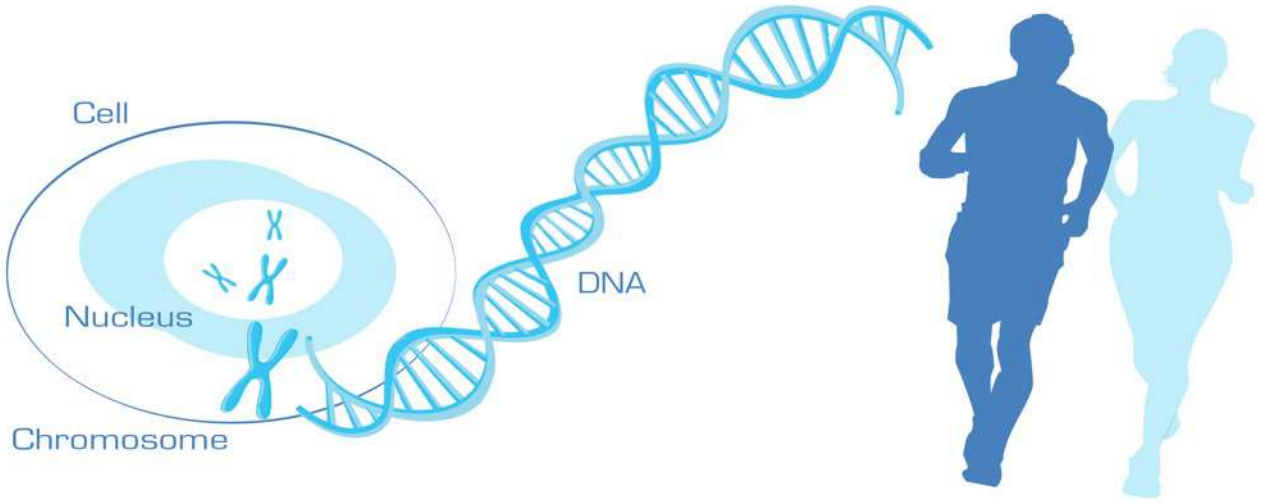
The RevoluGen vision is to revolutionize rapid diagnostics

Fire Wolf – 16S rRNA bacterial recognition at a single base pair accuracy level



17th November 2018

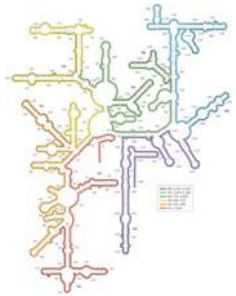
Fire Wolf has competitive advantages – it offers a rapid and highly accurate diagnostic technology

Speed
Isothermal amplification, RNA to result in 30mins

Live & Active
Focuses only on the live bacteria that matter giving a real-time snapshot of the relevant microbiome

Accuracy
Identifies down to a single base pair difference between similar bacteria in the same family

Multiplex
Easy to multiplex which cuts down on costs



16S rRNA target
Gold standard for bacterial identification and microbiome abundance ratio calculations

Design ease
Detection probes designed straight from digital code. Very useful for bacteria that cannot be grown in Lab.

Quantitative
Bacterial type abundance ratio calculations possible for selected groups of bacteria

Thermodynamic flexibility
Detection probes work in in genomic areas where other systems like Molecular Beacons cannot operate.

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The Microbiome bacterial networks are complex – everywhere and everything has a microbiome

The Question - What matters ?

Good bacteria or microbiome modulators enter the system

Are the bacteria destroyed by stomach acids?

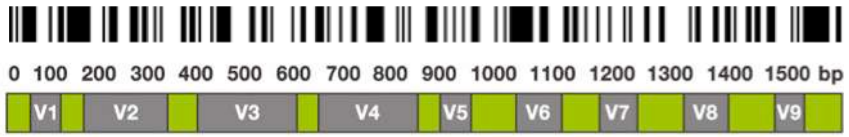
Do the bacteria colonise the gut?

Are they live and active?

What effect do microbiome modulators have?

- Microbiome-modulation **is not personalised**

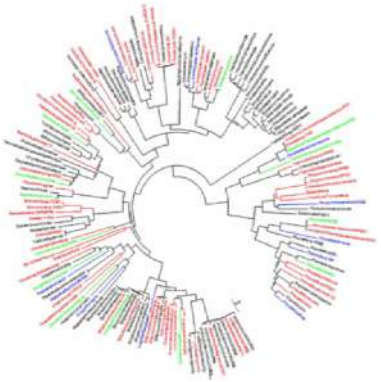
The Research – DNA sequencing

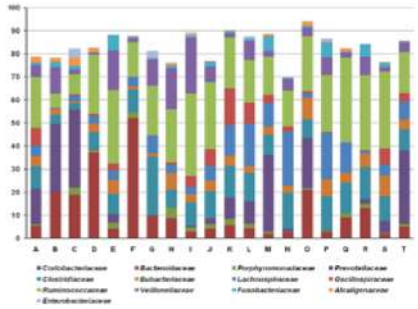


16S rRNA Variable (V) regions 1-9

- 16S rRNA is the bacterial identification barcode
- 16S rDNA sequencing analyses DNA from all bacteria in the sample, **whether they are dead or alive**
- Sequencing is a **specialised**, **long** and **costly** process

The Answer – A complex mix





Relative abundance ratios

- Dead bacteria distort relative abundance ratios
- 16S rDNA sequencing data offers little information on activity

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Fire Wolf – Microbiome applications as specific diagnostic tests are designed to commercialise our Microbiome understanding

The Microbiome

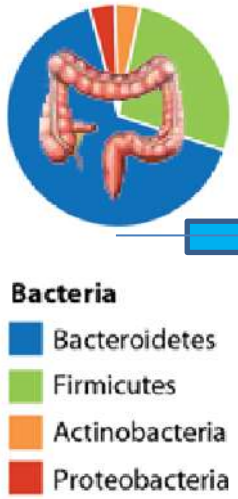
- Many bacteria in the mix
  Many bacteria cannot be cultured

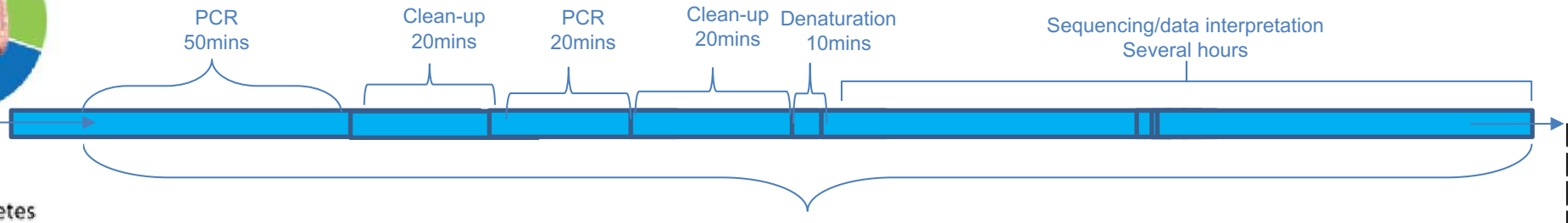
DNA sequencing identifies all 16S barcodes

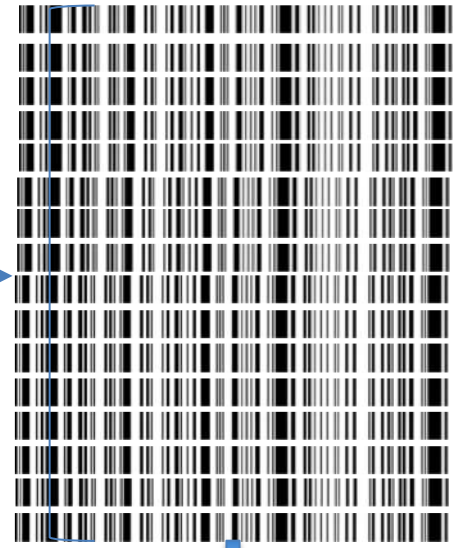
- Research tool is slow and expensive
- Does not distinguish between live/active or dead bacteria

16S Barcodes for each bacteria

- Lists all bacteria present in sample
- Calculates abundance ratios







A process of > 5hrs

A Fire Wolf diagnostic test for any of these bacteria can be designed from just the 16S Barcode without needing to grow the bacteria.

Fire Wolf identifies specific barcodes

- Research & diagnostic tool
- Calculates relative abundance ratios
- Focuses on live/active bacteria

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Fire Wolf is a combined sequence amplification and identification technology – a diagnostic test

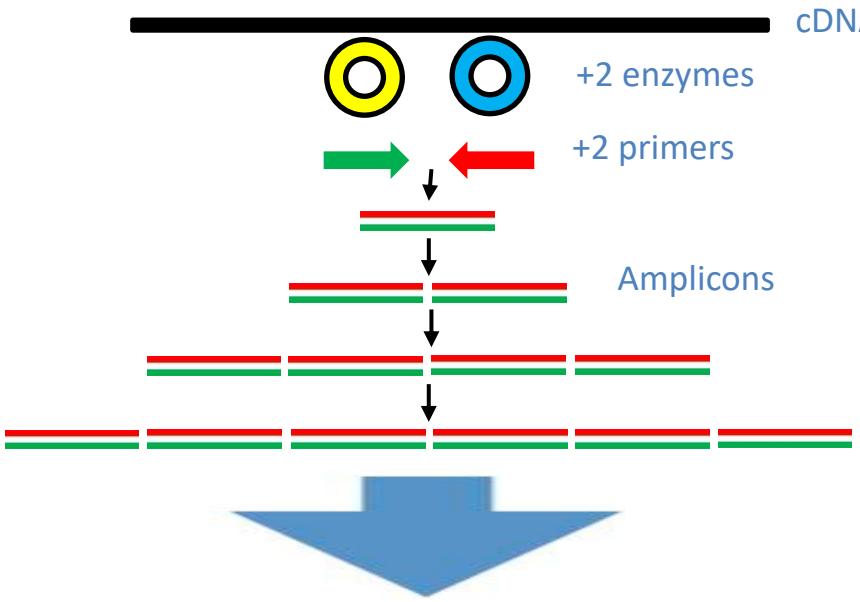
Fire Wolf combines two technologies - a DNA amplifier (Leon) and an sequence assay system (Milo)

Step 1 Leon

Leon performs at least $10x10^4$ multiplications within 10 minutes

Leon is faster to read out than PCR

Leon is the isothermal amplification part of Fire Wolf

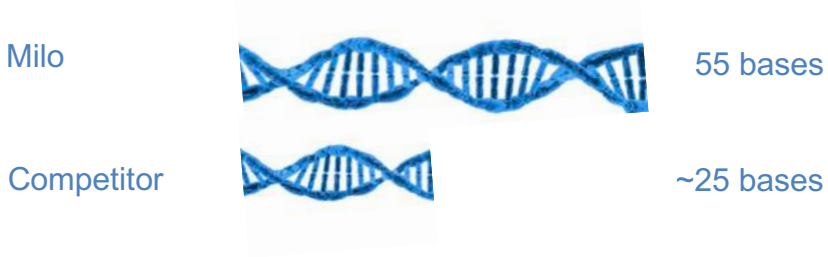


Step 2 Milo

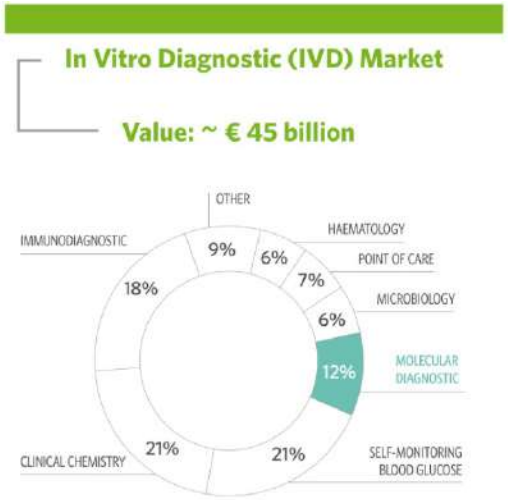
Milo utilises a unique long recognition system up to 55 nucleotides

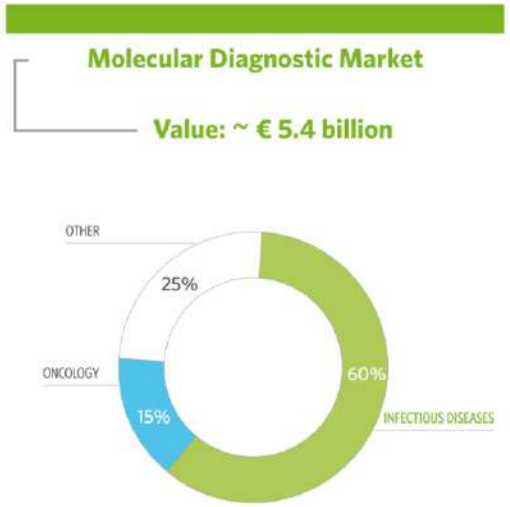
Specificity down to a single base discrimination

Milo is the sequence identification part of Fire Wolf



Molecular diagnostics global market is € 5.4bn
And growing at about 9% per annum.





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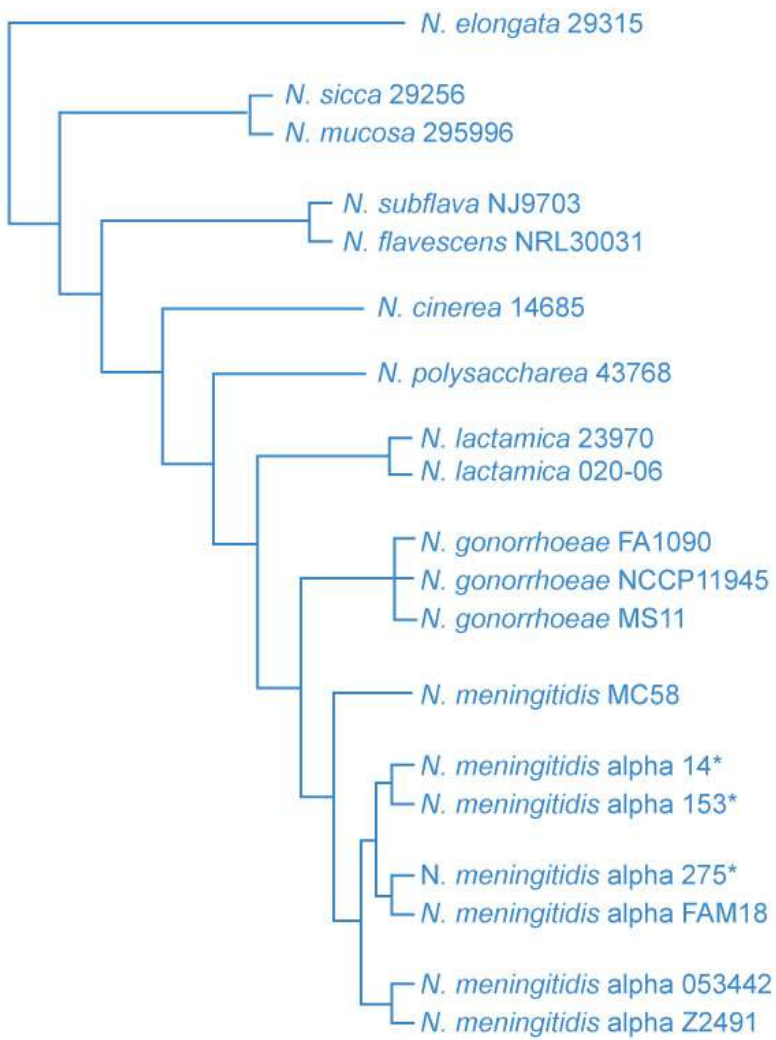
Fire Wolf's first target is a Neisseria species panel diagnostic – 13 strains of the Neisseria Genus affect man



This Phylogenetic tree lists all the Neisseria species in the *Neisseria* family (the *Neisseria* Genus) that afflict man. It shows how closely each species is related to each other based on concatenating the DNA sequences of all 896 core *Neisseria* genes. The genome of *Neisseria* species and strains consists of some 2.2 million base pairs which code for a total of about 2,500 proteins.

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This *Neisseria* genus contains several species believed to be commensal, or nonpathogenic including these listed below. However, some of these can infrequently be linked with disease. These benign bacteria confuse present tests and lead to wrong results which is why the world's first panel of tests to accurately identify all of them would have commercial value. Gonorrhoea testing alone is about a $35bn per year market worldwide.

Neisseria bacilliformis
Neisseria cinerea
Neisseria elongata
Neisseria flavescens
Neisseria lactamica
Neisseria macacae
Neisseria mucosa
Neisseria polysaccharea
Neisseria sicca
Neisseria subflava
Neisseria flava

Neisseria is a large genus of bacteria that colonize the mucosal surfaces of many animals. Of the 11 species that colonize humans, only two are pathogens, the strains of *N meningitidis* and *N gonorrhoeae.* Most *gonoccocal* infections are asymptomatic and self-resolving, and epidemic strains of the *meningococcus* may be carried in >95% of a population where systemic disease occurs at <1% prevalence.

Major application for Fire Wolf - the Microbiome needs a quantitative relative abundance signal of living bacteria

- The number of bacterial 16S rRNA copies is proportional to growth rate to meet the demand for protein synthesis, while the number of 16S rDNA remains stable
- Fire Wolf output is directly proportional to the number of 16S rRNA copies
- 16S sequencing and 16S real-time PCR are streamlined to utilise 16S rDNA
- DNA from dead cells can be present in a sample, while RNA will be found in live cells
- Recent reports have found that phylum relative abundance percentages can significantly vary when comparing DNA to RNA sequencing:

www.nature.com/scientificreports

SCIENTIFIC REPORTS

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Received: 05 February 2016
Accepted: 28 July 2016
Published: 31 August 2016

Direct 16S rRNA-seq from bacterial communities: a PCR-independent approach to simultaneously assess microbial diversity and functional activity potential of each taxon

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Phylum	Day 154 sampling			Day 189 sampling		
	% (by PCR)	% (by RNA)	Ratio by PCR/by RNA	% (by PCR)	% (by RNA)	Ratio by PCR/by RNA
Acidobacteria	1.877	0.084	22.256	3.062	0.053	57.819
Actinobacteria	4.530	0.015	308.903	13.604	0.046	298.464
Bacteroidetes	1.359	0.151	9.020	1.590	0.399	3.989
Armatimonadetes	0.302	5.697	**0.053**	0.412	6.652	**0.062**
Chlorobi	23.598	2.595	9.093	10.984	1.648	6.662
Chloroflexi	2.071	0.867	2.387	1.914	0.978	1.953
Firmicutes	0.669	0.008	81.510	0.383	0.027	14.079
Gemmatimonadetes	4.659	0.206	22.612	6.302	0.177	35.642
Planctomycetes	24.892	87.362	**0.285**	14.694	86.459	**0.170**
Proteobacteria	34.060	2.705	12.593	43.463	3.301	13.166
Candidate Division BRC1	0.388	0.042	9.233	0.736	0.023	32.872
Candidate Division TM7	0.863	0.006	144.614	0.942	0.030	31.799

Table 1. Percentage of sequences obtained by PCR-based analysis and by rRNA-seq at the two sampling times and ratio of the two values pairs (% by PCR/% by rRNA-seq). Only phyla for which a minimum of 10 sequences were available are reported in the table. Ratios resulting in values <1 are highlighted in **boldface**.

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Fire Wolf has high specificity – this enables the design of the *Neisseria* genus panel

The working limits of the Fire Wolf technology

Example 1 – maxed out at a 25% difference

A 25% sequence difference (14/55nt)
produced a signal with a 100% differential

Example 2– smallest difference possible is still detected

A 3% sequence difference (1/35nt)*
produced a signal with a 72.5% differential

***The Sigma-Aldrich online tool (OligoArchitect) failed to design a molecular beacon for this particular Single Nucleotide Polymorphism (SNP) due to thermodynamic challenges.**

Homologous nucleotide
(i.e. same in both bacteria)

Non-homologous nucleotide
(i.e. different in each bacteria)

Non-homologous nucleotides are randomly distributed
along the 16S rRNA variable regions

Neisseria panel design – clinical need to select two sequences

Target area A

Minimum: 7.5% sequence difference (3/40nt)

Target area B

Minimum: 13% sequence difference (4/30nt)

Neisseria panel design – all other 12 *Neisseria* are within Fire Wolf's test range

Maximum: 30% sequence difference (12/40nt)

Maximum: 53% sequence difference (16/30nt)

- In line with the British Association for Sexual Health and HIV (BASHH) recommendations, two 16S molecular target areas (A and B) have to be used to identify *Neisseria gonorrhoea* (NG) against all the other 12 *Neisseria* species found in human
- Target area A has a minimum of 7.5% and a maximum of 30% sequence difference between NG and all the other *Neisseria* species found in humans
- Target area B has a minimum of 13% and a maximum of 53% sequence difference between NG and all the other *Neisseria* species found in humans

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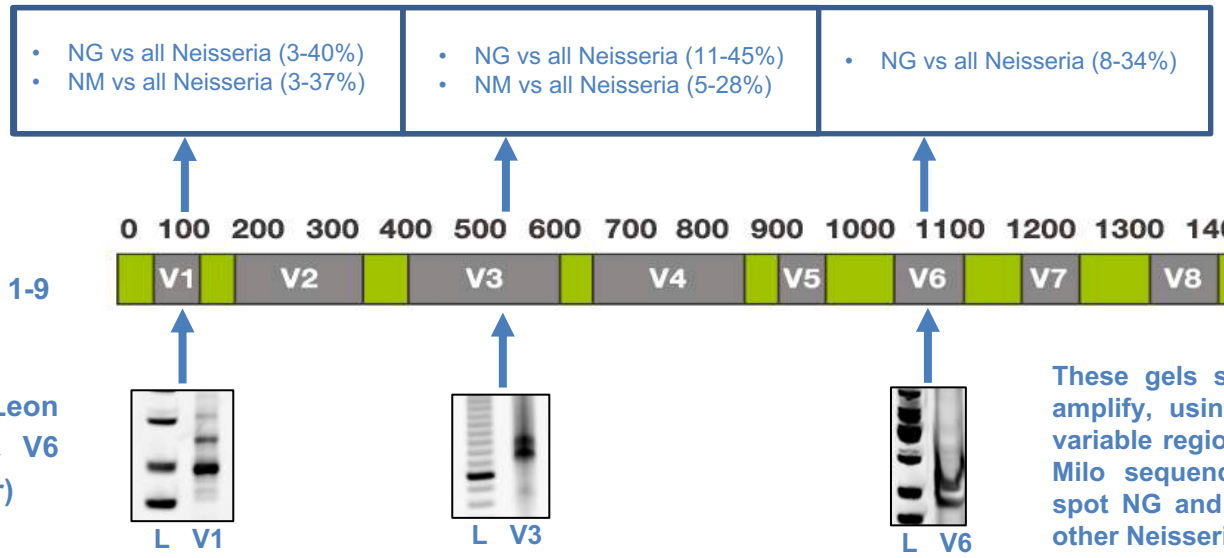
Three molecular targets to distinguish between NG and all different Neisseria species

How much of a difference in base pair sequences is present between Neisseria species in these regions (appx. % sequence difference):



These PAGE gel analyses show Leon amplifications of the V1, V3 & V6 regions of the 16S rRNA (L:ladder)

These gels show that RevoluGen can amplify, using Leon, the parts of the variable regions that are needed by the Milo sequence recognition system to spot NG and NM in any mix of all the other Neisseria species

- BASHH recommends the use of **2 molecular target sequences** for identifying NG in extra-genital sites
- We have identified **3 molecular target sequences** on what is considered to be the gold standard for bacterial identification, the 16S rRNA sequence
- Using the Leon technology, we can amplify three variable regions of the 16S rRNA sequences for discriminating NG from all other *Neisseria* species. These are the V1, V3 and V6 regions
- Using the Milo technology, we can design a specific probe for members of the Neisseria genus in each of these three regions. Milo can be used to discriminate a **single base mutation** generating a differential signal of **~75%** in comparison to the signal generated for the wild type region
- This works on sequence areas where molecular beacons cannot be designed to work at all for any level of mutational analysis
- In the Milo system, a **single base difference** equates to appx **3%** sequence difference. **9%** sequence difference generates a differential signal of **~97%.** The V1, V3, and V6 regions show sequence differences of **3%** right up to **45%** between the NG, NM and all the other Neisseria species. This makes every species separately identifiable in any mix of species.
- Stage 1 - The first IVD panel incorporating this technology that RevoluGen will consist of a set of three independent diagnostic tests for NG. These tests will identify NG in any mix of all the other Neisseria species. There is currently no approved diagnostic test that has been approved for this application, with this detection power and for use in all body sites
- Stage 2 - The second IVD panel will identify NM in any mix of all the other Neisseria species. The Stage 2 panel will be based on a set of probes within 2 variable regions of the 16S rRNA (V1, V3)
- NM in genital sites has been reported to be an emerging STI epidemic