How to Develop a Clinical NGS Assay Without Losing Your Mind or Your Shirt

Genetic Testing Labs: Seize the Moment!

A genomics revolution has been underway for nearly a decade, and clinical laboratories are the catalyst.

An exponentially growing body of clinical evidence shows doctors can diagnose and treat some of the most deadly and persistent forms of cancer by looking to the genome. And as the new world of pathway-based therapeutics intersects with faster and cheaper DNA/RNA sequencing technologies, physicians, researchers, and patients are turning toward molecular pathology laboratories to lead the way.

You want to expand your use of the new diagnostic tools for your patient population. And to do so, you are developing clinical NGS-based assays and planning to bring them online as **quickly** and **cost effectively** as possible.

The Path to a New NGS Assay

If your lab is like most, the pathway you follow when bringing on new clinical tests looks like the one described in Figure 1.

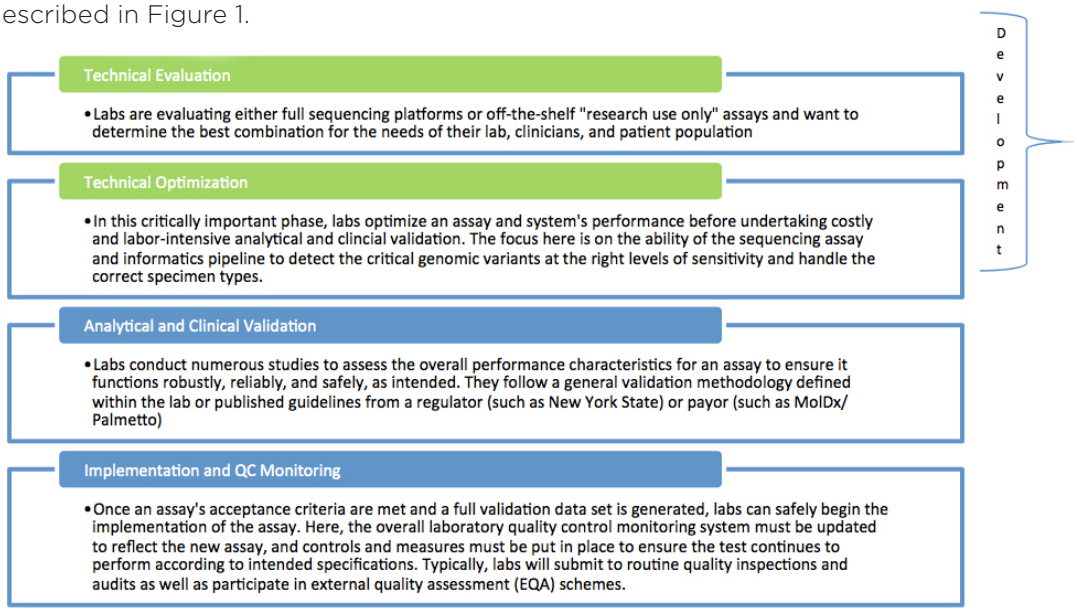

Figure 1: Generalized process clinical labs will follow when developing a novel NGS-based assay.

In this white paper, we will focus on the first two steps: ***the development phase****. Drawing from the expertise and experience of the clinical genomics specialists at SeraCare, we'll show you how your lab can complete this stage quickly, developing high-performing clinical NGS-based assays within your budget.*

Why is the Development Phase So Critical?

The development phase is not only where you and your team choose the most appropriate assay to deploy in your clinical laboratory. It's also where you can identify and optimize the assay's robustness before proceeding to the detailed validation of its performance so you can demonstrate its safety and effectiveness.

If, during the development phase, you assess too few variants and variant types and you don't take the time to establish critical preliminary performance characteristics, it can jeopardize both your timeline and the cost of bringing an NGS assay into production.

2 Ways to Develop an NGS Assay

A few years ago, there weren't many commercial options available to clinical genomics laboratories considering bringing on a new NGS-based assay. Now there are several. While not yet true *in vitro* diagnostic (IVD) systems, these commercial options are making it easier to purchase all the critical components of an assay and match them to one of several NGS instrument platforms in use today such as Illumina's MiSeqDx™ or the Ion PGM™.

Some labs, on the other hand, continue to custom-develop their assays — right down to the primer designs and gene coverage strategies — rather than buy pre-packaged kits. Moreover, they may have customized analysis pipelines and reporting systems underpinning their data analysis.

> ***Whether your lab uses fully customized or off-the-shelf kits, it remains your responsibility to ensure each test is rigorously developed and suitable for safe and effective implementation in your local context.***

This means examining the entire workflow of your assay and any and all specimens and specimen types likely to be used during its implementation.

As we'll see in the next section of this paper, this is easier said than done.

The Top 3 Challenges Facing Your Lab in the Development Phase

Increasingly, labs are adopting large gene panel assays to cover a wider number of clinically important disease-causing variants. The benefit of clinical NGS platforms is that they are highly multiplexed and can monitor hundreds of genes simultaneously. However, it is precisely this benefit that can be troublesome if your lab is trying to adopt these tests into clinical practice.

There are three overriding challenges with comprehensive gene panel assays:

1. **The sheer number** of different genes and clinically important variants they can detect.
2. **The relative rarity** of many of these clinically important variants and how long it takes to collect specimens containing them.
3. **The high cost** of evaluating large numbers of specimens, especially if you are sequencing sequentially.

In the next section, we'll examine each challenge and suggest a solution.

1. How Many Variants?

One popular comprehensive cancer panel assay from Illumina — the TruSight Tumor 170 assay® — covers 170 genes and several hundred possible genomic variants, both RNA and DNA. Your lab may not opt for such a gargantuan panel, but the challenges hold true even for moderately sized panels of 50 or 60 genes.

The first thing to understand is that *not all variants perform the same in each assay*. For any given variant, these factors all contribute to some level of variability of the sequencing:

- Different primer designs
- Variant callers
- Regions of different genomic complexity
- Resultant sequencing density

As you would expect, harder variants are harder to sequence than easier variants. Clinically important genomic variants come in all shapes and sizes, from the easy-to-detect single nucleotide variants to insertions, deletions of varying sizes, gene fusions, and large structural rearrangements.

Another important fact to realize is (as Sarah Sandmann and her research colleagues found recently) that different variant callers perform differently with different types of variants.

Our Advice

Generally speaking, you want to test enough of the tougher variants — particularly if they are clinically important in the disease area you are assessing. What is a tough variant? It depends on context: assay, variant caller, and sequencing quality.

If you don't have an idea before you begin assay development, it's best to try to test as many variants as you can get your hands on. In a recent BMC Genomics paper, Alexandra Buckley and her team showed the identification of INDELs can be more challenging than single nucleotide variants in submitted sequencing data. This has also been observed in a recently published paper (De Abreu, et.al., JALM, 2017) looking at the results of running a common DNA hotspot reference material from seven different laboratories with independently validated CLIA LDTs. Table 1 highlights a summary data set illustrating that the more difficult variants of insertions and deletions can exhibit higher variability as compared to single nucleotide variants (SNVs).

Interlab study results

			Average of All Labs	
Variant Type	**Gene HGVS**	**Allele Frequency ddPCR**	**Allele Frequency NGS**	**%CV**
Deletion	EGFR c.2236_2250del15	9.3%	9.3%	15.7%
Deletion	PTEN c.800delA	9.2%	10.6%	29.8%
Insertion	APC c.4666_4667insA	9.3%	3.8%	48.7%
SNV	AVG of all SNVs	9.6%	10.0%	10.3%

Table 1: Results from an interlab study (DOI: 10.1373/jalm.2017.023085 Published August 2017) of seven different labs with different cancer profiling NGS LDTs running a common NGS reference standard at 10% allelic frequency. Note the highest variability (red) amongst some of the important insertion and deletion variants. Shown for comparison: an average variability of all the SNVs in the reference mix.

INDELs of different sizes also perform differently. Some guidelines recommend evaluating small INDELs (<10 bp), medium INDELs (11-70 bp) and large INDELs (>70 bp). **So you should ensure you are targeting sufficient numbers of insertions and deletions.**

Your goal should be to determine how well your assay detects these known variants. Which brings up another point.

You need to run specimens that have been characterized by another validated method. You should set these validated specimens as "truth."

More on truth sets later.

2. Where Can You Find Rare Variants?

Unfortunately, clinically important variants do not all occur with the same frequencies in the same cancers. This makes accumulating sufficient numbers in a biobank very difficult.

Unless you are a major cancer center such as Memorial Sloan Kettering Cancer Center or MD Anderson Cancer Center, how are you going to find specimens with all these variants in them? And even if you have all the variants handy, you'll need a validated alternative method to test each specimen to ensure you know what the "truth" is, plus sufficient materials to run multiple times.

It may be easier than you think to find rare variants. Table 2 includes a summary of the variants available with SeraCare's Seraseq™ technology.

Variants types and genes available with Seraseq™ Biosynthetic NGS Reference Materials	
# Tumor genes represented	50
# Variants represented	135
SNV	86
Insertion	19
Deletion	22
INDELs	5
HP	3
ITD	2
Gene fusion	2
Exon skipping	1
RNA fusion	26

Table 2: SeraCare's Seraseq Technology: Available Variants

So should you skip the rare or hard-to-find variants in the development phase?

Our Advice

Whether it's rare or common, an assay should reliably detect a variant when it's present and fail to do so when it's absent. Looking at three or four of the rarer and hard-to-find variants during this phase is appropriate and necessary.

Your options for rare variants include:

- Remnant patient specimens with a known test result (orthogonal)
- Cancer cell lines
- Biosynthetic NGS reference materials

Remnant Patient Samples

The good: Remnant patient samples are most similar to the material you will run when the test is live.

The bad: You probably have a limited amount of material and could run out. It could be very old or degraded. The material could be untested by an equally sensitive or validated method. A remnant patient sample could be more valuable in the clinical validation phase.

Or it could be the unicorn: the MET exon 14 skipping RNA fusion or some other rare-but-important variant you've only read about in JMD papers.

Cancer Cell Line Mixes

The good: Many characterized cell lines exist with relatively wide ranges of variants. They are fairly affordable and you can store them safely for longer periods of time.

The bad: How characterized is the background? You may encounter spurious variants, false positives, or random background mutations acquired during cell culture and growth. Generally, you'll have only one variant per cell line — not exactly the most efficient. Using cancer cell line mixes tends to bias for variants that are easier and more stable.

Biosynthetic NGS Reference Materials (Our Recommendation)

The good: These can be engineered to contain any variant at any allelic frequency and are mixed with a highly characterized isogenic cell line reference gDNA. These perform the same as patient or cancer cell line gDNA. They are highly characterized, highly multiplexed, reproducibly manufactured lot after lot, and come in sufficient volumes for any size project.

The bad: It takes GMP-level manufacturing capabilities and upfront investment to develop a large variant library. (You can't homebrew these.) There is a cost to these types of materials.

3. How Can You Control Costs?

Bringing a comprehensive gene panel test on board is going to require an investment in resources. It is important to understand that during the development phase, your lab needs to draw a line between dedicating too much of your resources toward assessing and optimizing the assay or doing too little investigation before entering the most costly phase, validation.

Our Advice

The costs during the development phase consist mainly of acquiring sufficient numbers of specimens and the number of sequencing reactions you will need to run to collect the data. Other costs include the acquisition and deployment of any bioinformatics pipelines and data handling infrastructure. These are the areas you should look at for cost savings.

The Best Way to Save Money and Time on Assay Development

The single best approach you can take to achieve both a comprehensive understanding of an assay's performance and robustness while smartly managing time and resources is to use materials that:

- **Are readily available**
- **Are highly multiplexed**
- **Cover the range of variants and variant types**

The best biosynthetic NGS reference materials meet all three requirements. They can significantly speed up and improve your laboratory's assay development phase — even if you have large characterized specimen banks.

For example, Seraseq™ reference materials for clinical genomics applications provide a highly characterized and highly multiplexed tool for DNA and RNA-based genomics assays. They are expert-designed and include significant numbers of important and challenging variants across many different variant types and formats.

More importantly, these are **truth sets** — reagents that have very high lot-to-lot reproducibility and whose presence and allelic frequencies have been confirmed by sequencing and digital PCR methods. Your lab can use them, again and again, to measure changes to an assay's performance after tweaking or changing any parameter, whether it's the reagent or in the bioinformatics pipeline.

Unlike leftover patient specimens, the performance of high-quality commercial materials will be consistent over long periods of time.

If you are currently developing an NGS-based clinical assay or are interested in improving the characterization and robustness of an existing assay, contact your SeraCare representative at 800.676.1881 or visit www.seracare.com. We will gladly provide a customized assessment of your current situation and show you how some of our leading-edge materials can save you time and money while improving your confidence in your comprehensive assay.

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