable pair, as these targets appear endogenously and might vary from sample to sample. To avoid accidental collisions with endogenous targets, four O. anatinus (platypus) microRNAs were considered for temperature measurement, oan-mir-1357, oan-mir-1421aa-5p, oan-mir-458-5p and oan-mir-1397-5p. These microRNAs have no human or rodent homolog. For each candidate, a number of mismatch variants was designed, in each case identifying one whose melting curve straddled the plate temperature. In each case, it was found that substitutions at position 8,9 and 10 from the 5’ end resulted in a match / mismatch pair with a temperature range that straddled the plate temperature.
The protocol for using the match / mismatch pair was to add a low concentration of the platypus target into the hybridization buffer, a relatively simple modification to the assay.

To test the performance of the newly developed probe pairs, a particle panel containing probes for human microRNAs let-7a-5p, let-7c-5p, let-7d-5p and let-7g-5p was manufactured, along with the platypus temperature measurement probes and their variants. An amplicon containing only let-7a-5p target was spiked into multiple replicates on a plate, and the particles were hybridized with the amplicon.

In principle, only the let-7a-5p probe should register signal in each well. However, due to the inevitable non-specific binding of let-7a-5p target to the homologous members of the let-7-5p family, other probes also registered signal (Figure 55).

![Figure 55. Raw signal for let-7x-5p probes across plate, let-7a-5p spike-in](image-url)
When temperature correction is applied using a target temperature of the hottest well on the plate, the let-7x-5p family members in each well were brought into alignment with the hottest well. As a result, the let-7a-5p signal changed little (high $T_m$) but the let-7{c,d,g} signals all decreased to the lowest values on the plate (Figure 56).

![Figure 56. Temperature-corrected signals for let-7x-5p probes, using oan-mir-1357 mismatch probe pair for temperature estimation](image)

Concurrent with the reduction in unwanted signal, there was a decrease in variability. The maximum CoV decreased from 57% in the raw data to 6% in the corrected data.

Corrections were carried out with a single probe pair, two probe pairs or three probe pairs. The corrected data was of similar quality in each case. For a plate with a particularly wide temperature range, it might be necessary to use a probe pair with a higher $T_m$ for an upper range of temperatures, and a probe pair with a lower $T_m$ for a
lower range of temperatures. For the 5 °C temperature range seen here, a single probe
pair sufficed. Each 1 °C results in a 2X intensity drop, so a mismatch probe can vary by
~32X across a plate. The assay has a dynamic range of 10,000:1 so a 32:1 ratio is easily
accommodated.
Chapter IV
Discussion

Most hybridization-based assays rely on Watson-Crick base pairing for sensitivity, specificity and reproducibility. Reproducible performance from well to well is important for detecting changes in the level of an analyte when comparing samples. A t-test or Anova test depends on comparing variation between groups to the variation within a group. If the latter is high, the ability of the assay to discriminate subtle changes is impaired.

As discussed under Materials and Methods, the FirePlex microRNA assay achieves its sensitivity due to a PCR amplification step, which captures microRNAs from a biological sample into an amplified pool of predominantly single stranded biotinylated cDNA by asymmetric PCR. A library of this cDNA is then mixed with DNA probes bound within hydrogel particles to retain molecules that hybridize to these probes. Bound molecules are labeled by streptavidin-PE-Cy5 conjugate and quantified by cytometry. The assay achieves excellent specificity through the double capture step, once from the biological sample, and then again from the PCR amplicon, although specificity between targets contained within the panel is only as good as the last hybridization step (see discussion under Materials and Methods). Data on specificity and reproducibility were presented in the Results section. In the following discussion, we describe how the results showed that specificity and reproducibility were linked, and how improved specificity and reduced variability were achieved using a combination of experimental and numerical methods.
Origin of variability

Variability of capture signals for some probes in the assay was found to be quite high [Figure 7, Figure 10, Figure 11]. Experimentally it was demonstrated that

- Certain probes [Figure 7] in replicate wells vary from well to well to a greater extent than others [Figure 8] (quantified by coefficient of variation)
- The spatial variation of the different probes is correlated, even when the magnitude of the variations was quite different [Figure 9, Figure 10]
- The same probes are consistently more variable or less variable from run to run [Figure 12]
- Increasing the assay temperature by 2 °C reduces assay signal, from as little as a few percent to as much as a 20-fold reduction, depending on the probe [Figure 15]. The probes most reduced by temperature are also the probes that showed the highest variability at a single plate temperature [Figure 15 compared to Figure 11].
- Increasing the assay temperature increases the number of variable probes above a certain CoV and expands the magnitude of variation
- The magnitude of variation due to temperature exceeds other technical factors, such as pipetting errors or variance in other assay component composition

These facts together led to a model of probe variability primarily driven by temperature variation between wells.

Scrupiny of the most variable probes showed that in almost all cases they were probes for targets that belonged to microRNA families with close homology between members [Figure 11, Figure 16, Figure 17]. The possibility of cDNA cross-talk onto
different probes was then verified by spike-in experiments that showed that introducing a single target would cause multiple family members to report signal [Figure 19, Figure 20, Figure 21].

Combining the observation of cross-talk with the temperature-driven variability leads to the model of probe variability illustrated in Figure 23 and elaborated in Figure 24. On-target binding has $T_m$ above the assay temperature, leading to reproducible readings. However, some probes bind to cDNA from off-targets related by homology, with a melting temperature which is lower than on-target binding and can fall in the range of plate temperatures. If wells on the plate have different temperatures, the degree of off-target binding will differ between replicate wells, leading to high signal variability.

Strategies to address non-specific binding and variability

To reduce non-specific binding, new probes were designed that had deliberate mismatches, strategically placed to destabilize off-target binding without undue impact to on-target binding. To further reduce variability, numerical corrections for the varying well temperatures across a plate were developed.

Reducing probe cross-talk with mismatch probes

To reduce non-specific binding, it was hypothesized that lowering the $T_m$ of non-specific probes below a threshold defined by assay temperature would eliminate most non-specific binding. An important consideration was not to lower on-target binding of such probes to their target cDNA. Novel probes were designed and tested in order to reduce probe cross-talk. In addition to measuring cross-talk directly using the particle-
based hybridization assay, potential probes were screened using numerical modeling and with a SYBR melting temperature assay.

Ideally, probe screening would be quite simple and fast if numerical modeling accurately predicted probe melting temperatures. However, simulated melting temperatures proved to be a limited guide to melting temperature of the probe in a SYBR assay, or performance of a probe in a particle hybridization. Simulated \( T_m \) correlated well with measured \( T_m \) as long as the probe and target were perfectly matched, albeit a correction factor of 47% had to be applied to simulated temperatures [Figure 33]. Simulated melting temperatures between probes that contained one or more substitutions relative to a matched probe also had reasonable correlation with measured melting temperatures, although the correction factor needed to be changed to 35% [Figure 34]. Simulated melting temperatures between probes that contained insertions or deletions relative to a matched probe had weak correlation to measured melting temperatures [Figure 40, Figure 41]. The discrepancies observed between measured and simulated melting temperatures may arise from several factors, including

- Lack of thermodynamic data for the effect of intercalating agents on DNA duplex formation
- Lack of thermodynamic data for multiple adjacent substitutions in mismatch duplex hybridization
- Limited thermodynamic data on hybridization between strands containing bulges or gaps introduced by insertion or deletion of a base
• Reported nearest-neighbor thermodynamic values are consensus values obtained from multiple different sequences. Consensus values may not accurately represent the thermodynamics of a particular sequence.

• Predicted melting temperatures result from the subtraction of large numbers; the enthalpy difference $\Delta H$ for a 23-mer microRNA is of the order of -190 kcal/mole, while the entropy term $T\Delta S$ is of order -160 kcal/mole. The Gibbs free energy at room temperature is then just -30 kcal/mole. Even a small difference in either enthalpy or entropy estimated from the training data [Sanalucia2004] will give rise to a large shift in predicted $T_m$.

As a compromise between accuracy and speed, new probes were primarily screened using a SYBR assay. The SYBR assay showed that deletions, substitutions and abasic sites were found to have similar impact on the melting temperature. A range of base deletions at different probe loci were tested, with particular emphasis on G and C deletions to maximize the reduction in melting temperature. Insertions of C and T were also tested to destabilize the bound duplex. The SYBR probe screening yielded several candidate probes that reduced cross-talk from $\sim75\%$ to as low as 2-3% when tested on particles in the hybridization assay [Figure 42, Figure 43].

While no deterministic program for identifying good probes can be guaranteed, the following approach was found useful. The conserved region between members of a family is first identified. A set of 12-24 candidates is created by deleting G or C from one or more GC dinucleotide loci in the conserved region, starting in the center and
working outwards, first as single deletions, then in paired deletions, then if necessary, triple deletions. The candidate pool is subjected to a rough screen by simulation, to estimate their on-target and off-target binding, then the better candidates are synthesized and screened using a SYBR assay. A shortlist of 3-4 most promising candidates is conjugated onto particles and tested in the hybridization assay. In each case tested, at least one candidate with substantially reduced cross-talk resulted by following this program. In practice, a modification that improved a probe for one member of a family also proved to be useful when applied to other members of a family. Thus, the burden of reducing all potential \( N \times N \) cross-talk combinations between in a family containing \( N \) members was minimized, and required a single screen of probes for one representative family member.

Temperature correction

Even after reducing non-specific binding, some variability in probe signals due to temperature remains. As shown in Figure 15, comparing signal intensity at two plate temperatures 2 °C apart showed that almost all probes were sensitive to temperature to some degree. Meanwhile, the experiments on variability showed that the range of temperatures on a single plate [Figure 47, Figure 49] could be as large as 5 °C. The temperature range on a nominally uniform plate inside a heated shaker is shockingly large and invites disbelief. However, combining the 30-fold variation seen between replicates for some probes with the two-fold change in hybridization for every ~1 °C temperature difference seen in Figure 31, makes it difficult to avoid the conclusion that the well temperature on a plate truly spans a 5 °C range. The manufacturer specifies that
the average plate temperature is controlled to within 0.5 °C, but offers no specification for variation across a plate [Labnet2018]. Temperature-driven variability may be an inevitability of running hybridization reactions in a large filter plate and in a small plate shaking device with non-uniform temperature.

The methodology developed here to correct temperature variation has two requirements in the assay. One was the presence of at least a pair of probes whose ratio could be used as a “thermometer” in each well when the corresponding target is spiked into the wells. An “off-species” probe pair is desirable for such a thermometer, so that biological differences between samples do not generate spurious temperature signals. Probes complementary to platypus microRNAs were found to provide useful temperature correction information for human tissue samples. The second requirement is a database of probe melting temperatures. Such a database can be generated in situ for each experiment by spiking in an amplicon that stimulates all probes into 8-12 replicate wells. Alternatively, to avoid losing valuable plate real estate, accumulated information from previous runs can be used to bypass the need for replicates, after a number of plates have been run with the same panel. Variability between replicates was reduced from a median of 20-30% CoV before correction to 5-6% CoV after correction [Figure 53, Figure 55, Figure 56].

Temperature correction and non-specific binding

Temperature correction can reduce the variability of the assay with or without cross-talk between analytes being present. One example was shown in Figure 56. However, temperature correction does not itself compensate for cross-talk. In a well with
a mixture of two cross-talking analytes, it is challenging to ascertain which contribution each analyte provides to the measured signals.

To illustrate the challenge, consider for instance a hot well and a cold well, where the signal in each well is composed of a relatively consistent contribution from binding of the desired target, and a highly variable contribution from temperature-dependent binding of non-specific targets [Figure 24]. In principle, there is reason to hope to be able to extract the separate components in each well, because each well has two measurements (the signals for each analyte) and two unknowns (the actual concentrations of each analyte).

To see how such a compensation might be attempted, assume first that the assay is perfect linear, so the relationship between measured values and actual values in a well could be written

\[ M_A = \alpha C_A + \epsilon C_B \]
\[ M_B = \beta C_B + \delta C_A \]

where \( M_A \) is the measured value for target A, and \( C_A \) is the actual abundance of A. Similarly, \( M_B \) is the measured value for target B, and \( C_B \) is the actual abundance of B.

The coefficient \( \alpha \) is the sensitivity of probe A to target A, and \( \epsilon \) is the sensitivity of probe A to target B. Since B is not a matched target, we should have \( \epsilon < \alpha \). The coefficient \( \beta \) is the sensitivity of probe B to target B, and \( \delta \) is the sensitivity of probe B to target A. Since A is not a matched target, we should have \( \delta < \beta \).

Next assume that the matched probes for A and B have the same response to targets A and B, respectively, at any temperature, so that \( \alpha = \beta \). Without loss of generality we can take \( \alpha = \beta = 1 \), since the proportionality constant between molarity and fluorescent intensity is not of interest. The coefficient \( \epsilon \) is then the relative
sensitivity of probe A to target B compared to probe A to target A, also known as the cross-talk coefficient, and similarly for $\delta$. Both coefficients vary with temperature, because off-target binding will have a lower $T_m$ than on-target binding, so the ratio is driven by the difference of the two melt curves. The pair of relations can be written as a matrix equation

$$\begin{bmatrix} M_A \\ M_B \end{bmatrix} = \begin{bmatrix} 1 & \epsilon \\ \delta & 1 \end{bmatrix} \begin{bmatrix} C_A \\ C_B \end{bmatrix}$$

If we further assume that $\delta \epsilon < 1$, that is to say, the cross-talk is less than 100%, the matrix can be inverted, and the actual concentrations can in principle be derived from the measured signals:

$$\begin{bmatrix} C_A \\ C_B \end{bmatrix} = \frac{1}{1 - \delta \epsilon} \begin{bmatrix} 1 & -\epsilon \\ -\delta & 1 \end{bmatrix} \begin{bmatrix} M_A \\ M_B \end{bmatrix}$$

Converting back to algebraic notation

$$C_A = \frac{1}{1 - \delta \epsilon} [M_A - \epsilon M_B]$$
$$C_B = \frac{1}{1 - \delta \epsilon} [M_B - \delta M_A]$$

For example, if there is symmetrical cross-talk of 30% between A and B, the correction for A would amount to subtracting 30% of the B signal from A and adjusting upward by 9%, and similarly for B. If the homology family has more than two members A and B, the matrix equation can be extended in the obvious way, using a 5x5 matrix for family with five members, for instance.

So far, so straightforward. However, when cross-talk compensation is attempted in practice, the results are very unsatisfactory even for just two cross-talking probes, giving noisy and often negative values for the probe concentrations. The primary reason for the failure is that many of the simplifying assumptions made in the analysis above are
known to be untrue. Like most assays, the assay is not perfectly linear; a two-fold dilution generally results in less than a two-fold reduction of signal. Moreover, the nonlinearity is different for different probes; consider the different response between probes miR-15b-5p and let-7a-5p generated by a 25% reduction in sample concentration [Figure 14]. One probe is reduced by 25% as one would expect, but the signal for the other is reduced almost 75%.

To sharpen the point of the challenge, consider the cross-talk for the mixed analyte in Figure 44, using matched probes. In the first four wells, there is no miR-17-5p present but the signal for miR-17-5p is almost as high as for miR-93-5p, which is in fact present. In the last two wells, the signal for miR-17-5p is half as high as that of miR-93-5p. If zero abundance of miR-17-5p gave a signal comparable to miR-93-5p, what abundance would give a signal only half as high as miR-93-5p? Only a negative value would suffice mathematically, but a negative value is physically impossible.

For a cross-talk compensation algorithm to be effective, it would be necessary to collect the non-linear response curve for each probe to its target over the target’s entire range of expression, as well as the response curve of each probe to relevant non-specific targets. Such a data collection task would be onerous, compared to simply designing probes with reduced cross-talk, as done above.

In summary, temperature correction can be effective for reducing variability in the assay, even when non-specific binding is present, exemplified by Figure 56. However, temperature correction does not resolve the question of how much of the signal, however consistent it might be, is due to each analyte that might bind that probe. Only re-
designed probes that minimize non-specific binding, as described earlier, can truly resolve that ambiguity.

Contributions and conclusions

The main contributions of this work are

- The identification of off-target binding coupled with temperature variation between wells on a plate as a major source of variability
- The development of an in situ methodology to measure well temperature in each well across a plate
- The development of a methodology to correct probe intensities for temperature variation across the plate
- The development of more specific probes containing gaps and insertions to reduce binding to closely homologous targets
- The introduction of SYBR assay as a screening tool for candidate probes
- Data describing the relationship between solution $T_m$ and $T_m$ measured by binding within hydrogel particles

The use of mismatch probes allows a more reliable discrimination between analytes in a mixed sample containing closely homologous analytes. It also reduces the variability of lower concentration targets in the presence of higher concentrations of a homologous target.
Even with more specific probes, there remains variability between identical samples on the same plate due to well to well temperature variation. Such variability can be further mitigated by temperature correction, using temperature estimated with a match / mismatch probe pair and knowledge of the probe melting temperatures.

Using temperature correction does not preclude other methods of normalization. Temperature correction reduces the probe-specific variability due to temperature variation across a plate. It can be combined with other normalization techniques that correct for overall sample to sample variation from other technical sources that affect all probes to the same degree (for instance, variations in collected sample dilution).

As a parting note, the benefit obtained by temperature correction in the particle hybridization is compared to best-in-class normalization methods in other technologies. Microarrays are expensive and require substantial sample volumes, but they have the advantage of measuring hundreds of thousands of probes, providing many conserved targets between samples to assist in normalization. A white paper from Affymetrix [Affymetrix 2018] illustrates the uniformity that can be achieved between replicate samples. The CoV varies from a median of 8% to a median of 15%, depending on the algorithm used. (The raw CoV is not reported). The paper recommends their GCCN algorithm, which corrects for variable intensity signals between probes due to variable GC content between probes, and results in a median CoV of 15%.
Coefficient of variation between replicate samples using an Affymetrix microarray [Affymetrix2018], four data processing algorithms compared.

Corresponding data for the FirePlex hybridization assay used here is shown in Figure 58. A plate of 16 replicates of the cardiology amplicon was hybridized with the cardiology panel at 38.5 °C. The CoV of raw data, data normalized by geometric mean, data normalized by temperature correction, and data normalized by temperature correction followed by geometric mean are compared. Each bar shows the mean CoV of all probes expressed above background (median CoV is slightly lower than mean), while the dots are individual probe CoVs. The median CoV for raw data was 22%. Geometric mean normalization reduced the median CoV to 10%. Temperature correction was significantly better, reducing the median CoV to 5%. Adding geometric mean normalization to temperature correction resulted in a median CoV of 4%.
The hybridization assay was able to retain the advantages of high throughput and very low sample volume while achieving equal or better CoV to microarray data, using the methods developed in this thesis.

Figure 58. CoV between replicate samples using FirePlex hybridization assay, raw and normalized
Appendix 1. Definition of Terms

Abasic site: A locus on a DNA or RNA strand where the sugar on the sugar-phosphate backbone has no nucleobase bound

Amplicon: the result of applying polymerase chain reaction (PCR) to a mixture of DNA or RNA

Anova: analysis of variation, a statistical test to determine if different sample groups are significantly different from one another. The test compares variation between groups to variation within a group to determine significance

Argonaute: an enzyme in the cell cytoplasm that inhibits the transcription of mRNA transcripts using a microRNA guide strand for specificity

cDNA: complementary DNA: a DNA strand synthesized from single-stranded RNA, usually through the action of a reverse transcriptase enzyme. The single stranded DNA resulting from polymerase chain reaction is often referred to as cDNA, although strictly speaking only the first few strands are complementary to RNA, later strands are formed by DNA-DNA polymerization through the action of a DNA polymerization enzyme, usually Taq polymerase

C. elegans: abbreviation of Caenorhabditis elegans, a nematode widely used as a model organism in biological studies

Coefficient of variation (CoV): the standard deviation of a set of measurements, relative to its mean

Cross-hybridization: see non-specific binding

Cross-talk: see non-specific binding
Dicer: an enzyme in the cytoplasm that cleaves single-stranded microRNAs from a double-stranded pre-microRNA

DNA sequencing: the process of reading the base sequence of a DNA strand

Drosha: an enzyme in the cell nucleus that cleaves a pre-microRNA from a pri-microRNA that may contains multiple pre-microRNAs

DNA: deoxyribonucleic acid, a long polymeric molecule with a sugar-phosphate backbone and a series of varied bases that carries the genetic code from one generation to another

dsDNA: double-stranded DNA. DNA is normally found as a double helix, but can be separated into single strands

FFPE: formalin fixed paraffin embedded samples. Many biological samples are fixed for long term storage in formalin and embedded in paraffin.

Hybridization assay: a solution containing single-stranded DNA or RNA probes labeled in some way is incubated with a biological target, allowing the matching strands to bind one another. When unbound strands are washed away, the DNA/RNA content of the original solution can be determined by quantifying the labeled probes

Intercalating agent: a chemical compound that fits between adjacent base pairs in a nucleotide double helix

Matched probe: a DNA probe containing a sequence that is a complete reverse complement of its intended target, without substitutions, insertions or deletions

Melting temperature ($T_m$): when double stranded DNA or RNA is heated, at a certain temperature it will begin to spontaneously separate into two strands. At any temperature there is an equilibrium between the bound and unbound strands. The melting temperature
is the temperature at which half of strands remain bound (half of the lower concentration strand if they are present at different concentrations)

Microarray: a silicon chip with tens or hundreds of thousands of DNA or RNA oligomers bound to its surface, used for multiplex hybridization assays

MicroRNA: short single strands of RNA, 21-23 bases long, which regulate the expression of most genes

Mismatch probe: a DNA probe which contains an imperfect reverse complement to its target, containing either substitutions, insertions or deletions relative to a matched probe

mRNA: messenger RNA. Active genes are copied from DNA in the nucleus onto a temporary RNA transcript, which is exported to the cytoplasm and translated by the ribosome into a protein

Multiplex assay: an assay which measures multiple analytes at once in the same volume

NGS (next generation sequencing): technique to determine the sequence the entire genome or transcriptome of an organism in a matter of hours

Non-specific binding: a DNA probe complementary to a particular oligomer target in solution may bind to a different oligomer if the second oligomer has a closely related sequence

Normalization: the process of removing systematic bias and variation caused by technical artifacts while maintaining the important biological variation of interest in assay data

O. anatinus: abbreviation for Ornithorhynchus anatinus, or duck-billed platypus

Off-target binding: non-specific binding between a probe and an unintended target. Not to be confused with mismatch probe, where a mismatch is intentionally introduced to reduce melting temperature
Oligomer: a short sequence of DNA or RNA nucleotides, typically 10-100 long

On-target binding: specific binding between a probe and its intended target

Plex: the number of different targets a multiplex assay can detect simultaneously in one sample volume

PCR: polymerase chain reaction, a chemical reaction used to amplify selected DNA or RNA strands

RNA: ribonucleic acid, a long polymeric molecule with a sugar-phosphate backbone, similar to DNA but with a modified sugar and thymine bases replaced by guanine

RNAi: RNA interference, a phenomenon where introducing endogenous or exogenous strands of RNA into a cell inhibits expression of genes whose mRNA is complementary to the RNA introduced. microRNAs are an important class of RNAi molecules

RNase: an enzyme that digests RNA

RNaseq: DNA sequencing applied to the RNA content of the cell

Spike-in target: target oligomer introduced into a well to investigate response of different probes

ssDNA: single-stranded DNA

SYBR: an intercalating dye that preferentially binds double-stranded DNA. IUPAC formula N,N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine. Described at more length in Materials and Methods.
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