High throughput screening of buffer, enzyme and proprietary enhancers to develop a high performance, inhibitor resistant multiplex qPCR MasterMix.

Ellie Kirby PhD, Sam Brown BSc, Jim Wicks PhD
YouSeq Ltd, 8 Moorside Place, Winchester, United Kingdom, SO23 7FX

Abstract
YouSeq carried out a large-scale research and development project to create a qPCR MasterMix with superior performance characteristics to all other market leading products. The ideal characteristics of a 2X qPCR MasterMix were defined as; resistant to high levels of PCR inhibition whilst retaining high sensitivity in detection of targets and parallel multiplex amplification capability. Lastly, we wanted to show that this MasterMix retained these performance metrics following lyophilisation/freeze drying. Model systems, designed to include inhibitors, were used to "stress test" each of these characteristics in a high throughput screening method for trialling of multiple buffers, enzymes, proprietary additives, and PCR enhancers. The strongest performing candidate mixes were then optimised. The project identified one outstanding formulation capable of highly sensitive, multiplex detection in the presence of blood, faeces, or soil. The mix was also shown to be suitable for freeze drying and delivers market leading sensitivity, even in multiplex settings. The Tetra™ MasterMix range is available to purchase now at www.youseq.com

Introduction
qPCR is the 21st century's method of choice for infectious disease diagnostics. The recent SARS-Cov-2 (COVID 19) global pandemic has accelerated, this already dominant molecular diagnostic technique, even further to the forefront of the diagnostic laboratory's armoury. It is common practice to use a ready-made, so called, "MasterMix" to simplify the qPCR workflow in a modern diagnostic laboratory. This is typically a 2X mix containing all of the necessary components (the Taq polymerase, dNTPs, buffer, enhancers, proprietary additives, etc.) required for an optimised qPCR reaction. The user then just adds primers/probes and an extracted DNA sample to create a ready optimised qPCR reaction.

The YouSeq research and development team have extensive experience in the field of qPCR and have designed a robust qPCR MasterMix to suit the needs of a modern molecular diagnostics laboratory. PCR inhibition is a common cause of diagnostic test failure and although most PCR protocols extract and purify the nucleic acid, a residual level of 'carry over' inhibitors will always remain. PCR inhibition can severely reduce the sensitivity of a test and the efficiency of PCR amplification leading to false negative results or delayed detection. Inhibition becomes especially challenging when the starting material is a highly complex matrix of organic and inorganic compounds. Soil contains the known inhibitor humic acid, blood contains haemoglobin, iron and lacotoferin and faeces a multitude of inhibitors including polysaccharides, bile and lipids amongst others. An ideal qPCR MasterMix can operate with high efficiency in the presence of such inhibitors and evade the underlying mechanisms of enzyme inhibition.

To drive both time and cost saving it is advantageous to "multiplex" the detection of multiple targets in a PCR reaction. In order to have four or five PCR amplifications occur in unison within the same reaction, the MasterMix must have the capacity and processing power to drive all reactions with equal efficiency. Although PCR primer design is the main driver of PCR efficiency, a poor performing MasterMix can also cause a loss of PCR efficiency, especially in a multiplex reaction. This results in a loss of assay sensitivity.

Shipping and logistics are an important and practical consideration in the design of an ideal qPCR MasterMix. It is common for laboratories to source diagnostics reagents from overseas. In these instances, cold-chain shipping of frozen, temperature sensitive reagents are costly and requires constant in-transit monitoring to meet the quality assurance demands of diagnostically validated reagents. The preferred use of Dry Ice (Carbon dioxide) is also environmentally undesirable. The ideal qPCR MasterMix is therefore manufactured in a lyophilised (freeze dried) format so that ambient shipping is possible without any detrimental impact on reagent performance.

Material and Methods

Development of model systems for MasterMix benchmark testing

The YouSeq YS-qP-CT.NG.TV-100 kit was used as a model system for four-way multiplex detection of Chlamydia trachomatis (CT), Neisseria gonorrhoea (NG) and Trichomonas vaginalis (TV) as well as the endogenous control primers and probe for human RNaseP. The standard qPCR reaction set up was 10µl candidate MasterMix, 1µl Primer/probe mix, 4µl RNase/DNase free water, 5µl target DNA. PCR cycling conditions were 50 cycles: Hot start 95°C 10 mins, Denaturation 95°C 10seconds, Annealing and extension 60°C 60 seconds. A QuantStudio 5.0 (ThermoFisher Scientific) qPCR instrument was used.

Positive control material was manufactured by combining equimolar concentrations of CT, NG and TV synthetic DNA template (YouSeq Ltd) to a final concentration of 200,000 copies (each target) per microlitre. This was used to create serial standard curves from 200,000 copies per microlitre down to 2 copies per µl. 5µl of standard was used in a PCR reaction giving final copy numbers of 1,000,000, 10,000 and 100 copies (Fig.1).

Preparation of complex biological samples

Whole blood (human) was diluted 1:50 to create a stock solution that could be added to a PCR reaction at a final concentration of 0.4% (fig1a). 0.5g of soil was resuspended in 500µl of TE buffer to create a stock and diluted 1:50 to give a working solution. saliva was diluted 1:20 to create a working solution. In each case 4µl of the working solution was added to each PCR reaction.

Complex biological samples were tested in singleplex reactions using primers and probes directed against a Cucumis sativus target gene (Fig.2). They were also tested in multiplex scenarios (data not shown).

Screening of Taq Polymerases

Initial screening of Polymerase enzymes etc was carried out using YouSeq's standard 2X qPCR MasterMix buffering system without any additional optimisation.

A large range of both proprietary and commercially available Taq Polymerases were combined with this buffer system to create 5 candidate mixes. Each mix was then used to detect a 6-point serial dilution of synthetic template. The mixes were screened for multiplex detection of CT, NG and TV within a dynamic range of $1x10^6$ to 10 copies. The best performing Taq polymerases are defined as having the highest PCR efficiency for all targets in a multiplex scenario.

Screening of >1000 buffer, formulations by qPCR

A multidimensional checkerboard method was used to screen several reagent variables, including $MgCl_2$ concentration, Final buffer pH, dNTP concentration, glycerol, and proprietary components.

The resulting formulation were used to detect $1x10^6$ and 10 copies of CT, NG and TV in multiplex in Blood, Faces and Soil preparations as described above. Semi-automated dispensing of 384 well plates was implemented to expedite experimental throughput.

The best performing candidate mixes as defined by PCR efficiency, sensitivity and early Cq Value detection were then taken through multiple rounds/iterations of improvements to fine tune the factors that delivered the biggest benefits to PCR performance.

Screening of proprietary additive and candidate PCR enhancers.

The top 3 performing candidate mixes were then taken forward for further optimisation by the addition of candidate PCR enhancers including but not limited to, Bovine Serum Albumin, SSB4 protein, DMSO and Betain.

Comparison with other market leading 2X qPCR MasterMixes

Benchmark testing was performed against a range of leading competitor products.

Development of freeze-drying protocol

A wide range of polysaccharide incipient stabilisers were screened for their ability to protect the MasterMix components during the freeze-drying process. A range of drying protocols were tested to ensuring drying to <5% hydration within a 24-hour dying cycle. Further optimisations were preformed to produce a freeze dried 'cake' that was stable, easy to dissolve and had a long shelf life during stability studies (fig.4).

Results

Comparison with other market leading 2X qPCR MasterMixes

Benchmark testing was performed against a range of leading competitor products. In comparison with the market leader from suppler "T" YouSeq Tetra 2X qPCR MasterMix achieved earlier detection of the target pathogens in multiplex (mean delta Ct gain 1.2 Cq values). End point fluorescence was a mean 23% higher with Tetra 2X qPCR MasterMix vs competitor "T".

Tetra 2X qPCR MasterMix performance in the presence of whole blood

In the presence of 0.4% blood (a level far higher than expected in a correctly extracted clinical sample – and a level that is visibly "red" to the naked eye - Fig2b). PCR efficiency was maintained at 98.2% despite a small delay in Cq values (mean 0.92 Cqs) vs control template - Fig 2.

A small reduction in end point fluorescence was observed – This is likely down to the impact of the blood colour on the fluorescent properties of the reaction.

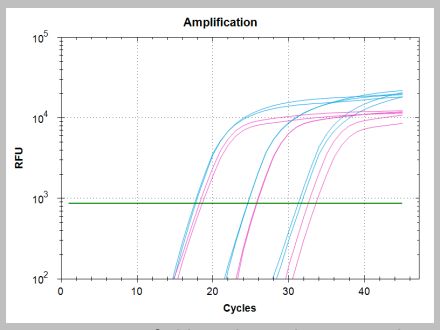
Tetra 2X qPCR MasterMix performance in the presence of Saliva

In the presence of saliva 1:20 the PCR performance remained identical to when compared to control template. With no loss of Cq or PCR efficiency. Fig3.

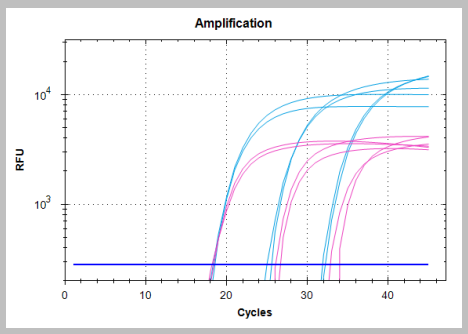
Comparison of Tetra 2X qPCR MasterMix performance in lyophilised form vs frozen

Comparison of PCR performance between traditional frozen formulation and novel lyophilised format showed

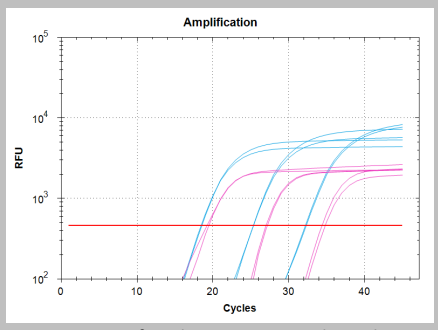
PCR performance remained identical between the two formulations, with no loss of Cq value or PCR efficiency Fig 4.



a. Detection of Chlamydia trachomatis in the FAM channel



b. Detection of Neisseria gonorrhoea in the HEX channel



c. Detection of Trichomonas vaginalis in the ROX channel

Fig 1. Comparison of YouSeq Tetra 2X qPCR MasterMix (blue) with Competitor T (pink) mix in the YS-qP-CT.NG.TV-100 test. The assay was a 3-way multiplex qPCR assay for detection with RNaseP as the endogenous control (data not shown). These dyes show earlier PCR detection and better reproducibility with the Tetra MasterMix.

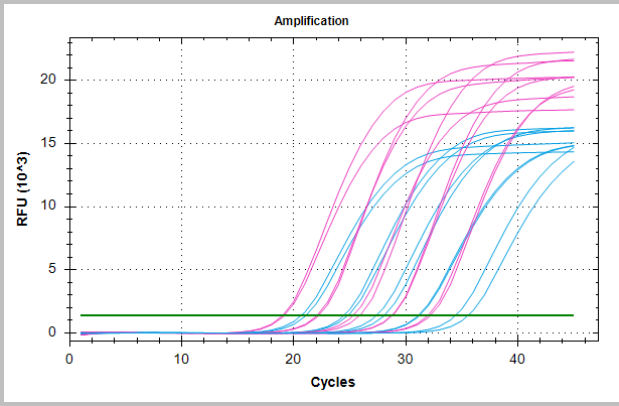


Fig 2a. Detection of *Cucumis sativus* erf115 gene in presence of blood (blue) vs ctrl (pink). Mean loss of 0.92 Cq value with efficiency maintained at 98.2%

Fig 2b. photograph showing visual appearance of template prior to PCR containing 0.4% blood by volume.

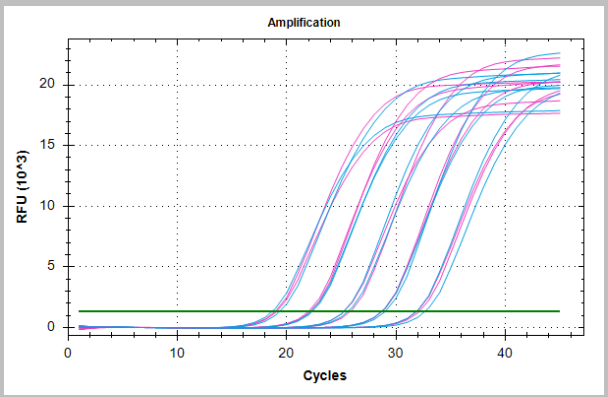


Fig 3. Standard curve detection of the *Cucumis sativus* erf115 gene in presence of saliva (blue) vs ctrl (pink). No loss of sensitivity of efficiency 99.2%

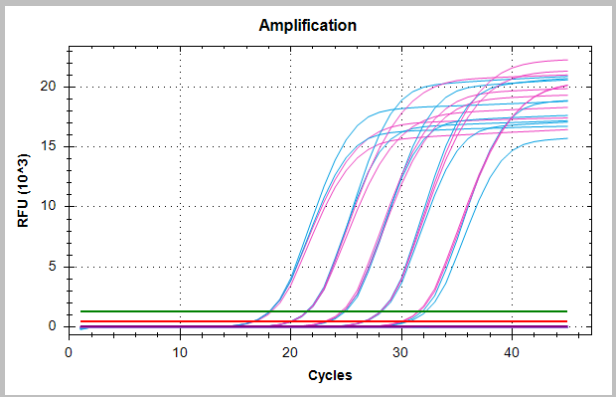


Fig 4. Standard curve Detection of *Cucumis sativus* erf115 gene using Tetra 2X qPCR MasterMix in frozen form (blue) vs (lyophilised form). Identical performance

Discussion

For this study we have utilised a highly effective multiplex design, to validate the Tetra MasterMix and perform benchmark comparisons against competitor products (fig.1). Our results demonstrate that across a range of indicators the YouSeq Tetra™ had improved performance overall. Firstly, the Cq values are earlier when using the YouSeq Tetra™ formulation, but it was also clear that duplicate test traces are more closely aligned indicating better reproducibility in the test. The end point fluorescence was also higher, demonstrating a clearer signal and improved signal to noise ratio. Across a range of parameters, the YouSeq Tetra™ MasterMix out-performed leading competitors in this multiplex scenario.

qPCR testing is increasingly being deployed to test a wide range of samples, from different biological substrates (e.g blood and saliva) to environmental samples such as soil or even sewage water. Blood is considered one of the hardest contaminants for PCR to amplify efficiently as the iron found in blood is a known strong inhibitor of PCR amplification. We were able to demonstrate no effect from saliva, soil or faeces on the PCR reaction and a slight increase in the Cq detection in the presence of blood. However even in 0.4% blood, Tetra™ MasterMix was still able to deliver highly sensitive 10 copy detection (fig.2a), a concentration that is sufficient to turn the reaction itself red (fig. 2b). The Tetra™ MasterMix is highly resistant to the effects of contaminating PCR inhibitors that are commonly found in biological samples, which often diminish the sensitivity of commercially available mixes.

Work was undertaken to lyophilise the mix and demonstrate the efficacy of the drying process in not denaturing any of the critical PCR components.

Fig.4 shows that this critical goal was also achieved within this study.

This study showed that all components of a 2X qPCR MasterMix when not ideally optimised can have dramatic detrimental effect on basic PCR performance. Conversely, when all optimised under suitable model system conditions it is possible to bring about massive improvements in PCR sensitivity and efficiency.

The Tetra 2X qPCR MasterMix demonstrates superior analytical performance across a wide range of PCR scenarios. This powerful MasterMix combines the potential for massive parallel amplification in multiplexed applications with an enviable resistance to PCR inhibitors. It can be supplied as a room stable lyophilised component that can be integrated into a wide range of automated and non-automated workflows.

References

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