The Four Most Commonly Encountered Problems in Multiplex Panel Design

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Abstract

Genome sequencing methods have provided a treasure trove of information about the diversity of infectious diseases, genetic disorders, cancers, and human variation. There is a growing need for new methods that utilize genome sequence information to create better diagnostics that are more sensitive, more specific, and more tolerant to sequence variations. In addition, market forces have dictated the need to simultaneously measure multiple analytes in a single run, which in turn has led to an explosion in the number of multiplexed assays. This will only continue to increase in breadth and depth as molecular databases advance. In this white paper, we will discuss the four most frequently encountered problems in multiplex panel design: false negatives, false positives, coverage, and getting everything to "play well together". This white paper will also discuss a new product called *PanelPlex*™ and how it solves the four multiplex design problems.

Introduction

Developing multiplex panels is a complex endeavor. Primer design is constrained by the target sequences, target secondary structure, cross-hybridization interactions between primers and probes, different hybridization rates, false amplicons, varying solution conditions, temperature cycling parameters, and enzyme characteristics. As a result, poor primer design is the main reason for multiplex failures or suboptimal performance of panels.

As the size of the multiplex reaction increases, the complexity of primer design increases exponentially. For example, suppose you have 30 different panels and for each panel you have 10 primer design candidates for each panel; then the number of possible multiplex combinations is 10^{30} (!!!) so a brute force computational approach is not going to work. In addition, an empirical approach can only sample a small fraction of these possibilities and thus is destined to fail. Even "small-plex" reactions (e.g. 2-10 plex) can be difficult to design properly due to the enormous number of combinations of undesired interactions.

> "When there are multiple reactions working simultaneously the complexity increases exponentially. It gets to a point where it is impossible for a human being to rationally figure it out in any logical or realistic way. You can get lucky, or use trial and error but you are just fooling yourself."
>
> Arjang Hassibi, Ph.D., CEO, Insilixia.

PanelPlex utilizes sophisticated algorithms and massive cloud-based computing to resolve the complexity of multiplex panel primer design.

Multiplex Design Challenge #1:

False Negatives

Poor amplification is caused by a number of effects that can lead to low sensitivity or false negative assays (Figure 1). Some effects such as target secondary structure and undesired unimolecular extension reactions are present in even singleplex reactions. Other effects, such as false amplification and undesired primer-amplicon interactions get much worse with larger multiplex sizes.

Causes of False Negatives

A. Target secondary structure (both DNA and RNA targets)
B. False amplification (primer dimers and false amplicons) -depletes rxn. resources
C. Primer-amplicon interactions (exponentially worse for multiplex)
D. Unimolecular extension

(Also sequence variation - see "Coverage" section)

Figure 1: False negatives impact assay sensitivity.

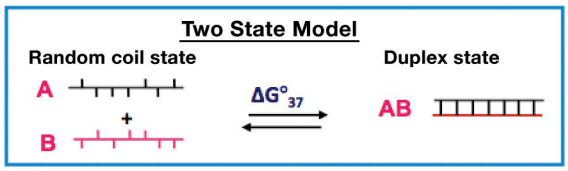
1A: Target secondary structure

Secondary structure of the target(s) can inhibit primer-binding thermodynamics and kinetics. This is particularly true for RNA targets, but often true as well for DNA targets. Such folding can cause uneven amplification since different targets or primers are not folded uniformly. A direct consequence of uneven amplification is that some amplicons (i.e. the ones with little secondary structure competition) can "take over" the multiplex by consuming the nucleotide triphosphates (NTPs), thereby preventing the amplification of other targets.

A second mechanism for shutting down the PCR is caused by amplicon reannealing of sense and antisense strands for the fast-amplified targets, which in turn preferentially bind to polymerase enzyme, thereby inhibiting the DNA polymerase from binding and extending other low-abundance primer-target hybrids.[1-2] The solution is to use software that can accurately predict target secondary structure, use a multi-state model to solve for the amount bound (discussed below), and thereby to identify thermodynamically accessible sites and result in uniform amplification efficiency for all the targets in the multiplex reaction.

Most users when considering primer hybridization use a two-state model (i.e. random coil state vs. duplex state) along with the nearest-neighbor parameters to predict the melting temperature, Tm (Figure 2, Left).[3] However, such a 2-state model neglects competing structure in both the target and the oligonucleotide.[2] A better approach is to account for all the competing secondary structures by using a multi-state coupled equilibrium model. For example, in Figure 2 (Right) the hybridization region of the target is green and involved in a hairpin structure that needs to be unfolded before probe hybridization can occur. In the example given, the probe can also fold (i.e. hairpin labeled "Folded Probe DNA"). The multi-state model properly accounts for these competing interactions, and solves for the amount bound in the hybridized duplex, which is directly proportional to the signal in the assay.

The 2-state model is not sufficient!

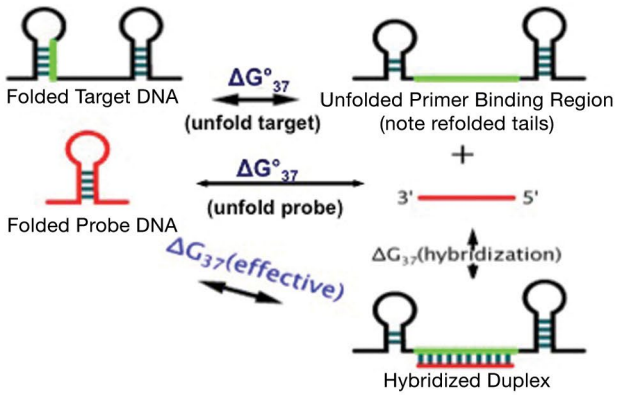


Problem: There is an energetic cost to break secondary structure.

- Folding causes low sensitivity
- Folding causesuneven amplification in multiplex PCR

Solution: Use software to solve for amount bound and identify the thermodynamically accessible sites.

Multistate Coupled Equilibrium Model



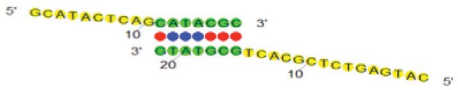
- ΔG (effective = solve coupled equilibria)
- Solve for the concentration of all species

Figure 2: (Above left) Two-state model, which neglects the effect of target and oligonucleotide secondary structure. (Above right) Multi-state coupled equilibrium model, which fully accounts for competing secondary structure in the target and oligos. The multi-state model provides the concentrations of all the species shown, including the amount bound hybridized duplex, which is directly proportional to assay signal.

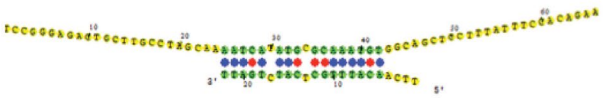
1B: False amplification

Other causes for false negatives include false amplifications due to primer dimers; false amplicons involving background genomes, primer-amplicon interactions and unimolecular polymerase extension. Sequence variation can also cause false negatives (discussed in section 3: Coverage). False amplification can also cause the shutdown of the multiplex PCR due to the depletion of primers and NTPs.

A. Primer dimers and false amplicons
- Depletes PCR primers and NTPs

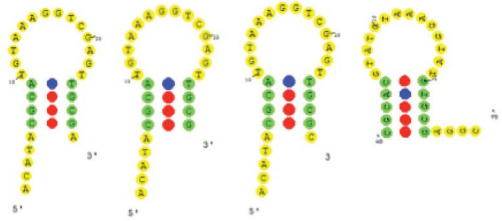


B. Primer-amplicon interactions
- Many more primers and amplicons
- Probability of occuring goes as the square of plex size
- Ruins the amplicon, probe doesn't bind



Example: Influenza primer binding to Zika amplicon

C. Unimolecular extension
- Which of these structures is extensible?



Considerations:
Not all mismatches are equally extensible
3'-exonuclease (proof reading)
Important to design 5'-end too!

Figure 3: False amplification reactions. A. Primer dimers due to hybridization at the 3' ends. B. Undesired primer-amplicon interactions (i.e. cross-hybridization). C. Undesired unimolecular extension reactions.

Formation of Primer Dimers

By accident, two primers may have pairing at their 3'-prime ends that a polymerase would extend. Such primer-dimer extension depletes that primer so that it does not properly amplify its desired target. Most primer design software packages try to avoid combinations of primers that form dimers. However, most such programs have not been informed by experimental data as to how much hybridization is sufficient for a polymerase to extend (e.g. how many base pair are required and how many mismatches can be tolerated?). Knowledge of such rules is critical to proper design and yet, to date, there are essentially no published data on these requirements for primer-dimer extension. DNA Software has acquired the necessary data to construct complete rules for which primer-dimer structures are extensible by a variety of polymerases (H. SantaLucia and J. SantaLucia, unpublished results).

Primer-Amplicon Interactions

Figure 3B shows an amplicon from the Zika virus, but there is a primer binding site for one of the other target primers in the multiplex (e.g. an influenza primer). The number of such primer-amplicon cross-hybridization interactions increases dramatically as the size of the multiplex increases. Such primer-amplicon interactions are deleterious to multiplex PCR because they consume primers and produce incorrect amplicons that would not allow for the proper TaqMan or Beacon probe to bind, thereby causing a false negative. Most multiplex design software packages do not account for this crucial effect.

Unimolecular Extension

Surprisingly, all the structures illustrated in Figure 3C are polymerase extensible, though some are easier to extend than others. The second structure is stable and its 3'-end is base paired, and most scientists know that such a structure is suboptimal for PCR. The third structure has a terminal AC mismatch, which is a substrate for extension by most polymerases. The first structure has an AA mismatch, which is usually not a substrate for many polymerases. However, if the polymerase has 3'-exonuclease activity, then such polymerases would remove the 3'-dangling end nucleotides and then extend.

3'-exonuclease activity is useful for high-fidelity replication and long amplicons, but in multiplex PCR such 3'-exonuclease activity is disastrous. This is because there are many cross-hybridization reactions most of which are not immediately extensible, but the presence of 3'-exonuclease would make essentially all cross-hybridization reactions extensible and thus ruin the multiplex. For multiplex applications it is essential to choose a polymerase that lacks 3'-exonuclease activity.

The final structure on the right of Figure 3C is the most surprising. Most users do not usually think about the importance of designing the 5'-end of an oligonucleotide. However, during amplification that 5'-end is copied into its complement and THAT sequence may fold into a structure that has its 3'-end hybridized – such structures cause the formation of inverted-repeat concatemer amplicons that shut down the PCR.

Multiplex Design Challenge #2:
False Positives

For a successful multiplex PCR, it is critical to minimize the formation of false-positive amplifications and false-positive signal formation. As the number of primers and amplicons increases in a multiplex reaction, mishybridization grows exponentially. Reducing false positives requires that primers are designed with maximum specificity.

Assay developers designing multiplex panels need to detect all the targets of interest in a panel, the inclusivity list, while making certain there are no false-positive amplicons from either the host genome (e.g. human genome) or from the genomes of organisms that are phylogenetically similar to the pathogen, which may be present in the sample from the host microbiome or from contaminating environmental organisms. We call the host genome the "background" and the near-neighbor organisms the "exclusivity list".

> "The challenge is having the detection of the intended target versus detection of nonintended targets; making sure every time you detect the target you want but only the target you want."
>
> Aude Argillier, Senior Scientist, Design and Assay Development
> Manchester, MDx Assay Development, R&D Europe

The most commonly used tool for checking primer specificity is *BLAST* (Basic Local Alignment Search Tool).[4] However, *BLAST* is meant to determine sequence similarity to infer common evolutionary ancestry and thereby infer function. To use *BLAST* for primer design, users need to use a workaround where they take the complement of the primer and then use *BLAST* to find hits that are similar to the primer complement.

This feature of *BLAST* of detecting sequence similarity immediately compromises the results for determining primer specificity. *BLAST* gives the wrong ranking of hits because it is based on an evolutionary scoring model, not based on the thermodynamics of hybridization. *BLAST* misses about 80% of the thermodynamically stable hits; thus, many of the sequences that could cause a false amplification reaction are not caught. *BLAST* is often used against a large database, such as the nucleotide (nt) database collection, and as a result gives too many irrelevant hits. Lastly, *BLAST* does not distinguish between hits that are extensible by polymerase versus those that are not. *BLAST* also does not have the capability of detecting all the amplicons that result from all the primer hits.

Sequence similarity does not equal thermodynamic stability

Figure 4 summarizes several of the deficiencies of *BLAST* for determining primer specificity. The workaround of using *BLAST* to scan for similarities to the primer complement, is tantamount to assuming that G-C base pairs are equal in stability to A-T base pairs, which is not correct. To accurately predict melting temperature and ΔG thermodynamics, a nearest-neighbor model is needed.[3]

BLAST scores all mismatches the same; it does not know that different mismatches have different stabilities. For example, a G-T mismatch, known to be stabilizing, is scored the same by *BLAST* as a destabilizing C-C mismatch.[5] These mismatches differ in thermodynamic equilibrium constant by more than a factor of 2,000, a huge effect. *BLAST* also scores gaps incorrectly; it scores them as insertions and deletion events, whereas they should be thought of as unpaired nucleotides. *BLAST* also ignores dangling ends, which are the extra nucleotides at the ends of a base pair duplex that contribute significantly. But the most important point is that a *BLAST* search has a minimum word length of seven consecutive perfect matches. If the hybridization does not contain seven consecutive perfect matches it will not be detected by *BLAST* (Figure 5).

DNA Software's *ThermoBLAST*-Cloud Edition has the speed and database capabilities of *BLAST*, but ranks hits on thermodynamic affinity, and has several other features (Figure 6). *ThermoBLAST* has completely different seeding and extension algorithms, and thus does not have the limitations found with *BLAST* such as minimum word length. *ThermoBLAST* analyzes hits for polymerase extensibility, and automatically detects all the amplicons.

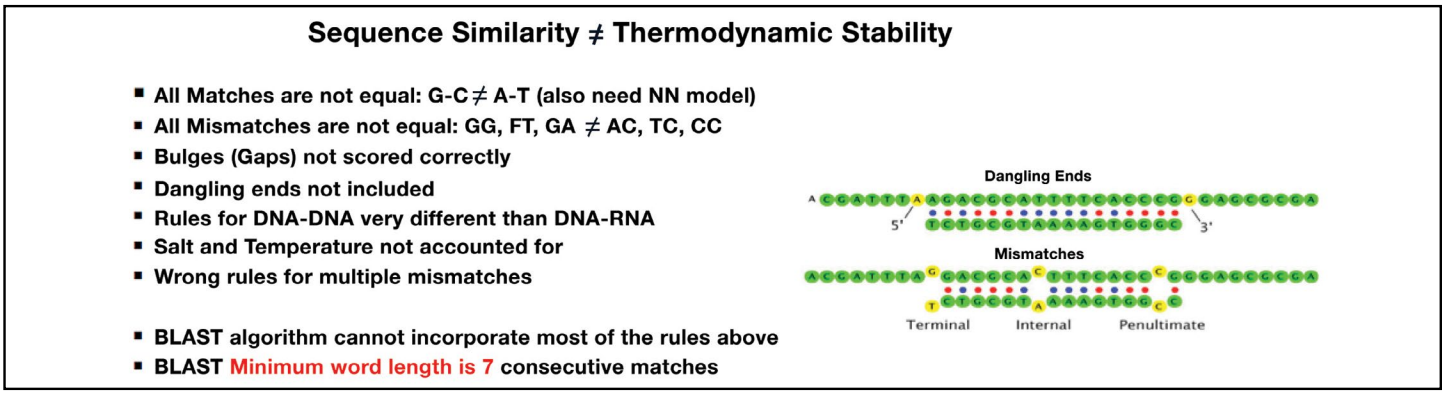


Figure 4: Effects that are not properly accounted for by *BLAST*.

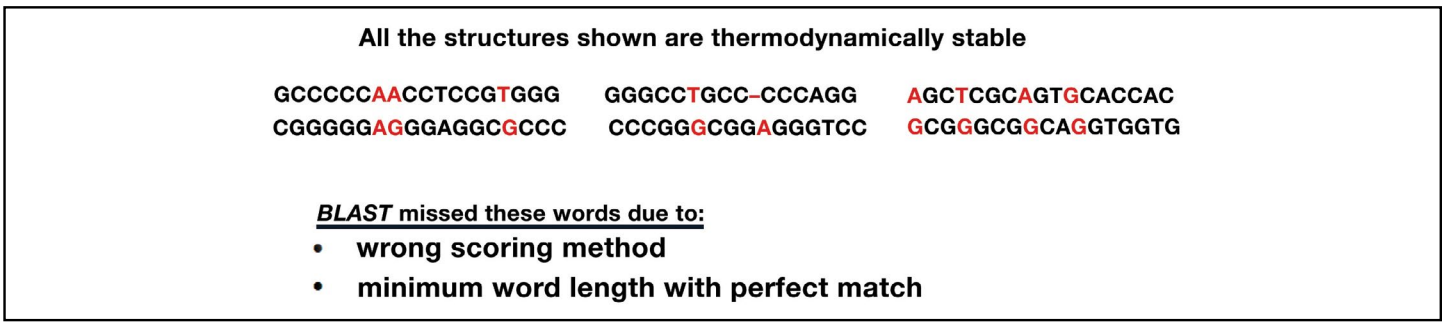


Figure 5: Typical structures missed by *BLAST*.

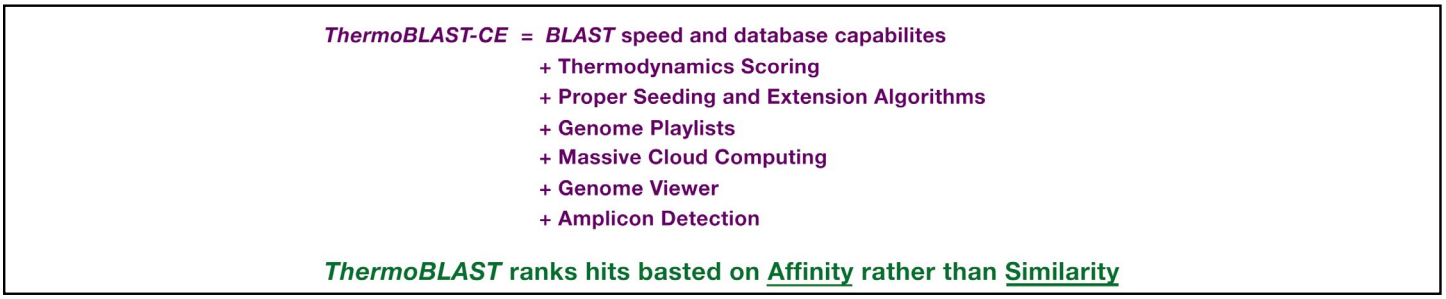


Figure 6: DNA Software's *ThermoBLAST*-Cloud Edition has the speed and database capabilities of *BLAST*, but ranks hits on thermodynamic affinity rather than sequence similarity.

Multiplex Design Challenge #3:
Coverage

The third multiplex design challenge is the issue of "coverage" and getting all the primers and probes to "play well together". We define two different types of coverage. The first type of coverage is for different variants of the target, which is called consensus design. We want a minimal number (preferably one) of primer sets that will bind to all variants of the given target, called the "inclusivity list". For example, there are currently 168 known variants of the Zika virus genome and we would like to find the minimum number of primers that would amplify a region of all 168 variants.

The second type of coverage is when you have multiple targets that are very different from each other. For example, suppose you wanted to amplify 100 different genes from the human genome simultaneously (e.g. all the genes from a pathway involved in cancer). Since they are all very different from each other that would require 100 primer sets to cover the whole multiplex.

Let's talk about consensus design and coverage in that context. Generally, we are interested in finding the regions that show the least variability in all members of the inclusivity list. Such low variability regions are likely to be locations where one could design primers that can bind to most, if not all, the members of the inclusivity list. Fundamentally, we are trying to answer the question, "Where should I target the design of my oligos in that huge virus?". Traditionally, people would use a multiple sequence alignment (MSA) algorithm to identify the conserved regions. We will see why that is not a very good approach. Another question that comes up is "What are the criteria for considering a target to be 'covered'?" Lastly, on the topic of multiplexing, how do you get all of the primers to work well together?

What's wrong with using a multiple sequence alignment for consensus design?

Figure 7 summarizes the issues with using a MSA for consensus design. The first problem with the MSA approach is that current computer hardware has insufficient memory and CPU. The sequences that are present in GenBank are growing exponentially every year, and the multiple sequence alignment algorithms do not scale well for large databases, both in terms of length of the sequences and the number of sequences. Most MSA algorithms cannot handle 1,000 different sequences that are the full-length genomes. To make the problem more computationally trackable, users will manually parse out a region of the targets and they will use the MSA for a limited subset of the inclusivity list. Such laborious manual steps are symptoms that the MSA is not the right tool for the job.

What's wrong with using multiple sequence alignment (MSA)?

1. MSA does not scale well for large databases (length and number of sequences).
2. Global Pairwise alignments are poor. Multiple alignments even worse. When you see 1 and 2 nt. Insertions and deletions in coding region, you know the alignment is wrong.
3. Sequence similarity is wrong metric.
4. Nucleotide sequence is information poor (only 4 letters).

MSA is the wrong approach!

Figure 7: The MSA approach is not appropriate for consensus design.

Another problem is the poor quality of the pairwise alignments that are used to make the multiple sequence alignment. Look at any multiple sequence alignment of DNA or RNA targets and look in the protein coding regions. Every single place where you see a single nucleotide insertion or deletion, or a two nucleotide insertion or deletion, you immediately know that that alignment cannot be correct, because these insertions or deletions would imply non-sense mutations that cause premature stop codons. You commonly see such non-sense insertions and deletions in MSA, which indicates that the alignments are junk.

Lastly, sequence similarity is the wrong metric. One of the reasons why the MSA alignments are not very good is that nucleotide sequences are information poor. You only have four different letters, A, C, G, and T. For viruses and bacteria, there is a lot of sequence variation. Because of the high level of sequence variation and low information content of DNA and RNA, the MSAs do not work that well, particularly for primer design. Instead, what we need to find is a region where thermodynamic binding complementarity is conserved across all members of the inclusivity list.

What does it mean to be covered?

What are the criteria for determining whether a primer will bind sufficiently to a target (i.e. one of the members of the inclusivity list) such that that target is efficiently amplified? To answer that question, we need to know a lot more about polymerase extensibility rules. How stable does a hybridization have to be in order for extension to occur? What mismatches are tolerable and yet retain extensibility and also high efficiency of amplification? Those are rules that most users do not know, but DNA Software has been investigating these topics experimentally for >15 years, and we have incorporated that information into our software. Above we discussed that *BLAST* is the wrong approach for such problems, and multiple sequence alignments the wrong approach; what is the right approach?

ThermoBLAST is very good at properly computing inclusivity coverage. It uses a proper thermodynamic scoring for duplex complementarity, it analyzes hits for polymerase extensibility, and it automatically detects the amplicons that are created by pairs of primers. Figure 8 shows a coverage table. For all the members of an inclusivity list, we can see how the primers that we designed cover them, and we can see the locations where there are mismatches (shown in RED). The primer designs have been optimized to put these mismatches in places that are tolerable by DNA polymerases.

Target Name	Accession	FP region	Probe region	RP region	RT region
target 1	KX694532	GACCACTCAGACTC	GCAGCATCGACGACT	ATCATTACCGGCAT	TGAGTCGGCATCGCAT
target 2	KX838904	GACGACTAAGACTC	GCAGCATCGACGATT	ATCATTACCAGCAT	TGAGTCGGCATCGCAT
target 3	KY014321	GACGACTCAGTCTC	GCAGCATCGACGACT	ATCATTACCGGCAT	TGAGTCGGCATCGAAT
target 4	KU681082	GACGACTCAGACTC	GCAGCATCGACGACT	AACATCACCGGCAT	TGAGTCGGCATCGCAT

Figure 8: Coverage table. Mismatched locations are shown in Red. *PanelPlex* produces a similar output.

PanelPlex Software for Consensus MultiPlex Design

PanelPlex is a solution from DNA Software that incorporates all of the design principles described in this white paper. The automated-design process is easy to use and eliminates iterative trial-and-error design cycles, greatly reducing developmental time and cost. *PanelPlex* combines the strengths of state-of-the-art modeling, advanced search algorithms, and massive cloud computing to deliver highly optimized designs in a few hours. *PanelPlex* consists of 4 compute engine modules (i.e. *DESIGNER*, *TargAn*, *ThermoBLAST*, and *MultiPick*) integrated into one interface (Figure 9).

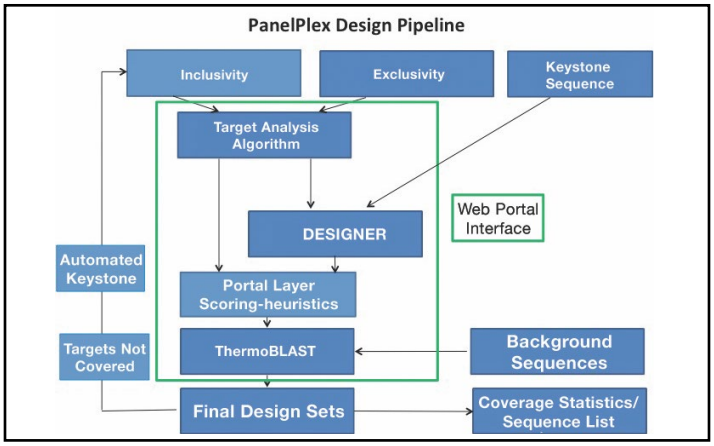


Figure 9: *PanelPlex* integrates four compute engines into one easy-to-use interface. The user only needs to input the inclusivity, exclusivity, and background playlists as well as the primer concentrations, annealing temperature, and buffer conditions, and *PanelPlex* automatically does the rest.

DESIGNER is the core engine for creating the primer and probe candidates and computing the thermodynamic aspects of design, such as target unimolecular folding, primer folding, bimolecular hybridization, and solving the multi-state coupled equilibria for the amount bound for the desired bimolecular duplex. *DESIGNER* designs all primers and probes against one target sequence, called the "keystone". Thus, all the primer designs are a perfect match to the keystone and the top designs are kept for further analysis. However, the primer designs could have mismatches to other members of the inclusivity list.

To minimize that mismatch possibility, a target analysis algorithm (i.e. *TargAn*) precomputes the coverage scores for forward and reverse primers and inputs that information into *DESIGNER*. This is accomplished by breaking up the keystone sequence into all of its 21mer sub-sequences, and using *ThermoBLAST* to determine how well each of those 21mers covers all the members of the inclusivity list and thereby generates a coverage score and an exclusivity score. *PanelPlex* combines each primer score with the *TargAn* score for the 21mer that has the same 3'-end as each primer candidate from *DESIGNER*, thereby favoring primers that have high coverage of the inclusivity list and low amounts of false hybridization to the exclusivity set.

In addition to thermodynamic scoring based on the amount bound (using the multi-state coupled equilibrium model), each primer and probe design is analyzed for heuristic properties such as sequence complexity, polyG test, oligo length penalty, etc. Each of the above effects are multiplied by weighting factors and combined into an overall score for each primer/ probe set (Figure 10).

$$PrimerScore = PercentBound * CoverageScore - \sum_{i=1}^{N} W_i P_i$$

Figure 10: Heuristic properties are multiplied by weighting factors and combined into an overall score. Where i is an index designating each of the N different penalty terms (or bonuses), Wi is the weight of each scoring term, and Pi is the penalty for each scoring term. The weighting factors are user adjustable.

PanelPlex performs a mixing and matching of primer and probe candidates to make "solution sets" consisting of forward primer, probe, and reverse primer (and optionally for RNA targets, a reverse transcription primer). The score of these solutions is calculated by averaging the scores of each of the oligos and adding in additional scoring terms, such as the amplicon length, amplicon folding penalty, etc.

The oligos in the solution sets are *ThermoBLASTed* against the combined exclusivity and background sequences. Penalties are then applied based upon the number of hits for each primer, and on the false amplicons formed by pairwise combinations of primer hits. This step maximizes specificity by penalizing primers that have strong off-target hybridizations.

The actual inclusivity coverage of the final candidate primer pairs is then determined by *ThermoBLAST*. Due to sequence variation within the inclusivity list, some targets may not be covered by a single set of primers. These non-covered targets are resubmitted. A new keystone sequence is automatically chosen from the new list and a new set of primers designed to improve the coverage. Multiple iterations are performed to find the best multiplexed primers to maximally cover the inclusivity but without interfering with one another.

Applications of *PanelPlex* for Viruses, Bacteria, mRNAs, SNP Sites, etc.

PanelPlex can design primers in three modes: Whole Target, Design Region, or Junction. The "Whole Target" option allows for inclusivity panels where each target is less than 40,000 nucleotides. For Whole Target, *PanelPlex* designs primers across the entire target and outputs primer/probe combinations that utilize the most conserved and most thermodynamically accessible sites. The Whole Target option is applicable to most viruses, single genes from higher organisms, or parsed out intergenic regions.

In the "Design Region" mode, *PanelPlex* allows the user to specify the range of nucleotides for design within a larger target (such as a region within a bacterium, eukaryotic pathogen, or even a human chromosome). Importantly, the user only needs to specify the design region for the keystone sequence, *PanelPlex* then automatically determines the corresponding locations for the design regions in all members of the inclusivity list.

The "Junction" option allows the user to specify a single location within the keystone of the inclusivity list and automatically sets up the design regions of the forward primer, probe, and reverse primer (or RT primer). The Junction option has applications for targeting the design to an exon-exon boundary in spliced mRNA (helps avoid amplification of genomic DNA), targeting to a recombinant DNA insertion site, or an SNP site.

Multiplex Design Challenge #4:
Getting everything to "play well together"

Multiplex PCR is a nonlinear complex system with many interacting variables, and there are myriad reasons for failure. In multiplex PCR, there are many targets and thousands of primer candidates, which results in a combinatorial explosion in the number of cross-hybridization interactions and false amplicons. It is impossible for a human to account for all such interactions. In addition, if the singleplex reactions are not designed correctly, then unequal amplification rates can also affect the dynamics of the multiplex reaction (that can be minimized using the principles from section 1). Finding the combinations of primers that do not interfere with one another is an optimization problem with a multidimensional landscape with a huge number of possibilities.

The Typical Empirical Approach to Multiplex Design

> "A lot of time and effort goes into designing assays yet a lot are on version 2 or 3. Sometimes after a product is introduced you start seeing false negatives and portions of the assay require rework."
>
> Jaime Prout, Developmental Scientist II, Beckman Coulter

Figure 11 summarizes the typical empirical approach. Most researchers resort to the empirical approach because they do not recognize that there is an alternative. Such an empirical approach often starts with optimization of individual singleplexes. These singleplexes are then combined into larger and larger multiplexes until a failure is identified (i.e. a member of the multiplex does not amplify efficiently or false amplicons are produced). The members of the multiplex are then changed (without knowing the actual reason why they failed) and the modified multiplex is then tested again.

This "linear 1-dimensional" search does not work. The reason why this fails is that Multiplex PCR is NOT a linear system! Instead, multiplex PCR is a non-linear multidimensional landscape with complex interactions among the variables. It is not unusual to have a 7-plex PCR assay working only to have it completely fail when an eighth primer pair is added. Efforts to make higher multiplexes often completely fail even with highly qualified teams expending large resources. The process is much like the arcade game "Whack-a-mole" where you knock down one problem just to have another problem pop up. This is practically the definition of insanity!

Empirical approach:

1. **Start with optimization of individual singleplexes.**
2. **Try to combine the singleplexes.**
3. **Then fix singleplexes that do not work together, without knowing WHY failure occured.**

Typically, it takes a Ph.D. level scientist with associates 3-6 months to get a 10-plex PCR to work.

It is not unusual to have a 7-plex PCR working only to have it completely fail when an 8th primer pair is added.

Why does this fail?

1. **Multiplex PCR is a complex system, with many interacting variables.**
2. **Cross-hybridization can cause artifacts.**
3. **The individual PCRs are optimized to work at different conditions.**
4. **The amplicons are amplified at different rates, such that one amplicon can take over the reaction.**

Figure 11: Failure of the empirical optimization approach. This one-dimensional approach is like "Whack-a-mole" and is doomed for multiple reasons.

Computational Approach to Multiplex Design

Let's think about the computational scale of multiplexing. Suppose you have 30 different panels and for each panel you have 10 primer design candidates that work in singleplex, then the number of possible multiplex combinations is 10^{30} (!!!). Thus, a brute force computational approach is not going to work. We need a 21st century approach to solve this kind of problem.

Figure 12 shows the design pipeline used by the *MultiPick* algorithm (i.e. a module in *PanelPlex* for multiplex design). The core algorithm (contained in the box "Find Superior Multiplex Combinations") uses a depth-first search with pruning and is implemented in cache memory to enhance computational efficiency. The algorithm is exhaustive and is guaranteed to produce the top N solutions out of all of the possible multiplex combinations.

The pruning is a key part of the algorithm that makes it so the actual number of multiplex candidates that are checked is much less than the 10^{30} possible combinations. This elegantly solves the combinatorial explosion. Each multiplex candidate is scored based on its combined singleplex scores (contains the singleplex thermodynamics metrics and heuristics (refer to Figure 9)). The *MultiPick* algorithm also excludes all primer-dimers and all primer-amplicon interactions and penalizes all the primers in a given multiplex candidate for any false amplicons against the background list (Figure 12).

MultiPick also has the capability of "custom constraints" that can be used to force the algorithm to use designs that are known to be high performing and to exclude primer designs that have poor performance – that capability is very useful for minimizing the experimental iterations so that high performing assays are developed quickly.

Summary and Outlook

PanelPlex represents the best of what DNA Software is known for: primer design for difficult targets, high-level multiplexing, computational assay optimization and minimizing of trial-and-error to help companies more efficiently build better diagnostics. *PanelPlex* is easy to use and provides completely automated design of multiplex PCR with unprecedented sensitivity, specificity, and coverage. *PanelPlex* is the result of more than 15 years of investigation into the mechanism of PCR and careful experiments to identify the sources of failure of PCR. *PanelPlex* has been rigorously validated for the detection of numerous viruses, bacteria and human targets.

The current version of *PanelPlex* is focused on single-panel consensus design with low-level multiplexing (under 10-plex) for applications such as: infectious disease variants for bacteria, viruses, human genomic targets, and mRNA profiling. The *MultiPick* algorithm is complete and experimentally validated on a variety of multiplexes in the 30-100 plex range, but *MultiPick* is currently run in console mode and is only for internal use by DNA Software. A new version of *PanelPlex* will incorporate *MultiPick* and will allow for large scale multiplexes and is due for release by September, 2017. This algorithm is useful for applications such as target enrichment for next-generation sequencing and highly-multiplexed molecular diagnostics. We are also developing a version of *PanelPlex* to distinguish SNP variants (due for release by the end of 2017).

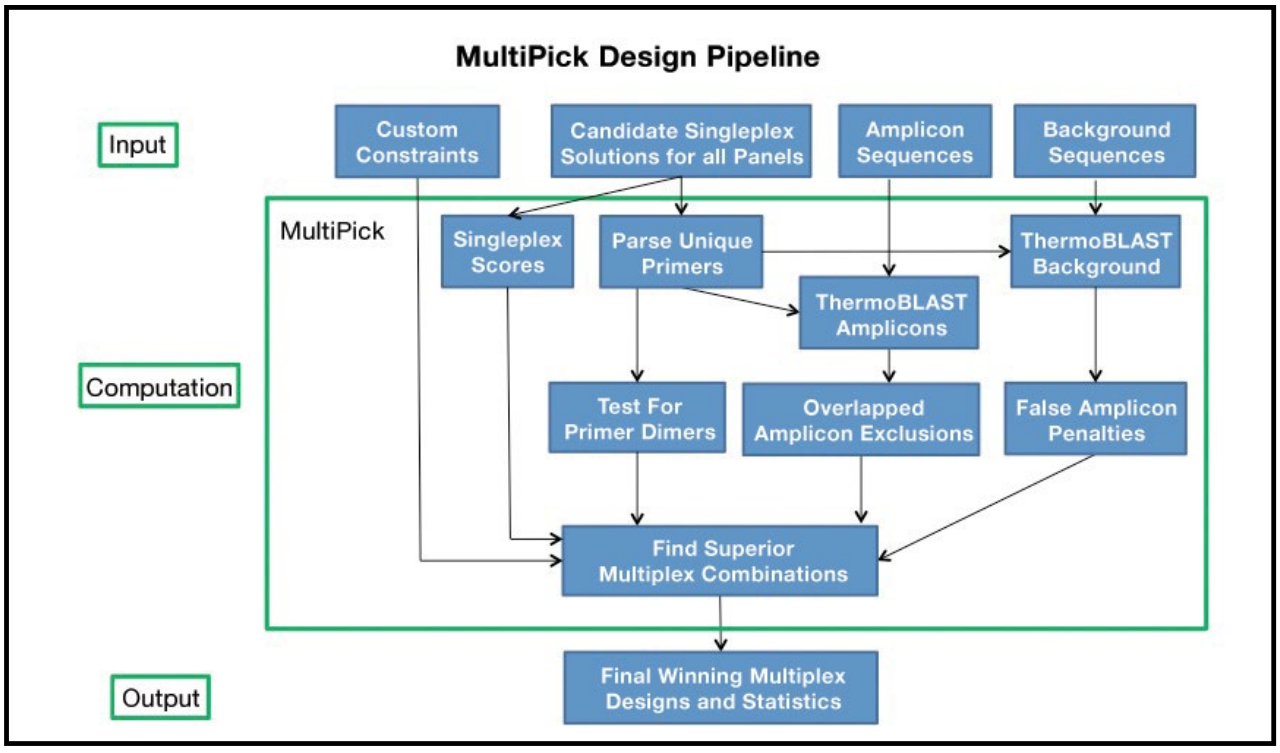


Figure 12: Design pipeline for *MultiPick*.

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