Guide

Guidance for optimisation and validation of custom oligonucleotides

For Research Use Only. Not for use in diagnostic procedures.

BIOSEARCH™ TECHNOLOGIES

Guide

Guidance for optimisation and validation of custom oligonucleotides

Contents

Guide

Guidance for optimisation and validation of custom oligonucleotides

Introduction

This document provides guidance on working with custom oligonucleotides including how to handle these in the laboratory, optimisation and validation of assays, and batch control. Where appropriate, we have referenced specific online tools that are part of our compiled oligo toolbox, designed to help you get the most from your oligos.

1. Oligonucleotide handling

1.1 Characterisation of your oligonucleotides

Upon receiving your custom oligos from LGC Biosearch Technologies, we recommend documenting key information as this will assist with any downstream troubleshooting that may be required. This information can be found on the certificate of analysis (CoA) document (figure 1). This document can be requested at time of purchase or you can contact us (see section 7) to request this for a completed order.

Appendix 8.1 provides an example table that can be adapted to your specific laboratory. Information that should be recorded includes:

- Sales Order (SO) number
- Reference number (Sequence Set number - SS)
- Purification method
- Calculated stock concentration of oligos, including the method used to calculate this.

1.2 Resuspension of your oligonucleotides

Custom oligonucleotides may be supplied in buffer or dried down. Before use, dried oligos need to be resuspended in a suitable buffer. Most oligos resuspend easily in aqueous solution, but those containing modifications such as fluorophores may take longer to fully solubilise.

1.2.1 Resuspension buffer

During transit, dry pelleted oligos can become dislodged from the bottom of the tube. We recommend brief centrifugation (30 seconds) of oligo tubes before proceeding with resuspension to ensure no dislodged pellet is lost when the tube is first opened as this can result in loss of yield.

For oligonucleotide suspension, we recommend preparing stock and working solutions using a TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5 or pH 8.0) made with nuclease-free water. This maintains the pH and protects against acidic or basic induced degradation. The EDTA serves to protect against microbial contamination. If your experiment cannot tolerate EDTA, you may use 10 mM Tris-Cl buffer. Molecular biology grade nuclease-free water at a physiological pH is a suitable alternative. DEPC treated water is not recommended because it can be acidic and result in depurination of oligos.

1.2.2 Resuspension concentration

For most applications (e.g. PCR, qPCR, dPCR) a stock concentration of 100 µM is both straightforward to calculate and convenient for downstream dilutions. PCR protocols typically require 10–50 pmol of each primer per reaction and with a 100 µM stock concentration, 1 µL of solution contains 100 pmol of oligo. In some scenarios it may be convenient to prepare working dilutions from the resuspended oligo stocks, yet it is critical to ensure that the working solution for other potential applications is not a higher concentration.

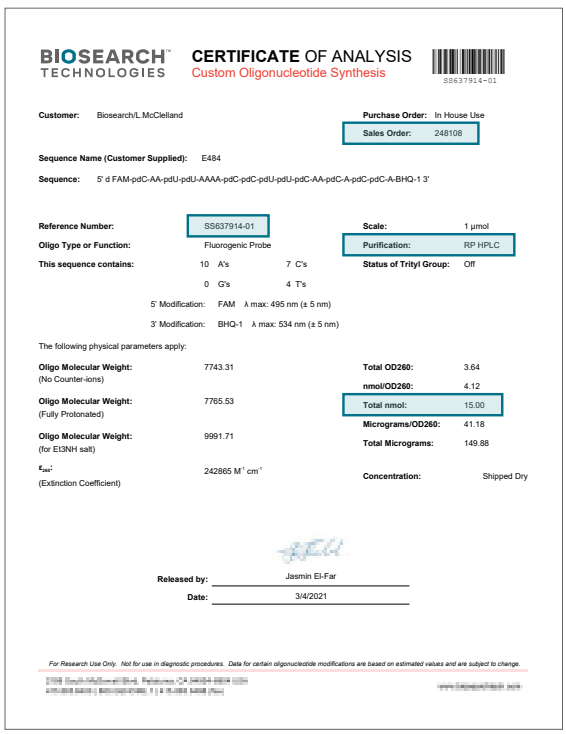
1.2.3 Calculations for a stock solution

Our online oligo resuspension calculator can be used to calculate how to dissolve your own oligo into the required concentration of stock solution. You will require the amount of oligo delivered (nmol) in your tube (found on the Certificate of Analysis), and your desired stock solution in µM.

If you prefer to calculate stock solutions manually, please follow these steps (for a 100 µM solution):

a) Determine the total oligo yield for your oligo on the unique Certificate of Analysis (CofA) document.
b) Multiply the yield figure by 10.
c) The value from step b is the volume of buffer (µL) to add to the oligo tube to achieve a 100 µM stock solution.

The CofA for your oligos will be provided alongside your physical oligos. An example is shown in figure 1.



BIOSEARCH TECHNOLOGIES

CERTIFICATE OF ANALYSIS
Custom Oligonucleotide Synthesis

SS637914-01

Customer: Biosearch/L.McClelland

Purchase Order: In House Use
Sales Order: 248108

Sequence Name (Customer Supplied): E484

Sequence: 5' d FAM-pdC-AA-pdU-pdU-AAAA-pdC-pdC-pdU-pdU-pdC-AA-pdC-A-pdC-pdC-A-BHQ-1 3'

Reference Number: SS637914-01
Oligo Type or Function: Fluorogenic Probe
This sequence contains: 10 A's 7 C's 0 G's 4 T's
5' Modification: FAM λ max: 495 nm (± 5 nm)
3' Modification: BHQ-1 λ max: 534 nm (± 5 nm)

Scale: 1 µmol
Purification: RP HPLC
Status of Trityl Group: Off

The following physical parameters apply:

Oligo Molecular Weight: (No Counter-ions) 7743.31
Oligo Molecular Weight: (Fully Protonated) 7765.53
Oligo Molecular Weight: (for Et3NH salt) 9991.71
ε_{260}: (Extinction Coefficient) 242865 M^{-1} cm^{-1}

Total OD260: 3.64
nmol/OD260: 4.12
Total nmol: 15.00
Micrograms/OD260: 41.18
Total Micrograms: 149.88
Concentration: Shipped Dry

Released by: Jasmin El-Far
Date: 3/4/2021

For Research Use Only. Not for use in diagnostic procedures. Data for certain oligonucleotide modifications are based on estimated values and are subject to change.

Figure 1. An example Certificate of Analysis (CofA) document. A CofA is provided for each oligo. The blue boxes identify where the sale order number, reference number, purification and total oligo yield (nmol) information can be obtained.

Locate the total oligo yield (nmol) information on your Certificate of Analysis (CofA) document (figure 1).

A worked example:

In the example CofA (figure 1), the oligo yield is listed as 15 nmol. Using the guidance in section 1.2.3, the required buffer volume to generate a 100 µM stock solution would be 150 µL (15 nmol × 10 = 150).

1.2.4 Encouraging resuspension

Some oligos are more resistant than others to resuspension, for example those containing fluorophores or hydrophobic molecules. To further encourage resuspension, warm the oligo at 55 °C between 1 and 5 minutes, then vortex the tube and centrifuge to spin down the contents.

If any precipitate remains after this remedial action has been taken, it is likely to be stray remnants from the synthesis process. These may be removed by column purification or more simply by transferring the supernatant to a clean tube and discarding the remaining precipitate.

1.2.5 Measuring the concentration of the prepared stock solution

LGC Biosearch Technologies recommend deriving the oligo concentration of a prepared stock solution from the raw absorbance value at 260 nm (OD260), using a traditional spectrometer, a 96-well microplate and a phosphate buffer.

To calculate the oligo concentration using the OD260, the unique extinction coefficient for each oligo is required. This can be found on the CofA document.

To quantify an oligo stock solution by spectrophotometer:

a) Prepare a 25x dilution of the 100 µM oligo stock, achieving a concentration in the range of 4 µM. Dilute 40 µL of the 100 µM oligo stock in 960 µL TE or PBS to achieve a total volume of 1000 µL of diluted oligo stock.
b) Vortex (or repeatedly pipette up and down) for 15 seconds to ensure the dilution is thoroughly mixed.
c) Using a spectrometer, read the absorbance of the diluted aliquot at 260 nm (OD260). Take three reads per sample and use the average value in downstream calculations.
d) Either
    1. Use the Beer-Lambert Law $A = \varepsilon \times L \times C$ to calculate the concentration of the oligo where:
        - A = average of measured absorbance at 260 nm (OD260)
        - ε = extinction coefficient of the oligo at 260 nm (found on the CofA or in this file for oligo modifications) in M^{-1} cm^{-1}
        - L = path length of the spectrometer in centimetres (cm)
        - C = the concentration in Molar (M)

OR

2. Use (nmol/OD260) × (average OD260) × (Dilution Factor) = [C], concentration in µM (micromolarity) where:
   - nmol/OD260 is listed on the CofA for the oligo
   - average OD260 is the average of the spectrometer OD readings for the oligo acquired in step c above.

Important note: use the same method to calculate oligo concentration between experiments to limit variability.

Our online oligo concentration calculator can be used to calculate the concentration of oligos in solution, using the absorption measurement (OD260) and the extinction coefficient.

1.2.6 Expectations for oligo stock concentration values

Measured concentrations of resuspended oligos should meet the following guidelines:

- Research-only grade oligos: *After diluting the stock of oligos to the appropriate range, measuring the OD260 and calculating the concentration according to the instructions listed above, the oligo quantity calculated should be within +/- 10% of the stated nmol delivered on the CofA.*
- GMP grade oligos: *After diluting the stock of oligos to the appropriate range, measuring the OD260 and calculating the concentration according to the instructions listed above, the oligo quantity calculated should be within +/- 5% of the stated nmol delivered on the CofA.*

Please note that calculations of oligo concentration using any other approach, such as using a unit of weight, may arrive at a different value from that provided with the purchased oligo.

1.2.7 A note on droplet-style spectrometers

Biosearch Technologies do not recommend performing concentration measurements of oligos using a droplet style spectrometer such as the NanoDrop (Thermo Fisher Scientific). When using a droplet style spectrometer, it is common to witness discrepancies between replicate concentration measurements. This relates to limitations in the instruments themselves, and is explained more in this document.

Scientists at Biosearch Technologies have observed that when measuring concentrations of synthetic oligos, the linear range of accurate concentration determination is much more limited than advertised. This is particularly applicable to labelled oligos.

In addition, it is important to calculate oligo concentration manually in absolute units of molarity, by using the raw OD260 data in conjunction with the unique extinction coefficient for the given oligo (section 1.2.5). When a droplet-style spectrometer provides the concentration in units of ng/µL, it is using a general purpose constant which may provide a poor approximation for synthetic oligos. Additionally, these instruments may apply unusual algorithms that are absent from other spectrometers. For example, the manual for the NanoDrop 1000 refers to Spectrum Normalisation:

"The baseline is automatically set to the absorbance value of the sample at 340 nm, which should be very nearly zero absorbance. All spectra are referenced off of this zero."

Many fluorophores and quenchers will absorb in the range of 340 nm and so will confound this baseline subtraction.

1.2.8 Diluting a stock concentration to make a working stock

The desired concentration of both stock and working stocks may vary depending on the desired final reaction volume.

Working stocks can be made easily from storage stocks using our online oligo dilution calculator. To calculate the dilution of storage stocks and create aliquots of working concentration oligos, follow these steps:

a) Enter the stock concentration and select the appropriate units.
b) Enter the desired working concentration and select the appropriate units.
c) Enter volume of working concentration oligo required and select the appropriate units.
d) Click the Calculate button.
e) The tool will determine the volumes of both stock oligo and buffer (or water) needed to dilute the storage stock concentration to produce the required working stock concentration.

2. Preparing for assay optimisation and validation

2.1 The assay lifecycle

All newly designed assays require optimisation and validation before they can be routinely used in experiments. Figure 2 illustrate the lifecycle of an assay.

- Optimisation: identifies the conditions under which the assay performs best. This is achieved by identifying compatible buffers, optimal oligo concentrations, the most appropriate annealing temperatures and the ideal PCR cycling conditions.
- Validation: refers to a set of performance criteria which define the acceptable performance data for the assay. When validation criteria have been identified, they are utilised for subsequent quality control (QC). This provides a baseline for QC assurance each time a component of the assay procedure is changed e.g. new production batches of master mix or oligos or adopting new nucleic acid isolation chemistries. Once the change has been implemented, the initial QC data are used for comparison with data obtained after the proposed change to confirm that the assay is still performing as expected.

More details on both optimisation and validation of assays can be found in sections 3 and 4.

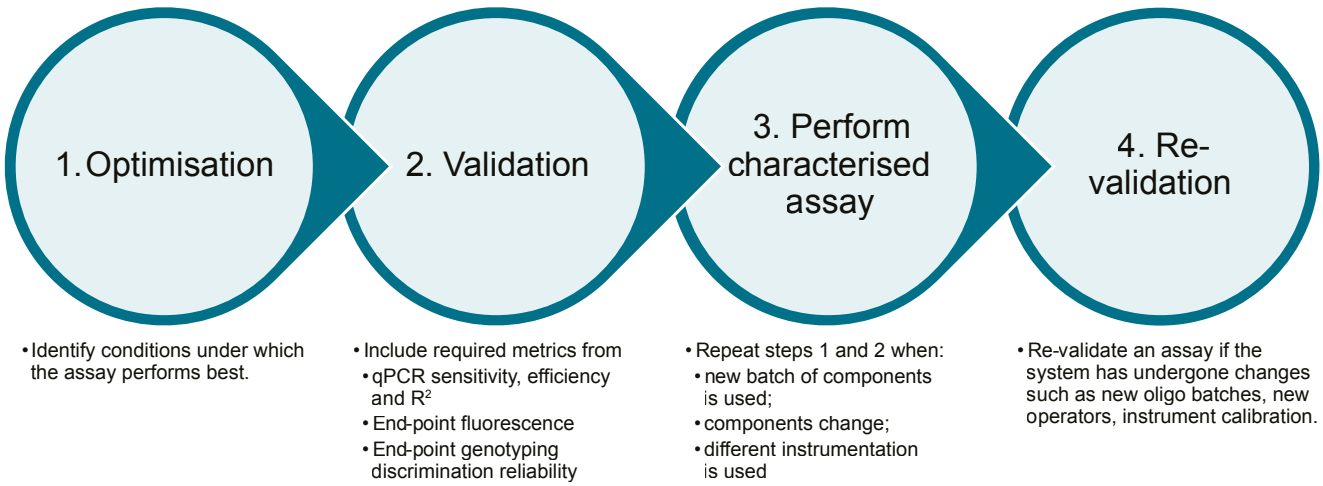


Figure 2. The lifecycle of an assay.

2.2 Control template material for validation and optimisation

Optimisation and validation of assays requires access to suitable control material. It is essential to verify that commercially supplied control materials include the specific amplicon sequence of the detecting assay. Ideally, control materials will mimic the true test samples that will be used once when the assay is ready for routine use. There are, however, experimental circumstances when this is not possible such as when only small amounts of sample are available or when samples are isolated from multiple sources using different nucleic acid isolation procedures. In such situations it may be appropriate to select synthetic DNA or RNA templates for assay optimisation and validation. For assays designed to detect multiple targets, synthetic concatemerised templates that include all targets are available from specialist suppliers. Synthetic templates are spiked into a negative sample matrix (such as saliva, blood, or urine) to mimic the test sample environment. The nucleic acids can then be isolated in the same way as the true test samples will be, and the isolated DNA or RNA template used in assay optimisation and validation.

2.3 Managing contamination risk

Assay validation and optimisation experiments often require the use of highly concentrated templates that can become aerosolised and result in contamination of laboratory spaces. It is essential to employ good laboratory practices when working with highly concentrated templates to prevent contamination of the environment with PCR template.

Biosearch Technologies recommend using a dedicated work area for each stage of the process:

a) Sample preparation area: distinct area of the laboratory where nucleic acid isolation can take place.
b) Pre-PCR hood; used to prepare reaction reagents for downstream PCR. This area should be distinct from the sample preparation area (at least 3 metres away).
c) A high-template hood: a designated hood for working with high concentrations of PCR template such as synthetic controls. This should be in a different room.
d) Post-PCR area: Amplified PCR products should never be in contact with the sample preparation area or pre-PCR hood.

The protocol below is provided to demonstrate suggested steps to follow to prevent template contamination in the laboratory during PCR set up.

1. Fill an ice bucket, remove reagents stored at -20 ºC from the freezer and place into the prepared ice bucket on the laboratory bench.
2. Prepare the pre-PCR hood by wiping down all surfaces with 70% ethanol solution. The pre-PCR hood should be located at least 3 metres away from the edge of lab bench.
3. Transfer the ice bucket containing reagents to into the pre-PCR hood.
4. Whilst in the pre-PCR hood:
   a) Prepare reaction mix
   b) Aliquot reaction mix into 96 well plates or strip tubes
   c) Aliquot the negative control
5. Securely cover the prepared reaction plates or tubes ready to transport to the high-template hood and place into the ice bucket.
6. Remove the ice bucket from the pre-PCR hood.
7. Wipe down all surfaces of the pre-PCR hood with 70% ethanol. Switch on the UV light for 10-15 minutes.
8. Move to the room containing the high-template PCR hood. Wipe down all surfaces in the high-template PCR hood with 70% ethanol.
9. Place the ice bucket inside the prepared high-template PCR hood.
10. Remove the plate or tube seals and aliquot the PCR template into the prepared wells.
11. Appropriately cover reaction plates or tubes and seal in preparation for PCR.
12. Place prepared reactions into the PCR or qPCR instrument and start the reaction.
13. Return to the high-template PCR hood. Wipe all surfaces with 70% ethanol. Switch on the UV light for 10-15 minutes.
14. Once the reactions have completed, dispose of reaction plates in a rubbish collection system adjacent to the thermal cycling instrument.
15. All thermal cycling instruments should be inspected monthly for routine cleaning and maintenance.

3. Assay optimisation

In some instances, well designed assays may perform as desired under a wide range of conditions. An initial analysis reaction is a worthwhile step as it enables the performance of the assay to be determined. A serial dilution and standard curve analysis provide several important pieces of data and consequently is the recommended process for qPCR assay evaluation.

There are four key steps in assay optimisation:

1. Standard curve analysis
2. Assay optimisation (if required)
3. Assay validation (if required)
4. Evaluation of new batches oligos or reaction reagents as required

3.1 Standard curve analysis

3.1.1 Protocol for standard curve analysis

At the initial evaluation stage, all primers and probes are kept separate because the optimal concentration for each is yet to be determined. In the first test, Biosearch Technologies recommend including forward and reverse primers at 450 nM and probes at 200 nM.

The standard curve is constructed from six log10 dilutions, where the lowest concentration contains 5-10 copies of template per reaction. This will give an indication of the potential sensitivity of the reaction.

At least four replicates of each concentration should be run in the qPCR. This will give an indication of the reproducibility of the assay and its expected variability, particularly as the assay nears its limit of detection (LOD). This information will also indicate how reliable resulting data may be.

3.1.2 Prepare a template dilution series

When working with concentrated template stocks, take extreme care as these present a very high contamination risk. For more details on this, please see section 2.3.

The calculations below illustrate how to prepare a template dilution series, and utilises Avogadro's constant (1 mole of substance is equal to 6.022×10^{23} units).

Stock synthetic template: 100 µM = 6.022×10^{13} copies per µL

Initial dilution: 8.3 µL template + 491.4 µL diluent = **1×10^{12} copies per µL**

Subsequent dilutions:

1. 1 in 100 dilution of the initial dilution 1×10^{12} = **1×10^{10} copies per µL**
2. 1 in 100 dilution of the 1×10^{10} dilution = **1×10^{8} copies per µL**
3. 1 in 100 dilution of the 1×10^{12} dilution = **1×10^{6} copies per µL**

Table 1 illustrates a 1 in 10 dilution series of synthetic template, generated from the **1×10^{6} dilution created in iii. above.** This dilution series can then be trialled in the qPCR, using 10 µL of template per reaction.

Initial concentration (copies per reaction)	Dilution	Created concentration	Copies per reaction when 10 µL is used as template in the reaction
1×10^{6}	1 in 10	1×10^{5}	1×10^{6}
1×10^{5}	1 in 10	1×10^{4}	1×10^{5}
1×10^{4}	1 in 10	1×10^{3}	1×10^{4}
1×10^{3}	1 in 10	1×10^{2}	1×10^{3}
1×10^{2}	1 in 10	1×10^{1}	1×10^{2}
1×10^{1}	1 in 10	1×10^{0}	1×10^{1}

Table 1. A 1 in 10 dilution series of synthetic template.

If the ultimate objective is to run a multiplex assay, it can be informative to run both singleplex and multiplex reactions on the same plate to determine if any of the primers are affected when run in multiplex.

3.1.3 Evaluate the linear efficiency of the standard curve

Within the qPCR analysis software for your specific instrument, the software will generate the standard curve and associated details, assuming that the serial dilution samples have been defined as a standard curve.

Robust assays should have the following values:

- Efficiency of 95-105%
- An R^2 value of approximately 0.995-1.0
- Achieves desired limit of detection

The results for linear efficiency can be used to determine whether or not further optimisation is required (see figure 3).

If the assay meets the above parameters, then no further optimisation is required. Likewise, if all tested assays meet the above parameters in combination, then no further optimisation is required. It may be beneficial, however, to determine the range of concentrations the assay is successful. This information is useful for multiplex optimisation and characterising the overall robustness of the assay. Robust assays tolerate variability which is inevitable.

If you are trialling more than one assay, you may find that some assays meet the above parameters whilst others do not. If this is the case, then proceed to assay optimisation. If all assays fail to meet the above parameters, verify assay design and performance characteristics before proceeding to assay optimisation.

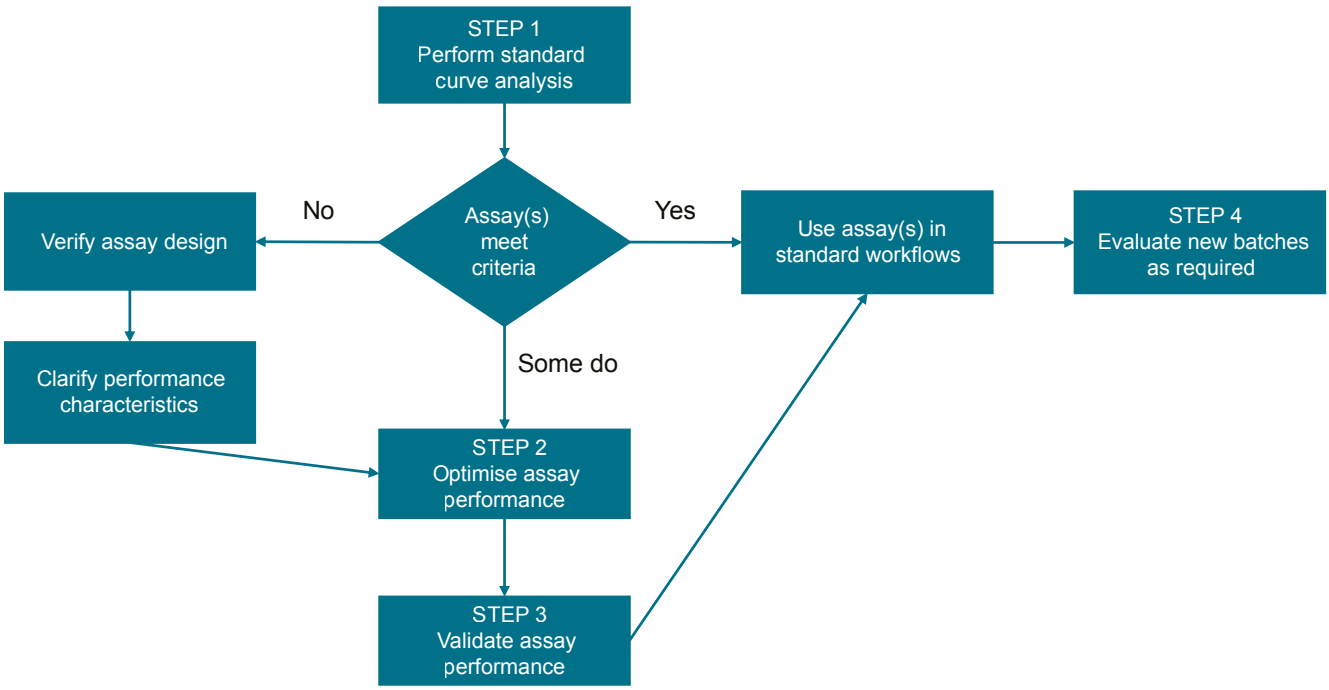


Figure 3. Decision tree to determine next steps after standard curve analysis of assay dilutions.

3.2 Assay optimisation

Assay optimisation is performed through modifications to the oligo concentrations, adjustments to the annealing temperatures or a combination of both. Changes to these parameters affect the hybridisation thermodynamics of the oligos. In turn, this either enhances or diminishes the efficiency of the reaction. For multiplex assays that share the same thermocycling protocol, evaluating a range of primer concentrations is a key area to focus optimisation efforts. Note that optimisation of annealing temperature requires a gradient PCR block.

3.2.1 *Primer concentration optimisation*

The optimal concentration of the individual primers and the probe is determined using a concentration grid (figure 4) using a set concentration of template; the amount of template should be sufficient to yield a C_q of 20-25. Each assay should be run in triplicate using varying concentrations of each primer, with 200 nM probe. It is also possible to perform primer optimisation using a master mix that contains an intercalating dye, such as SYBR Green. This approach permits omission of the probe from the reactions, thus making the optimisation process more cost effective and efficient. If this approach is taken, care must be taken to review melting curves of assays to ensure that only the targeted amplicon is considered in the qPCR data (figure 5).

If optimising primers without the probe, the melt curve should be analysed to ensure that there is only one peak present for the target amplicon. If multiple peaks are present, reaction efficiency, R_2 and LOD values may not reflect the characteristics of the hydrolysis probe-based assay. Repeat the studies using the hydrolysis probe-based approach without SYBR green.

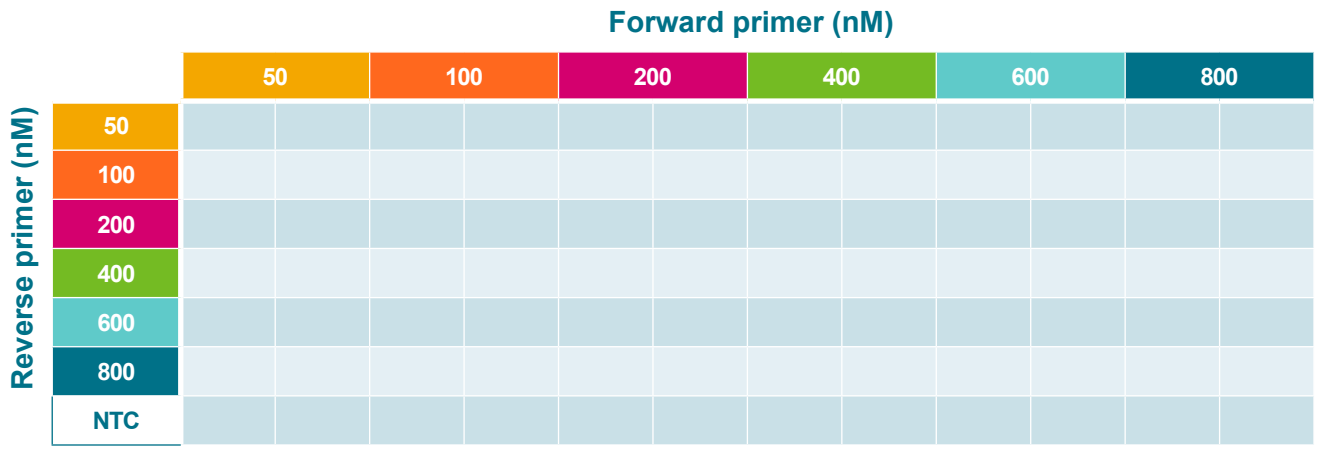


Figure 4. A typical concentration grid used to optimise the concentration of each primer. Following analysing of the data, the optimal combination of primer concentrations should be selected and used for subsequent probe concentration optimisation.

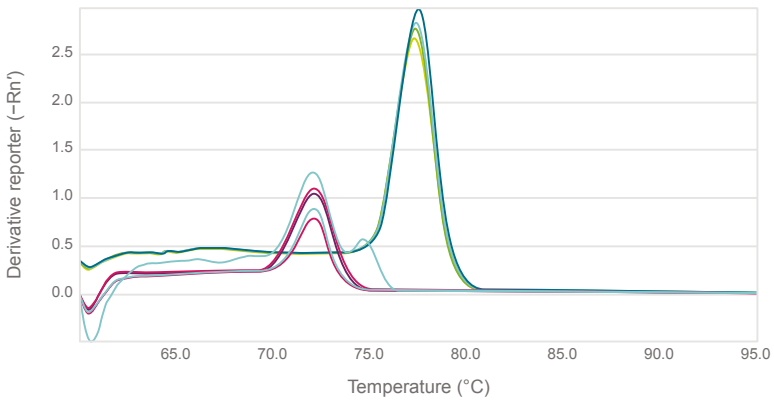


Figure 5. Example melt curve from a qPCR experiment. There is one main peak, representing the target amplicon, and some smaller peaks indicating additional PCR products.

Following data analysis, select the primer concentrations that result in the lowest C_q and the highest fluorescence values but without showing signal in the no template control (NTC) wells. Using these established primer concentrations, different probe concentrations can then be tested, for example a range from 50 nM to 800 nM. An example set of results for primer concentration optimisation is shown in figure 6.

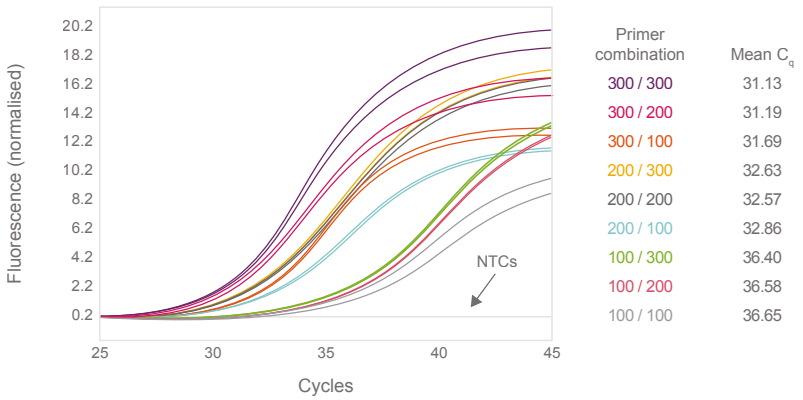


Figure 6. qPCR amplification curves illustrating the effects of primer concentration on assay performance. For this assay, 300 nM of both forward and reverse primer (purple lines) results in the lowest C_q value (most sensitive detection) and the highest fluorescence.

This process of primer optimisation will often rescue an apparently poorly performing assay. Once the primer and probe concentrations for the assay have been successfully optimised, repeat the standard curve analysis (section 3.1) using the optimal primer and probe concentrations and the same sample dilution series (section 3.1.2) and a minimum of 4 replicates for each point. Confirm that the efficiency and R^2 values fall within the expected values (section 3.2). The assay sensitivity is the highest dilution for which all replicates are detected.

If diminished performance is observed in the multiplex assay following optimisation of individual assay, systematically evaluate the assays and determine if the source of reduced performance can be attributed to a specific assay or oligo. If candidate oligos are found to reduce performance in the multiplex, rebalance those specific primer in the multiplex to optimise the multiplex.

Once optimal primer and probe concentrations have been determined for your assay, ensure that this information is recorded alongside the other assay characterisation information (section 1.1).

3.2.2 Limiting primer concentrations for a control assay

Control assays are an essential part of an experiment as they enable verification that the assay workflow is successful. Typically, ubiquitous targets found in the sample material (e.g. RNaseP, Actin, MS2) are chosen as control assays. When run in a multiplex assay, these targets are often expressed at high concentrations and can result in the control assay depleting reaction components such as dNTPs and polymerases. It is recommended to limit the primer concentrations of the control assay; this allows the assay to generate signal but limits amplification preventing the control assay from depleting reaction components that may reduce performance of target assays.

Guidelines for limiting primer concentrations:

1. Limit primer concentration for abundant genes;
2. Do not limit a primer that is required for reverse transcription (RT). Limiting the RT primer may result in failure to generate cDNA and consequently result in failed control assays;
3. Primer concentration can be limited from 50-300 nM.
4. Using the primer optimisation approach (section 3.2), select the conditions that produce reliable replicate data but at a higher C_q value than the optimal conditions.

4. Validating a custom PCR or qPCR assay

Once the primer and probe concentrations are determined and the custom assay is further optimised, this information can be used to characterise assay performance. A standard curve should be generated by plotting the C_q values against template concentration. From this, reaction efficiency can be calculated. Assays with high PCR efficiencies ensure consistent assay performance, reproducible data and accurate quantification of unknown samples.

A standard curve can be generated following the guidelines below:

1. Prepare a 96-well plate containing a 10-fold dilution series (10^1 to 10^7) of assay template. A row of NTC wells must also be included.
2. Perform the PCR using the optimal primer and probe concentrations determined as detailed in section 3.
3. Plot template concentration against C_q values and generate the standard curve. The software for qPCR instruments can generate this for you if samples are defined as standard curve samples.

   The formula is y = mx + C where m = slope of standard curve; C = y-intercept (theoretical C_q for 1 copy of template)

The m value from the standard curve is used to calculate reaction efficiency. A slope of -3.32 indicates a reaction with 100% efficiency, meaning the PCR product doubles every cycle during the geometric phase of the reaction. Again, qPCR instrument software can generate reaction efficiency for you if samples are defined as standard curve samples.

$E=(10^{(-1/m)}-1) \times 100$

The slope, assay efficiency and limit of detection values should be recorded alongside the other assay characterisation information (section 1.1).

Figures 7 and 8 illustrate amplification plots and standard curves for an efficient assay and for a less robust assay respectively.

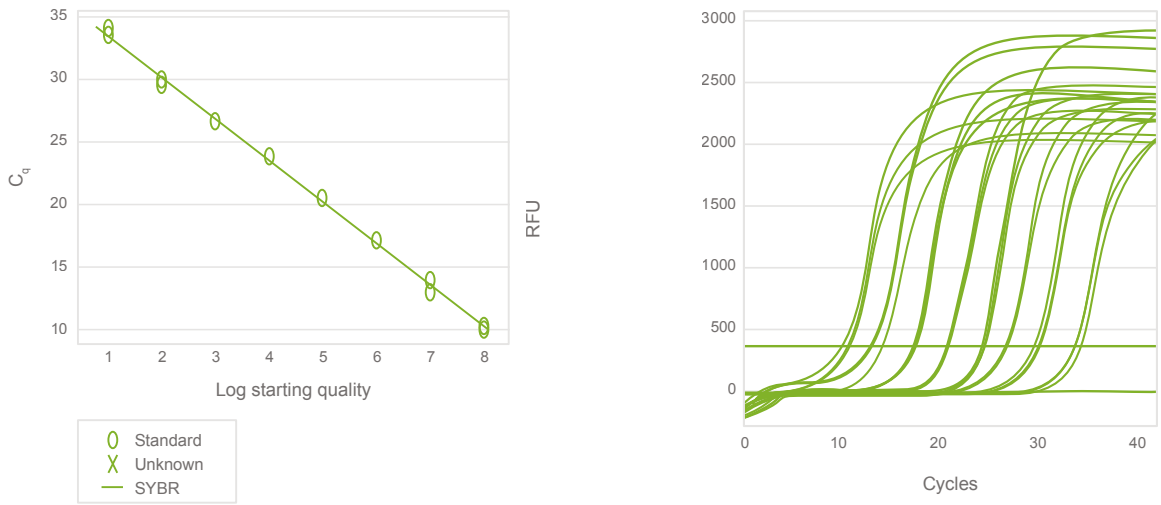


Figure 7: Standard curve and amplification plots for an efficient assay. Template serial dilution ranges from 10^8 to 10^1 copies. E = 100.9%, R^2 = 0.998, m = -3.300 and C_q = 36.775. Amplification plots show reliable data is captured at the lowest concentration of template tested (10 copies).

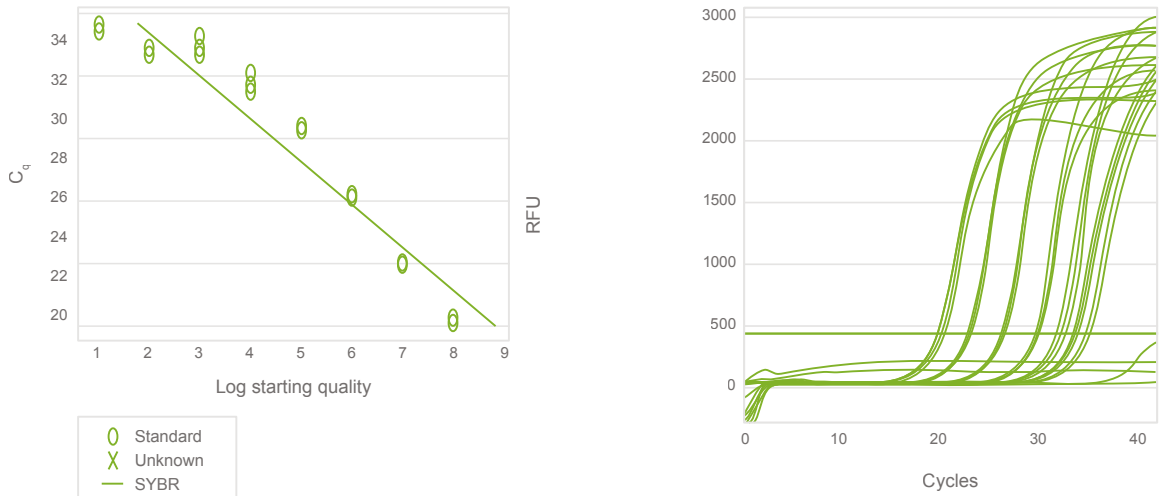


Figure 8: Standard curve and amplification plot for a less robust assay. Template serial dilutions ranged from 10^8 to 10^1 copies. E = 184.8%, R^2 = 0.922, m = -2.200 and C_q = 38.948. Amplification plots and standard curve data for 10, 100 and 1000 copies of template did not significantly differ, making 10,000 copies the lowest reliably detected concentration (or the LOD, limit of detection).

When working with template material that differs from the template material used during assay validation, it is recommended to repeat the validation exercise with template material that represents the new test sample type. Use of different templates can impact the performance of the assay and the data generated. Table 2 illustrates the impact of using a modified template type on the assay validation characteristics.

	LOD	Efficiency	R^2	m	C
Unmodified template	10 copies	100.9%	0.998	-3.300	36.775
Modified template	10,000 copies	184.8%	0.922	-2.200	38.948

Table 2. Assay validation characteristics for one assay when validated with two different templates.

Likewise, if a change of reaction component is required, it is important to re-validate the assay performance under these new conditions.

Once a custom assay is optimised and validated as detailed in sections 3 and 4, please contact the Assay Design and Development team at Biosearch Technologies (ADD@LGCgroup.com) for review. This step is required to qualify for re-design of custom assays in the case of an underperforming assay.

5. Evaluating assay oligonucleotide batches

After an assay has been optimised and is utilised in routine applications, one or more of the oligos from the original batch (referred to as batch 1) may begin to run out. In advance of running out of stock, it is recommended to put aside sufficient oligo to enable several plates worth of subsequent batch testing.

When the new oligo or oligos arrive (referred to as batch 2), repeat the oligo concentration steps described in section 1.2. Ensure concentration equivalence for the working solutions of oligos from batch 1 and batch 2.

When running comparison studies, every effort should be made to limit the test to one variable. For analysis purposes, the variable in an experiment is referred to as V. For a variable, there are typically two options e.g. two batch numbers of a forward primer, that are referred to as V1 and V2. The performance of V1 and V2 are compared within the comparison study. The null hypothesis of the study (referred to as H0) is that there is no difference in performance between V1 and V2. The alternative hypothesis (referred to as H1) is that the performance differs between V1 and V2. Within the comparison study, either H0 or H1 will be true. The specific performance measures to be compared will depend on the assay type; for end-point assays the values for cluster position and total fluorescence will be appropriate whereas for qPCR assays the C_q values, assay efficiency and LOD will be relevant.

In the example experimental design for testing new batches of oligo (table 3), ideally a single new oligo replaces the one from the previous batch and is run alongside the other assay oligos using all batch 1 oligos. This enables any change in performance to be attributed to a single oligo.

Experiment	Forward primer batch number	Reverse primer batch number	Probe batch number
Control (original)	1	1	1
Test: Forward primer	2	1	1
Test: Reverse primer	1	2	1
Test: Probe	1	1	2

Table 3. Suggested experimental design for testing new oligo batches. Ideally, only one new oligo should be tested in each experiment whilst all other oligos remain from the current batch.

When comparing batches, it is important to ensure that the manufacturing conditions (e.g. purification process) remain constant between the current batch and the new batch to minimise variables in downstream testing.

As well as testing for oligo batch changes, alternative oligo types or experimental conditions can also be compared. In some of these possible scenarios, it is inevitable that multiple variables are present. For example, comparing oligos obtained from different manufacturers will result in additional 'batch' differences that cannot be fully controlled for. Comparing oligos from different manufacturers requires independent optimisation of the oligos from each manufacturer. Due to differences in production methods and impurity profiles, optimal primer concentrations will vary between manufacturers i.e. the optimal primer concentration from manufacturer A may differ from the optimal primer concentration from manufacturer B. To make meaningful comparisons, ensure that optimised assays from both manufacturers are being compared.

Typical comparisons include:

- Batch 1 oligo vs batch 2 oligo from the same manufacturer
- Oligo manufacturer 1 vs oligo manufacturer 2
- A specific modification e.g. MGB vs LNA modified probes
- Different dyes – note that these have different fluorescent properties thus preventing direct comparison
- Master mix 1 vs master mix 2
- Instrument 1 vs instrument 2

For any comparison experiment, proceed to run a standard curve protocol as described in section 3.1 for both the control and the test scenarios. Ideally, both sets of reactions are run on the same plate, side by side, to minimise variability. When testing different oligo batches or types, ensure that sufficient volume of master mix is prepared for all control and test scenarios to minimise variability.

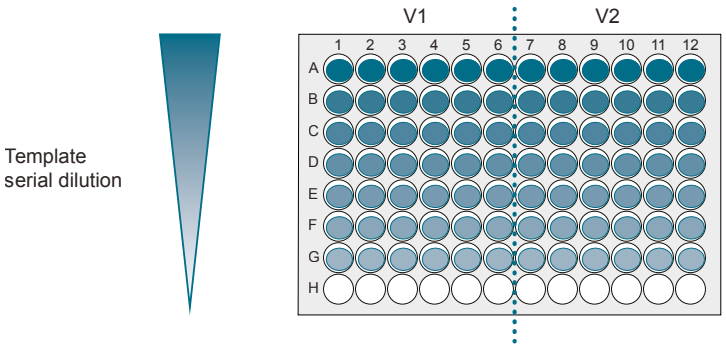


Figure 9. Suggested plate layout for side-by-side comparison of two oligo batches. Running both batches on the same reaction plate, using the same prepared master mix, minimises variability. V = variable being tested.

In the suggested experimental design for side-by-side comparison of two oligo batches, each concentration of the standard curve is replicated six times for each variable (V) (see figure 9). Design of the experiment to include six replicates provides for natural variance within each condition and ensures that statistical analyses detect only true variation for the condition being tested.

When comparing two syntheses of the same oligo, the first synthesis is V1 and the second synthesis is V2. The data generated for V1 and V2 are independently analysed to evaluate the replicates, the standard curve and theoretical assay sensitivity. These values give a measure of the assay optimisation and (potentially) how well the reaction plate was prepared. After this, results for V1 and V2 are compared to determine if the null (H0) or alternative (H1) hypothesis is true.

Table 4 illustrates example data for a comparison study, comparing two designs of an assay for a range of performance measures; reaction efficiency, assay R^2, end-point fluorescence values and C_q. A student's T-test was performed for each performance measure to determine if H0 or H1 was true, and thus whether performance was equivalent between the two assay designs.

	Assay efficiency		Assay R^2		End-point fluorescence (dilution 1)		C_q value (dilution 1)	
	Design 1	**Design 2**	**Design 1**	**Design 2**	**Design 1**	**Design 2**	**Design 1**	**Design 2**
Array 1	97.93	90.47	0.99	0.99	0.54	0.15	21.50	24.68
Array 2	95.75	88.36	1.00	0.99	0.51	0.15	21.05	22.22
Array 3	98.64	93.45	1.00	0.99	0.49	0.16	20.90	22.38
Average	97.43	90.76	0.99	0.99	0.51	0.15	21.15	23.09
Standard deviation	1.23	2.09	0.00	0.01	0.02	0.00	0.26	1.13
T-test P value	0.01>p<0.05		0.34		0.00		0.09	
H0: V1=V2	FALSE		TRUE		FALSE		TRUE	

Table 4. Results from a comparison study for two alternative assay designs. Design 1 represents V1 and design 2 represents V2. The null hypothesis (H0), that there is no performance difference between the two designs, was tested for four performance measures. Design 1 was statistically more efficient and exhibited higher end-point fluorescence (dilution 1) than design 2, thus proving H0 to be false.

6. Troubleshooting qPCR data

Troubleshooting an underperforming assay can be laborious and frustrating. However, taking steps to optimise and understand the assay can prevent problems from occurring or ensure that sufficient data is available to reveal underlying issues.

Our qPCR troubleshooting blog article defines the three phases that a standard qPCR amplification curve should have, and details a range of abnormal plots including potential causes and corrective steps.

7. Further support

If you require any further support please contact our technical support team at techsupport@lgcgroup.com or submit a request for support directly into our case system.

8. Appendix

8.1 Custom oligo characterisation

The table below can be adopted for use in your laboratory to characterise an individual custom oligo.

Value		Notes
Custom oligo name		
Sales Order number		
Lot ID		
Purification method		
nmol delivered		[Found on the Certificate of Analysis] Section 1.2.3
Stock concentration prepared		[Typically 100 µM] Section 1.2.3
Buffer used to prepare stock		[Typically TE] Section 1.2.1
Calculated oligo concentration		[Include method used to calculate this] Section 1.2.5
Working stock concentration		Section 1.2.8
To be use with these other oligos		[Note any other oligos that this oligo should be used with to generate an assay]

8.2 Characterisation of an assay

The table below can be adopted for use in your laboratory to characterise a qPCR assay.

Value		Notes
Oligos included in the assay		
#1	Name+type	[Note if it is forward, reverse, probe, modifications, dyes]
#2	Name+type	[Note if it is forward, reverse, probe, modifications, dyes]
#3	Name+type	[Note if it is forward, reverse, probe, modifications, dyes]
#4	Name+type	[Note if it is forward, reverse, probe, modifications, dyes]
#5	Name+type	[Note if it is forward, reverse, probe, modifications, dyes]
#6	Name+type	[Note if it is forward, reverse, probe, modifications, dyes]
Optimised working concentrations		[Detail the optimised primer and probe concentrations] Section 3.2.2
Standard curve linear efficiency: efficiency (%)		[Performed with control template] Section 3.2
Standard curve linear efficiency: R^2		[Performed with control template] Section 3.2
Assay validation: efficiency (%)		[Performed with specific template type, 10-fold dilution series] Section 4
Assay validation: efficiency (R^2)		[Performed with specific template type, 10-fold dilution series] Section 4

For Research Use Only. Not for use in diagnostic procedures.

@LGCBiosearch | biosearchtech.com

BIOSEARCH™ TECHNOLOGIES