



Thermo Scientific
DyNAmo Capillary SYBR Green qPCR Kit

Technical Manual

F-420S 100 reactions (20 μ l each)

F-420L 500 reactions (20 μ l each)

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1. Description

Thermo Scientific DyNAmo Capillary SYBR Green qPCR Kit is designed for quantitative, real-time analysis of DNA samples from various sources with instruments using glass capillaries. Quantitative PCR (qPCR) is a useful technique for the investigation of gene expression, viral load, pathogen detection, and numerous other applications.

The performance of the DyNAmo Capillary™ SYBR® Green qPCR Kit is based on a hot-start version of a modified *Thermus brockianus* DNA polymerase and SYBR Green I fluorescent dye. A nonspecific DNA binding domain has been fused to the *Tbr* DNA polymerase. This domain lends physical stability to the polymerase-DNA complex. The initial denaturation step in the PCR protocol activates the modified hot-start *Tbr* polymerase. SYBR Green I is specific for double-stranded DNA and fluoresces when bound to the amplified double-stranded PCR product, thereby permitting the direct quantification of amplified DNA without labeled probes. The reaction buffer is optimized for use with glass capillaries.

The reaction chemistry of DyNAmo Capillary SYBR Green qPCR Kit is applicable to capillary-based instruments, such as Roche LightCycler™. For block-based real-time instruments we recommend DyNAmo™ Flash SYBR® Green qPCR Kits (F-415 or F-416). When RNA is used as the starting material for producing cDNA, we recommend Thermo Scientific DyNAmo cDNA synthesis Kit (F-470) to ensure high-quality results.

2. Kit components

DyNAmo Capillary SYBR Green qPCR Kit	F-420S	F-420L
2x master mix (contains a hot-start version of a modified <i>Tbr</i> DNA polymerase, SYBR® Green I, optimized PCR buffer, 6 mM MgCl ₂ , dNTP mix including dUTP)	2 x 500 µl (sufficient for 100 reactions of 20 µl)	10 x 500 µl (sufficient for 500 reactions of 20 µl)

Material safety data sheet (MSDS) is available at www.thermoscientific.com/fzmsds.

3. Shipping and storage

The DyNAmo Capillary SYBR Green qPCR Kit is shipped on gel ice. Upon arrival, store all kit components at -20°C. When using the 2x master mix, the leftover thawed mix can be refrozen and stored at -20°C without affecting the performance of the kit.

4. Notes about reaction components

Table 1. General recommendations.

Categories	Comments
Kit storage	Store at -20°C.
Consumables	Capillary reaction vessels and capillary closures.
Reaction volume	20 µl
Amplicon size	< 500 bp
Template amount	Depends on template type and quality. In general, do not use more than 200 ng of genomic DNA in a 20 µl reaction.
Primer design	Use primers with matched T _m . Avoid inter-primer and intra-primer complementary sequences. We recommend calculating T _m by the nearest-neighbor method as described by Breslauer <i>et al.</i> (1986) <i>Proc. Nat. Acad. Sci.</i> 83: 3746–50. Instructions for T _m calculation and a link to a calculator using the nearest-neighbor method can be found on the Thermo Scientific website (www.thermoscientific.com/pcrwebtools).
MgCl ₂	1x master mix contains 3 mM MgCl ₂ .

4.1 DNA polymerase

The 2x qPCR master mix in the DyNAmo Capillary SYBR Green qPCR Kit includes a hot-start version of a modified *Thermus brockianus* DNA polymerase. The modified polymerase incorporates a nonspecific DNA binding domain that lends physical stability to the polymerase-DNA complex. The modified *Tbr* polymerase is chemically engineered to be inactive at room temperature. The inactivation prevents the extension of nonspecifically bound primers during reaction setup and therefore increases PCR specificity. The reaction setup can be performed at room temperature. The initial denaturation step in the PCR protocol reactivates the polymerase (hot start).

4.2 PCR primers

Careful primer design is particularly important to minimize nonspecific primer annealing and primer-dimer formation, since fluorescence from SYBR Green I increases strongly upon binding to any double-stranded DNA. Standard precautions must be taken during primer design to avoid primer-dimer or hairpin loop formation. Most primer design software tools will yield well-designed primers for use in qPCR. In most cases, good results are obtained using a concentration of 0.5 µM for each primer. The optimum primer concentration is usually between 0.1 and 1 µM.

4.3 Template preparation and quality

Purity of nucleic acid templates is particularly important for qPCR, as contaminants may interfere with fluorescence detection. Most commercial DNA purification kits give satisfactory results for qPCR.

4.4 Standards

Standard curve is needed for absolute quantification and for analyzing the efficiency of the qPCR reaction (see Section 6.2). Correlation coefficient (R²) of the standard curve indicates how well the standard curve fits the measured data and therefore reflects the reliability of the assay.

The absolute amount of the target nucleic acid (expressed as a copy number or concentration) is determined by comparison of C_p values to external standards containing a known amount of nucleic acid. (C_p = crossing point, the cycle number at detection threshold). The external standards should contain the same or nearly the same sequence as the template of interest. It is especially important that the primer binding sites are identical to ensure equivalent amplification efficiencies of both standard and target molecules.

4.5 UNG (UDG) treatment

Due to the high sensitivity of qPCR even minute amounts of contaminating DNA can lead to false positive results. If dUTP is used in all qPCR reactions, the carry-over contamination from previous PCR runs can be prevented by treating the reaction samples with UNG prior to PCR. UNG (uracil-N-glycosylase) digests dU-containing DNA, and the digested DNA cannot act as a template in qPCR (Longo, M.C. *et al.* (1990) *Gene* 93: 125–28). UNG is inactivated during the first denaturation step in PCR. The UNG treatment step (50°C for 2 min) has no negative effect on qPCR performance because the hot start *Tbr* DNA polymerase is not reactivated at 50°C. All Thermo Scientific DyNAmo qPCR Kits contain dUTP and therefore UNG treatment can be used.

To minimize contamination risk in general, capillaries containing reaction products should not be opened or analyzed by gel electrophoresis in the same laboratory area which is used to set up reactions.

4.6 Reaction volume

A reaction volume of 20 µl is recommended.

4.7 MgCl₂ optimization

Generally, it is not necessary to optimize the MgCl₂ concentration with the DyNAmo Capillary SYBR Green qPCR Kit. For most reactions, we recommend a final concentration of 3 mM MgCl₂, as provided in the master mix. However, in some rare cases, better results may be obtained with higher MgCl₂ concentrations. Excessive MgCl₂ concentrations can lead to the amplification of nonspecific products and primer-dimers, however. Usually no more than 5 mM MgCl₂ is required by any amplicon.

4.8 Quantification of RNA

To determine the quantity of mRNA, a reverse transcription (RT) reaction must be performed before qPCR. We offer DyNAmo cDNA Synthesis Kit (F-470) for quantitative reverse transcription. DyNAmo Capillary SYBR Green qPCR Kit has been optimized using the DyNAmo cDNA Synthesis Kit.

For additional information about the reverse transcription step, see Appendix I: cDNA synthesis.

5. Reaction setup and cycling protocols

- Perform the reaction setup in an area separate from nucleic acid preparation and PCR product analysis.
- As the hot-start DNA polymerase is inactive during PCR setup, it is not necessary to do the setup on ice.
- Pipette with sterile filter tips.
- Minimize the exposure of the qPCR master mix to light.
- Minimize pipetting errors by using calibrated pipettes and by preparing premixes to avoid pipetting very small volumes.
- Avoid touching the optical surface of the capillaries.
- Capillaries should be centrifuged before starting the cycling program to force the solution to the bottom of the capillary and to remove any bubbles.
- Use molecular biology grade H₂O.

Reaction setup

1. Program the cycler as outlined in Table 3. Thaw the template DNA, primers and master mix. Mix the individual tubes to ensure homogeneity. This is especially important for the master mix.
2. Prepare a PCR premix by mixing the master mix, primers and H₂O. * Mix the PCR premix thoroughly to ensure homogeneity. Dispense appropriate volumes into capillaries.
3. Add template DNA (<200 ng per 20 µl reaction) to the capillaries containing the PCR premix. * For two-step qRT-PCR, the volume of the cDNA added (from the RT reaction) as the template should not exceed 10 % of the final PCR volume.
5. Insert capillaries into the carousel and centrifuge them, or alternatively centrifuge capillaries in microcentrifuge adapters before inserting them into the carousel.
6. Place the carousel in the thermal cycler and start the cycling program.

* Alternatively, complete PCR mixes can be prepared in separate tubes by mixing template DNA to PCR premix. PCR mixes are then mixed thoroughly and dispensed into capillaries (Note that some excess volume is needed). This enables better mixing of PCR premix and DNA template.

Table 2. Reaction setup.

Components (In order of addition)	20 µl reaction	Final concentration	Comments
2x Master mix	10 µl	1x	Mix thoroughly.
Primer mix (in H ₂ O)	X µl	0.5 µM fwd 0.5 µM rev	Titrate from 0.1 to 1 µM if necessary.
Template DNA	X µl	<10 ng/µl	Do not exceed 10 ng/µl in the final reaction.
H ₂ O	add to 20 µl		

Cycling protocol

Table 3. Cycling protocol for Light Cycler.

Step	Purpose	Temp	Hold time	Slope (°C/s)	Data acquisition	Comments
Initial denaturation						
	UNG incubation					Optional, see below.
1	Initial denaturation	95°C	10 min	20	None	
Amplification 35–45 cycles						
2	Denaturation	95°C	10 s	20	None	
3*	Annealing	X°C	20 s	20	None	5°C below lower primer T _m .
4	Extension	72°C	10–20 s	20	Single	Longer extension time may be necessary for amplicons >500 bp.
Melting curve						
5	Denaturation	95°C	0 s	20	None	
6	Reannealing	57°C	15 s	20	None	
7	Denaturation	98°C	0 s	0.1	Continuous	Temperature is increased gradually to melt the DNA
Cooling						
8	Cooling of the carousel	40°C	10 s	20	None	Carousel is cooled for user safety.

* Use the T_m calculator at www.thermoscientific.com/pcrwebtools to determine T_m of the primers. Use 50 mM KCl and 0.5 μM primer concentration (or the primer concentration in your reaction if optimized to other than 0.5 μM) when calculating T_m. Due to the characteristics of the modified DNA polymerase, it is often possible to use higher annealing temperatures than with other enzymes.

UNG incubation (optional)

If UNG enzyme is used, incubate 2 min at 50°C. This step does not negatively affect qPCR performance because the hot-start *Tbr* DNA polymerase is not active at 50°C. If heat-labile UNG is used, decrease the incubation temperature and increase time in accordance with the manufacturers' instructions.

Initial denaturation / reactivation

Initial denaturation at 95°C for 10 min is needed to ensure a complete reactivation of the hot-start *Tbr* DNA polymerase and denaturation of the template. If high initial activity of the enzyme is needed for example in cases with very high initial copy numbers or if genomic DNA is used as a template, longer initial denaturation might be needed, up to 15 min.

Denaturation

Denaturation at 95°C for 10 s is sufficient in most cases. To minimize cycle time it is often possible to use even shorter denaturation time. In many cases as short as 0 s works, but each amplicon must be individually tested.

Annealing

For most amplicons, annealing for 20 s at 5°C below the lower T_m of the two primers works well as a starting point. In many cases, 60°C can be used with success for a wide range of primer pairs. Due to the unique characteristics of the modified hot start DNA polymerase it is often possible to use higher annealing temperatures than with other enzymes and thereby minimize the chances of primer-dimer formation or amplification of nonspecific products.

To minimize cycle time it is often possible to use shorter than recommended 20 s annealing, but other parameters such as annealing temperature and extension time might need to be reoptimized.

These guidelines are based on T_m values (50 mM salt and 0.5 μ M primer) calculated by the nearest-neighbor method as described by Breslauer *et al.* (1986) *Proc. Nat. Acad. Sci.* 83: 3746–50. Instructions for T_m calculation and a link to a calculator using the nearest-neighbor method can be found on the Thermo Scientific website (www.thermoscientific.com/pcrwebtools). Different software may give different T_m values.

If primer-dimers are observed, the easiest solution is often to redesign primers. Another alternative is to optimize the annealing temperature by performing additional runs, varying the annealing temperature in each by 2°C.

Extension

Extension temperature should be 72°C for most reactions. In cases where the melting point of the product is near or lower than 72°C, a lower extension temperature (e.g. 68°C) should be used.

Depending on the amplicon a combined annealing/extension step can also be used (two-step PCR). For most amplicons shorter than 250 bp, 10 s extension time generally gives good results. For amplicon longer than 250 bp, 4s/100 bp extension time is recommended. If annealing temperature is low and annealing time is short, slower temperature ramp time to extension temperature can give better results (e.g. slope = 5 °C/s).

Data acquisition

If primer-dimers are observed, it may be helpful to perform a data acquisition step at an elevated temperature to minimize the interference of primer-dimers with quantification. The temperature used should be sufficiently higher than the T_m of any primer-dimer (usually $<80^\circ\text{C}$) and lower than that of the specific product.

Number of cycles

For most applications, 40 cycles of amplification should be sufficient even when the template is present at a very low copy number. An excessive number of cycles can lead to nonspecific amplification, which manifests itself in undesirable products seen during melting curve analysis.

Melting curve

A melting curve is used to check the specificity of an amplified product. When the temperature is gradually increased, a sharp decrease in SYBR Green fluorescence is observed as the product undergoes denaturation. Specific products can be distinguished from the nonspecific products by the difference in their melting temperatures. The recommended temperature ramp time is stated in Table 3. If a faster protocol is preferred, the ramp time of the melting curve can be increased, although this may affect resolution.

6. Analysis

6.1 Melting curve

Melting curve analysis is typically included in the analysis software of real-time fluorescence detection instruments. The melting point of the product depends mainly on base composition and length. When the decrease in SYBR Green fluorescence during the temperature increase is plotted as a negative first derivative, the temperature of the peak is defined as the T_m , or the melting temperature of the product.

If primer-dimers or other nonspecific products are observed, the efficiency of the PCR should be checked. Varying efficiency leads to incorrect quantification.

6.2 Absolute quantification

Absolute quantification is performed by plotting samples of unknown concentration on a standard curve generated from a dilution series of template DNA of known concentration. Typically, the standard curve is a plot of the cycle numbers of crossing points (Cp) against the logarithm of the amount of DNA. A linear regression analysis of the standard plot is used to calculate the amount of DNA in unknown samples. The slope of the equation is related to the efficiency of the PCR reaction. The PCR efficiency should be the same for standards and samples for quantification to be accurate. The PCR efficiency of the samples can be determined by doing a dilution series of these samples.

For a graph where cycle number is on the y axis and log(DNA copy #) on the x axis:

$$\text{PCR efficiency} = \left(10^{\frac{-1}{\text{slope}}} - 1\right) \times 100 \%$$

The slope of -3.322 corresponds to 100 % efficiency.

6.3 Relative quantification

Relative quantification is used to determine the ratio between the quantity of a target molecule in a sample and in the calibrator (healthy tissue or untreated cells, for example). The most common application of this method is the analysis of gene expression, such as comparisons of gene expression levels in different samples, for example. The target molecule quantity is usually normalized with a reference gene (see 'Reference genes' in Appendix I: cDNA synthesis).

If the amplification efficiency of a reference gene is the same as that of the target gene, the comparative $\Delta\Delta C_p$ method can be used for relative quantification. Both the sample and the calibrator data are first normalized against variation in sample quality and quantity. Normalized (ΔC_p) values are calculated by the following equations:

$$\Delta C_p(\text{sample}) = C_p(\text{target}) - C_p(\text{reference})$$

$$\Delta C_p(\text{calibrator}) = C_p(\text{target}) - C_p(\text{reference})$$

The $\Delta\Delta C_p$ value is then determined using the following formula:

$$\Delta\Delta C_p = \Delta C_p(\text{sample}) - \Delta C_p(\text{calibrator})$$

The expression of the target gene normalized to the reference gene and relative to the calibrator = $2^{-\Delta\Delta C_p}$

7. Troubleshooting

Possible causes	Comments and suggestions
No increase in fluorescence signal	
Error in cycler setup	<ul style="list-style-type: none"> • Make sure that the instrument settings are correct for the experiment.
Missing components (e.g. primers or template) or pipetting error	<ul style="list-style-type: none"> • Check the assembly of the reactions. • Check the concentrations and storage conditions of the reagents.
Missing essential step in the cycler protocols	<ul style="list-style-type: none"> • Check the cycler protocol.
Late increase in fluorescence signal	
Error in cycler setup	<ul style="list-style-type: none"> • Make sure that the instrument settings are correct for the experiment.
Missing components or pipetting error	<ul style="list-style-type: none"> • Check the assembly of the reactions. • Check the concentrations and storage conditions of the reagents.
Insufficient activation of the hot start DNA polymerase	<ul style="list-style-type: none"> • Make sure 95°C 10 min was used for the initial reactivation/denaturation step. • Make sure cycler temperature is accurate.
Insufficient starting template	<ul style="list-style-type: none"> • Check the calculation of the template stock concentration; increase the template amount if possible.
qPCR primer design not optimal	<ul style="list-style-type: none"> • Check primer design. See Section 4.2.
qPCR primer concentration too low	<ul style="list-style-type: none"> • Increase qPCR primer concentration (to a maximum of 1 μM each).
Annealing temperature too high	<ul style="list-style-type: none"> • Decrease annealing temperature in 2°C decrements.
Insufficient extension time for the amplicon size	<ul style="list-style-type: none"> • We recommend 10 s or 4 s/100 bp s extension time for longer than 250 bp amplicons.
PCR protocol not optimal	<ul style="list-style-type: none"> • Make sure you are using the recommended PCR protocol. If necessary, optimize using the recommended protocol as a starting point.
Normal fluorescence signal, but melting curve analysis shows primer-dimers or nonspecific products only	
Missing components or pipetting error	<ul style="list-style-type: none"> • Check the assembly of the reactions. • Check the concentrations and storage conditions of the reagents.
Primer–dimers from a previous run contaminating the reaction	<ul style="list-style-type: none"> • Perform UNG treatment before PCR cycling.
Annealing temperature of qPCR primers too low	<ul style="list-style-type: none"> • Increase annealing temperature in 2°C increments.
qPCR primer design not optimal or very low template concentration	<ul style="list-style-type: none"> • Check primer design. See Section 4.2.

Normal fluorescence signal, melting curve analysis shows both primer-dimer or nonspecific product and specific product peaks	
Low initial template concentration	<ul style="list-style-type: none"> • Increase template amount.
qPCR primer design not optimal	<ul style="list-style-type: none"> • Check primer design. See Section 4.2.
Primer concentration too high	<ul style="list-style-type: none"> • Optimize primer concentration.
Annealing temperature of qPCR primers too low	<ul style="list-style-type: none"> • Increase annealing temperature in 2°C increments.
Primer–dimers or PCR products from previous run contaminating the reaction	<ul style="list-style-type: none"> • Perform UNG treatment before PCR cycling.
Co-amplification of primer-dimers with the specific product	<ul style="list-style-type: none"> • Perform a second data acquisition at an elevated temperature to minimize the interference of primer-dimers.
Extension time in qPCR too long	<ul style="list-style-type: none"> • Decrease extension time.
Non-linear correlation between Cp and log of template amount in the standard curve	
Template dilution inaccurate	<ul style="list-style-type: none"> • Remake dilution series and make sure the samples are well mixed.
Template amount too high	<ul style="list-style-type: none"> • Reduce template amount. • Increase reaction volume.
Template amount too low	<ul style="list-style-type: none"> • Increase template amount.
Insufficient activation of the hot start DNA polymerase	<ul style="list-style-type: none"> • Make sure 95°C 10 min was used for the initial reactivation/ denaturation step in qPCR. • Make sure the cycler temperature is accurate.
Co-amplification of primer-dimers with the specific product	<ul style="list-style-type: none"> • Perform a second data acquisition at an elevated temperature to minimize the interference of primer-dimers.
High initial fluorescence signal, gradually decreasing over the first 10–20 cycles	
Template amount too high	<ul style="list-style-type: none"> • Reduce template amount.
Insufficient denaturation of template	<ul style="list-style-type: none"> • Make sure 95°C 15 min is used for the initial denaturation step in qPCR. • Make sure the cycler block temperature is accurate.

Appendix I: cDNA synthesis

The cDNA synthesis step is very critical in qRT-PCR. The efficiency of reverse transcription varies and can be low in some cases. The expression level of the target RNA molecule and the efficiency of the RT reaction must therefore be considered when determining the appropriate amount of the starting template for subsequent PCR steps. The volume of cDNA template should not exceed 10 % of the qPCR reaction volume, as elevated volumes of the template may reduce the efficiency of the PCR amplification. A dilution series of the template can be done to optimize the volume of the starting material used.

Since RNA quantification involves a number of variables, and each experiment is inherently different, careful experiment design is very important. Useful information and guidelines for experiment design, normalization, RNA standards, etc. can be found in the following review articles:

Bustin S.A. (2000) *Journal of Molecular Endocrinology* 25: 169–193

Bustin S.A. (2002) *Journal of Molecular Endocrinology* 29: 23–39.

We recommend using the DyNAmo cDNA Synthesis Kit (F-470) for the reverse transcription step. This kit has been specifically optimized for quantitative reverse transcription.

RT Primers

Random hexamers, oligo(dT) or specific primers can be used for the RT step. A good starting point is to use random hexamers for cDNA synthesis. Random hexamers transcribe all RNA, producing cDNA that covers the whole transcript. Oligo(dT) primers can be used to transcribe poly(A)⁺ RNAs, and gene-specific primers to transcribe only the particular RNA of interest. Using specific primers can help to decrease background. Random hexamers and oligo(dT) primers are useful if several different amplicons need to be analyzed from a small amount of starting material.

Primers for qPCR step

PCR primers in qRT-PCR experiments should be designed to anneal to sequences in two exons on opposite sides of an intron. A long intron inhibits the amplification of the genomic target. Alternatively, primers can be designed to anneal to the exon-exon boundary of the mRNA. With such an assay design, the priming of genomic target is highly inefficient.

DNase I

If primers cannot be designed to anneal to the exon-exon boundaries or in separate exons, the RNA sample must be treated with RNase-free DNase I.

Minus RT control

A minus RT control should be included in all qRT-PCR experiments to test for DNA contamination (such as genomic DNA or PCR product from a previous run). Such a control reaction contains all the reaction components except for the reverse transcriptase. RT reaction should not occur in this control, so if PCR amplification is seen, it is most likely derived from contaminating DNA.

Reference genes

When studying gene expression, the quantity of the target gene transcript needs to be normalized against variation in the sample quality and quantity between samples. To ensure identical starting conditions, the relative expression data have to be normalized with respect to at least one variable, such as sample size, total amount of RNA, or reference gene(s), for example. A gene used as a reference should have a constant expression level that is independent of the variation in the state of the sample tissue. Examples of commonly used reference genes are beta actin, GAPDH and 18S rRNA. A problem is that, even with housekeeping genes, the expression usually varies to some extent. That is why several reference genes are usually required, and their expression needs to be checked for each experiment. For relative quantification ($\Delta\Delta Cq$ method), see Section 6.3.

The amplification efficiency of a reference gene should be the same as the amplification efficiency of the target gene, i.e. the slopes of their standard curves are the same. For efficiency calculation using the slope, see Section 6.2 (Absolute quantification).

Appendix II: general molecular biology data

Table 4. Spectrophotometric conversions for nucleic acid templates.

1 A ₂₆₀ unit*	Concentration (µg/ml)
Double-stranded DNA	50
Single-stranded DNA	33
Single-stranded RNA	40

* Absorbance at 260 nm = 1 (1 cm detection path).

Table 5. Molar conversions for nucleic acid templates.

Nucleic acid	Size	pmol/µg	Copies/µg*
1 kb DNA	1 000 bp	1.52	9.1 x 10 ¹¹
pUC19DNA	2 686 bp	0.57	3.4 x 10 ¹¹
Lambda DNA	48 502 bp	0.03	1.8 x 10 ¹⁰
<i>Escherichia coli</i>	4.7 x 10 ⁶ bp	3.2 x 10 ⁻⁴	1.9 x 10 ⁸
Human	3.2 x 10 ⁹ bp	4.7 x 10 ⁻⁷	2.8 x 10 ⁵

* For single-copy genes.

Product use limitation

This product has been developed and is sold exclusively for research purposes and in vitro use only. This product has not been tested for use in diagnostics or drug development, nor is it suitable for administration to humans or animals.

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SYBR is a registered trademark of Life Technologies Corporation and its affiliated companies.

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**Thermo Scientific DyNAmo Capillary SYBR Green qPCR Kit
Technical Manual**



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