VariantPro™ Capture Technology

LC Sciences

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VariantPro™ Capture Technology

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Introduction

Somatic mutations such as SNPs are unlikely to be best detected using regular whole genome sequencing and genotyping by sequencing (GBS) in any large genome species requires reduction of genome complexity.

Target enrichment has become a significant tool in focused genomic studies. Multiplexing PCR is a simple way to extract genomic regions of interest for various genetic tests such as variant analysis or genotyping.

Currently available methods for targeted next-generation sequencing assays involve multiple hands-on steps and have limitations with respect to sensitivity, specificity, uniformity, and sequence drop out.

LC Sciences has developed a new multiplexing PCR approach with a significantly simplified workflow and significantly improved robustness. When applied to sequencing target enrichment application, the workflow for producing an amplicon sequencing library involves only one hands-on step and one PCR run.

Sequencing library adapters, sample barcodes and molecular tags are all incorporated during this single PCR run.

The approach is designed to require low sample input and to produce superior amplicon uniformity and sequence specificity.

The approach involves a novel primer design and a proprietary reaction composition. A PCR run consists of two functionally separated reaction phases, namely target capture and library amplification, without any hands-on step in between.

Multiple Innovations

VariantPro™ capture technology is a novel multiplex PCR technology that combines major innovations to facilitate simple operation and high performance in the preparation of targeted sequencing libraries. The innovations include

1. **Relay-PCR™** - an elegant PCR method that combines multiple target capture and monoplex PCR amplification into a single reaction and therefore simplifies targeted sequencing library preparation.

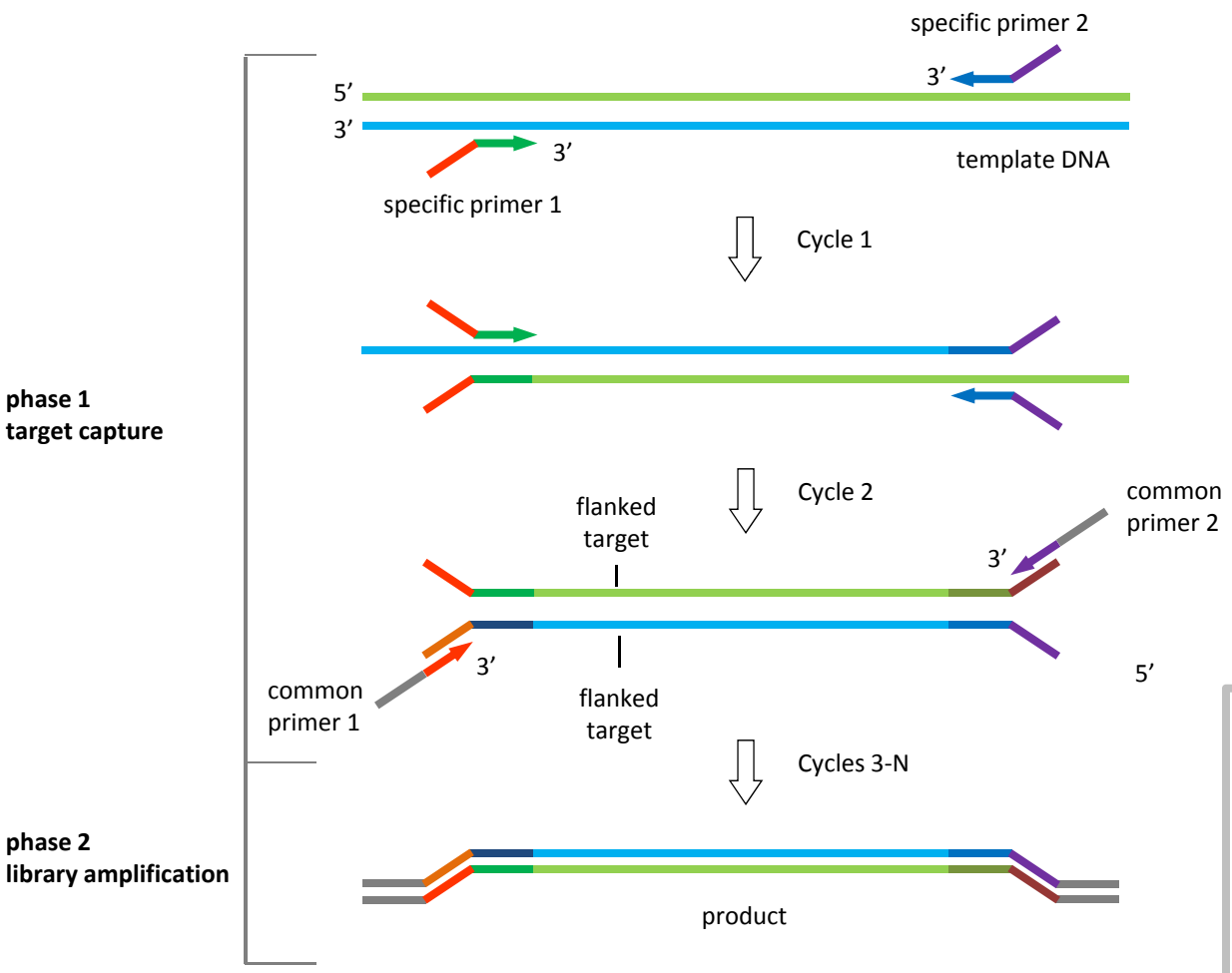
2. **Omega Primer™** - is a novel segmented primer design that combines the robustness advantage of long hybridization probes with the specificity advantage of short PCR primers.

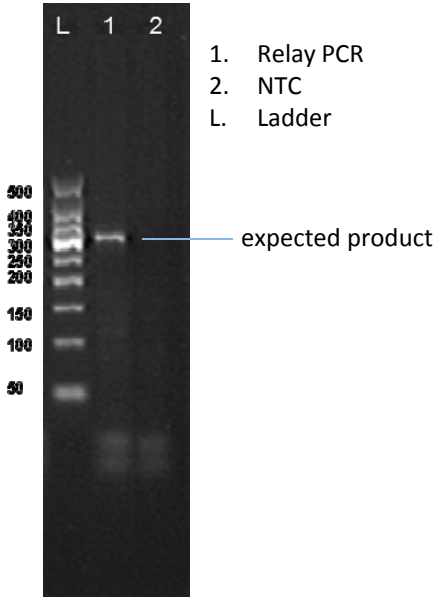
3. **Molecular tag** – is used to identify individual PCR template molecules. Relay-PCR™ and Omega Primer™ in combination provides a perfect vehicle to carry the molecular tags into amplicons.

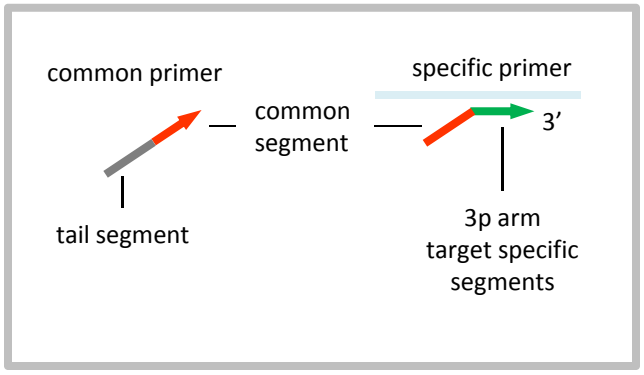
4. **New Design Principles -** We have developed new approaches to perform quantitative prediction of PCR primer performance and incorporated into design software.

Relay-PCR™

Relay-PCR™ is a form of multiplex PCR that combines multiple pairs of target specific primers and one pair of common primers into a single tube. The concentrations of the specific primers are significantly lower than that of common primers, by orders of magnitudes. Thus, as polymerase cyclic reactions proceed, primer competition induces an automatic switch from specific primer initiated target capture reactions to common primer initiated amplification reactions.







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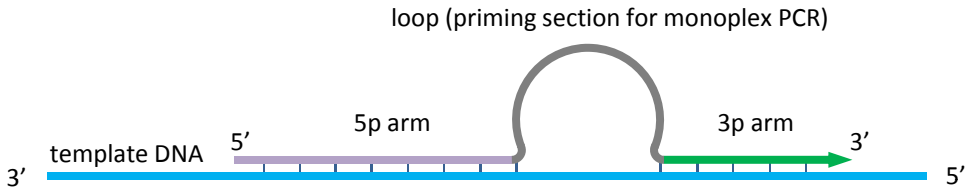
Omega Primers™

An omega primer consists of three functional sections:

1. A 5p arm functions as an anchor attaching to a DNA template.
2. A 3p arm functions as an initiator in a polymerase extension reaction.
3. A loop separates the two arms. In a relay PCR the loop is used as a priming section for monoplex PCR.



This design has fundamental advantages over conventional primers in the following aspects

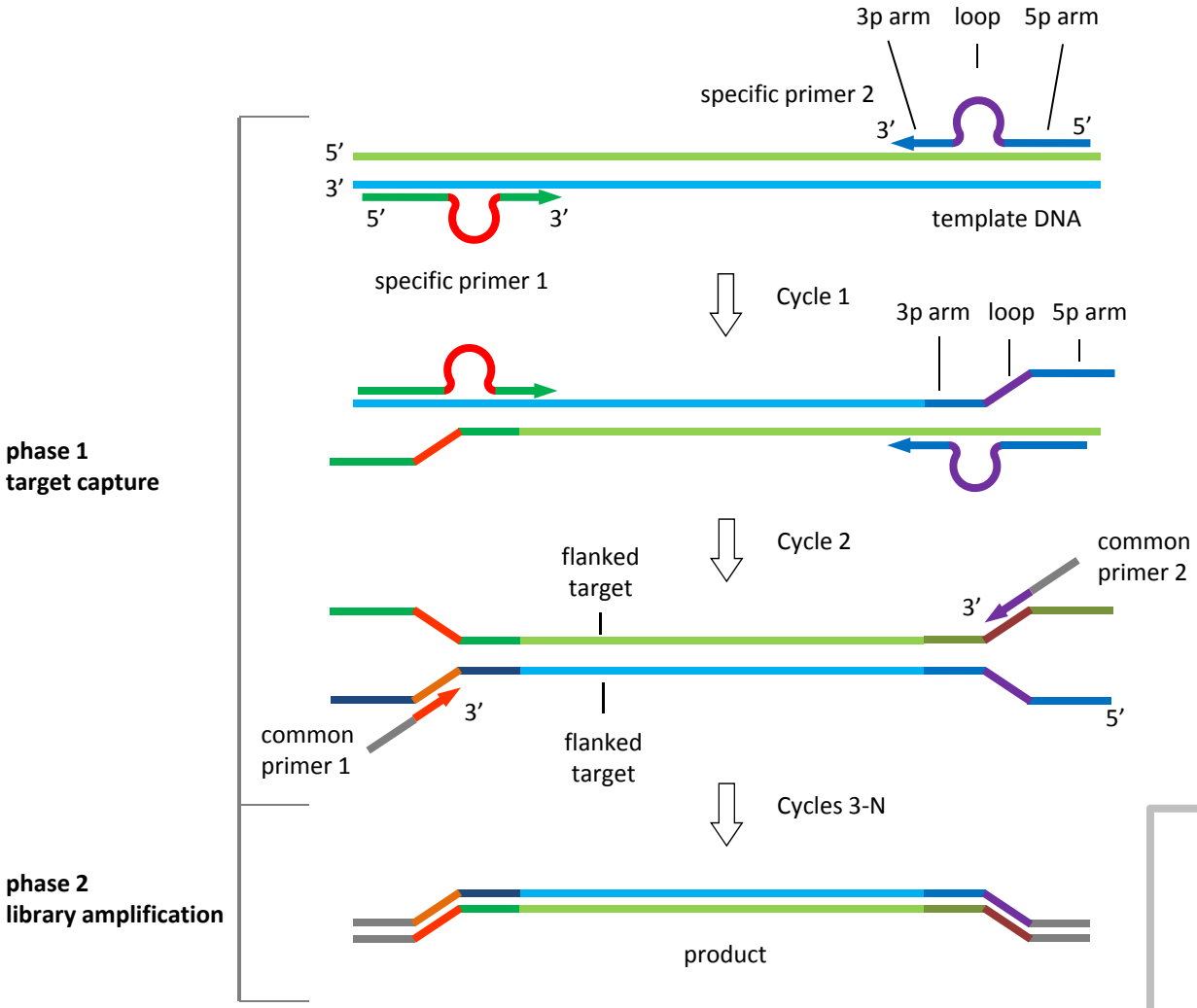
1. Specificity – A viable omega primer requires specific bindings of both 3p arm and 5p arm. This requirement results in a significant increase of specificity as compared to conventional primers (a calculation will be provided in a later slide).

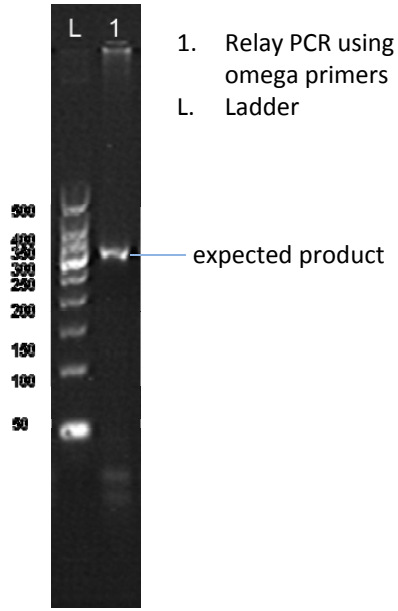
2. Ability to tolerate certain template variations – In some applications, the 5p arm is intentionally made long so that it stays hybridized to a template even when a mismatch (e.g. a SNP) is present in the template.

3. Reduced primer dimer formation – Amplifiable primer-primer dimmers are formed only when 3p arms of two primers are cross-hybridized with at least one primer having its 3' end complementarily bind to the other primer. Omega primers generally have much shorter 3p arms (16 nt) than that of regular primers (35 nt) therefore have statistically a much less probability of forming the amplifiable primer-primer dimers.

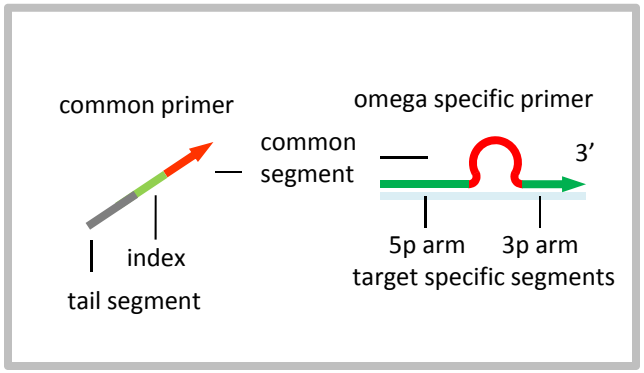
4. Higher % of native sequence in an amplicon – An amplicon sequencing read consists of native and primer sequence sections. Primer 3p arms are incorporated into amplicons becoming the primer sections. Since omega primers have much shorter 3p arms (~16 nt) than that of regular primers (~35 nt), omega primer produced amplicons have higher percentage of native sequence sections.

Combine Relay-PCR™ & Omega Primer™





1. Relay PCR using omega primers
L. Ladder



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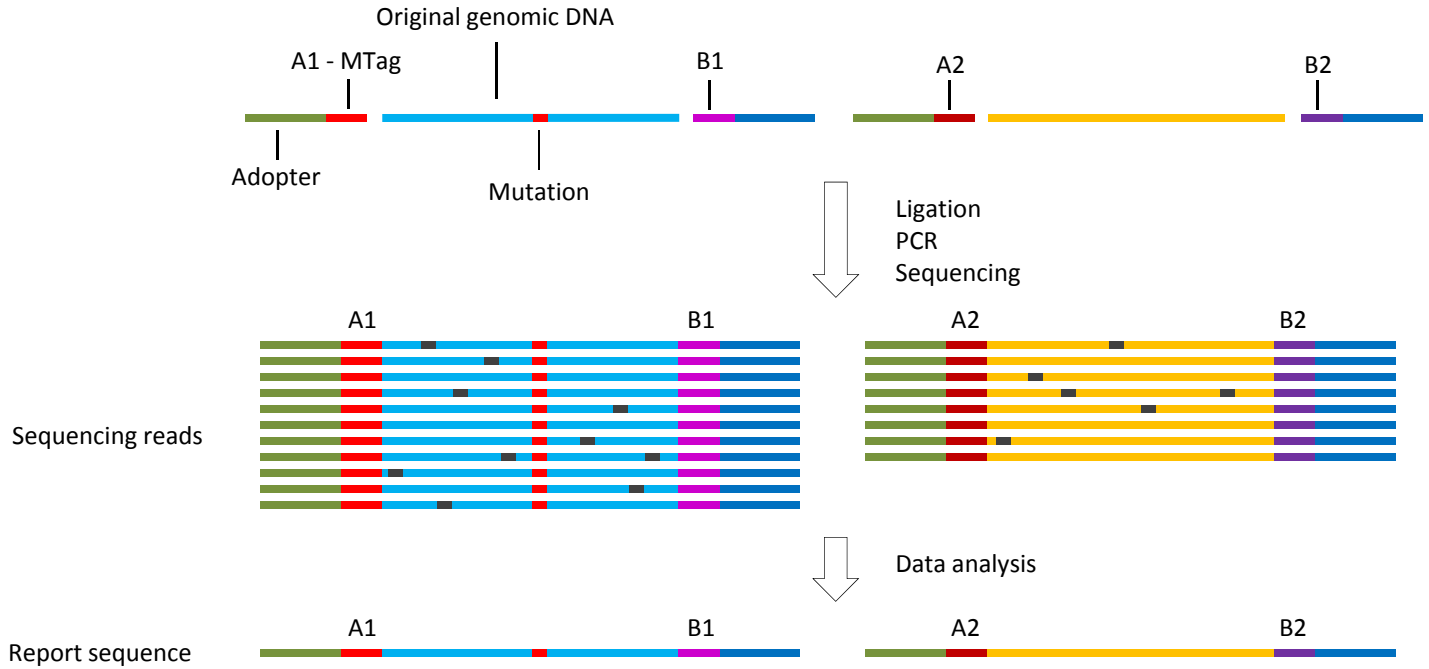
Molecular Tags

In NGS applications the use of molecular tags (MTag) to label individual genomic DNA templates prior to PCR amplification provides valuable benefits.

1. Original DNA molecules are uniquely identified, counted, and/or analyzed.
2. PCR and sequencing induced biases and errors are detected and then removed analytically, reducing false positive rate.
3. Variant calling accuracy is increased resulting in improved low allele frequency detection limit.

The MTags are commonly introduced through adapter ligation



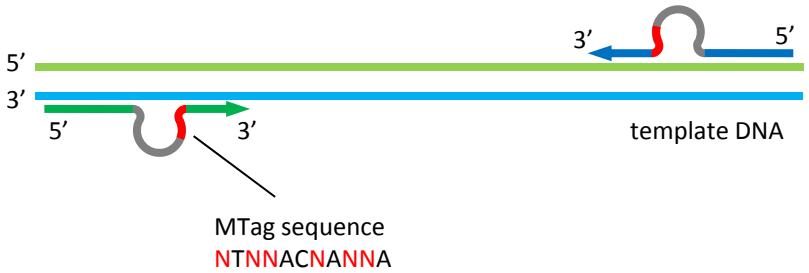
Molecular Tag Design Considerations

1. In a Relay-PCR™ specific primers participate only the first two extension reactions. Their participations in amplification reactions are negligible. Therefore, they are the right vehicles to deliver the molecular tags into amplicon seeds.

2. Six quaternarily degenerated nucleotides are used in each tag forming 4^6=4,096 unique tag sequences which would then form 4^{6x2}=16,777,216 unique paired tag sequences. This size is sufficient to largely avoid having 2 starting DNA copies carrying the same tag sequence at an input level of 10 ng human genomic DNA (3,000 copies).

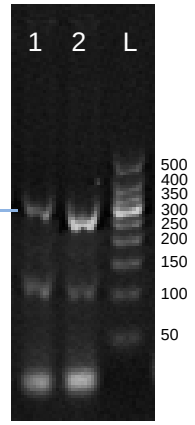
3. Non-degenerate nucleotides are mixed with the degenerate nucleotides in each MTag to make the tag somewhat rigid. The type and the arrangement of the non-degenerate nucleotides are carefully designed so as to minimize the probabilities of folding formation within individual Omega primers and cross-hybridization between the tag sequences and all primers involved.

Introduction of MTags into amplicon seeds though Omega primers



1. With MTag
2. Without MTag
L. Ladder

expected product



New Primer Design Principles

A significant aspect of PCR primer design is to ensure a sufficient primer annealing efficiency. The annealing efficiency at annealing step can determine PCR yield.

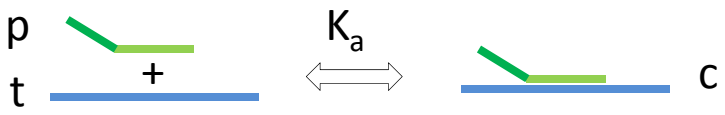
- We have developed primer/amplicon design tools based on **rigorous** chemical equilibrium equations and thermodynamic calculations which are capable of producing quantitative predictions of primer annealing efficiency. Our design ensures the annealing efficiency of each primer to reach a threshold level (e.g. >0.95) at a predetermined annealing temperature.

- As comparison, current PCR primer designs are generally based on melting temperature (Tm) calculation. The method is largely **empirical** and does not provide quantitative assessment of primer-template interactions at various PCR stages. For example, by knowing the Tm alone of a primer sequence one may not know exactly what annealing efficiency will be at a predetermined annealing temperature.

LC Primer Design – Equilibrium Equation

Hybridization reaction is a simple second order reaction in which two reactants react forming one product.

$$t + p \overset{K_a}{\Leftrightarrow} c$$



where t, p, and c are template, primer, and primer-template complex. Let f_a be the association fraction of the template to a corresponding primer, which we usually annealing efficiency.

Mass balance equations

$C_c = f_a \times C_{to}$, equilibrium concentration of primer-template complex

$C_t = (1 - f_a) \times C_{to}$, equilibrium concentration of template

$C_p = C_{p0} - f_a \times C_{to}$, equilibrium concentration of template

Reaction equilibrium equation

$$K_a = \frac{C_c}{C_t \times C_p} = \frac{f_a}{(1 - f_a) \times (C_{p0} - f_a \times C_{t0})}$$

Thermodynamic calculation

$$K_a = e^{\frac{-\Delta G}{RT}}$$

where, ΔG is free energy primer-template hybridization, R is gas constant, and T is temperature.

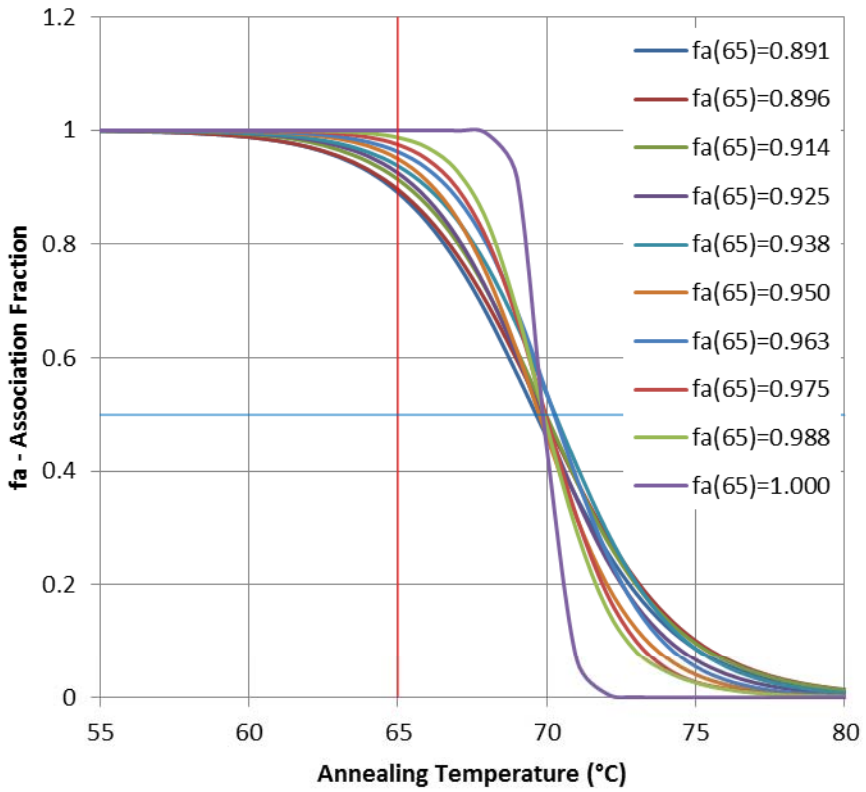
$$f_a \approx \frac{C_{p0} \times K_a}{1 + C_{p0} \times K_a}, when\ f_a C_{t0} \ll C_{p0}$$

This method was first used by Miura, F. et al of University of Tokyo and colleagues in Japan on PCR primer performance prediction (2005) Bioinformatics 21 4363

Conventional Primer Design – Tm Method

DNA sequence Tm is defined as the temperature of an equilibrium system in which 50% of the complementary sequence pairs remain associated to each other as double strand sequences.

Tm versus primer-template association fraction (fa)



10 primer sequences are selected from human PIK3CA12, LMTK2, LTK, TP53 genes with a narrow Tm range of 69.6 to 70.3 °C.

fa varies from 0.891 to 1.000 at an annealing temperature of 65 °C. After 20 PCR cycles, the contributions to the final product yields from the primers would vary from 0.891^{20} = 0.099 to 1, which is more than 10-fold variation.

PCR buffer composition is cPrm=500 nM, cTpl=1 fM, Tanneal=65°C, cNa=0.06M, and cMg=0.0015M. GC contents of the sequences are between 0.2 and 0.8.

For multiplex PCR applications Tm method is clearly inadequate for designing primers to producing uniform amplicon coverage.

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Conventional vs Relay Multiplex PCR

Conventional multiplex PCR is known to have two main issues.

1. Amplicon yield (f_{amp}) variations among different targets
2. Primer dimer formation

The causes of these issues are associated with the following.

1. Amplicon yield variation is determined by
   a. Primer initiation efficiency ($f_{int} \leq f_a$) – different primers may have different initiation efficiencies
   b. Primer extension efficiency (f_{ext}) – template sequences of high AT contents and/or strong secondary structures can interrupt the extension

$$f_{amp} = {f_{int}}^{n} \times {f_{ext}}^{n}, \quad n: PCR\ cycle\ number, f_{int} \leq 1, f_{ext} \leq 1$$

2. Primer-dimer formation ($C_{prm\text{-}prm}$) is determined by
   a. Primer sequence cross-hybridization reaction ($K_{prm,i-prm,j}$)
   b. Primer concentration (C_{prm})

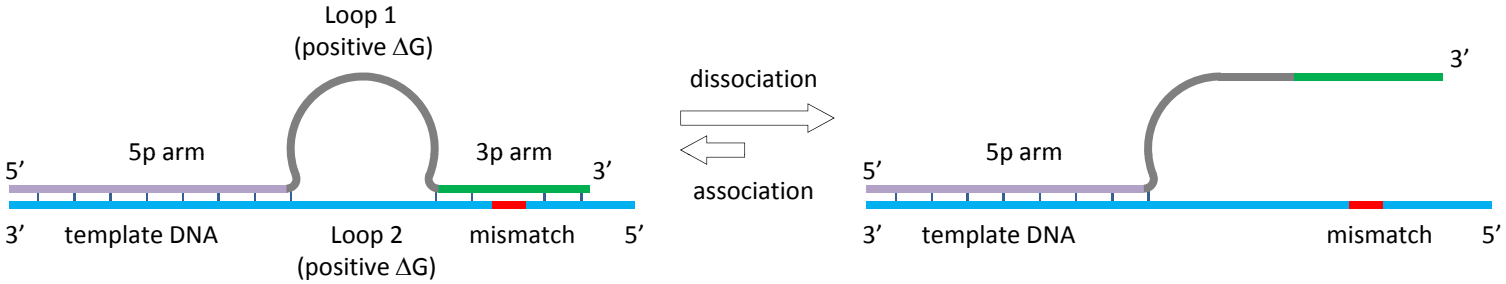
$$C_{prm-prm} = \sum K_{prm,i-prm,j} \times C_{prm,i} \times C_{prm,j}$$

Relay multiplex PCR uses much lower concentrations of specific primers and thus essentially excludes them from contributing to amplification and quadratically reduces dimer formation

$$f_{amp} = f_{int} \times {f_{ext}}^{n}$$

Specificity Enhancement - Omega Primer™

An omega primers is designed to hybridize with corresponding template forming a stable looped structure. Mismatches between 3p arm and a template destabilize the looped structure and cause the dissociation of the 3p arm from the template and reduces the probability of polymerase extension of the primer. Additionally, the 3p arm is short and by itself does not provide a sufficient inter-molecular binding with a template and does not sustain any significant polymerase extension reactions.



An extension reaction of an omega primer requires hybridization of both 5p arm and 3p arm. In a typical design, the binding efficiency of the 5p arm to a template is the same as that of priming segment of a regular primer. The specificity enhancement of the omega primer is attributed to the requirement of additional recognition site for the 3p arm. In a typical design, median 3p arm length is 16. Assume that minimum 12 out of 16 template nucleotides must match in order to achieve a significant binding. We have the probability of significant but wrong binding in a complete random sequence:

$$P = \frac{3^4 C(16,12) + 3^3 C(16,13) + 3^2 C(16,14) + 3^1 C(16,15)}{4^{16}} = 3.8 \times 10^{-5}$$

This means more than 1/P = 26,000 times specificity enhancement

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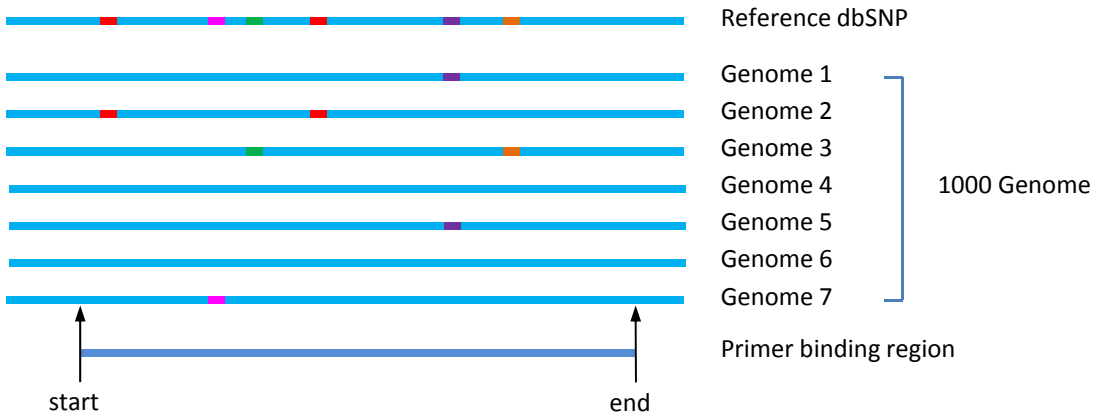
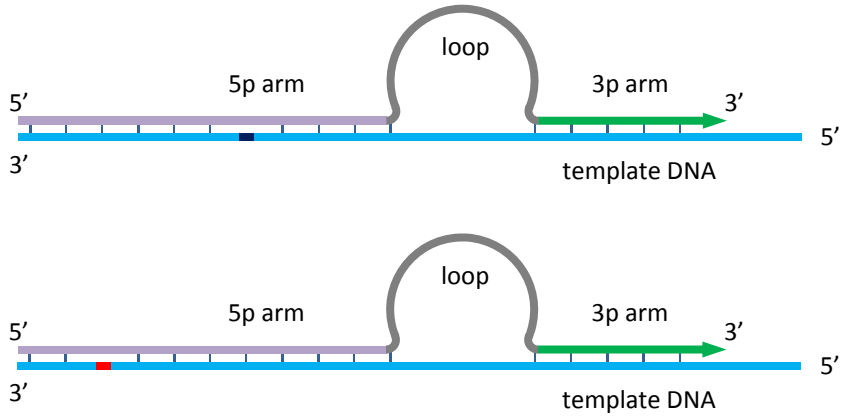
Primer design in variant regions

There are 6 million high allele frequency (>1%) SNPs in human genome of 3 billion bp long, an average of 1 SNP in every 50 bp. Omega primers are designed to work well even when they have to be placed in SNP containing regions. For human DNA sequences, omega primer 5p arms are designed to tolerate SNPs above a predetermined allele frequency of 1000 Genome database as well as user provided pathogenic variants. This approach allows us to design assays of predictable population coverage.



5p arms are intentionally made long enough to sustain the presence of SNPs in template. **This approach combines the variant tolerant advantage of hybridization methods with the sequence specificity advantage of PCR methods.**



Minimize allele drop-out

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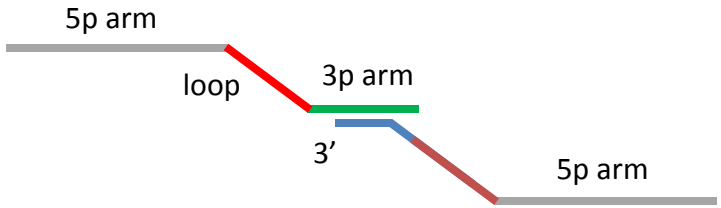
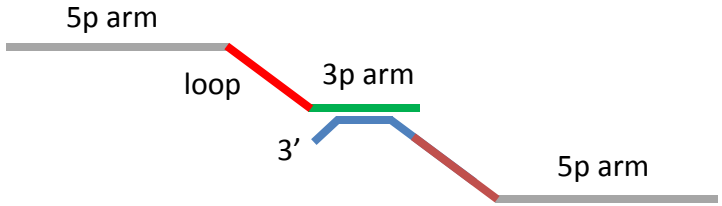
Primer-Primer Cross Types

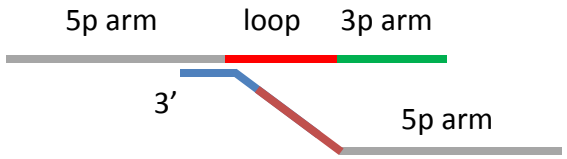
Amplifiable primer-primer dimers are formed only when 3p arms of two primers are cross-hybridized with at least one primer having its 3’ end complementarily bind to the other primer. Omega primers generally have much shorter 3p arms (16 nt) than that of regular primers (35 nt) therefore have statistically a much less probability of forming the amplifiable primer-primer dimers.



Type 1: Extendable cross hybridization in 3p arm sections. This will produce an amplifiable product and is the most undesirable primer-primer cross.



Type 2: Non-extendable cross hybridization to any section of the primers. This will not produce any extension product and will usually not cause a great problem unless tight primer-primer cross bindings are formed which compete with on-target primer-template bindings.



Types 3: Extendable cross hybridization to 5p arm or loop sections. This will produce an extension product. Blast-hybridization analysis against specific primers and library primers will further determine the conversion of these products into: non-amplifiable and amplifiable products in cycle 2 of PCR reaction.

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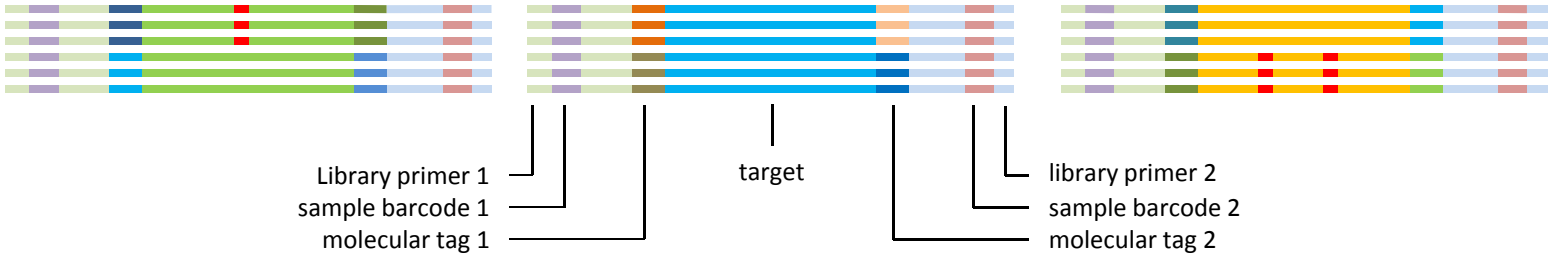
Workflow

Genomic DNA Sample Relay-PCR Next-Gen Sequencing

1 hands-on step

Amplicon library



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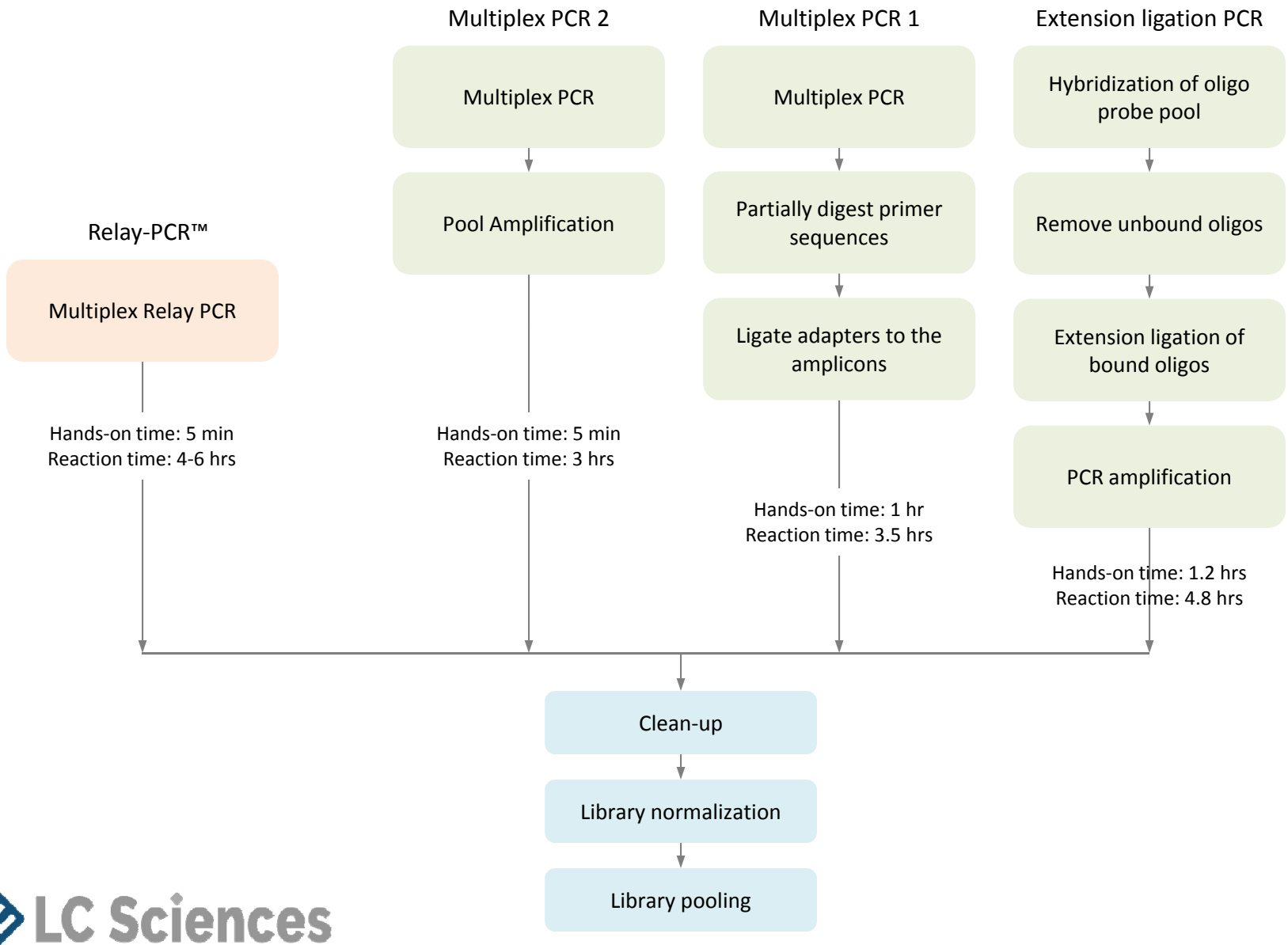
Workflow Comparison



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Experimental Validation

Relay-PCR using Omega Primers was validated on human genomic DNA.

PCR product bands in gel image **A** demonstrate the successful amplification of correct products by capturing six individual targets at six different genome locations.

PCR product band in gel image **B** demonstrates the successful amplification of all six targets by mixing six pairs of Omega primers plus one pair of common primers in a single tube.

Sequences of the PCR products was validated using a custom microarray (data no shown here).

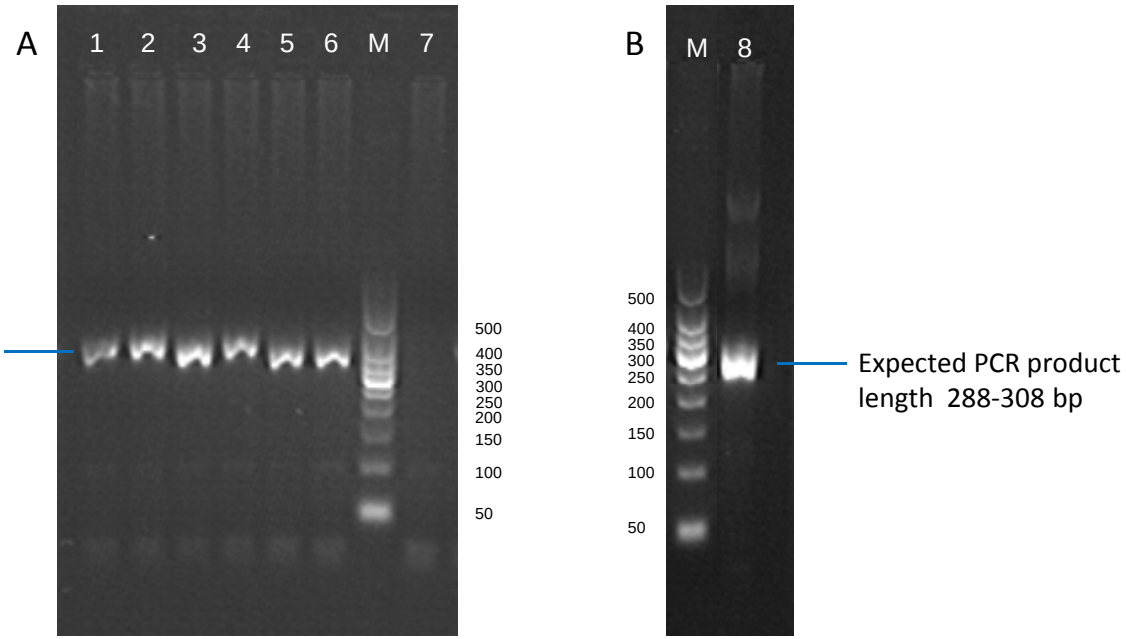
Agarose gel (3%, Cybr Gold) image of 7 PCR runs of 25 cycles.

1. Relay PCR, TP53-T3, pair 1
2. Relay PCR, TP53-T1 pair 2
3. Relay PCR, PIK3CA12, pair 3
4. Relay PCR, KRAS1, pair 4
5. Relay PCR, APC1, pair 5
6. Relay PCR, APC2, pair 6
7. Relay PCR, No specific primer control
8. Relay PCR, pairs 1 to 6

M. 50 bp marker

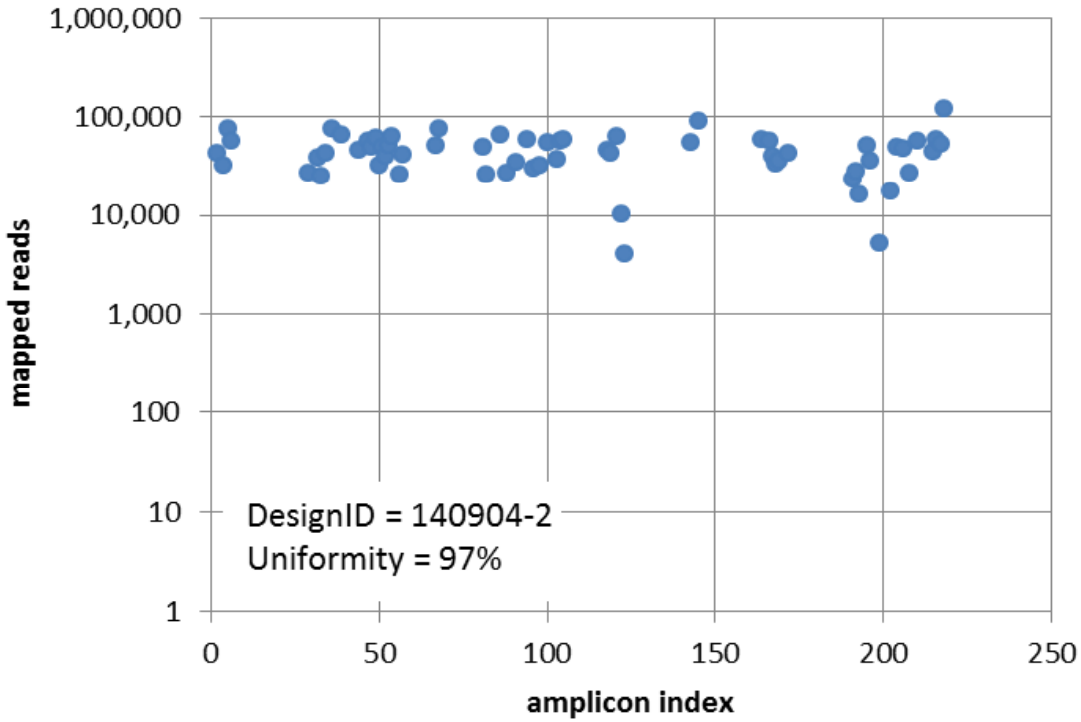
Expected PCR product length

1. 293 bp
2. 308 bp
3. 290 bp
4. 307 bp
5. 288 bp
6. 290 bp



Sequencing Results

The following plot is obtained from 63 amplicons of cancer genes.



In the reaction assay all Omega primers are added at the same concentration, without any adjustment for compensating final product yield.

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VariantPro™ Product Specifications

Capture Method	The VariantPro™ system incorporates novel Relay-PCR™ and Omega Primer™ technologies to form a one-step multiplex PCR based workflow.
Number of Targets	Projected - 20,000
Amplicon Length	Depends on sequencing platform
DNA Sample Source	Frozen tissue, whole blood, FFPE
Input DNA Required	10 ng per tube for genomic DNA
Number of Tubes	1-2
Assay Time	4-6 hours
Hands-on Steps	1
Hands-on Time	5 minutes
Sequencing Platform	Illumina, Ion Torrent, (Pac-Bio coming soon)
Multiplexing Capability	Up to 3024 samples in one lane
Coverage Uniformity	> 99% at 0.2 x mean
Dropout Rate	0

Product Service Options

1. Custom Amplicon Library Kits
2. Standard Panels
3. Full Service

    Kit Design & Synthesis

    Capture

        DNA sample QC/quantitation

        Concentration normalization

        Relay-PCR

        Bioanalyzer of amplicons

        Normalization and pooling

        Purification and bioanalyzer of amplicon pool

    Illumina Sequencing

    Basic Data Handling

        De-multiplexing

        Alignment & mapping

        Optional advanced analysis available

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Product Service Options

The VariantPro kit is composed of two parts:

1. The specific primers for target capture (priced per amplicon)
2. The library kit which includes: indexing primers, bar-codes, sequencing adapters, enzyme mix, purification beads (priced per rxn).

Specific Primers

- Priced per amplicon - Kit size - 9,600 rxns
- For synthesis of the specific primers, we can only go so low on the synthesis scale.

Library Kit (common primers)

- Priced per sample – Kit size varies
- Because the library primers are not unique, they can be delivered in various kit sizes depending on the total number of samples and how many you plan to multiplex in a single run.

Product Service Options

1. Target specific primers - Multiplexed target specific primers are designed, synthesized, and delivered in tubes of pooled primers in ready-to-use concentrations.

Part No.	Description	Size
VP-SP	VariantPro™ specific primers for one amplicon	9,600 reactions
VP-SP-MTag	VariantPro™ specific primers with molecular tags for one amplicon	9,600 reactions

2. Library kit for Illumina sequencing use Library kit for amplicon library preparations for Illumina sequencers. Contents include index library primers, polymerase, purification beads and reaction buffers.

Part No.	Description	Size
VP-LB-0048	Library kit for amplicon library preparations for Illumina sequencers	48 reactions
VP-LB-0096	Library kit for amplicon library preparations for Illumina sequencers	96 reactions
VP-LB-0192	Library kit for amplicon library preparations for Illumina sequencers	192 reactions
VP-LB-0384	Library kit for amplicon library preparations for Illumina sequencers	384 reactions
VP-LB-1000	Library kit for amplicon library preparations for Illumina sequencers	1000 reactions
VP-LB-1500	Library kit for amplicon library preparations for Illumina sequencers	1500 reactions
VP-LB-2000	Library kit for amplicon library preparations for Illumina sequencers	2000 reactions

Thank You