

Guidelines for comparing SYBR Green master mixes

Take the master mix challenge—compare your current master mix to Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix using this simple protocol. In our own experiments, we found that PowerUp SYBR Green Master Mix outperformed other master mixes when comparing specificity, PCR efficiency, dynamic range, and precision over multiple targets.

This protocol is for the comparison of PowerUp SYBR Green Master Mix to one other master mix using 20 µL reactions in triplicate. Adjust volumes as needed.

Reaction preparation

1. Prepare 10-fold dilution series of template.

Dilution	Template	Nuclease-free water
Undiluted	40 µL of 20 ng/µL DNA	–
10 ⁻¹	4 µL of undiluted DNA	36 µL
10 ⁻²	4 µL of 10 ⁻¹	36 µL
10 ⁻³	4 µL of 10 ⁻²	36 µL
10 ⁻⁴	4 µL of 10 ⁻³	36 µL
10 ⁻⁵	4 µL of 10 ⁻⁴	36 µL
10 ⁻⁶	4 µL of 10 ⁻⁵	36 µL

General guidelines

- Use the manufacturer’s recommended primer and template DNA concentrations
- Use the manufacturer’s recommended thermal cycling parameters
- Run a standard curve to compare PCR efficiency, sensitivity, dynamic range, and precision
- Run reactions in triplicate to measure reproducibility
- Perform a melt curve to analyze specificity

2. Prepare enough master mix for 26 x 20 µL reactions for each qPCR master mix to be tested, according to the volumes in the table below. Mix thoroughly.

Component	PowerUp SYBR Green Master Mix	Master mix for comparison
qPCR master mix, 2X	260 µL	260 µL
Forward primer, 10 µM	26 µL*	Manufacturer’s recommendation
Reverse primer, 10 µM	26 µL*	Manufacturer’s recommendation
Nuclease-free water	78 µL (final volume 390 µL)	Adjust to 390 µL

* Volumes shown are for 500 nM primer. The recommended primer concentration for PowerUp SYBR Green Master Mix is 300–800 nM.

- Pipet 15 μL of the PowerUp SYBR Green mix prepared in step 2 to each well of a 96-well optical plate according to the plate map below.
- Pipet 5 μL of the indicated dilution from step 1 into each well of the plate according to the plate map below. For no-template control (NTC) reactions, use 5 μL of nuclease-free water.

	1	2	3	4	5	6	7	8	9	10	11	12
A					Undiluted	Undiluted	Undiluted					
B					10^{-1}	10^{-1}	10^{-1}					
C					10^{-2}	10^{-2}	10^{-2}					
D					10^{-3}	10^{-3}	10^{-3}					
E					10^{-4}	10^{-4}	10^{-4}					
F					10^{-5}	10^{-5}	10^{-5}					
G					10^{-6}	10^{-6}	10^{-6}					
H					NTC	NTC	NTC					

- Repeat steps 3–4 for the other master mix to be compared, using a new plate. Reactions can be loaded on the same plate if the thermal protocols are identical.
- Mix the components thoroughly. Seal the plates, and briefly centrifuge to remove any bubbles.

Real-time PCR

- Place the plate in the instrument.
- Set the thermal cycling conditions.
 - For PowerUp SYBR Green Master Mix, use one of the following conditions:
 - Standard cycling (for most instruments and primer $T_m \geq 60^\circ\text{C}$)

Step	Temperature	Time	Cycles
UDG activation	50°C	2 min	Hold
Polymerase activation	95°C	2 min	Hold
Denaturation	95°C	15 sec	40
Annealing/extension	60°C^*	1 min	

* For primers with $T_m < 60^\circ\text{C}$, please refer to the PowerUp SYBR Green Master Mix instruction manual.

- Fast cycling (for Applied Biosystems™ ViiA™ 7, QuantStudio™, 7500 Fast, StepOne™, and StepOnePlus™ instruments and primer $T_m \geq 60^\circ\text{C}$)

Step	Temperature	Time	Cycles
UDG activation	50°C	2 min	Hold
Polymerase activation	95°C	2 min	Hold
Denaturation	95°C	1* or 3† sec	40
Annealing/extension	$60^\circ\text{C}^\ddagger$	30 sec	

* For ViiA 7 or QuantStudio instruments.

† For 7500 Fast, StepOne, or StepOnePlus instruments.

‡ For primers with $T_m < 60^\circ\text{C}$, please refer to the PowerUp SYBR Green Master Mix instruction manual.

- Include conditions for melt curve analysis after the PCR stage. On Applied Biosystems™ instruments, use the default continuous melt curve setting, which on most newer instruments are:

Stage	Step	Ramp rate	Temperature	Time
Melt curve stage	Step 1	1.6°C/sec	95°C	15 sec
	Step 2	1.6°C/sec	60°C	1 min
	Step 3 (dissociation)	0.15°C/sec	95°C	15 sec

- For Applied Biosystems instruments, use the following settings:

Experiment type:	Standard curve
Reagent:	SYBR Green reagents
Reporter:	SYBR
Quencher:	None
Passive reference dye:	ROX™
Ramp speed:	Standard or Fast (choose the corresponding thermal cycler profiles in step 2 above)
Melt curve ramp increment:	Continuous

See the instrument instruction manual for more information or if using a different instrument.

3. Run the plate. Repeat the run setup for the other master mix being tested using the manufacturer's recommended thermal cycling profile.

Data analysis

In most qPCR systems, the data can be analyzed by the qPCR system software.

Slope and amplification efficiency: The amplification efficiency is calculated using the slope of the regression line of the standard curve. A slope of -3.323 indicates optimal 100% PCR amplification efficiency. Reactions with efficiencies of $100\% \pm 10\%$ over a broad dynamic range are needed to reliably use the $\Delta\Delta C_t$ method for gene expression analysis.

Caution: Don't be fooled by lower C_t values when comparing master mixes. The only way to compare the sensitivity and performance of two master mixes is by performing a dilution series and examining the amplification efficiency, linearity, and precision across the dilution range.

R² value (correlation coefficient): The R^2 value is a measure of the closeness of fit between the regression line and the individual C_t data of the standard curve reactions. A value of 1.00 indicates a perfect fit between the regression line and the data. An R^2 value >0.99 is desirable.

Standard deviation (precision): Calculate the standard deviation of the C_t values for each dilution. To be able to quantify a 2-fold dilution with $\geq 95\%$ confidence, the standard deviation of a dilution should be ≤ 0.250 .

Melt curve (specificity): View the melt curve using a derivative reporter plot (a plot of derivative reporter ($-R_n$) vs. temperature). A single peak indicates specific amplification, whereas multiple peaks or shoulders indicate nonspecific amplification or primer-dimer formation.

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